Novel role of p66Shc in ROS-dependent VEGF signaling and angiogenesis in endothelial cells

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Angiogenesis is involved in normal and postnatal development as well as reparative neovascularization that is associated with wound healing, ischemic heart and limb diseases, and chronic inflammatory diseases such as atherosclerosis. In endothelial cells (ECs), vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating EC migration and proliferation primarily through the autophosphorylation of VEGF receptor type-2 (VEGFR2). We have shown that ROS derived from Rac1-dependent NADPH oxidase are involved in VEGFR2 autophosphorylation and angiogenic-related responses in ECs. However, a role of p66Shc in VEGF signaling and physiological responses in ECs is unknown. Here we show that VEGF promotes p66Shc phosphorylation at Ser36 through the JNK/ERK or PKC pathway as well as Rac1 binding to a nonphosphorylated form of p66Shc in ECs. Depletion of endogenous p66Shc with short interfering RNA inhibits VEGF-induced Rac1 activity and ROS production. Fractionation of caveolae-enriched lipid raft demonstrates that p66Shc plays a critical role in VEGF-induced phosphorylation in caveolae/lipid rafts as well as downstream p38MAP kinase activation. This in turn stimulates VEGF-induced EC migration, proliferation, and capillary-like tube formation. These studies uncover a novel role of p66Shc as a positive regulator for ROS-dependent VEGF signaling linked to angiogenesis in ECs and suggest p66Shc as a potential therapeutic target for various angiogenesis-dependent diseases.

reactive oxygen species; vascular endothelial growth factor

ANGIOGENESIS IS INVOLVED in normal and postnatal development as well as reparative neovascularization that is associated with wound healing, ischemic heart and limb diseases, and chronic inflammatory diseases such as atherosclerosis. In endothelial cells (ECs), vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating EC migration and proliferation primarily through the autophosphorylation of VEGF receptor type-2 (VEGFR2, KDR/Flk1; VEGFR2-pY), which initially occurs in part in caveolae/lipid rafts (19, 23, 32). Reactive oxygen species (ROS) function as key signaling molecules to mediate various biological responses such as cell migration, proliferation, and gene expression (11, 15, 37, 47). We and others (7, 48) reported that ROS derived from NADPH oxidase play an important role in VEGF2 signaling and angiogenic responses in ECs, as well as ischemia-induced neovascularization in vivo (9, 17, 42, 45). However, the underlying mechanisms are poorly understood.

The adaptor protein p66Shc is a life span determinant that integrates metabolic and longevity pathways and a key regulator for oxidative stress-mediated aging and cardiovascular diseases (3, 8, 10, 13, 26, 28, 29, 36). The Shc protein is expressed as three isoforms with relative molecular mass of 46, 52, and 66 kDa (34). They consist of a phosphotyrosine binding domain (PTB), a collagen homology domain (CH1), and a C-terminal Src homology domain (SH2). In addition, p66Shc contains a unique N-terminal collagen homology domain (CH2) that includes a Ser36. p66Shc phosphorylation at Ser36 through the JNK/ERK or PKC pathway as well as Rac1 binding to a nonphosphorylated form of p66Shc and that p66Shc is binding to a nonphosphorylated form of p66Shc in ECs. Depletion of endogenous p66Shc with short interfering RNA inhibits VEGF-induced Rac1 activity and ROS production. Fractionation of caveolae-enriched lipid raft demonstrates that p66Shc plays a critical role in VEGF2 phosphorylation in caveolae/lipid rafts as well as downstream p38MAP kinase activation. This in turn stimulates VEGF-induced EC migration, proliferation, and capillary-like tube formation. These studies uncover a novel role of p66Shc as a positive regulator for ROS-dependent VEGF2 signaling linked to angiogenesis in ECs and suggest p66Shc as a potential therapeutic target for various angiogenesis-dependent diseases.

METHODS

Materials. Antibodies to VEGFR2, phosphotyrosine (pY99), and paxillin were from Santa Cruz Biotechnology. Antibody to phospho-Ser36-p66Shc was from Calbiochem. Antibodies to phospho-VEGFR2 (pY1175), phospho-p38MAPK, and phospho-ERK1/2 were from Cell Signaling. Anti-caveolin-1 and Shc antibodies were from BD Biosciences. Human recombinant VEGF165 was from R&D Systems. Oligofectamine and Opti-MEMI reduced-serum medium were from Invitrogen. Other materials were purchased from Sigma.
Cell culture. HUVECs were grown in Endo-Gro (Millipore) containing 5% FBS.

Immunoprecipitation and immunoblotting. Growth-arrested HUVECs were stimulated with VEGF (20 ng/ml), and cells were lysed in lysis buffer at pH 7.4 (in mM: 50 HEPES, 5 EDTA, and 100 NaCl), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mmol/l PMSF, and 10 μg/ml leupeptin), and phosphatase inhibitors (in mmol/l: 50 sodium fluoride, 1 sodium orthovanadate, and 10 sodium pyrophosphate). Cell lysates were used for immunoprecipitation and immunoblotting, as described previously (49).

H₂O₂ measurement. HUVECs grown on glass coverslips were incubated with 20 μM 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCF-DA; Invitrogen) for 6 min at 37°C and then Vectashield mounting medium with DAPI was dropped on them. Cells were observed by confocal microscopy using the same exposure condition in each experiment. Relative DCF-DA fluorescence intensity with DAPI-positive cells was recorded and analyzed using Image J.

Sucrose gradient fractionation. Caveolae/lipid rafts fractions were separated, as described previously (32, 38). Briefly, HUVECs (5.0 × 10⁵ cells) were homogenized in a solution containing 0.5 M sodium carbonate (pH 11), 1 mM sodium orthovanadate, and protease inhibitors. The homogenates were adjusted to 45% sucrose by addition of 90% sucrose in a buffer containing 25 mM MES (pH 6.5) and 0.15 M NaCl. A 5–35% discontinuous sucrose gradient was formed above and below the homogenate. The homogenates were incubated with 20 ng/ml VEGF (20 ng/ml) for 5 min. Cell lysates were incubated on Rac-GTP affinity plates for 30 min at 4°C, and Rac1 activation was measured by luminometry.

Modified Boyden chamber migration assay. Migration assays using a modified Boyden chamber method were conducted in 24-well Transwell chambers as described previously (49).

Wound scratch assay. HUVECs were grown to confluence in six-well plates, and a scratch was applied with a plastic pipette tip to mimic wound injury. After 24 h in a 37°C CO₂ incubator in growth arrest medium containing 50 ng/ml VEGF, migrating cells were assessed by the closure of the wound area, as described previously (21, 52).

Cell proliferation assay. HUVECs (10⁵ cells) were seeded in sixwell plates, and cell number with and without VEGF in 0.2% FBS containing culture medium was determined by counting with a hemocytometer as described before (49, 52).

Capillary network formation assay. HUVECs transfected with control siRNA or p66Shc siRNA were seeded on top of the thick growth factor-reduced Matrigel-coated wells (BD Biosciences) and incubated for 6 h at 37°C. Images were taken with a Nikon digital camera, and eight random fields per well were analyzed.

Short interfering RNA transfection. HUVECs were grown to 40% confluence in 100-mm dishes and transfected with 10 nM control short interfering (si)RNA (from Ambion) and p66Shc siRNA (sense: 5'-GAAGAGUCUCUGUACCCGU-3' and antisense: 5'-CGAGCAGAGACUACUAAGC-3' within the N-terminal CH2 domain, which is not found in p52 and p46 Shc) using Oligofectamine (Invitrogen), as described previously (52). Cells were used for experiments at 48 h after transfection.

Rac1 activity assay. Rac1 activation was measured using a G-LISA Rac activation assay kit (Cytoskeleton, Denver, CO) according to the manufacturer’s instruction. Briefly, serum-starved HUVECs were stimulated with VEGF (20 ng/ml) for 5 min. Cell lysates were incubated on Rac-GTP affinity plates for 30 min at 4°C, and Rac1 activation was measured by luminometry.

HUVECs were grown to 40% confluence in sixwell plates, and a scratch was applied with a plastic pipette tip to mimic wound injury. After 24 h in a 37°C CO₂ incubator in growth arrest medium containing 50 ng/ml VEGF, migrating cells were assessed by the closure of the wound area, as described previously (21, 52).

Cell proliferation assay. HUVECs (10⁵ cells) were seeded in sixwell plates, and cell number with and without VEGF in 0.2% FBS containing culture medium was determined by counting with a hemocytometer as described before (49, 52).

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Fig. 1. VEGF increases Ser36 phosphorylation of p66Shc through ERK, JNK, or PKC in endothelial cells (ECs). A: human umbilical vein ECs (HUVECs) were stimulated with VEGF (20 ng/ml) for 15 min. Lysates were measured for pS36-p66Shc (pS36-p66Shc) Ab or Shc Ab. (n = 3). Bottom: averaged data, expressed as fold change over basal (means ± SE). *P < 0.05 vs. control.

B: HUVECs were pretreated with 20 μM LY294002 (LY), 10 μM SB203580 (SB), 20 μM PD98059 (PD), 20 μM GF109203X (GF), or 10 μM SP600125 (SP) for 30 min and stimulated with VEGF (20 ng/ml) for 15 min. Lysates were measured for pS36-p66Shc (n = 5). *P < 0.05 vs. vehicle +VEGF.
Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed by Student’s paired two-tailed t-test or ANOVA on untransformed data, followed by comparison of group averages by contrast analysis using the Super ANOVA statistical program (Abacus Concepts, Berkeley, CA). A P value of <0.05 was considered to be statistically significant.

RESULTS

VEGF increases Ser36 phosphorylation of p66Shc through ERK/JNK or PKC in ECs. To address the role of p66Shc in VEGF signaling, we examined the effect of VEGF on Ser36 phosphorylation of p66Shc (pS36-p66Shc) in HUVECs. Figure 1A shows that VEGF increased pS36-p66Shc within 5 min, which peaked at 15 min and remained above baseline for up to 60 min. We examined the upstream pathways mediating pS36-p66Shc formation by VEGF. Figure 1B shows that the MEK inhibitor PD98059, the JNK inhibitor SP600125, or the protein kinase C inhibitor GF109203X, but not the phosphatidylinositol 3-kinase inhibitor LY294002 or the p38MAP kinase inhibitor SB203580, significantly inhibited VEGF-induced p66Shc phosphorylation. These results suggest that VEGF increases pS36-p66Shc at least in part through ERK/JNK or PKC in ECs.

VEGF increases ROS production through p66Shc that associates with Rac1 and regulates its activation in ECs. We next examined whether p66Shc is involved in VEGF-induced ROS production using DCF-DA that mainly detects intracellular H2O2 (32). Figure 2A shows that p66Shc siRNA selectively knocked down endogenous p66Shc protein expression without affecting the expression of p52Shc or p46Shc protein in HUVECs. Under this condition, p66Shc siRNA significantly inhibited VEGF-induced ROS production measured at 5 min (Fig. 2B) or 30 min (not shown). We confirmed that pretreatment of ECs with polyethylene glycol-catalase before loading DCF-DA abolished the basal and VEGF-induced fluorescence signals, as reported previously (32).

Since we previously demonstrated that VEGF-induced ROS production is dependent on Rac1, a cytosolic component of NADPH oxidase, we examined whether p66Shc binds to Rac1 and/or involved in VEGF-induced Rac1 activation. Figure 3A using coimmunoprecipitation analysis shows that VEGF stimulation increased association of Rac1 with p66Shc within 5 min, which continued above basal levels for ≥30 min. Rac1 immunoprecipitates failed to bind to pS36-p66Shc, indicating that Rac1 binds to nonphosphorylated form of p66Shc. Figure 3B shows that VEGF-induced Rac1 activation was significantly inhibited by p66Shc siRNA. These results suggest that VEGF promotes p66Shc association with Rac1, which may be required for activation of Rac1 and subsequent ROS production in ECs.

p66Shc is involved in VEGF-induced VEGFR2 autophosphorylation in caveolae/lipid rafts and p38MAP kinase activation in ECs. Since we previously demonstrated that ROS mediate VEGF-induced VEGFR2-pY, we next examined the role of p66Shc in VEGF signaling. Figure 4A shows that knockdown of p66Shc with siRNA significantly inhibited VEGF-induced VEGFR2-pY without affecting basal phosphorylation or total VEGFR2 protein expression. We next examined the role of p66Shc in major VEGF2 downstream signaling events such as p38MAPK and ERK1/2 activation, which are key mediators of VEGF-induced EC migration and proliferation, respectively (24, 40). Figure 4B shows that p66Shc siRNA markedly inhibited VEGF-induced phosphorylation of p38MAPK without affecting ERK1/2 phosphorylation.

Since initial VEGFR2-pY increase occurs in part in caveolae/lipid rafts (19, 23, 32) where a fraction of NADPH oxidase is activated (25, 46, 47, 53, 55), we next examined the role of p66Shc in this localized VEGFR2 activation. Sucrose gradient fractionation confirmed that VEGFR2 and Nox2 were localized in caveolin-1-enriched, caveolae/lipid raft fractions 4–6 (Fig. 5A). Under this condition, VEGF stimulation rapidly increased VEGFR2-pY in caveolae/lipid rafts, which was sig-

p66Shc is involved in VEGFR2 autophosphorylation in caveolae/lipid rafts in ECs. To determine the functional significance of p66Shc-mediated EC migration and proliferation, we examined the role of p66Shc in VEGF-induced capillary network formation on Matrigel. Figure 7A shows that depletion of p66Shc significantly reduced the number of capillary tube branches, branching sprouts as well as tube length, suggesting that endogenous p66Shc plays an important role in VEGF-induced angiogenic responses in ECs.

Fig. 3. VEGF stimulates p66Shc association with Rac1 and its activation through p66Shc in ECs. A: HUVECs were stimulated with VEGF (20 ng/ml) for indicated minutes. Cell lysates were IP with anti-Rac1 or Shc Abs or normal IgG, and IB with anti-Shc or pS36-p66Shc Abs. Lysates without IP (No IP) were IB with anti-Rac1 Ab. A, bottom: averaged data for fold change in p66Shc–Rac1 association, expressed as -fold change over basal. *P < 0.05. B: Rac1 activities were measured in HUVECs transfected with control or p66Shc siRNAs with or without 20 ng/ml VEGF stimulation for 5 min (n = 3). *P < 0.05.

p66Shc is involved in VEGF-induced angiogenic related responses in ECs. To determine the functional significance of p66Shc in VEGF signaling, we examined the role of p66Shc in VEGF-induced EC migration and proliferation. Wound scratch assay (Fig. 6A) and modified Boyden chamber assay (Fig. 6B) show that depletion of p66Shc with siRNA significantly inhibited VEGF-induced directional EC migration. We also found that VEGF-induced EC proliferation was also significantly inhibited by p66Shc siRNA (Fig. 6C). To determine the func-

Fig. 4. p66Shc is involved in VEGF-induced VEGFR2 autophosphorylation and p38MAPK activation in ECs. HUVECs were transfected with control or p66Shc siRNAs. A: cells were stimulated with VEGF (20 ng/ml) for 5 min, and lysates were IP with anti-VEGFR2 Ab and IB with anti-phospho-tyrosine (pTyr) Ab. Same lysates were IB with anti-VEGFR2 or Shc Abs (n = 4) *P < 0.05. B: cells were stimulated with VEGF for indicated minutes and lysates were IB with phospho-p38MAPK or phospho-ERK1/2 or p66Shc expression. *P < 0.05.
DISCUSSION

Accumulating evidence reveals that p66Shc is a key regulator for various oxidative stress-dependent pathologies such as aging and cardiovascular diseases (3, 8, 10, 13, 26, 28, 29, 36). We (49) demonstrated that ROS derived from Rac1-dependent NADPH oxidase are involved in VEGFR2 autophosphorylation and angiogenic-related responses in ECs. However, a role of p66Shc in VEGF signaling and physiological responses in ECs is unknown. The present study demonstrates that VEGF stimulation increases pS36-p66Shc at least in part through ERK/JNK or PKC pathway as well as p66Shc association with Rac1, a component of NADPH oxidase in ECs. Moreover, depletion of p66Shc with siRNA blocks VEGF-induced Rac1 activation and ROS production, thereby inhibiting VEGF2-pY in caveolae/lipid rafts as well as p38MAPK activation. Functionally, p66Shc siRNA inhibits VEGF-induced EC migration, proliferation, and capillary tube formation. These findings suggest that p66Shc functions as a positive regulator for ROS-dependent VEGF2 signaling linked to angiogenesis in ECs (Fig. 7B).

p66Shc is shown to be phosphorylated at Ser36 by oxidative stress and insulin, EGF, endothelin-1 (5, 12, 20, 31), or angiotensin II (39) in various systems other than ECs. To our knowledge, the present study provides the first evidence that a key angiogenesis growth factor, VEGF, stimulates Ser36 phosphorylation of p66Shc. In response to stress factors, p66Shc is phosphorylated at Ser36 in N-terminal CH2 domain, which is not found in p46Shc and p52Shc, leading to H2O2 generation (14, 35). The upstream kinases responsible for pS36-p66Shc formation have been reported in other systems, including PKCB (14), PKCβ (16), ERK (18), or JNK (1). Consistent with previous reports, we found that inhibitors for MEK, JNK, or PKC, but not for phosphatidylinositol 3-kinase or p38MAPK, significantly reduce VEGF-induced pS36-p66Shc formation in ECs. Since Ser36 in p66Shc lies in the MAPK consensus phosphorylation motif, ERK or JNK may be the kinases that phosphorylate p66Shc. We confirmed that VEGF increases ERK and JNK phosphorylation within 5 min (unpublished observations). Thus our results suggest that VEGF increases pS36-p66Shc at least in part through ERK/JNK or PKC pathway in ECs. Detailed analysis of relationships among ERK, JNK, and PKC and identification of the subtypes of PKC involved in VEGF-induced pS36-p66Shc formation are the subjects of future studies.

We and others (48) reported that VEGF stimulation of ECs rapidly increases ROS by activation of Rac1-dependent NADPH oxidase. In this study, we show that VEGF stimulation rapidly promotes Rac1 association with nonphosphorylated form of p66Shc and that depletion of p66Shc with siRNA inhibits VEGF-induced Rac1 activation and ROS production. Given that Rac1 is a component of NADPH oxidase in ECs (2), these results suggest that p66Shc is at least involved in VEGF-induced ROS production via regulating Rac1-NADPH oxidase pathway. Consistent with our results, p66Shc is shown to mediate Rac1 activation through regulating a Rac-GEF (guanine nucleotide-exchange factor) such as son of sevenless 1 (Sos1; Ref. 22) or β-Pix (5). Tomilov et al. (43) recently demonstrated that O2 production by NADPH oxidase from macrophage is decreased in p66Shc−/− mice. Of note, S36-p66Shc phosphorylation has been shown to be involved in mitochondrial ROS production (8, 14, 26, 35), and most recent study (51) shows that VEGF stimulation increases mitochon-
p66Shc binding to Rac1 is required for VEGF-induced NADPH oxidase activation, while pS36-p66Shc is involved in mitochondrial ROS production in ECs. This point is currently under investigation.

Since ROS are highly diffusible molecules, localized ROS signal is important for efficient and specific activation of redox signaling events (6, 41, 47). VEGF-induced ROS are involved in activation of VEGFR2 (48), which occurs in part at caveolae/lipid rafts (19, 23, 32). In this study, we demonstrate that p66Shc siRNA significantly inhibits VEGF-induced increase in VEGFR2-pY in total lysates and caveolin-enriched lipid rafts where a fraction of Nox2 is found. Given that NADPH oxidase is activated in part at lipid rafts in ECs (25, 47, 53, 55), our result suggests that the p66Shc-mediated increase in ROS through NADPH oxidase promotes VEGFR2-pY via oxidative inactivation of PTPs DEP-1/PTP1B. Furthermore, Guo et al. (16) reported that the α1-adrenergic receptor promotes p66Shc-YY239/240 phosphorylation in caveolae via a ROS-dependent manner in cardiac myocytes. However, VEGF does not induce tyrosine phosphorylation of p66Shc in HUVECs (unpublished observations). These results suggest that VEGF-stimulated p66Shc/Rac1/ROS pathways may induce oxidative inactivation of PTPs localized at caveolae/lipid rafts, thereby promoting VEGFR2 activation.

We also examined the role of p66Shc in key VEGFR2 downstream signaling kinases involved in angiogenesis including p38MAPK and ERK1/2, which mainly mediate EC migration and proliferation, respectively. Knockdown of p66Shc significantly inhibits phosphorylation of p38MAPK but not ERK1/2, by VEGF. This in turn reduces VEGF-induced EC migration, proliferation, and capillary network formation, a key component of angiogenesis in ECs. Consistent with our data, a previous report (50) shows that p66Shc is involved in androgen-induced prostate cancer cell proliferation. The lack of p66Shc siRNA effect on VEGF-induced ERK activation...
suggests that other p66Shc downstream targets linked to EC proliferation than ERK1/2 may exist. Akt and/or forkhead related transcription factors FOXO3a/FOXL1 (5, 16, 18, 30) or mammalian target of rapamycin-S6 kinase (33) will be additional possible targets of p66Shc in VEGF signaling.

Previous studies (3, 4, 8, 10, 14, 26, 28, 35, 36) using p66Shc knockout or knockdown approaches implicate a pathological role of p66Shc in mediating apoptosis and endothelial dysfunction involved in oxidative stress-associated aging and cardiovascular diseases. Indeed, p66Shc activation has been shown to induce oxidative stress and endothelial dysfunction, while p66Shc inhibition exhibits vasoprotective effects (3, 13). By contrast, our findings suggest a physiological function of p66Shc in angiogenic signaling in ECs. Consistent with our data, Zaccagnini et al. (54) reported that p66Shc−/− mice subjected to ischemia/reperfusion show a more decreased capillary density in skeletal muscles than wild-type mice. Naldini et al. (27) showed that hypoxia-induced angiogenic response is impaired in p66Shc−/− T cells. Given that p66Shc−/− mice are not embryonic lethal, these results suggest that p66Shc is involved in postnatal angiogenesis in response to injury but not developmental angiogenesis.

In summary, we demonstrate that VEGF stimulation promotes pS36-pp66Shc formation at least in part through the ERK/JNK or PKC pathway as well as association of a non-phosphorylated form of p66Shc with Rac1 in ECs. We also found that p66Shc is involved in VEGF-induced Rac1 activation and ROS production, thereby promoting VEGFR2 activation in caveolae/lipid rafts, which in turn stimulates angiogenic responses in ECs. In other words, a low concentration of ROS is required for biological responses such as cell migration, while excess an amount of ROS produced in pathological conditions induce apoptosis or cytotoxic effects. Both cases may be mediated through p66Shc. Thus, under the condition in which VEGF increases ROS that act as signaling molecules in VEGFR2 signaling, p66Shc functions as a positive regulator for angiogenic-related responses in ECs.
REFERENCES


