The mitochrondial antioxidant manganese superoxide dismutase (Mn-SOD) plays a critical cytoprotective role against oxidative stress. Vascular endothelial growth factor (VEGF) was shown previously to induce expression of Mn-SOD in endothelial cells by a NADPH oxidase-dependent mechanism. The goal of the current study was to determine the transcriptional mechanisms underlying this phenomenon. VEGF resulted in protein kinase C-dependent phosphorylation of IκB and subsequent translocation of p65 NF-κB into the nucleus. Overexpression of constitutively active IκB blocked VEGF stimulation of Mn-SOD. In transient transfection assays, VEGF increased Mn-SOD promoter activity, an effect that was dependent on a second intronic NF-κB consensus motif. In contrast, VEGF-mediated induction of Mn-SOD was enhanced by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and by dominant negative Akt and was decreased by constitutively active Akt. Overexpression of a constitutively active (phosphorylation-resistant) form of FKHRL1 (TM-FKHRL1) resulted in increased Mn-SOD expression, suggesting that the negative effect of PI3K-Akt involves attenuation of forkhead activity. In co-transfection assays, the Mn-SOD promoter was transactivated by TM-FKHRL1. Flavoenzyme inhibitor, diphenyleneiodonium (DPI), and antisense oligonucleotides against p47phox (AS-p47phox) inhibited VEGF stimulation of IκB/NF-κB and forkhead phosphorylation, supporting a role for NADPH oxidase activity in both signaling pathways. Like VEGF, hepatocyte growth factor (HGF) activated the NADPH oxidase activity in both signaling pathways. Like and forkhead phosphorylation, supporting a role for constitutively active IκB blocked VEGF stimulation of Mn-SOD. In transient transfection assays, VEGF increased Mn-SOD promoter activity, an effect that was dependent on a second intronic NF-κB consensus motif. In contrast, VEGF-mediated induction of Mn-SOD was enhanced by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and by dominant negative Akt and was decreased by constitutively active Akt. Overexpression of a constitutively active (phosphorylation-resistant) form of FKHRL1 (TM-FKHRL1) resulted in increased Mn-SOD expression, suggesting that the negative effect of PI3K-Akt involves attenuation of forkhead activity. In co-transfection assays, the Mn-SOD promoter was transactivated by TM-FKHRL1. Flavoenzyme inhibitor, diphenyleneiodonium (DPI), and antisense oligonucleotides against p47phox (AS-p47phox) inhibited VEGF stimulation of IκB/NF-κB and forkhead phosphorylation, supporting a role for NADPH oxidase activity in both signaling pathways. Like VEGF, hepatocyte growth factor (HGF) activated the P13K-Akt-forkhead pathway. However, HGF-P13K-Akt forkhead signaling was insensitive to phosphodioidonum and AS-p47phox. Moreover, HGF failed to induce phosphorylation of IκB/NF-κB or nuclear translocation of NF-κB and had no effect on Mn-SOD expression. Together, these data suggest that VEGF is uniquely coupled to Mn-SOD expression through growth factor-specific reactive oxygen species (ROS)-sensitive positive (protein kinase C-NF-κB) and negative (P13K-Akt-forkhead) signaling pathways.

VEGF is an endothelial cell-specific mitogen and chemotactic agent that is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, vascular protection, and hemostasis (for review, see Refs. 1–3). In addition to its mitogenic and chemotactic effects, VEGF also acts to promote endothelial cell survival (4). The VEGF family of proteins binds to three receptor-type tyrosine kinases, Flt-1 (VEGF receptor-1), KDR/Flk-1 (VEGF receptor-2), and VEGFR-3. VEGFR-1 and -2 are normally expressed in vascular endothelial cells, 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 endothelial cell proliferation, migration, and permeability.

VEGF has been shown to activate a number of different intracellular signaling pathways, including PKC, PI3K and Akt, MEK1/2, p38 MAPK, and phospholipase Cγ (5–9). VEGF may alter endothelial cell phenotype through transcriptional and/or posttranscriptional mechanisms. Among the transcription factors that have been implicated in VEGF signaling are NF-κB, Egr-1, NFAT-1, Ets-1, and Stat-3/5 (10–14). More recently, we reported that VEGF results in P13K-Akt-dependent phosphorylation and nuclear exclusion of the forkhead family of proteins (15).

In previous studies, we provided evidence for a link between VEGF signaling and the redox state of the cell (16, 17). Specifically, we demonstrated that VEGF induces the activity of NADPH oxidase and that NADPH oxidase-derived reactive oxygen species (ROS) are, in turn, required for VEGF-mediated induction of cell migration, proliferation, and manganese superoxide dismutase (Mn-SOD) expression. Subsequent studies have confirmed the importance of ROS in VEGF signal transduction (18, 19).

The SOD family includes cytosolic Cu,Zn-SOD, mitochondrial manganese SOD, and extracellular Cu,Zn-SOD. By converting superoxide (O2•−) to H2O2 and O2, these enzymes inhibit free radical reactions that lead to oxidative damage. Mn-SOD is
Mean either an empty expression vector (pECE) or an expression vector encoding TM-FKHRL1. The expression levels were normalized to pRL-CMV. E expressing control (Clonetics, San Diego, CA) at 37°C grown in endothelial growth medium-2-MV (EGM-2-MV) BulletKit (HCAEC) and human umbilical vein endothelial cells (HUVEC) were infected with adenoviruses expressing control Adv, phosphorylation-resistant TM-FKHRL1, or WT-FKHRL1. C Northern blot analysis of Mn-SOD was carried out using total RNA from HCAEC infected with 20 multiplicity of infection adenoviruses expressing control (Ad-βGal), dominant negative Akt, or constitutively active Akt and treated in the absence or presence of 50 ng/ml VEGF for 4 h. D, same as in B except that HCAEC were infected with 3 multiplicity of infection adenoviruses expressing control Adv, phosphorylation-resistant TM-FKHRL1, or WT-FKHRL1. E Northern blot analysis of Mn-SOD mRNA from HCAEC infected with adenoviruses expressing control Adv, TM-FKHRL1, or WT-FKHRL1 and grown in medium containing 5% fetal bovine serum (FBS). F, co-transfection of HCAEC with pGL3-Mn-Luc and either an empty expression vector (pECE) or an expression vector encoding TM-FKHRL1. The expression levels were normalized to PRL-CMV activity and expressed as fold induction relative to pGL3-Mn-Luc plus pECE co-transfection alone. Experiments were carried out in triplicate. Mean ± S.D. of three independent experiments is shown.

encoded by the nuclear SOD2 gene and is localized in mitochondria, the major site for oxidative phosphorylation. ROS have been shown to induce mitochondrial damage and dysfunction, leading to an impaired Krebs’ cycle and induction of apoptotic pathways (for review, see Refs. 20 and 21). Mn-SOD plays a critical cytoprotective role against oxidative stress (22). Its importance in homeostasis is evidenced by the lethal cardiomyopathy that develops in neonatal mice that are null for Mn-SOD (23). Conversely, overexpression of Mn-SOD in transgenic mice protects against oxidative injuries caused by ischemia-reperfusion in the brain, oxygen therapy-induced inflammation in the lungs, and drug-induced toxicity in the heart (24–26).

In the present study, we wished to extend our previous findings by elucidating the mechanisms underlying VEGF stimulation of Mn-SOD. We show that the effect of VEGF on Mn-SOD expression involves a negative PI3K-Akt-forkhead pathway and a positive PKC-NF-κB pathway, both of which are sensitive to NADPH oxidase activity. The presence of two opposing pathways, one negative and the other positive, is likely to render the VEGF-Mn-SOD axis highly modulatable by the extracellular environment.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human coronary artery endothelial cells (HCAEC) and human umbilical vein endothelial cells (HUVEC) were grown in endothelial growth medium-2-MV (EGM-2-MV) BulletKit (Clonetics, San Diego, CA) at 37°C and 5% CO2. Endothelial cells from passage 3–6 were used for all experiments. Cells were serum-starved in 0.5% fetal bovine serum prior to treatment with 50 ng/ml human VEGF165 or 40 units/ml hepatocyte growth factor (HGF) (Pepro Tech Inc., Rocky Hill, NJ). Where indicated, cells were preincubated for 30 min with 50 μM LY294002, 1 μM GF109203X, 50 μM PD98059, or 10 μM diphenyleneiodonium (DPI) (Biomol, Plymouth Meeting, PA).

Western and Northern Blot Analyses—Endothelial cells were harvested for total protein, and Western blots were carried out as described previously (15). The following phospho-specific antibodies were used: Ser-256 FKHR, Ser-473 Akt, ERK1/2, c-Jun NH2-terminal kinase (JNK), IκBα, NF-κB, and CREB/activating transcription factor (ATF) antibodies were obtained from Cell Signaling (Beverly, MA); antiphospho-Tyr 4G10 from UBI (Waltham, MA); and anti-β-actin from Sigma. Anti-ERK1/2, anti-Akt, anti-FKHR, anti-IκBα, anti-NF-κB (p65), and anti-CREB/activating transcription factor (ATF) antibodies were obtained from Cell Signaling. RNA extraction and Northern blot assays were performed as described previously (17).

Immunolocalization Studies—HCAEC were plated onto 4-well chamber slides (Lab-Tek, Christchurch, New Zealand) at a density of 20,000 cells/well. The cells were grown in EGM-2-MV for 48 h and were fixed in ice-cold 3.7% paraformaldehyde for 10 min, washed with phosphate-buffered saline, and subsequently incubated with primary anti-p65 antibody (1:100 dilution) for 2 h. Following extensive washes in phosphate-buffered saline, the cells were incubated with a fluorescein isothiocyanate-labeled secondary antibody (1:200 dilution) for 1 h. Following additional phosphate-buffered saline washes, the slides were mounted in Aquamount (Vector, Burlingame, CA) and viewed under confocal fluorescence microscopy. To-Pro (Molecular Probes, Eugene, OR) was used for identification of nuclear co-localization.

Adenoviruses—HCAEC were infected with adenoviruses encoding the cDNAs of β-galactosidase (Adv), dominant negative T308A/S473A-
A. Northern blot analysis of Mn-SOD. HCAEC were serum-starved overnight, pretreated with or without PD98059 (50 \mu M) or GF109203X (1 \mu M) for 30 min, and then incubated in the absence or presence of 50 ng/ml VEGF for 4 h. The same blot was stripped and reprobed for 18 S RNA as a loading control (bottom). B. Northern blot analysis of Mn-SOD from HCAEC infected with adenoviruses expressing \( \beta \)-galactosidase (\( \beta \)-Gal), wild type or dominant negative PKC\( \delta \) or PKC\( \zeta \), or constitutively active IxB\( \beta \) and treated with or without VEGF. C. as same as in B except that the cells were infected with adenoviruses expressing \( \beta \)-galactosidase (A\( \beta \)), TM-FKHRL1, WT-FKHRL1, or CA-IxB. D. transfection of HCAEC with a 3340-bp Mn-SOD promoter without (Mn) or with a 369-bp second intronic sequence (Mn2; Mn2R, reverse orientation) coupled to a luciferase reporter gene. The transfected cells were grown for 36 h and then incubated for 12 h either in serum-starved (S/S) or serum-rich (S/R) medium. The expression levels were normalized to pRL-CMV activity and expressed as fold induction relative to pGL3-Mn-Luc or pGL3-Mn2-Luc alone. Experiments were carried out in triplicate. Mean \( \pm \) S.D. of three independent experiments is shown. E. same as in D except that the reporter used was Mn2, Mn2 with the C/EBP site mutated (mt-C/EBP), or Mn2 with the NF-\( \kappa \)B site mutated (mt-NF\( \kappa \)B).

A total of 0.05 pmol of the Mn-SOD reporter plasmid, 50 ng of a control plasmid containing the Renilla luciferase reporter gene under the control of a cytomegalovirus enhancer/promoter (pRL-CMV), and 0.075 pmol of theforkhead expression vector (or control vector, pECE) were incubated with 2 \mu l of FuGENE 6 (Roche Applied Science). Twenty-four h later, the cells were washed with phosphate-buffered saline and cultured for 12 h in EBM plus 0.5% fetal bovine serum. The cells were then incubated in the presence or absence of VEGF for 6 h, at which time they were lysed and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega, Madison, WI) and a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Experiments were performed in triplicates and repeated at least three times.

**Transfection with Antisense Oligonucleotide p47\( \text{phox} \)-HAEC**:—HAEC were grown to 70–80% confluency in 10-cm plates and transfected with 200 nm phosphorothioate antisense p47\( \text{phox} \) oligonucleotide in Opti-MEM containing Lipofectin (10 \mu g/ml) for 4 h. The cells were then incubated in EGM-2 for 24 h and serum-starved in 0.5% serum for 12–18 h before VEGF treatment for the times indicated. The antisense sequence (5'- TTGTCTGGTTGTTGTTGGG-3') was complementary to nucleotides 394–413 of human p47\( \text{phox} \) mRNA (29) and was phosphorothioate-modified and high pressure liquid chromatography-purified (Sequitur, Natick, MA).

**Assay for NADPH Oxidase Activity in HCAEC and HUVEC**:—The cells were washed with ice-cold phosphate-buffered saline, collected by a cell scraper, and homogenized with a Dounce homogenizer in a buffer containing 20 mM KH\( \text{PO}_4 \) (pH 7.0), 1 mM protease mixture inhibitor (Sigma), 1 mM EGTA, 10 \mu g/ml aprotinin, 0.5 \mu g/ml leupetin, 0.7 \mu g/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride. NADPH oxidase activity of the cell lysate was measured using a modified assay as described previously (30). Briefly, photon emission from the chromogenic substrate lucigenin as a function of acceptance of electron\( \text{Cu}^2+ \) generated by the NADPH oxidase complex was measured every 15 s for 20 min in a Berthold luminometer. NADPH oxidase assay buffer containing 250 mM HEPES (pH 7.4), 120 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO\( _4 \), 1.75 mM CaCl\( _2 \), 11 mM glucose, 0.5 mM EDTA, 100 \mu M NADH, and 5 \mu g lucigenin was used. The data were transformed to relative light units/min/mg of protein using a standard curve generated with xanthine/xanthine oxidase.

**Measurement of Intracellular ROS Generation**—The changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell-permeable 2,7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes Inc.) to fluorescent dichlorofluorescin (DCF) by fluorescence-activated cell sorter analysis as described previously (17).
RESULTS

VEGF-mediated Induction of Mn-SOD Is Attenuated by the PI3K-Akt-Forkhead Signaling Pathway—Previous studies in non-endothelial cells have demonstrated that insulin inhibits Mn-SOD expression via a PI3K-Akt-forkhead-dependent pathway (31). In contrast, we have shown that VEGF, although capable of inducing phosphorylation and nuclear exclusion of forkhead in endothelial cells in a PI3K-Akt-dependent manner (15), results in a net increase in Mn-SOD mRNA and protein levels (17). Together, these findings suggest that VEGF-mediated induction of Mn-SOD involves an interplay between positive and negative pathways. To test this hypothesis, HCAEC and HUVEC were preincubated for 30 min in the absence or presence of the PI3K inhibitor LY294002 (50 μM) or wortmannin (100 nM), treated with VEGF (50 ng/ml) for 4 h, and then processed for Northern blot analysis of Mn-SOD. Inhibition of PI3K increased basal and VEGF-induced Mn-SOD expression in both types of endothelial cells (Fig. 1A). Lower doses of LY294002 (0.1–10 μM) blocked VEGF-mediated phosphorylation of Akt and enhanced VEGF stimulation of Mn-SOD expression without altering basal Mn-SOD levels (Fig. 1B). Together, these findings strongly suggest that PI3K plays a negative role in the VEGF-Mn-SOD signaling pathway.

To determine whether PI3K exerts its negative effect on Mn-SOD expression through Akt, a serine-threonine kinase downstream of PI3K, HCAEC and HUVEC were infected with adenoviruses expressing β-galactosidase, CA-Akt, or DN-Akt, and were incubated in the absence or presence of VEGF. Overexpression of DN-Akt accentuated the effect of VEGF on Mn-SOD expression, whereas CA-Akt had the opposite effect (Fig. 1C). Together, these findings suggest that PI3K-Akt attenuates VEGF-mediated induction of Mn-SOD in endothelial cells.

Previous studies of Caenorhabditis elegans and mice have implicated thedaf-2/insulin-forkhead signaling pathway in regulating Mn-SOD expression (32, 33). The human Mn-SOD gene contains two forkhead consensus sites in the upstream promoter region, one at −1249 (GTAAACAA; inverse of TT-GTTTAC) and another at −997 (TTGTTTAA) (31). In non-endothelial cells, insulin has been shown to induce PI3K-Akt-dependent phosphorylation and nuclear exclusion of FKHRL1 and to inhibit Mn-SOD expression (31). To determine whether Mn-SOD lies downstream of VEGF-PI3K-Akt-forkhead in endothelial cells, HCAEC and HUVEC were infected with adenoviruses overexpressing β-galactosidase, WT-FKHRL1, or a constitutively activated phosphorylation-resistant TM-FKHRL1. The overexpressing cells were serum-starved and then treated in the presence or absence of VEGF for 4 h. VEGF-mediated induction of Mn-SOD was accentuated by overexpression of TM-FKHRL1 but not Adv or WT-FKHRL1 (Fig. 1D). Together, these data suggest that forkhead-mediated induction of Mn-SOD is dependent on VEGF- or serum-mediated activation of positive pathway(s). In co-transfection assays, the overexpression of TM-FKHRL1 resulted in a 6.6 ± 0.3-fold induction of the 3340-bp human Mn-SOD promoter (Fig. 1F). These findings support the conclusion that the PI3K-Akt signaling pathway negatively regulates expression of Mn-SOD via phosphorylation and nuclear exclusion of forkhead.

VEGF-mediated Induction of Mn-SOD Is Positively Regulated through PKC and IκB/NIκB—Although VEGF triggers a negative PI3K-Akt-forkhead signaling pathway, VEGF signaling results in the net induction of Mn-SOD mRNA and protein. To identify the positive pathway(s) responsible for this effect, endothelial cells were preincubated with other inhibitors of signaling and then treated in the absence or presence of VEGF. As shown in Fig. 2A, preincubation of endothelial cells with the PKC inhibitor GF109203X (1 μM) resulted in marked inhibition of VEGF induction of Mn-SOD, whereas the MEK1/2 inhibitor PD98059 (50 μM) had no such effect. To determine which PKC isoforms are involved in mediating the VEGF response,
HCAEC were infected with either WT or DN isoforms of PKC and treated in the absence or presence of VEGF. VEGF induction of Mn-SOD was completely abrogated by DN-PKCα, significantly inhibited by DN-PKCγ (Fig. 2B), and unchanged by DN-PKCα or DN-PKCε (data not shown). These findings suggest that VEGF-mediated induction of Mn-SOD is dependent on novel and atypical PKC isoforms.

Previous studies have demonstrated a link between PKCζ/δ signaling and NF-κB activity in endothelial cells (27, 34, 35). Moreover, NF-κB has been shown to mediate inducible expression of Mn-SOD in a variety of cell types (22, 36–38). Therefore, we hypothesized that VEGF induces Mn-SOD via a PKC-NF-κB-dependent signaling pathway. Consistent with this hypothesis, VEGF-mediated induction of Mn-SOD was blocked by overexpression of a constitutively active, phosphorylation/ubiquitination-resistant form of IκB (Fig. 2, A and C). Previous studies have pointed to the importance of an NF-κB consensus element in the second intron (at +2758 nucleotides, relative to the start site of the second intron). In transient transfection assays, VEGF induced Mn-SOD promoter activity in the presence, but not the absence, of the second intronic sequence (Fig. 2D). Mutation of the NF-κB motif (mt-NF-κB) resulted in a loss of induction, whereas mutation of the C/EBP site (at +2648 nucleotides; mt-CEBP) had no such effect (Fig. 2E).

The transcription factor NF-κB is normally sequestered in the cytoplasm by IκB. NF-κB activation requires phosphorylation-induced ubiquitination and degradation of cytoplasmic IκB, with subsequent release and nuclear translocation of NF-κB. Incubation of HCAEC with VEGF resulted in increased phosphorylation of IκB in the cytoplasm at 15 min and decreased total cytoplasmic IκB at 30 and 60 min (Fig. 3A). Moreover, VEGF promoted the nuclear translocation of p65 NF-κB at 15 and 30 min (Fig. 3B). Preincubation with the PKC inhibitor GF109203X blocked VEGF-mediated phosphorylation of IκB (Fig. 3C) and nuclear translocation of NF-κB (Fig. 3D). In immunofluorescent assays, VEGF induced the nuclear translocation of p65 NF-κB, an effect that was similarly blocked by GF109203X (Fig. 4). Taken together, these results suggest that VEGF-mediated induction of Mn-SOD is dependent on a PKC-NF-κB signaling pathway.

**VEGF-Forkhead-mediated Attenuation and VEGF-NF-κB-mediated Induction of Mn-SOD Are Dependent on NADPH Oxidase Activity**—We reported previously that VEGF signaling induces NADPH oxidase activity in endothelial cells and that VEGF-mediated induction of Mn-SOD mRNA and protein is dependent on NADPH oxidase-derived ROS (16, 17). In the next series of experiments, we examined the role of NADPH oxidase in regulating the VEGF-forkhead-Mn-SOD and VEGF-NF-κB-Mn-SOD pathways. We generated an antisense oligonucleotide to the p47phox component of NADPH oxidase (AS-p47phox). To confirm efficacy of the antisense oligonucleotide, HCAEC and HUVEC were either pretreated with the flavoenzyme inhibitor DPI or transfected with AS-p47phox. As shown in Fig. 5A, DPI and AS-p47phox each resulted in significant inhibition of NADPH oxidase activity in HCAEC and HUVEC. Moreover, consistent with our previous results using DPI and DN-Rac1 (17), AS-p47phox blocked VEGF-mediated induction of Mn-SOD in both endothelial cell types (Fig. 5B shows HCAEC). As shown in Fig. 5C, VEGF-mediated phosphorylation of FKHR was inhibited by AS-p47phox or DPI. Moreover, VEGF-mediated phosphorylation of IκB and nuclear translocation of p65 NF-κB were blocked by AS-p47phox or DPI (Figs. 4 and 5D). Taken together, the above data suggest that NADPH oxidase activity is required for VEGF-induced forkhead and IκB/NF-κB signaling in endothelial cells.

**HGF Signaling Is Coupled to Forkhead but Not to NF-κB Activity in Endothelial Cells**—Previous studies in non-endothelial cells have shown that activation of the insulin receptor results in forkhead-dependent down-regulation of Mn-SOD (31). These results raise the question of whether the induction of Mn-SOD observed with VEGF is specific to this growth factor or is a general property of endothelial cells. We have consistently failed to demonstrate an effect of insulin or insulin-like growth factor on forkhead phosphorylation and activity in endothelial cells (data not shown). Thus, we decided to study HGF/scatter factor. HGF plays a role in endothelial cell proliferation, migration, and angiogenesis (39, 40). The addition of HGF (40 ng/ml) to HCAEC and HUVEC did not result in significant changes in Mn-SOD expression (Fig. 6A shows HCAEC). In Western blot analyses, HGF treatment resulted in increased phosphorylation of CREB, Erk1/2, c-Jun NH2-terminal kinase, Akt, and forkhead proteins (Fig. 6B). However, in contrast to VEGF, HGF failed to induce IκB phosphorylation in HCAEC (Fig. 6C). These data suggest that although both VEGF and HGF activate a number of common downstream signaling pathways, only VEGF induces NF-κB activity and Mn-SOD expression. Finally, pretreatment of HCAEC with DPI or transfection with p47phox failed to block HGF-mediated phosphorylation of Akt and forkhead (Fig. 6, D and E). These data suggest that the requirement for NADPH oxidase activity
is a function of the growth factor (VEGF) and not the down-stream pathway per se (Akt-forkhead).

**DISCUSSION**

Although VEGF plays a prominent role in endothelial cell migration, proliferation, and barrier function, it also functions in other important ways to maintain homeostasis. For example, VEGF enhances the survival of endothelial cells, alters the expression of hemostatic factors, and modulates selectin levels. Recent findings from our lab as well as others suggest that VEGF signaling is coupled to the redox state of the cell (16–19, 41). VEGF induces NADPH oxidase activity, which in turn is necessary for VEGF function and induction of Mn-SOD expression (17).

Several lines of evidence suggest that protein kinase receptor signaling normally serves to inhibit Mn-SOD mRNA and protein. First, forkhead factors have been shown to activate Mn-SOD expression (31–33). Second, many growth factors, including insulin, IGF-1, epidermal growth factor (EGF), VEGF, and erythropoietin, have been shown to phosphorylate forkhead proteins, resulting in their exclusion from the nucleus and secondary reduction in the expression of forkhead-responsive target genes (15, 42). Finally, in non-endothelial cells, insulin suppresses Mn-SOD levels via a PI3K-Akt-forkhead-dependent signaling pathway (31). In the current study, we have shown that although VEGF does indeed trigger PI3K-Akt-forkhead signaling, the tendency to repress Mn-SOD expression is overridden by a positive PKC-NF-κB signaling axis. The observation that HGF neither activates NF-κB nor induces Mn-SOD expression suggests that the interplay between positive and negative pathways is more a function of the mediator than of the cell type.

An interesting question is why VEGF, as distinct from other growth factors, results in a net increase in Mn-SOD. Given that VEGF signaling, but not HGF signaling, is critically coupled to the redox state of the cell, perhaps the increased levels of Mn-SOD are important for modulating ROS levels and/or protecting the endothelial cell against excessive oxidative stress. Alternatively, by converting short lived superoxide in the mitochondria into membrane-permeable hydrogen peroxide, increased levels of Mn-SOD may ultimately link signaling between mitochondria and cytosol. Finally, both NF-κB and Mn-SOD have been implicated in the control of cell survival (22, 37, 43–45), raising the possibility that the VEGF-PKC-NF-κB-Mn-SOD axis plays an important role in inhibiting endothelial cell apoptosis.

It is also interesting to speculate whether the VEGF PKC-NF-κB pathway evolved to overcome a “default” PI3K-Akt-forkhead pathway or whether the presence of positive and negative signaling provides the system with an additional level of regulation. For example, it is possible that extracellular mediators that are comparatively more efficient in activating PI3K-Akt (e.g. angiopoietin-1) (46, 47) will inhibit VEGF-mediated induction of Mn-SOD, whereas endogenous inhibitors of PI3K-Akt may have the opposite effect. Alternatively, extracellular signals that preferentially affect NF-κB nuclear translocation and/or DNA binding activity may influence the VEGF PKC-NF-κB-Mn-SOD axis. In other words, at any given point in time and space, the net effect of VEGF on Mn-SOD expression will depend on the relative activity or set point of the negative and positive pathways.

There are conflicting data in the literature regarding the role of NF-κB in VEGF signaling. One group has been unable to...
demonstrate an effect of VEGF on nuclear translocation of p65 NF-κB, DNA-protein binding, or IκB protein levels in HUVEC (14, 48). Others have shown that VEGF does indeed induce NF-κB binding in HUVEC (49). A role for NF-κB in VEGF signaling is further supported by studies employing nonspecific inhibitors of the transcription factor (13, 50, 51). In this report, we have shown unequivocally that VEGF induces the phosphorylation of IκB and the nuclear translocation of p65 NF-κB. Moreover, VEGF-mediated induction of the Mn-SOD gene was blocked by a repressor of NF-κB, and VEGF stimulation of Mn-SOD promoter activity was ablated by mutation of the second intron NF-κB consensus element. Taken together, these findings support a functional role for NF-κB in the VEGF signaling pathway.

A previous study demonstrated that VEGF induces the expression of intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1, and E-selectin in endothelial cells (49). The data supported a role for NF-κB in mediating this effect (49). Interestingly, chemical inhibition of PI3K resulted in superinduction of the selectin genes, pointing to a negative role for PI3K-Akt (49). In another report, VEGF-mediated induction of tissue factor was enhanced by PI3K inhibition (46). Although these latter studies did not address the role for forkhead transcription factors in this process, they suggest that VEGF target genes other than Mn-SOD are regulated through the coordinated action of positive and negative pathways.

The finding that TM-FKHRL1 induces Mn-SOD expression only in the presence of VEGF or serum contrasts with our previous observations that TM-FKHRL1 alone is sufficient for inducing p27kip1 (15). These data suggest that TM-FKHRL1-mediated transactivation of Mn-SOD requires the presence of additional positive factor(s), such as NF-κB and/or other transcription factors or co-activators. In co-transfection assays, the forced overexpression of TM-FKHRL1 resulted in increased promoter activity, whether or not the cells were serum-starved. At the present time, we cannot explain the discrepancy in results between the endogenous gene and the promoter; namely, in the absence of VEGF or serum, overexpression of TM-FKHRL1 induces promoter activity, but not the endogenous gene. Possibilities include, but are not limited to, differ-
flamatory role for this molecule? The answers to these questions should provide important new insights into the mechanisms of VEGF signaling in the endothelium.

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