NADPH Oxidase Activity Selectively Modulates Vascular Endothelial Growth Factor Signaling Pathways**§

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Vascular endothelial growth factor (VEGF)² plays a critical role in endothelial survival, migration, and proliferation. VEGF has been implicated in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, vascular protection, and hemostasis (1–8). VEGF binds to two receptors, VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR2 (Flk-1/KDR). VEGF has been shown to activate several downstream signaling pathways, including protein kinase C, phosphoinositide 3-kinase (PI3K), Akt, extracellular signal-regulated kinase-1 and -2 (ERK1/2), mitogen-activated protein kinase (MAPK) p38, and phospholipase C (PLC)-γ (9–13). VEGF may alter endothelial cell phenotypes through transcriptional and post-transcriptional mechanisms. The effect of VEGF on gene transcription may be explained, at least in part, by its ability to activate nuclear factor (NF)-κB, early growth response factor-1, nuclear factor of activated T cells-1, Ets-1, and signal transducers and activators of transcription-3/5 (14–18). VEGF induces phosphorylation of forkhead transcription factors (e.g. FKHR), resulting in their nuclear exclusion and transcriptional inactivation (19).

Reactive oxygen species (ROS) have long been implicated in the pathogenesis of cardiovascular diseases, including atherosclerosis, hypertension, and diabetes (reviewed in Ref. 20). In addition, there is a growing appreciation for the role of ROS in physiological signaling in many cell types, including endothelial cells. NADPH oxidase is the primary source of superoxide in endothelial cells (21–24). NADPH oxidase was originally identified and characterized in phagocytes, where it contributes to host defense. The NADPH oxidase complex consists of two membrane-bound components, gp91phox (also known as Nox2) and p22phox, and several cytosolic regulatory subunits, including p40phox, p47phox, p67phox, and the small GTPase Rac (Rac1 or Rac2). Upon enzyme activation, the cytoplasmic units translocate to the cell membrane where they are assembled with gp91phox/p22phox. The resulting multisubunit complex transfers electrons from NAD(P)H to molecular oxygen. Each of the components of the neutrophil NADPH complex has been identified in endothelial cells (25–27). In addition, endothelial cells express a homologue of gp91phox/Nox2, termed Nox4 (28, 29). The endothelial NADPH oxidase differs from its leukocyte counterpart in several important ways. First, it is pre-assembled and displays constitutive low level activity. Second, agonist-mediated stimulation of NADPH oxidase results in slower and less potent activation of the enzyme (22). The enzyme com-

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; siRNA, short interfering RNA; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; HCAEC, human coronary artery endothelial cell; JNK, c-Jun N-terminal kinase; FACS, fluorescence-activated cell sorter; DN, dominant-negative; FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cell; BSA, bovine serum albumin; DPI, diphenyleneiodonium; DAF, decay-accelerating factor; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; HBSS, Hanks’ balanced saline solution; LCB, lower confidence bound; AS, antisense; CA, constitutively active; TM, triple mutant.
plex is localized in the perinuclear region of endothelial cells and produces an intracellular influx of ROS (30, 31).

Many different agonists have been shown to induce endothelial NADPH oxidase activity, including hemodynamic forces (32–34), VEGF (21, 22, 24), angiopoietin-1 (Ang1) (21, 35, 36), tumor necrosis factor-α (37), thrombin (38), angiotensin II (37, 39), endothelin-1 (40), transforming growth factor-β (41), oxidized low density lipoprotein (27), and high potassium (42). Activation of NADPH oxidase is mediated by post-translational modification and/or increased transcription of the regulatory subunits.

We previously reported that VEGF induces NADPH oxidase activity and that NADPH oxidase activity is required for VEGF-mediated migration and proliferation of endothelial cells (21, 22). These results were confirmed and expanded upon by other groups. For example, PI3K and Rac1 were shown to be required for the VEGF-dependent oxidative burst in porcine aortic endothelial cells expressing VEGFR2/KDR (23). Ushio-Fukai et al. (24) demonstrated an important role for gp91phox in mediating the effect of VEGF on endothelial cell migration and proliferation in vitro and in promoting angiogenesis in a mouse sponge implant model. Similar findings were observed in a hindlimb ischemia model (43). Moreover, we showed that NADPH oxidase-derived ROS are required for VEGF stimulation of manganese superoxide dismutase, activation of NF-κB, and inactivation of FKHR in endothelial cells (44).

The mechanisms by which NADPH oxidase-derived ROS influence VEGF are poorly understood. ROS may result in oxidation of cysteine residues on receptor and nonreceptor protein kinases and phosphatases. Indeed, previous studies have demonstrated a role for ROS in VEGFR2 autophosphorylation (23, 24). Here, we show that NADPH oxidase activity is required for some, but not all, downstream effects of VEGF in endothelial cells. These data argue against a global sensitivity of VEGF signal transduction pathways to the redox state of the cell, and suggest that therapeutic modulation of ROS will selectively influence the effect of VEGF on endothelial cell phenotypes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human coronary artery endothelial cells (HCAEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and grown in Endothelial Growth Medium-2-MV (EGM-2-MV) BulletKit (Clonetics, San Diego) at 37 °C and 5% CO2. Endothelial cells from passage 3 to 6 were used for all experiments. Cells were serum-starved in 0.5% fetal bovine serum for 12–16 h prior to treatment with 50 ng/ml human VEGF-A165 (PeproTech Inc, Rocky Hill, NJ). Where indicated, cells were preincubated for 30 min with 100 nmol/liter 1-NAME (Calbiochem), 50 μmol/liter LY294002 (Calbiochem), 50 μmol/liter PD98059 (Calbiochem), or 10 μmol/liter SB203580 (Calbiochem). Phenazine methosulfate and S2366 were from Calbiochem.

**Inhibition of NADPH Oxidase Activity by Targeting Endogenous p47phox**—HCAEC and HUVEC were grown to 70–80% confluence in 6-cm plates and transfected with 100 nm p47phox antisense oligonucleotide (5′-TTTGTCTCTCTTGTGTGGT-3′; Sequitur, Natick, MA), scrambled antisense (Scram-AS), siRNA against p47phox (5′-GGUCAUUCACAAGCUCUGTT-3′, Ambion, Austin, TX), or scrambled siRNA (Scramsi) in Opti-MEM containing 10 μg/ml Lipofectin (Invitrogen) for 4 h. The cells were then incubated in EGM-2-MV medium for 24 h and serum-starved in endothelial basal medium (EBM-2; Clonetics) containing 0.5% serum for 12–16 h prior to VEGF treatment. HP-validated siRNA against Src (catalog number S01223928) and FAK (catalog number S100287791) kinases were from Qiagen (Valencia, CA). ON-TARGETplus si-RNA against PLCγ-1 was from Dharmacon (Lafayette, CO).

**Assay for NADPH Oxidase Activity in HCAEC and HUVEC**—HCAEC and HUVEC were washed with ice-cold PBS, collected by a cell scraper, and Dounce-homogenized in buffer containing 20 mM KH2PO4 (pH 7.0), 1× protease mixture inhibitor (Sigma), 1 mM EGTA, 10 μg/ml aprotinin (Calbiochem), 0.5 μg/ml leupeptin (Sigma), 0.7 μg/ml pepstatin (Sigma), and 0.5 mM phenylmethylsulfonyl fluoride (Calbiochem). NADPH oxidase activity of the cell lysate was measured using a modified assay (45). Briefly, photon emission from the chromogenic substrate lucigenin as a function of acceptance of electron/O2 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 MgSO4(7H2O), 1.75 mM CaCl2(2H2O), 11 mM glucose, 0.5 mM EDTA, 100 μM NADH and 5 μM lucigenin was used. The data were converted to relative light units/min/mg of protein, using a standard curve generated with xanthine/xanthine oxidase. Lucigenin activity (light units/min/mg of protein) of control cells (Scram-AS-transfected) was arbitrarily set at 100%. Total intracellular levels of ROS were determined by FACS analyses of the oxidative conversion of cell-permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes Inc., Eugene, OR) to fluorescent dichlorofluorescein as described previously (22).

**Oligonucleotide Microarray Analysis of Gene Expression**—The transcriptional profile of control or VEGF-treated HCAEC with siRNA against p47phox was characterized by oligonucleotide microarray analysis using the human U133A Affymetrix GeneChip, according to previously described protocols for total RNA extraction and purification, cDNA synthesis, in vitro transcription reaction for production of biotin-labeled cRNA, hybridization of cRNA with U133A Affymetrix gene chips, scanning of image output files, analysis of gene expression data, and hierarchical and functional clustering algorithms (46). The scanned array images were analyzed by dChip (47). In the dChip analysis a smoothing spline normalization method was applied prior to obtaining model-based gene expression indices, also known as signal values. There were no outliers identified by dChip so all samples were carried out for subsequent analysis. When comparing two samples (groups) to identify the genes enriched in a given phenotype, we used the lower confidence bound (LCB) of the fold change between the experiment and the base line. If the 90% LCB of the fold change between the experiment and the base line was above 1.2, the corresponding gene was considered to be differentially expressed. An LCB of >1.2 typically corresponds with an “actual” fold change of at least 3 in gene expression. GOTree machine was used to identify gene ontology categories for the input gene set (48).
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Quantitative Real Time PCR—Real time PCR was carried out as described previously (49). Briefly, total RNA was prepared using the RNeasy RNA extraction kit with DNase-I treatment following the manufacturer’s protocol (Qiagen, Valencia, CA). To generate cDNA, total RNA (100 ng) from each of triplicate samples was converted into cDNA using random primers and SuperscriptIII reverse transcriptase (Invitrogen). All cDNA samples were aliquoted and stored at −80 °C. Primers were designed using the Primer Express oligo design software (Applied BioSystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). All primer sets were subjected to rigorous data base searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes. Amplicons generated from the primer set were analyzed for melting point temperatures using the first derivative primer melting curve software supplied by Applied BioSystems. The SYBR Green I assay and the ABI Prism 7700 sequence detection system were used for detecting real time PCR products from the reverse-transcribed cDNA samples, as described previously (50). 18 S rRNA, which exhibits a constant expression level across all the HCAEC samples, was used as the normalizer. PCRs for each sample were performed in duplicate, and copy numbers were measured as described previously (50). The level of target gene expression was normalized against the 18 S rRNA expression in each sample, and the data were presented as mRNA copies per 10⁸ 18 S rRNA copies.

Adenoviruses—HCAEC were transduced with replication-deficient adenoviruses encoding the cDNAs of β-galactosidase (Adv), triple mutant (TM)-FKHR (49), constitutively active (CA) Akt, or dominant-negative (DN) Akt (19) as described previously (49). The triple mutant version of FKHR contains (CA) Akt, or dominant-negative (DN) Akt (19) as described previously (49). The triple mutant version of FKHR contains T24A, S256A, and S319A and is resistant to agonist-induced phosphorylation.

Western and Northern Blot Analyses—HCAEC were harvested for total protein, and Western blots were carried out as described previously (19). Anti-Akt, anti-FKHR, anti-p38, anti-VEGFR2, anti-PLCγ-1, and anti-ERK1/2 antibodies and phosphospecific antibodies against Ser-256 FKHR, Ser-473 Akt, or dominant-negative (DN) Akt (19) as described previously (49). The triple mutant version of FKHR contains T24A, S256A, and S319A and is resistant to agonist-induced phosphorylation.

Immunolocalization Studies—HCAEC were grown in 4-well chamber slides (Lab-Tek, Christchurch, New Zealand) and treated with or without VEGF for the times indicated. Subcellular localization of FKHR and ERK1/2 was determined using anti-FKHR and anti-ERK1/2 antibodies, respectively, and a Cy3-conjugated secondary antibody as described previously (19). Quantitative analyses were carried out by counting 200 cells per time point.

Complement-mediated Cell Lysis Assay—HCAEC were plated in 24-well plates and incubated at 37 °C for 24 h prior to siRNA transfection. 24 h following siRNA transfections, the cells were serum-starved overnight and then incubated with or without VEGF for 24 h. 7 μmol/liter calcium acetate, calcium chloride, and magnesium chloride were added to each well. 30 min later, cells were washed with serum starvation medium containing 1% BSA and then incubated for 30 min with 250 μl of monoclonal anti-endoglin (CD105) antibody (IgG2a) (Covance, Berkley, CA) to opsonize the cells. HCAEC were washed with HBSS containing 1% BSA and incubated with 250 μl of 5–20% rabbit complement serum for 30 min. The supernatant from each well was transferred to a 96-well microtiter plate. The remaining HCAEC in the 24-well plate were washed with HBSS plus 1% BSA, and the calcein remaining in the cells was released by incubation with 250 μl of HBSS containing 1% BSA and 0.1% Triton X-100. The lysate was then transferred to another 96-well plate, and the calcein released by complement and detergent was estimated using a fluorescence plate reader (model 680; Bio-Rad). Percent specific lysis in triplicate wells was calculated as ((complement-mediated release − spontaneous release)/maximal release − spontaneous release) × 100%, where maximal release = complement-mediated release + detergent-mediated release.

**Assay for Thrombomodulin-dependent Activation of Protein C**—Functional assay for cellular thrombomodulin was carried out as described previously (51), with slight modifications. Briefly, HCAEC transfected with 75 nM siRNAs were seeded on 24-well tissue culture plates. After 24 h, HCAEC were serum-starved in EBM-2 containing 0.5% fetal bovine serum for 18 h and treated with 50 ng/ml VEGF for 14 h. Cells were washed with PBS and incubated in a reaction mixture containing 2.5 mM CaCl₂, 0.15 mM NaCl, 5 mM thrombin, and 5 nM protein C for 3 h. After withdrawing 100 μl at each time point, hirudin was
added to inhibit thrombin. Equal volume of the chromogenic substrate, 400 μM S2366, was added to the supernatant, and absorbance was measured at 405 nm.

**Statistical Analyses**—All values are presented as mean ± S.D. where appropriate. Statistical significance between two groups was determined by use of a paired t test, and values of *p* < 0.05 were considered significant.

**RESULTS**

**NADPH Oxidase Activity Is Abrogated by Down-regulation of p47phox Expression in Primary Human Endothelial Cells**—To inhibit NADPH oxidase in endothelial cells, HCAEC or HUVEC were transfected with antisense or siRNA against p47phox. Antisense (AS-p47phox) and siRNA (si-p47phox) resulted in significant (>90%) reduction in p47phox protein levels, compared with scrambled antisense (Scram-AS) and siRNA (Scram-si), respectively (supplemental Fig. 1, A and B). Transfection of HCAEC and HUVEC with AS-p47phox or si-p47phox also resulted in significant inhibition of NADPH oxidase activity as measured by lucigenin assay (Fig. 1A, HCAEC). As a negative control, treatment of endothelial cells with the nitric-oxide synthase inhibitor, L-NAME, had no effect on NADPH oxidase activity (Fig. 1A). To determine the effects of NADPH oxidase inhibition on intracellular ROS production in HCAEC, FACS analyses were performed using DCFH-DA. Transfection with si-p47phox significantly reduced intracellular levels of ROS (to 58.6 ± 6.5%) (Fig. 1B). Consistent with the findings of the lucigenin assays, ROS levels were unaffected by L-NAME. As a positive control, the superoxide donor, phenazine methosulfate (PMS) (5 μM), was used as positive control for ROS induction in HCAEC. All experiments were performed in triplicate, and the data shown are means ± S.D. *, *p < 0.05, relative to control.

**NADPH Oxidase Activity Is Required for Late but Not Early VEGF-mediated Tyrosine Phosphorylation of VEGFR2/KDR**—VEGF treatment of HCAEC resulted in a time-dependent increase in tyrosine phosphorylation of VEGFR2/KDR starting as early as 1 min and peaking at 10 min (data not shown). siRNA-mediated knockdown of p47phox resulted in partial inhibition of VEGF-mediated phosphorylation of VEGFR2/KDR at 10 min (to 69 ± 6.6%) but had no effect at 1 and 5 min (Fig. 2). Similar results were observed with AS-p47phox (supplemental Fig. 2A). These findings suggest that NADPH oxidase activity is not required for early phosphorylation of VEGFR2/KDR by VEGF but may play a role in maintaining the receptor in the phosphorylated state.

**Inhibition of NADPH Oxidase in Primary Human Endothelial Cells Has a Differential Effect on VEGF-
mediated Phosphorylation of Signal Intermediates—Incubation of HCAEC with VEGF resulted in time-dependent phosphorylation of Akt at serine 473 and threonine 308 residues, with maximal levels occurring at 10 min (Fig. 3A, Ser-473). VEGF-mediated phosphorylation of Akt was inhibited by transfection with si-p47phox (to 12% of control levels) (Fig. 3A). Similar results were observed with AS-p47phox (supplemental Fig. 2B). VEGF also induced phosphorylation of ERK1/2, with peak levels occurring at 5 min (Fig. 3A). However, VEGF-mediated activation of ERK1/2 was unaltered in p47phox-deficient endothelial cells (Fig. 3A and supplemental Fig. 2C). Similarly, VEGF-induced phosphorylation of JNK was unaffected by p47phox knockdown (Fig. 3B). In contrast, siRNA against p47phox reduced VEGF-mediated phosphorylation of p38 MAPK by \( \frac{1}{2} \) at all time points tested (Fig. 3B).

Consistent with a role for PI3K-Akt in mediating VEGF-inducible phosphorylation of the forkhead transcription factor, FKHR, si-p47phox abrogated the effect of VEGF on FKHR phosphorylation at Ser-256 (Fig. 4). Together, these findings suggest that VEGF triggers both ROS-sensitive (PI3K-Akt-FKHR and p38 MAPK) and ROS-insensitive (ERK1/2 and JNK) signaling pathways in endothelial cells.

Phosphorylated ERK1/2 is localized in the nucleus of endothelial cells (52). In keeping with the phosphorylation studies of ERK1/2, si-p47phox-mediated inhibition of NADPH oxidase had no effect on nuclear translocation of ERK1/2 in VEGF-treated cells (64% nuclear in VEGF-treated control cells versus 68% nuclear in VEGF-treated si-p47phox-transfected cells) (supplemental Fig. 3A and 3B). In accordance with the effect of NADPH oxidase inhibition on VEGF-induced FKHR phosphorylation, si-p47phox blocked VEGF-mediated nuclear exclusion of FKHR in HCAEC (27% nuclear in VEGF-treated control cells versus 72% nuclear in VEGF-treated si-p47phox-transfected cells) (supplemental Fig. 4A and 4B).

Inhibition of NADPH Oxidase in Primary Human Endothelial Cells Blocks VEGF-mediated Phosphorylation of Src and FAK—The above results indicate that NADPH oxidase activity is required for VEGF-mediated phosphorylation of Akt prior to any observable effect on VEGFR2/KDR phosphorylation (i.e., at 1 and 5 min). Based on these findings, we hypothesized that NADPH oxidase-derived ROS influence VEGF signaling at a level distal to VEGFR2/KDR and proximal to Akt. The nonre-
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**FIGURE 5.** VEGF-induced phosphorylation of Src and FAK, but not PLCγ-1, is sensitive to NADPH oxidase activity in HCAEC. **A**, Scram-si or si-p47phox-transfected HCAEC were serum-starved overnight and then treated with VEGF (50 ng/ml) for the times indicated. Western blot analysis was carried out using anti-phospho-Tyr-416 Src (p-Src) antibody, followed by phospho-specific anti-ERK1/2 antibody (positive control for VEGF response, negative control for NADPH oxidase inhibition), and total anti-ERK1/2 antibody (as control for loading). **Lower panel** shows quantitative analysis of three independent experiments (mean ± S.D. of fold changes versus control). *, p < 0.05, relative to time-matched control. **B**, same as in A except Western blots were carried out using anti-phospho-PLCγ-1 antibody, followed by stripping and reprobing of the membrane using anti-PLCγ-1 antibody as loading control. **C**, VEGF-induced Src phosphorylation was measured in lysates from Scram-si or si-p47phox-transfected HCAEC as described under “Experimental Procedures.” *, p < 0.05, VEGF-treated versus untreated in Scram-si transfected endothelial cells.

**FIGURE 6.** VEGF-induced phosphorylation of Akt is dependent on Src but not FAK or PLCγ-1. **A**, Scram-si, si-Src, or si-FAK-transfected HCAEC were serum-starved overnight and then treated with VEGF (50 ng/ml) for 10 min. Western blot analysis was carried out using anti-phospho-p473-Akt antibody. The membrane was stripped and subsequently reprobed using anti-phospho-ERK1/2 (p-ERK1/2), anti-FAK (p-FAK), and anti-Akt (p-Akt) antibodies. Total anti-Akt (Akt) antibody was used as a loading control. **Bar graphs** show quantitative analyses of three independent Western blot experiments (mean ± S.D.). *, p < 0.05, relative to control. **B**, Scram-si or si-PLCγ-1-transfected HCAEC were serum-starved overnight and then treated with VEGF (50 ng/ml) for 0, 5, and 15 min. Western blot analysis was carried out using anti-phospho-p473-Akt antibody. The membrane was stripped and subsequently reprobed using anti-PLCγ-1 antibody as loading control. **Bar graphs** show quantitative analyses of three independent Western blot experiments (mean ± S.D.).

VEGF-induced phosphorylation of Akt (by 58 ± 5.4%) but not ERK1/2 (Fig. 6A). Knockdown of Src significantly attenuated VEGF-mediated phosphorylation of Akt (by 58 ± 5.4%) but not ERK1/2 (Fig. 6A). Inhibition of FAK had no effect on VEGF-mediated phosphorylation of Akt or ERK1/2 (Fig. 6A). Similarly, siRNA knockdown of PLCγ-1 (>75%) failed to inhibit Akt phosphorylation (Fig. 6B). These findings suggest that NADPH oxidase may exert its permissive effect on
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VEGF-Pi3K-Akt signaling, at least partially, at the level of the nonreceptor tyrosine kinase Src.

Dependence of Src Kinase on NADPH Oxidase Activity Occurs at a Post-receptor Level—Phosphorylation of the tyrosine residue, Tyr-951, in the kinase insert domain of VEGFR2 has been shown to activate Src through its interaction with the T cell-specific adapter (TSAd/VRAP) molecule (61, 62). However, in immunoprecipitation assays, VEGF-induced phosphorylation of Tyr-951 was unaffected by inhibition of NADPH oxidase (Fig. 7A). These results suggest that NADPH oxidase-derived ROS modulate the activity of Src kinase (and thus, PI3K-Akt) independently of VEGFR2 Tyr-951 in endothelial cells.

Phosphorylation of the tyrosine residue, Tyr-1175, in the C-terminal region of VEGFR2 promotes activation of PLCγ (63). Activated PLCγ, in turn, generates inositol trisphosphate and diacylglycerol by hydrolyzing phosphatidylinositol 4,5-biphosphate, a common and rate-limiting substrate for both PLCγ and PI3K. In addition, Tyr-1175 provides a binding site for Shb, an adapter molecule that has been implicated in the activation of the PI3K-Akt signaling pathway in porcine aortic endothelial cells (63). Inhibition of NADPH oxidase by siRNA did not inhibit VEGF-mediated phosphorylation at Tyr-1175 (Fig. 7B), arguing against a role for this residue in mediating redox sensitivity of the PI3K-Akt signaling pathway.

Inhibition of NADPH Oxidase in Primary Human Endothelial Cells Has a Differential Effect on VEGF-mediated Gene Transcription—We next wished to determine whether the existence of NADPH oxidase-dependent and -independent VEGF signaling pathways is associated with differential sensitivity of downstream target genes. HCAEC were transfected with scrambled siRNA (control) or si-p47phox and then incubated in the absence or presence of 50 ng/ml VEGF for 4 h. Total RNA was extracted and processed for DNA microarray studies. The clusters were analyzed for a pool of VEGF-responsive genes that were inhibited by si-p47phox. A total of 1486 genes were induced by VEGF in scrambled siRNA-treated HCAEC (LCB 1.5). Of these, 402 were blocked by si-p47phox (at LCB 1.5; data not shown). The existence of distinct ROS-sensitive and -insensitive classes of VEGF-inducible genes was validated by real time-PCR of selected genes (Tables 1 and 2). As expected, VEGF treatment of HCAEC also resulted in reduced expression of certain genes (Table 3). In each case, VEGF-mediated gene repression was blocked by si-p47phox (Table 3). Selected examples of these three gene classes were verified by Northern blot experiments and are shown in Fig. 8.

VEGF Induction of NADPH Oxidase-sensitive Genes Is Mediated by ROS-dependent Signaling Pathways—We asked whether the sensitivity of VEGF-responsive genes to NADPH oxidase activity could be explained by the involvement of one or more of the ROS-dependent signaling pathways elucidated in this study. Indeed, VEGF induction of two NADPH oxidase-dependent genes, VCAM-1 and E-selectin, was significantly blocked by LY294002 (PI3K inhibitor) or SB203580 (p38 MAPK inhibitor) but not PD98059 (ERK1/2 inhibitor) (Fig. 9, A and B). Similarly, VEGF-mediated inhibition of GADD45A was attenuated by si-p47phox (see Table 3), LY294002, and SB203580 but not PD98059 (Fig. 9C). In contrast, expression of

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TABLE 1

NADPH oxidase-dependent genes that are induced by VEGF in endothelial cells as determined by RT-PCR analyses ( fold induction)

| Function | Control | VEGF |
|----------|---------|------|
| ACE-1    | 1       | $1.8 \pm 0.2^a$ |
| ADAMTS9  | 1       | $3.5 \pm 0.2$ |
| MGLAP    | 1       | $2.5 \pm 0.2^a$ |
| Endocan  | 1       | $2.4 \pm 0.1^a$ |
| HMOX1    | 1       | $1.8 \pm 0.2^a$ |
| SOD2     | 1       | $2.2 \pm 0.1^a$ |
| INS-R    | 1       | $1.8 \pm 0.1^a$ |
| PDGF-D   | 1       | $2.8 \pm 0.05^a$ |
| PCDH12   | 1       | $1.6 \pm 0.04^a$ |
| E-selectin | 1    | $10.6 \pm 0.3^a$ |
| VCAM-1   | 1       | $18.9 \pm 0.7^a$ |
| PMCH     | 1       | $5.8 \pm 0.02^a$ |
| TM       | 1       | $2.4 \pm 0.3^a$ |
| UBE2J1   | 1       | $3.1 \pm 0.5^a$ |

**Control** siRNA**p47phox**

| Function | Control | VEGF |
|----------|---------|------|
| Renin-angiotensin | 1 | $1.1 \pm 0.05^a$ | $0.9 \pm 0.06$ |
| Matrix regulation | 1 | $0.92 \pm 0.3$ | $0.82 \pm 0.2$ |
| Tumor endothelium | 1 | $1.2 \pm 0.1$ | $1.2 \pm 0.1$ |
| Insulin metabolism | 1 | $1.1 \pm 0.09^a$ | $1.2 \pm 0.09$ |
| Growth factor | 1 | $0.4 \pm 0.02^a$ | $0.6 \pm 0.02$ |
| Cell adhesion | 1 | $0.25 \pm 0.05$ | $0.4 \pm 0.02^a$ |
| Cell adhesion | 1 | $1.9 \pm 0.03^a$ | $1.4 \pm 0.03^a$ |
| Cell adhesion | 1 | $1.2 \pm 0.1$ | $1.2 \pm 0.1^a$ |
| Melanin regulation | 1 | $1.4 \pm 0.08$ | $1.2 \pm 0.09$ |
| Hemostasis | 1 | $1.6 \pm 0.3$ | $1.6 \pm 0.3$ |
| Protein metabolism | 1 | $2.8 \pm 0.05^a$ | $0.7 \pm 0.02^a$ |

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*a p < 0.05 is relative to scrambled siRNA-transfected uninduced/basal level.
*b p < 0.05 is relative to uninduced/basal level.
*c p < 0.001 is relative to basal level.
*d p < 0.05 is relative to si-p47phox-transfected basal level.
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TABLE 2
Vascular endothelial growth factor (VEGF) has been shown previously to induce inhibition of PI3K, p38 MAPK, and ERK1/2 in endothelial cells. Table 2 lists the effects of VEGF on gene expression.

| Gene        | Scrambled-siRNA | siRNA-p47phox | Function               |
|-------------|-----------------|---------------|------------------------|
| BMP-2       | 1.3 ± 0.07      | 3.7 ± 0.13    | Signal transduction    |
| DSCR1       | 1.6 ± 0.21      | 16.8 ± 0.87   | Signal transduction    |
| DAF         | 1.6 ± 0.07      | 3.7 ± 0.24    | Complement inhibition  |
| PDGF-A      | 1.1 ± 0.3       | 1.8 ± 0.22    | Growth                 |
| VEGF-C      | 1.6 ± 0.06      | 4.5 ± 0.24    | Growth/transcription   |
| Egr-1       | 1.4 ± 0.14      | 3 ± 0.25      | Transcription factor   |
| KLF5        | 2.98 ± 0.14     | 3.8 ± 0.27    | Cell adhesion          |
| ICAM-1      | 1.3 ± 0.04      | 1.5 ± 0.06    | Hemostasis             |
| PAI-1       | 3.1 ± 0.02      | 4.7 ± 0.1     | Hemostasis             |
| t-PA        | 0.7 ± 0.1       | 2.2 ± 0.3     | Signal transduction    |

* p < 0.001 relative to scrambled siRNA basal level.
* p < 0.05 relative to scrambled siRNA-transfected uninduced basal level.
* p < 0.05 is relative to scrambled siRNA-transfected, uninduced/basal level.
* p < 0.05 is relative to scrambled siRNA-transfected, VEGF-treated level.

TABLE 3
NADPH oxidase-dependent genes that are repressed by VEGF in endothelial cells as determined by real-time PCR analyses (-fold reduction)

The basal levels of expression for each gene in scrambled siRNA-transfected, unstimulated, serum-starved HCAEC were arbitrarily set at 1 (-fold) per 10^6 18 S mRNA copies. Numbers are expressed as -fold induction (or reduction if less than 1) over the basal levels. The abbreviations used are as follows: ADFP, adipose differentiation-related protein; BTG-1, B cell translocation gene 1; CDKN1B, cyclin-dependent kinase inhibitor 1B; GADD45A, growth arrest and DNA damage-inducible family, member 1; ICAM-1, intercellular adhesion molecule 1; PAI-1, plasminogen activator inhibitor 1; t-PA, plasminogen activator; tissue; SPRY4, Spry family protein 4.

| Gene        | Scrambled-siRNA | siRNA-p47phox | Function               |
|-------------|-----------------|---------------|------------------------|
| ADFP         | 1.6 ± 0.1       | 1.4 ± 0.2     | Adipose regulation     |
| BTG-1        | 1.1 ± 0.3       | 1.2 ± 0.1     | Cell cycle (G, to S)   |
| CDKN1B(p21^cip1) | 1.4 ± 0.1     | 1.1 ± 0.1     | Cell cycle (G, to S)   |
| GADD45A      | 2.3 ± 0.3       | 1.97 ± 0.2    | Cell cycle (G, to M)   |
| GADD45B      | 1.8 ± 0.3       | 1.6 ± 0.3     | Cell cycle (G, to M)   |
| CASP8        | 3.5 ± 0.4       | 3.5 ± 0.4     | Apoptosis              |
| INPP5D       | 1.3 ± 0.09      | 1.1 ± 0.07    | Signal transduction    |
| MAP2K7       | 1.5 ± 0.1       | 1.3 ± 0.1     | Signal transduction    |
| NFIB         | 0.98 ± 0.05     | 0.8 ± 0.02    | Signal transduction    |
| THSD1        | 1.8 ± 0.3       | 1.4 ± 0.2     | Signal transduction    |
| TNFRSF1B     | 1.5 ± 0.05      | 1.3 ± 0.4     | Signal transduction    |
| TNFSF10      | 3.4 ± 0.23      | 3.3 ± 0.03    | Signal transduction    |
| TXNIP        | 2.2 ± 0.5       | 1.6 ± 0.1     | Redox regulation       |

* p < 0.05 relative to scrambled siRNA-transfected, uninduced/basal level.
* p < 0.05 relative to scrambled siRNA basal level.
* p < 0.001 relative to scrambled siRNA-transfected (downregulated).
* p < 0.001 relative to scrambled siRNA basal level (upregulated).

an NADPH oxidase-independent gene, DAF, was unaffected by inhibition of PI3K or p38 MAPK (Fig. 9D). These results suggest that relative sensitivity of PI3K, p38 MAPK, and ERK1/2 may vary in part account for the differential effect of NADPH oxidase on VEGF-mediated gene expression.

Inhibition of Akt Phosphorylation in VEGF-treated NADPH Oxidase-depleted Endothelial Cells Is Sufficient for Modulating Downstream Gene Expression—We have demonstrated previously that VEGF reduces mRNA expression of GADD45A in endothelial cells through a mechanism that involves phospho-Akt-mediated exclusion of forkhead from the nucleus (49). Consistent with the redox sensitivity of Akt, this effect was reversed by si-p47phox, DN-Akt, or constitutively active triple-mutant (TM) FKHR (Fig. 10). In contrast, expression of a CA-Akt alone or in the presence of si-p47phox mimicked the effect of VEGF on GADD45A expression (Fig. 10). Data demonstrating a role for Akt-FKHR in mediating the expression of another redox-dependent gene, MnSOD, were obtained (data not shown). Taken together, these findings suggest that activation of the redox-sensitive signaling intermediates may modulate activity of downstream transcription factors, which in turn lead to alterations in target gene expression.

Inhibition of NADPH Oxidase in Primary Human Endothelial Cells Has a Differential Effect on VEGF-mediated Endothelial Cell Function—VEGF has been shown previously to induce the expression of thrombomodulin and DAF in cultured endothelial cells (49, 64, 65). VEGF-mediated induction of thrombomodulin, but not DAF, was blocked by si-p47phox (Tables 1 and 2 and Fig. 7). To determine whether differential redox sensitivity occurred at a functional level, we carried out in vitro assays for thrombomodulin and DAF activity. The thrombomodulin assay relies on its ability to activate exogenously supplied protein C, which in turn catalyzes a substrate 52366 (measured at 405 nm wavelength). Treatment of HCAEC with VEGF resulted in...
in >2-fold induction of thrombomodulin activity, an effect that was abrogated by si-p47phox (Fig. 11A), suggesting VEGF-mediated induction in the activity of thrombomodulin was NADPH oxidase-dependent. The DAF assay measures complement-mediated lysis of endothelial cells (49). Consistent with the gene expression data, VEGF-mediated protection against C3b complement was unaffected by si-p47phox (Fig. 11B). These findings suggest that VEGF induction of thrombomodulin activity but not DAF is sensitive to NADPH oxidase in endothelial cells.

**DISCUSSION**

Vascular NADPH oxidase is believed to play an important role in both physiology and pathophysiology. NADPH oxidase activity varies between vascular cells and between different sites of the vasculature. For example, vascular smooth muscle cells express high levels of Nox1 and Nox4, whereas endothelial cells express high levels of gp91phox/Nox2 and Nox4 (reviewed in Ref. 67). Cultured human microvascular endothelial cells display higher NADPH oxidase activity compared with HUVEC (68). Human saphenous veins were shown to express more Nox2 and p22phox, whereas internal mammary arteries expressed relatively high Nox4 (69). Many extracellular factors have been reported to induce NADPH oxidase activity in cultured endothelial cells. The nature of input signals varies at different sites of the vascular tree and over time. Together, these observations underscore the remarkable complexity of NADPH oxidase signaling in the intact vasculature.

Adding to this complexity is our finding that NADPH oxidase activity selectively modulates downstream signaling induced by a single agonist, namely VEGF. Differential effects of NADPH oxidase inhibition on VEGF signaling were observed at the level of signal transduction, transcription factor activation, target gene expression, and cell function.

Previous studies have implicated a role for ROS in mediating reversible receptor autophosphorylation in response to such ligands as insulin, epidermal growth factor, and platelet-derived growth factor (70, 71, 72). In addition, angiotensin II-mediated transactivation of the epidermal growth factor receptor and platelet-derived growth factor receptor in vascular smooth muscle cells was shown to be redox-sensitive (73, 74). Not all protein-tyrosine kinase receptors are similarly sensitive to ROS. For example, the NADPH oxidase inhibitor diphenyleneiodonium (DPI) or apocynin had no effect on Ang1-mediated phosphorylation of Tie2 (35). In this study, we demonstrated that si-p47phox-mediated knockdown of ROS had no effect on VEGFR2/KDR tyrosine phosphorylation at 1 and 5 min following VEGF treatment and only partially reduced phosphorylation at 10 min. Similar results were obtained with DPI (data not shown). Two previous studies have shown a role for ROS in autophosphorylation of VEGFR2/KDR. In one case, pretreatment of porcine aortic endothelial cells with catalase or the antioxidant NDGA partially inhibited VEGF induction of VEGFR2 phosphorylation between 10 and 15 min (23). These results are consistent with our 10-min data.
another study, preincubation of HUVEC with chemical inhibitors of ROS or transfection with antisense against gp91phox resulted in partial attenuation of VEGFR2/KDR autophosphorylation at 5 min (24). The reason for the discrepancy between the results of the latter report and the current study is not clear. Nevertheless, in neither case did ROS inhibition cause complete loss of VEGFR2/KDR autophosphorylation. Our results demonstrating insensitivity of VEGFR2 to NADPH oxidase activity at early time points are in accordance with the findings of Berk and co-workers (75). Our findings also suggest that inhibition of VEGFR2/KDR phosphorylation at later time points (10 and 30 min) was insufficient to completely abrogate signaling (as evidenced by the existence of a subclass of NADPH oxidase-independent pathways). The observation that NADPH oxidase is not required for VEGF-mediated phosphorylation of two major phosphorylation sites (Tyr-951 and Y Tyr-1175) argues against a tyrosine residue-specific effect of ROS on VEGF autophosphorylation. Although it is formally possible that other sites within the receptor are differentially sensitive to the redox state, a more likely explanation for our findings is that early and transient activation of VEGFR2/KDR is adequate for propagating downstream signals.

That VEGF activation of PI3K/Akt is redox-sensitive is consistent with previous findings. For example, in HUVEC, VEGF-mediated phosphorylation of Akt was blocked by DPI, apocynin, and catalase (76). Studies of Ang1 have yielded conflicting results. Chen et al. (35) demonstrated that Ang1-dependent phosphorylation of Akt at Ser-473 in porcine and murine endothelial cells was dependent on NADPH oxidase-derived ROS. In another study, Ang1-mediated phosphorylation of Akt in HUVEC was unaffected by overexpression of superoxide dismutase, catalase, or dominant-negative Rac1 (Rac1N17) (36). We have found that hepatocyte growth factor-mediated activation of Akt in HCAEC occurs independently of NADPH oxidase activity (supplemental Fig. 5). These findings suggest that the redox sensitivity of PI3K/Akt in endothelial cells is ligand- and receptor-dependent.

In time course experiments, siRNA-mediated inhibition of p47phox attenuated VEGF activation of Akt prior to any observable effect on VEGFR2/KDR phosphorylation. These data suggest that NADPH oxidase-derived ROS exert their positive effects on Akt at site(s) distal to the VEGF receptor. We have shown si-p47phox blocks VEGF-mediated phosphorylation and activity of Src. This finding is consistent with a previous investigation in which antioxidants attenuated VEGF-induced Src phosphorylation in HUVEC (77). Importantly, we also demonstrated that Src knockdown attenuates VEGF stimulation of Akt phosphorylation. Thus, NADPH oxidase influences Akt signaling via an effect on Src (Fig. 12). The precise mechanism underlying redox sensitivity of Src requires further study.
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FIGURE 12. Proposed model for the bifurcation of VEGF signals into redox-sensitive and redox-insensitive pathways downstream of VEGFR2. VEGF-induced activation of Src kinase is dependent on NADPH oxidase activity in endothelial cells. The redox-sensitive signaling pathway (shown in colored box) appears to deviate from the redox-insensitive pathway (PLCγ, ERK1/2) at the level of Src kinase. IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol.

We have demonstrated a role for NADPH oxidase activity in mediating the effect of VEGF on p38 MAPK but not ERK1/2 or JNK. These data contrast with those of Wu et al. (78) who showed that VEGF-mediated phosphorylation of JNK was oxidase-dependent. Moreover, previous studies in HUVEC have demonstrated a role for ROS in VEGF-mediated activation of ERK1/2 in HUVEC and porcine aortic endothelial cells (23, 36). The reason for the discrepancies between these latter reports and the present study is not clear. However, our findings that both AS- oligonucleotides and siRNA against p47phox had no effect on ERK1/2 or JNK phosphorylation in HUVEC and HCAEC in multiple independent time course experiments strongly argue against a role for NADPH oxidase-derived ROS in VEGF activation of these signal intermediates.

It is interesting to note that similar discrepancies exist regarding the role for ROS in Ang1-mediated MAPK signaling. One group demonstrated that overexpression of superoxide dismutase or Rac1N17 in HUVEC resulted in potentiation of Ang1-mediated phosphorylation of ERK1/2 but not p38 MAPK (36). Chen et al. (35), employing porcine coronary artery endothelial cells, as well as wild type and p47phox mouse heart microvascular endothelial cells, demonstrated a role for NADPH oxidase in Ang1-stimulated phosphorylation of ERK1/2.

These discrepancies notwithstanding, the effect of ROS on the MAPK family appears to be dependent on the agonist and cell type. For example, whereas the current results argue Against a role for NADPH oxidase in mediating VEGF activation of ERK1/2 in endothelial cells, previous studies support a role for the enzyme in angiotensin II-mediated activation of ERK1/2 in endothelial cells (79). Urotensin-II-mediated activation of ERK1/2, p38 MAPK, and JNK was inhibited by DPI and antisense to p22phox in human pulmonary artery smooth muscle cells (80). Thrombin-mediated activation of p38 MAPK but not ERK1/2 in vascular smooth muscle cells was p22phox-dependent (81). Endothelin-1-mediated activation of JNK, but not ERK1/2, in vascular smooth muscle cells was inhibited by DPI (82). In murine cardiac microvascular endothelial cells, hypoxia-reoxygenation-mediated phosphorylation of ERK1/2 and Akt was NADPH oxidase-dependent (83). Another study demonstrated a critical role for p47phox in tumor necrosis factor-α-mediated activation of ERK1/2 and p38 MAPK in cardiac microvascular endothelial cells (66).

Previously, we have shown that NADPH oxidase-derived ROS play an important role in mediating the effect of VEGF on the activity of the transcription factors, NF-κB and forkhead (44). The current study extends these observations by demonstrating an inhibitory action of si-p47phox on VEGF-mediated nuclear exclusion of FKHR. This study has also established a link between the relative redox sensitivity of the VEGF signaling intermediates (e.g. PI3K-Akt, p38 MAPK/ERK1/2) and the differential effects of NADPH oxidase-derived ROS on the expression of several VEGF-dependent genes. The data also supported the notion that redox-dependent alterations of a signaling molecule (e.g. Akt) by VEGF may result in the modulation of the activity of a downstream transcription factor(s) (e.g. FKHR), which in turn leads to modification of gene expression (e.g. GADD45A). Additionally, based on the results of the DNA microarrays, we predict the existence of a class of transcription factors whose activity is not sensitive to NADPH oxidase activity. Identification of those ROS-insensitive factors requires further studies.

Prior studies from our own group (21, 22), as well as others (23, 24, 43, 76), have demonstrated an important role for NADPH oxidase in mediating the effect of VEGF on endothelial cell migration and proliferation and angiogenesis. Here, we have shown that NADPH oxidase is also necessary for VEGF-mediated induction of thrombomodulin activity. However, inhibition of NADPH oxidase had no effect on VEGF-stimulated DAF function. These findings are in accordance with the differential effect of NADPH oxidase on downstream signaling pathways, transcription factors, and gene expression, and they provide compelling evidence for the existence of NADPH oxidase-dependent and -independent VEGF functions.

The finding that NADPH oxidase is necessary for some, but not all, functions of VEGF have important biological and therapeutic implications. The data suggest that basal and/or inducible ROS are not required for global VEGF signaling but rather serve as signal intermediates in selected downstream pathways. In this way, alteration of NADPH oxidase activity either in response to agonists or drugs will have highly selective effects on VEGF signal transduction and endothelial cell phenotypes. Such an effect may be leveraged for therapeutic gain. For example, it is plausible that the therapeutic inhibition of NADPH oxidase would inhibit VEGF stimulation of migration and proliferation, while retaining certain protective effects of VEGF signaling, e.g. protection against complement lysis.

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