

VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells

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Background. Vascular endothelial growth factor (VEGF) regulation of endothelial nitric oxide synthase (eNOS) and signaling pathways involved have not been well studied in glomerular endothelial cells (GENCs).

Methods. GENCs grown from tsA58 Immortomice[®] were used. Immunoblotting and in-cell Western blot analysis were employed to assess changes in VEGF receptor signaling pathway and eNOS phosphorylation of ser1177. Immunokinase assay and immunoblotting with phosphospecific antibodies were performed to assess activity of kinases.

Results. VEGF rapidly induced tyrosine phosphorylation of type 1 and type 2 VEGF receptors. Physical association between VEGF-receptor 2 (VEGF-R2) and insulin receptor substrate (IRS-1) and phosphatidylinositol 3'-kinase (PI3K) was induced by VEGF, which augmented PI3K activity in VEGF-R2 immunoprecipitates. VEGF stimulated Akt phosphorylation in a PI3K-dependent manner. VEGF increased eNOS phosphorylation on Ser1177. Activation of eNOS was associated with nitric oxide generation as measured by medium nitrite content. Signaling mechanisms involved in VEGF stimulation of eNOS were explored. VEGF-induced eNOS phosphorylation was abolished by SU1498, a VEGF-R2 inhibitor, LY294002, a PI3K inhibitor, and infection of cells with an adenovirus carrying a dominant negative-mutant of Akt, demonstrating the requirement of the VEGF-R2/IRS-1/PI3K/Akt axis for activation of eNOS. VEGF also activated extracellular signal-regulated protein kinase (ERK) in a time-dependent manner; and VEGF-stimulated eNOS phosphorylation on Ser1177 was prevented by PD098059, an upstream inhibitor of ERK, demonstrating that ERK was involved in VEGF regulation of eNOS. ERK phosphorylation was abolished by LY294002, suggesting ERK was downstream of PI3K in VEGF-treated GENC.

Conclusions. Our data demonstrate that in GENC, VEGF stimulates VEGF-R2/IRS-1/PI3K/Akt axis to regulate eNOS phosphorylation on Ser1177 in conjunction with the ERK signaling pathway.

Of the constituent cells of the glomerulus, the participation of mesangial and epithelial cells in physiologic and pathologic states has been examined in depth. However, studies on glomerular endothelial cells (GENCs) are lacking mainly because of the difficulty in initiating and maintaining them in culture. This limitation has compromised our understanding of the vital part this cell plays in glomerular physiology and pathology. Endothelial cells have been extensively studied in other vascular beds. Key signaling and metabolic pathways in arterial endothelial cells are altered contributing to endothelial dysfunction and atherosclerosis. Circulating growth factors and locally produced humoral agents such as endothelins and prostaglandins have been identified as important regulators of endothelial integrity and function.

Among growth factors, vascular endothelial growth factor (VEGF) has emerged as a prime mediator of endothelial cell survival and function. VEGFs are a family of polypeptides with diverse functions [1]. The vital importance of VEGF in integrity of vasculature is underscored by the observation that absence of a single allele of VEGF is embryonic lethal due to severe derangement in endothelial cell development [2, 3]. In addition to genesis of endothelium, organization of endothelial cells into vascular beds is regulated by VEGF and its cognate VEGF type 1 and type 2 receptors (VEGF-R1 and VEGF-R2). Mice lacking both alleles of VEGF-R1 or VEGF-R2 die in utero due to vascular disorganization, and failure of hematopoietic and endothelial development, respectively [4, 5]. VEGF is also called vascular permeability factor as it regulates passage of fluid across endothelial cell layers [6]. These observations emphasize the importance of VEGF and its signaling system in the survival, organization, and function of endothelial cells

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and blood vessels, and they have obvious implications for glomerular structure and function.

VEGFs are synthesized in several cell compartments of the nephron, including the visceral epithelial cells of the glomerulus, and the proximal tubular epithelial cells [7, 8]. Intense labeling of the renal cortex with labeled VEGF suggests presence of VEGF receptors in glomeruli and possibly cortical epithelial cells [9]. In vitro studies have shown that proximal tubular epithelial cells possess VEGF receptors and that VEGF regulates important metabolic functions of that cell, such as protein synthesis [10]. Although VEGF receptors have been identified on the surface of GENCs in vivo [11], to date, metabolic activities that are under the control of these receptors have not been identified. Recently, we were able to grow GENCs from the tsA58 immortalized mice that carry a temperature sensitive variant of SV40 T antigen [12]. With the ready availability of GENCs, our aim in the present study was to investigate a fundamental function of glomerular endothelium [i.e., expression and activation of endothelial nitric oxide synthase (eNOS) by VEGF]. eNOS is known to regulate glomerular hemodynamics by generation of nitric oxide; however, the regulatory pathways that govern its generation have not been elucidated. Accordingly, we investigated the signaling pathways involved in VEGF-regulation of eNOS in GENCs.

METHODS

GENCs culture

tsA58 Immortomice[®], transgenic for the temperature sensitive SV40 TAg transcribed under interferon- γ (INF- γ)-sensitive H-2Kb promoter, were used to isolate and clone cells from decapsulated glomerular explants; they have been extensively characterized [12]. For experiments described in this report, GENCs were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5 mmol/L glucose, 25% nutrient mixture F-12 Ham, and 10% fetal bovine serum (FBS). Monolayers of cells that were 90% confluent were serum-starved for 24 hours before experiments were performed. VEGF₁₆₅, the major form of VEGF expressed in the kidney [13], was used at 20 ng/mL for indicated durations of incubation; this concentration regulates metabolic functions in renal cells [10].

Immunoblotting and immunoprecipitation

Cells were washed twice with phosphate buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris HCl, pH 7.4, 150 mmol/L potassium chloride, 1 mmol/L dithiothreitol (DTT), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L glycerophosphate, pH 7.5, 50 mmol/L

sodium fluoride, 0.1 mmol/L sodium orthovanadate, 1 mmol/L ethylene glycol-bis (β -aminoethyl ether)-*N,N,N,N'*-tetra-acetic acid (EGTA), 2 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin). Cell debris was removed by centrifugation at 12,000 rpm for 20 minutes and concentration of protein was measured using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA, USA). Indicated amounts of lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and probed with various primary antibodies at the indicated dilutions followed by incubation with secondary antibodies that were fluorochrome-coupled or peroxidase-coupled for detection by Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA) or enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ, USA), respectively. For immunoprecipitation, 500 μ g lysates were incubated with the indicated antibodies at a 1:100 dilution overnight at 4°C with rotation. Immunoprecipitates were then incubated with a protein A/G agarose slurry for an additional hour at 4°C with rotation. Agarose beads were then washed three times with RIPA buffer, twice with cold phosphate-buffered saline (PBS), before being used for immunoblot as described above.

In-cell Western analysis

GENCs were plated in a 96-well plate and grown to 80% confluence, at which time they were incubated in serum-free medium for 20 hours before being treated as indicated. Immediately after treatment, cells were incubated in a fixing solution (4% formaldehyde in PBS) for 20 minutes at room temperature. Cells were then permeabilized by five washes of 5 minutes each in PBS containing 0.1% Triton X-100. Cells were then blocked by a 90-minute incubation in Li-Cor Odyssey blocking buffer at room temperature with gentle shaking. The primary antibodies were added at a concentration of 1:500 and incubated overnight at 4°C with gentle shaking. Cells were then washed 5 \times 5 minutes each in PBS containing 0.1% Tween. The secondary antibodies (IRDye800-conjugated or Alexafluor-conjugated) were added at a concentration of 1:500 and incubated 1 hour at room temperature with gentle shaking, after which cells were washed 5 \times 5 min in PBS containing 0.1% Tween. The plate was then scanned using the Odyssey Infrared Imaging System (169 μ m resolution, 2 mm offset, intensity setting of 5 for both channels). Label intensity was measured by densitometric analysis of the wells.

Phosphatidylinositol 3'-kinase (PI3K) assay

PI3K activity was measured as described in [14]. Briefly, control and VEGF-treated GENCs were homogenized

in RIPA buffer. Equal amounts of homogenates (500 μ g) were immunoprecipitated with an antiphosphotyrosine antibody, then protein A/G agarose slurry was added. The agarose beads were pelleted, washed, and incubated with 10 μ g phosphatidylinositol for 10 minutes at 25°C in PI3K assay buffer containing 20 mmol/L Tris/HCl, pH7.5, 100 mmol/L NaCl, and 0.5 mmol/L EGTA. Following addition of 20 mmol/L MgCl₂ and 5 μ Ci of ³²P-adenosine triphosphate (ATP) to the assay mixture, reactants were further incubated for 10 minutes at 25°C. Reaction was stopped by adding chloroform/methanol/11.6 N HCl mixture. Reactants were extracted in chloroform and the organic layer was washed with methanol/1 N HCl mixture. The reaction products were dried, resuspended in chloroform, separated by silica gel 60 thin layer chromatography. Phosphatidylinositol 3 phosphate spots were visualized by autoradiography.

Infection of GENCs with adenovirus was performed as described in [14]. Briefly, replication-defective adenovirus vector carrying mouse Akt containing a serine/threonine-to-alanine mutation at its activation sites (T308A and S473A) and a hemagglutinin (HA) tag was used. GENCs were infected with 100 multiplicity of infection (MOI) of adenovirus carrying the dominant-negative mutant of Akt (Ad-DN-Akt) or green fluorescent protein (Ad-GFP) for 1 hour at room temperature. After a 24-hour incubation at 37°C, cells were stimulated with VEGF for the indicated time.

Quantification of no release

GENCs were cultured in the 6-well plates in phenol-free medium. After being serum-starved for 24 hours, the cells were treated with VEGF for the indicated time. The amount of nitrite, the stable end product of nitric oxide oxidation, was used as an indicator of nitric oxide synthesis, catalyzed by eNOS. Nitrite concentration in the culture medium was measured using the Griess reaction with sodium nitrite as a standard, as described [15]. Data were expressed corrected for protein content.

Statistics

Data from a minimum of three experiments were expressed as mean \pm SEM and analyzed by analysis of variance (ANOVA) for comparison among multiple groups using Bonferroni post hoc test analysis (GraphPad Prism®; GraphPad Software, San Diego, CA, USA). *P* values of < 0.05 were considered significant.

RESULTS

VEGF increases tyrosine phosphorylation of VEGF-R1 and VEGF-R2 in GENCs

We examined whether VEGF regulates tyrosine phosphorylation of VEGF-R1 and VEGF-R2, which indi-

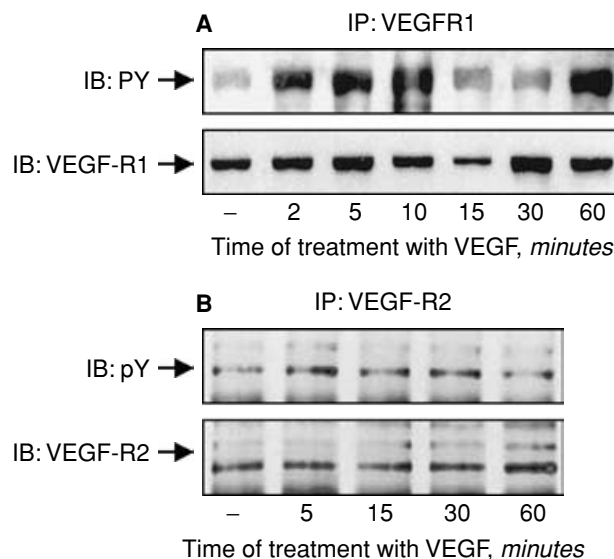


Fig. 1. Vascular endothelial growth factor (VEGF) induces tyrosine phosphorylation in VEGF-receptor 1 (VEGF-R1) and VEGF-receptor 2 (VEGF-R2) in glomerular endothelial cells (GENCs). (A) Equal amounts of lysates (500 μ g) from serum-starved GENCs treated with or without VEGF (20 ng/mL) for various durations were immunoprecipitated (IP) with a specific antibody against VEGF-R1 and run on a 7.5% gel. Following transfer to nitrocellulose membrane, the membranes were probed with antiphosphotyrosine (PY) antibody (upper panel) or the immunoprecipitating antibody (lower panel). A representative blot from three experiments is shown. (B) Same procedure was followed as in (A), except that antibody against VEGF-R2 was employed. A representative blot [immunoblot (IB)] from three experiments is shown.

cates activation of the receptors. Quiescent GENCs were stimulated with 20 ng/mL VEGF for up to 60 minutes, and whole cell lysates were used for immunoprecipitation with an antibody against VEGF-R1 or VEGF-R2. Immune complexes were immunoblotted with an antiphosphotyrosine antibody. VEGF-induced VEGF-R1 tyrosine phosphorylation was biphasic, starting at 2 minutes, and peaking between 5 and 10 minutes, almost returning to baseline at 15 and 30 minutes, and showing a second increment at 60 minutes (Fig. 1A). VEGF-induced tyrosine phosphorylation of VEGF-R2 was also rapid, starting at 5 minutes, and remained elevated for 30 minutes (Fig. 1B).

VEGF increases insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and its association with VEGF-R2

We focused on VEGF-R2 for the rest of the studies as it has been linked to most VEGF-related effects in endothelial cells [1]. IRS-1 is a docking protein that binds to cytoplasmic domains of several receptors. Following incubation with VEGF, IRS-1 rapidly associates with VEGF-R2 in renal epithelial cells and this binding is required for VEGF induction of protein synthesis [16]. We thus examined whether VEGF promotes IRS-1

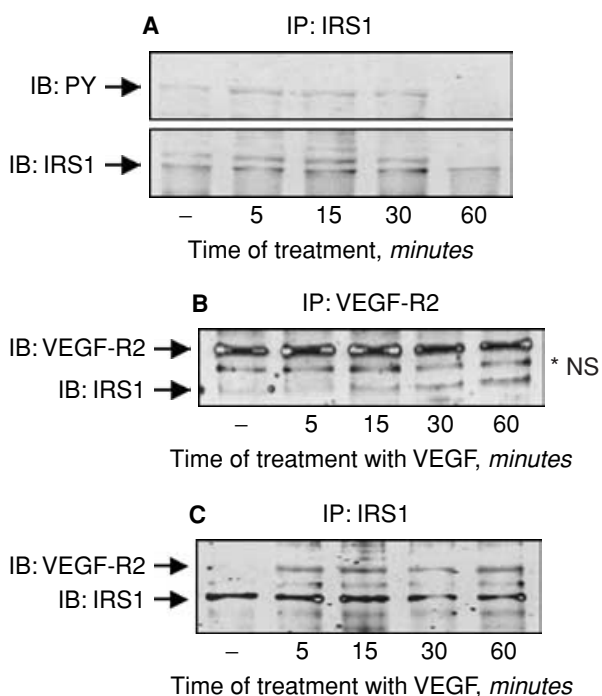


Fig. 2. Vascular endothelial growth factor (VEGF) induces insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and promotes association between IRS-1 and VEGF-receptor 2 (VEGF-R2). (A) Equal amounts of lysates (500 μ g) from serum-starved glomerular endothelial cells (GENCs) treated with or without VEGF (20 ng/mL) for various durations were immunoprecipitated (IP) with a specific antibody against IRS-1 and run on a 7.5% gel. Following transfer to nitrocellulose membrane, membranes were probed with antiphosphotyrosine antibody (upper panel) or the antibody against IRS-1 (lower panel). A representative blot [immunoblot (IB)] from three experiments is shown. (B) Equal amounts of lysates (500 μ g) from serum-starved GENCs treated with or without VEGF (20 ng/mL) for various durations were immunoprecipitated with a specific antibody against VEGF-R2 and run on a 7.5% gel. Following transfer to nitrocellulose membrane, membranes were probed with antibody against IRS-1 followed by antibody against VEGF-R2. Increasing density of IRS-1 band shows greater association with VEGF-R2 (arrow). NS refers to a nonspecific band. A representative blot from three experiments is shown. (C) The order of antibodies used in immunoprecipitation and immunoblotting was reversed in a separate set of experiments. A representative blot from three experiments is shown.

association with VEGF-R2 in GENCs. Cell lysates were used for immunoprecipitation with an antibody against IRS-1 following addition of VEGF and the immune complexes were used for Western blot with an antiphosphotyrosine antibody. Induction of IRS-1 phosphorylation was evident at 5 minutes and lasted for up to 30 minutes (Fig. 2A). We next examined whether VEGF promotes IRS-1 association with VEGF-R2. GENCs were incubated with VEGF for up to 60 minutes, and cell lysates were used for immunoprecipitation with an antibody against VEGF-R2. Immune complexes were immunoblotted with antibody against IRS-1. VEGF induced a time-dependent physical association between VEGF-R2 and IRS-1, which lasted for 60 minutes

(Fig. 2B). Qualitatively similar results were obtained when the order of antibody application was reversed, immunoprecipitating with antibody against IRS-1 and immunoblotting with antibody against VEGF-R2 (Fig. 2C).

VEGF stimulates PI3K activity in GENCs

In select receptor tyrosine kinase signaling systems (e.g., insulin receptor), downstream events of receptor activation depend in part on association of the IRS docking proteins and SH2 domain-containing proteins. It is the case for PI3K, which is recruited to receptor tyrosine kinase or docking proteins through its p85 regulatory subunit. VEGF is known to stimulate IRS-1 association with PI3K and induce activation of the enzyme in renal epithelial cells [16]. We examined whether VEGF promoted association between p85 and IRS-1 in GENCs. Cells were incubated with VEGF for up to 60 minutes, and cell lysates were used for immunoprecipitation with an antibody against IRS-1. Immune complexes were immunoblotted with an antibody against p85 subunit of PI3K. VEGF induced a rapid association between IRS-1 and p85 that began at 2 minutes and followed a biphasic course for up to 15 minutes, returning to baseline thereafter (Fig. 3A). To assess the functional relevance of the association between PI3K and IRS-1, we examined whether PI3K activity could be detected in phosphotyrosine immunoprecipitates. Figure 3B shows that phosphotyrosine-associated PI3K activity increased between 5 and 15 minutes of VEGF treatment. These data provide evidence that activated VEGF-R2 recruits the p85 regulatory subunit of PI3K, probably via IRS-1, resulting in activation of the enzyme.

VEGF-induced Akt phosphorylation requires PI3K activation

Activation of PI3K results in the formation of phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3). Binding of PtdInsP3 to Akt promotes its translocation to cell membrane where phosphoinositide-dependent kinase 1 and phosphoinositide-dependent kinase 2 (PDK1 and PDK2) phosphorylate Akt on Thr308 and Ser473, respectively, leading to its activation. In order to determine the role of the PI3K/Akt axis in VEGF-induced phosphorylation of eNOS, we first examined if VEGF induces Akt phosphorylation in GENCs. Although PI3K is acknowledged to be an upstream regulator of Akt phosphorylation, Akt can be activated independently of PI3K, as it has been reported in renal mesangial cells [17]. Immunoblotting of lysates from VEGF-treated GENCs with an antibody that specifically detects phosphorylated Ser473 demonstrated that VEGF induced phosphorylation of Akt within 2 minutes, peaked at 10 minutes (250% control) (three experiments) ($P < 0.01$) and waned by

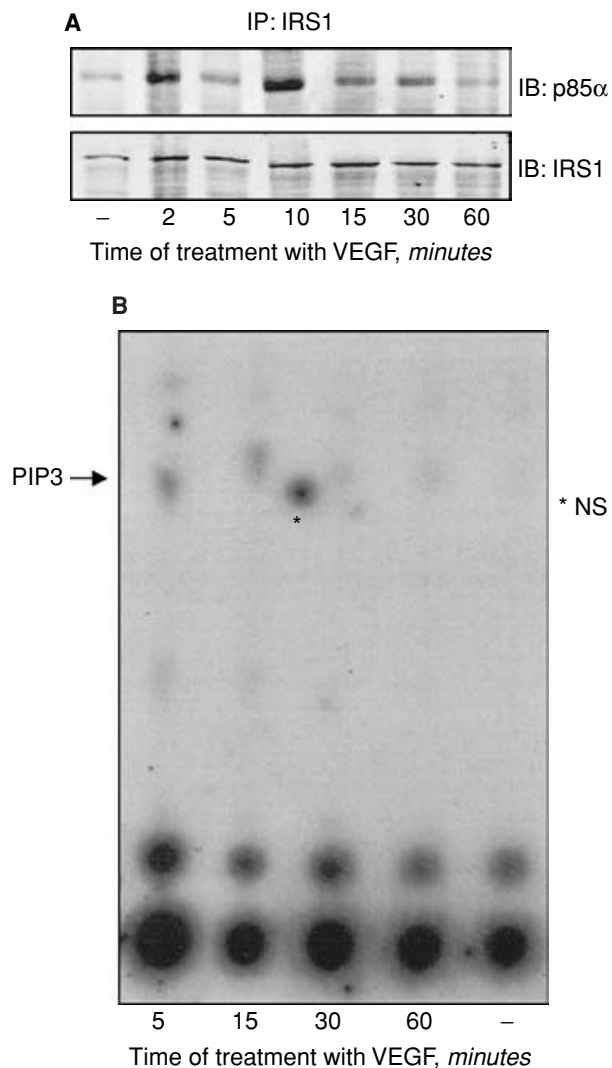


Fig. 3. Vascular endothelial growth factor (VEGF) promotes association between insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3'-kinase (PI3K). (A) Equal amounts of lysates (500 μ g) from serum-starved glomerular endothelial cells (GENCs) treated with or without VEGF (20 ng/mL) for various durations were immunoprecipitated (IP) with a specific antibody against insulin receptor substrate-1 (IRS-1) and run on a 7.5% gel. Following transfer to nitrocellulose membrane, the membranes were probed with an antibody against p85 subunit of PI3K or the antibody against IRS-1. A representative blot from three experiments is shown. (B) PI3K activity is increased in the VEGF-treated GENCs. Equal amounts of lysates (500 μ g) from serum-starved GENCs treated with VEGF for various durations were immunoprecipitated with an antiphosphotyrosine antibody. PI3K activity in immune complexes was measured by adding radiolabeled phosphatidylinositol (PtdIns), and measuring generation of radioactive PtdInsP3. Phospholipids were separated by thin-layer chromatography, and visualized by autoradiography. A representative chromatogram from four individual experiments is shown. Note that the control lane is to the right side of the figure. NS refers to an artifact.

15 minutes (Fig. 4A). Preincubation with LY294002, a selective PI3K inhibitor, abolished VEGF-induced Akt phosphorylation (Fig. 4B), showing that Akt activation by VEGF is PI3K-dependent.

VEGF induces phosphorylation of eNOS in GENCs

Phosphorylation of eNOS on Ser1177 is required for its activation. GENCs were incubated with VEGF for up to 60 minutes, and eNOS phosphorylation was assayed by in-cell Western blot analysis using an antibody that selectively detects eNOS phosphorylated on Ser1177 (p-eNOS). Actin immunoreactivity was measured in the same wells to assess employment of equal number of cells, and results were expressed as p-eNOS/actin ratio. VEGF stimulated phosphorylation of eNOS in a time dependent manner, with a peak as early as 2 minutes (200% of control) (four experiments) ($P < 0.05$ by ANOVA) followed by a second broader wave that peaked at 30 minutes (470% of control) (four experiments) ($P < 0.01$ by ANOVA) and waned by 60 minutes (Fig. 5A). In cells untreated with VEGF, no increase in basal eNOS phosphorylation was observed. Following phosphorylation, eNOS uses L-arginine to generate nitric oxide, which directly regulates hemodynamics of the capillary. Nitric oxide is promptly converted to nitrite, a stable end product. We measured nitrite in the media to test whether eNOS phosphorylation induced by VEGF in GENCs led to nitric oxide generation. Medium total nitrite content corrected to cell protein began to increase at 2 minutes; it reached significance at 10 minutes ($P < 0.01$) and remained elevated for 30 minutes ($P < 0.05$) (Fig. 5B) (four experiments).

Phosphorylation of eNOS by VEGF requires VEGF-R2 activation

We initiated studies on the signaling mechanisms recruited by VEGF in augmenting eNOS phosphorylation. As a first step, we examined the need for activation of VEGF-R2, the receptor that mediates most of VEGF effects. GENCs were pretreated with SU1498, a selective VEGF-R2 inhibitor, for 30 minutes before incubation with VEGF for 30 minutes. eNOS phosphorylation was measured by in-cell Western blot analysis. Figure 6, upper panel, shows that VEGF-induced eNOS phosphorylation was completely inhibited by SU1498 at 30 minutes (four experiments) ($P < 0.01$ VEGF vs. SU1498 + VEGF by ANOVA), but eNOS basal phosphorylation was not affected by SU1498. This was confirmed by immunoblot analysis of GENC lysates treated with VEGF for 30 minutes (time of maximum eNOS phosphorylation, as determined by in cell Western blot analysis) with or without preincubation with SU1498 (Fig. 6, lower panel). These data show that eNOS phosphorylation on Ser1177 requires VEGF-R2 activation. For analyzing changes in eNOS phosphorylation, we have observed that in-cell Western blot analysis is more sensitive and lends itself for better statistical analysis than SDS-PAGE followed by immunoblotting.

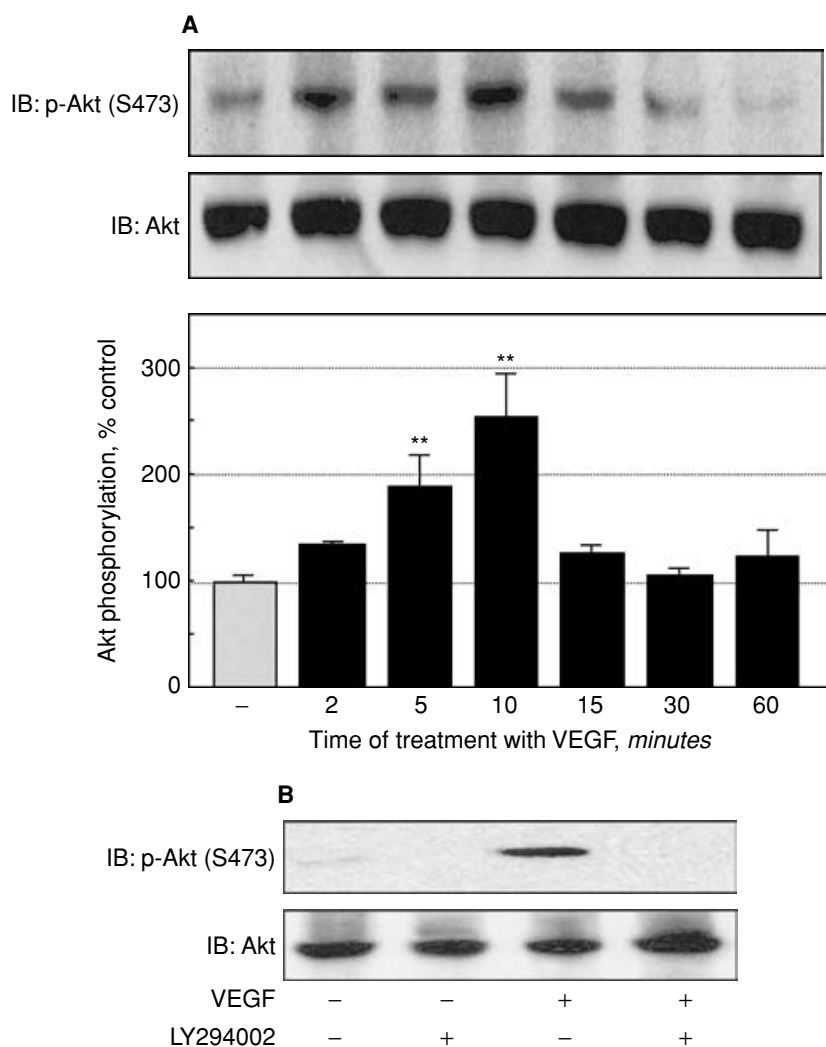


Fig. 4. Vascular endothelial growth factor (VEGF) induces phosphorylation of Akt that is dependent of phosphatidylinositol 3'-kinase (PI3K) activation. (A) Equal amounts of lysates (40 μ g) from serum-starved glomerular endothelial cells (GENCs) treated with VEGF (20 ng/mL) for various durations were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to nitrocellulose membrane, the membranes were probed the antibody against phosphorylated Akt (p-Akt). The membrane was stripped and reprobed with an antibody against total Akt to assess loading. A representative blot (IB) from three experiments is shown (upper panel). The lower panel represents densitometric analysis of VEGF-induced Akt phosphorylation from three individual experiments. Akt phosphorylation is expressed as percent of control ($N = 3$) (mean \pm SEM). ** $P < 0.01$ compared to control by analysis of variance (ANOVA). (B) GENCs were pre-treated for 30 minutes with selective PI3K inhibitor, LY294002 (25 μ mol/L), before VEGF (20 ng/mL) stimulation for 10 minutes. Akt phosphorylation of was detected by Western blot as described in (A). A representative blot from three experiments is shown.

Role of the PI3K/Akt axis in VEGF-induced eNOS phosphorylation

We sought to determine if phosphorylation of eNOS induced by VEGF was dependent on activation of PI3K/Akt axis. Preincubation with LY294002 completely blocked VEGF-stimulated phosphorylation of eNOS at 30 minutes (Fig. 7A, upper panel) (four experiments) ($P < 0.01$), but basal phosphorylation of eNOS was not affected by LY294002. An immunoblot performed on cells stimulated by VEGF for 30 minutes confirmed these findings (Fig. 7A, lower panel), showing that VEGF-induced eNOS activation is PI3K-dependent.

In order to examine the role of Akt in eNOS activation by VEGF, the cells were infected with an adenovirus carrying a dominant-negative construct of Akt (Ad-DN-Akt) in which the phosphorylation sites of Akt are mutated; an adenovirus carrying GFP (Ad-GFP) was used as control. VEGF induced phosphorylation of eNOS in control cells infected with Ad-GFP (Fig. 7B) (three experiments) ($P < 0.01$ VEGF vs. control by ANOVA),

but not in cells infected with Ad-DN-Akt ($P < 0.05$ by ANOVA, Ad-GFP cells + VEGF vs. Ad-DN-Akt cells + VEGF). Successful expression of Ad-DN-Akt was indicated by detection of HA tag by immunoblotting. Note that adenoviral infection did not affect basal expression of eNOS. These data demonstrate that Akt activation is required for eNOS phosphorylation on Ser1177 stimulated by VEGF.

Role of mitogen-activated protein kinase (MAPK) signaling pathway in eNOS phosphorylation

The role of extracellular signal-regulated protein kinase (ERK) activation in VEGF-regulation of eNOS is not clear with conflicting data in the literature. There are multiple putative sites of ERK-mediated phosphorylation in eNOS sequence [18], suggesting ERK could be a potential kinase for eNOS. Accordingly, we examined the role of ERK in VEGF regulation of eNOS. We first investigated whether VEGF induces ERK activation in

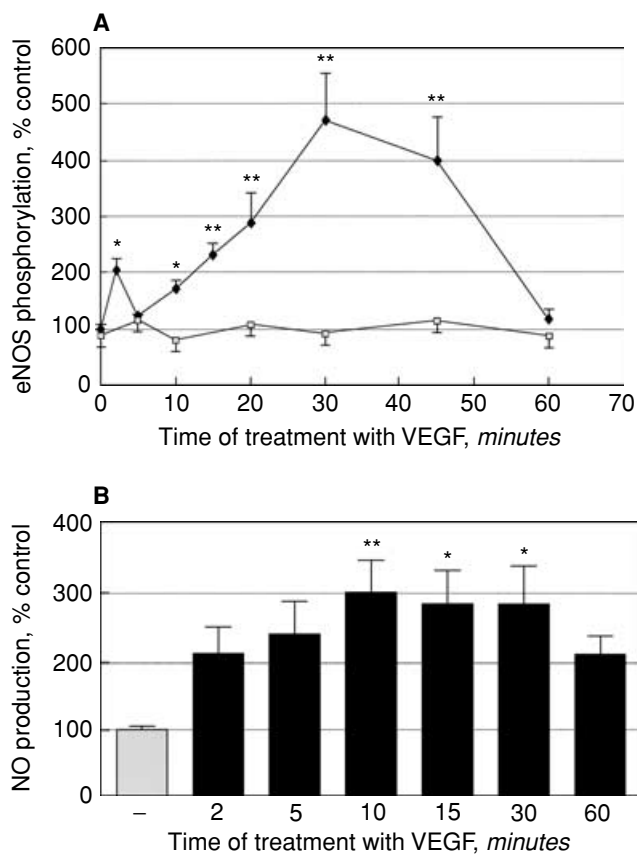


Fig. 5. Vascular endothelial growth factor (VEGF) induces phosphorylation of endothelial nitric oxide synthase (eNOS) and stimulates nitric oxide generation. (A) Serum-starved glomerular endothelial cells (GENCs) were treated with 20 ng/mL VEGF for up to 60 minutes. eNOS phosphorylation on Ser1177 was measured by in-cell Western blot analysis using a specific antibody as described in the Methods section. Quantification was performed by densitometric analysis of individual wells and was normalized for cell number in each well by actin immunoreactivity. eNOS phosphorylation is expressed as percentage of control (four experiments). ** $P < 0.01$; * $P < 0.05$ compared to control by analysis of variance (ANOVA). (B) Serum-starved GENCs were treated with 20 ng/mL VEGF for up to 60 minutes. The stable end product of nitric oxide, nitrite, was measured in the medium using Griess reaction. Medium total nitrite content corrected for protein content of the cell layer was estimated. Composite data from four experiments are shown relative to control (mean \pm SEM). ** $P < 0.01$; * $P < 0.05$ by analysis of variance (ANOVA).

GENCs by immunoblotting cell lysates with an antibody that only recognizes the phosphorylated form of the kinase. In quiescent GENCs, VEGF stimulated ERK phosphorylation, which peaked at 5 minutes (180% control) (three experiments) ($P < 0.01$ by ANOVA), and lasted for up to 15 minutes (Fig. 8A). In GENCs pretreated for 30 minutes with 50 μ mol/L of PD098059, an inhibitor of ERK kinase (MEK1/2), the direct upstream activator of ERK, VEGF failed to induce ERK phosphorylation at 10 minutes (Fig. 8B), indicating MEK was the upstream regulator of ERK and functional role of ERK in eNOS regulation in GENCs can be investigated using PD098059.

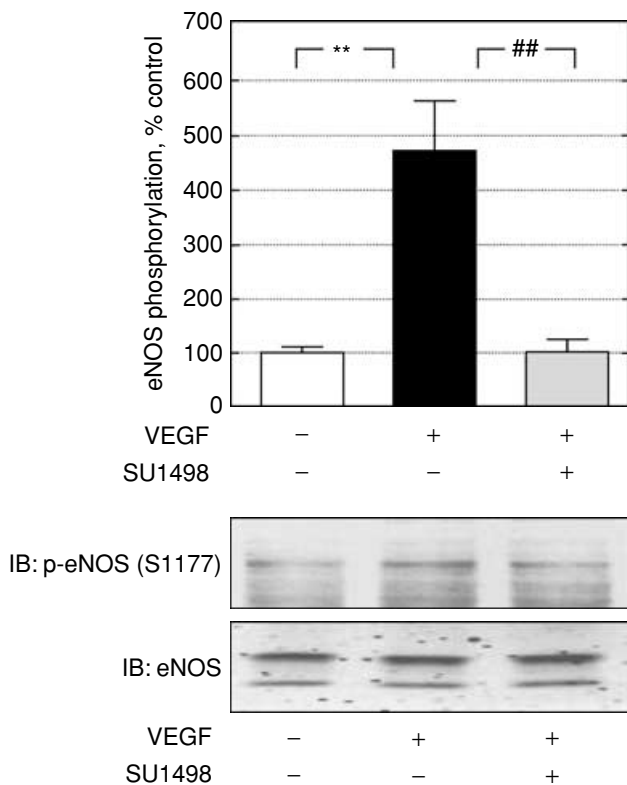


Fig. 6. Vascular endothelial growth factor (VEGF) stimulation of endothelial nitric oxide synthase (eNOS) phosphorylation is VEGF-receptor 2 (VEGF-R2)-dependent. (Upper) Glomerular endothelial cells (GENCs) were pretreated with a selective VEGF-R2 inhibitor, SU1498 (10 μ mol/L), for 30 minutes before incubation with 20 ng/mL VEGF for the indicated time. Phosphorylation of eNOS was detected by in-cell Western blot analysis as previously described. ** $P < 0.01$, VEGF vs. control; ## $P < 0.01$ VEGF vs. SU1498 + VEGF by analysis of variance (ANOVA). (Lower) Equal amounts of lysates from GENCs treated with or without VEGF (20 ng/mL) and preincubated with SU1498 (10 μ mol/L) for 30 minutes were immunoblotted (IB) with phospho-eNOS (p-eNOS) (ser1177)-specific antibody or antibody against total eNOS. A representative blot from three experiments is shown.

In order to assess the role of ERK in VEGF-induced eNOS phosphorylation, GENCs were pretreated with PD098059 for 30 minutes before stimulation with VEGF for 30 minutes. Figure 9A, upper panel, shows that PD098059 prevented VEGF-induced eNOS phosphorylation on Ser1177 at 30 minutes (three experiments) ($P < 0.01$ VEGF vs. PD09859 + VEGF). This result was confirmed by immunoblot at 30 minutes of treatment with VEGF (Fig. 9A, lower panel). These data show that inhibition of the MAPK pathway prevents eNOS phosphorylation on Ser1177, indicating ERK also mediates eNOS phosphorylation by VEGF.

As both PI3K and ERK pathways regulated eNOS phosphorylation, we investigated whether ERK was downstream of PI3K in GENCs treated with VEGF. Preincubation of GENCs with LY294002, the selective PI3K inhibitor abrogated VEGF-induced ERK

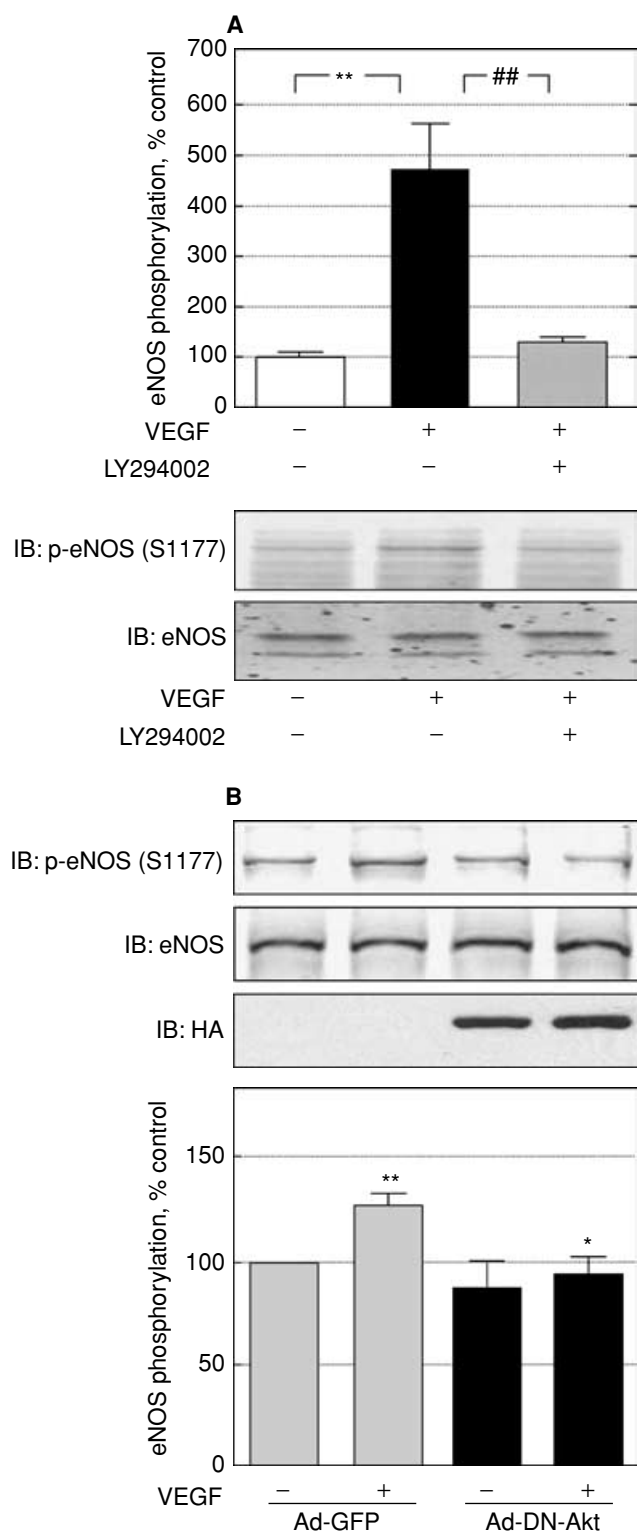


Fig. 7. Endothelial nitric oxide synthase (eNOS) phosphorylation induced by vascular endothelial growth factor (VEGF) is phosphatidylinositol 3'-kinase (PI3K)-dependent and Akt-dependent. (A) Glomerular endothelial cells (GENCs) were pretreated for 30 minutes with a selective PI3K inhibitor, LY294002 (25 μ mol/L), before stimulation with VEGF (20 ng/mL) for 30 minutes. Phosphorylation of eNOS was detected in-cell Western blot analysis as previously described (upper panel). ** $P < 0.01$ VEGF vs. control; ## $P < 0.01$ VEGF + LY294002 +

phosphorylation (Fig. 9B). These data suggest PI3K is upstream of ERK in VEGF-treated GENCs.

DISCUSSION

Our data demonstrate that VEGF promotes eNOS phosphorylation on Ser1177 in GENCs, resulting in enhanced generation of nitric oxide. Activation of eNOS by VEGF is dependent on recruitment of VEGF-R2, and activation of its downstream signaling events, including tyrosine phosphorylation of IRS-1 and activation of the PI3K/Akt signaling pathway. Additionally, VEGF also activates the ERK pathway in a PI3K-dependent manner. ERK is also involved in the control of eNOS phosphorylation on Ser1177. Thus, VEGF activates dual signaling pathways in GENCs (i.e., PI3K/Akt and PI3K/ERK), which converge on regulation of eNOS phosphorylation on Ser1177, resulting in activation of the enzyme (Fig. 10).

VEGF actions are mediated by VEGF-Rs, which function as tyrosine kinases. Our data show that GENCs in culture express VEGF-R1 and VEGF-R2, similar to glomerular endothelial cells in vivo. VEGF induced a robust and time-dependent phosphorylation of both VEGF-R1 and VEGF-R2 in GENCs. VEGF-R1 is implicated in macrophage migration and tumor angiogenesis [19, 20]. Most of the cellular actions of VEGF in endothelial cells (e.g., regulation of vascular permeability, fenestration of endothelial cells) are predominantly regulated by VEGF-R2 [21, 22]. Accordingly, we studied the role of VEGF-R2 in VEGF regulation of eNOS. VEGF time dependently induced tyrosine phosphorylation of VEGF-R2 in GENCs. Activated VEGF-R2 bound to IRS-1 and p85 regulatory component of PI3K. Association of IRS-1 with VEGF-R2 is not well known and is in agreement with our observations in renal nonendothelial cells [16]. In GENCs, VEGF promoted association between IRS-1 and PI3K via the p85 subunit of the latter. Our observations cannot determine whether PI3K binds to both VEGF-R2 and IRS-1 or solely to

VEGF by analysis of variance (ANOVA). Immunoblotting with phospho-specific and total eNOS antibodies was done in quiescent cells pre-incubated with LY294002 and incubated with VEGF for 30 minutes. A representative blot from three experiments is shown (lower panel). (B) GENCs were infected with an adenovirus carrying the dominant-negative construct of Akt (Ad-DN-Akt) or an adenovirus carrying green fluorescent protein (Ad-GFP) (as control) as described in the **Methods** section. eNOS phosphorylation on Ser1177 was measured by immunoblot (IB) using a phospho-specific antibody. Same cell lysates were assayed by immunoblot with anti-hemagglutinin (HA) antibody to assess infection efficiency. A representative blot from three independent experiments is shown (upper panel). The lower panel represents composite data of three independent experiments. Phosphorylation of eNOS is expressed as percentage of control (mean \pm SEM). ** $P < 0.01$ VEGF vs. control; * $P < 0.05$ Ad-GFP + VEGF vs. Ad-DN-Akt + VEGF by ANOVA.

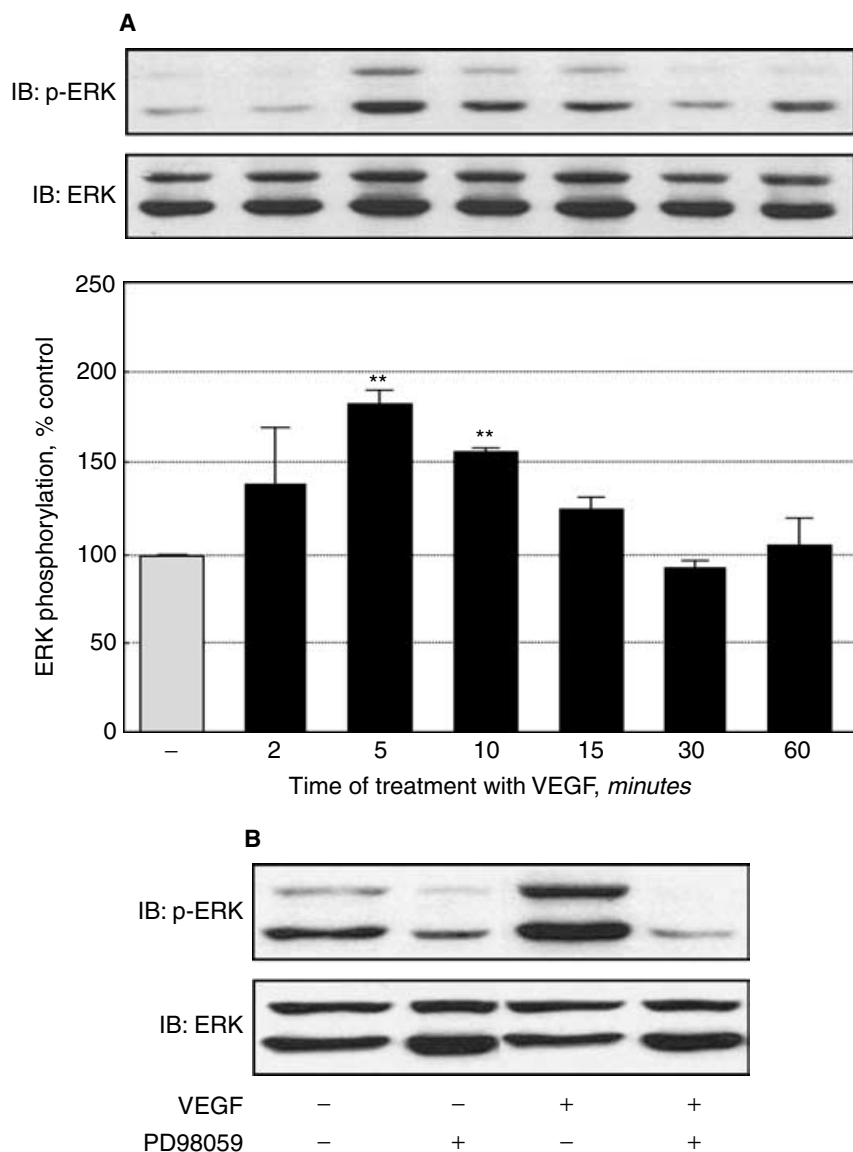


Fig. 8. Vascular endothelial growth factor (VEGF) stimulates extracellular signal-regulated protein kinase (ERK) phosphorylation. (A) Equal amounts of lysates (40 μ g) from serum-starved glomerular endothelial cells (GENCs) treated with or without VEGF (20 ng/mL) for various durations were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to nitrocellulose membrane, the membranes were probed the antibody against phospho-ERK (p-ERK). The membrane was stripped and reprobbed with an antibody against total ERK to assess loading. A representative blot [immunoblot (IB)] from three experiments is shown (upper panel). The lower panel represents densitometric analysis of VEGF-induced ERK phosphorylation from three independent experiments. ERK phosphorylation is expressed as percentage of control ($N = 3$) (mean \pm SEM). ** $P < 0.01$; * $P < 0.05$ compared with control, by analysis of variance (ANOVA). (B) GENCs were pretreated for 30 minutes with a selective ERK kinase (MEK) inhibitor, PD98059 (50 μ mol/L) before VEGF treatment. ERK phosphorylation was assessed by immunoblot as described earlier. A representative blot from three experiments is shown.

IRS-1. Recruitment of IRS-1 is likely to serve as a mechanism to amplify the signaling cascades downstream of VEGF-R2. Consonant with VEGF-stimulated binding of its p85 component with IRS-1, the activity of PI3K increased. VEGF also markedly stimulated the activity of Akt. As PI3K-independent pathways for Akt activation have been described in renal mesangial cells [17], whether prior activation of PI3K was required for Akt activation by VEGF was evaluated. LY294002, an inhibitor of PI3K, completely inhibited Akt activation by VEGF suggesting PI3K activation was needed for VEGF induction of Akt activity in GENCs.

eNOS was constitutively expressed by the GENCs, although the basal level of phosphorylation was minimal. VEGF increased ser1177 phosphorylation of eNOS within minutes and this was accompanied by prompt generation of nitric oxide. Ser1177 phosphorylation is critical

for eNOS activity [23, 24]. Incubation with LY294002, the PI3K inhibitor, or expression of dominant-negative mutant of Akt abrogated eNOS phosphorylation induced by VEGF. These data demonstrate that activation of PI3K-Akt axis is essential for VEGF activation of eNOS in GENCs.

We searched for additional signaling regulation of eNOS phosphorylation by VEGF. VEGF robustly induced activation of ERK in GENCs. There is indirect evidence that VEGF-induced ERK activation may be important in some functions of VEGF as upstream regulators of ERK, Raf, and Ras are involved in angiogenesis [25]. VEGF-induced ERK activation may be important for endothelial cell proliferation [1], which may be seen in physiologic and pathologic states of the kidney. Although ERK is also a serine kinase, there are conflicting reports on its regulation of ser1177 phosphorylation of eNOS.

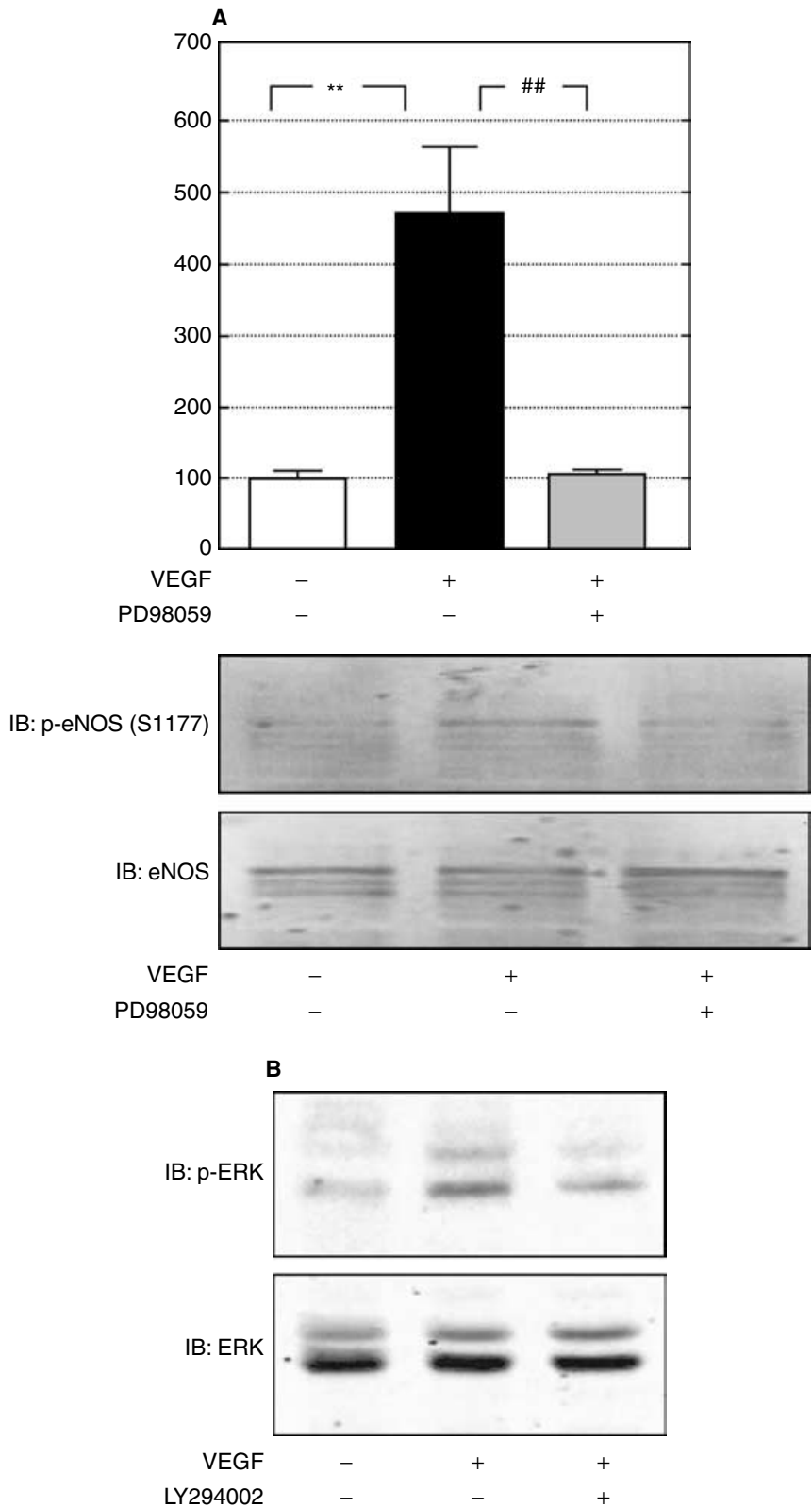


Fig. 9. Phosphorylation of endothelial nitric oxide synthase (eNOS) induced by vascular endothelial growth factor (VEGF) is extracellular signal-regulated protein kinase (ERK) kinase (MEK)/ERK dependent. (A) Glomerular endothelial cells (GENCs) were pretreated for 30 minutes with PD098059 (50 $\mu\text{mol/L}$) before VEGF treatment for 30 minutes. Phosphorylation of eNOS was detected by in-cell Western blot analysis as previously described (upper panel). $**P < 0.01$, VEGF vs. control; $##P < 0.01$, VEGF vs. PD098059 + VEGF by analysis of variance (ANOVA). Immunoblot (IB) analysis using the same antibody of quiescent cells preincubated with PD098059 and incubated with VEGF for 30 minutes (lower panel). A representative blot from three experiments is shown. (B) GENCs were pretreated for 30 minutes with selective phosphatidylinositol 3'-kinase (PI3K) inhibitor, LY294002 (25 $\mu\text{mol/L}$), before stimulation with VEGF (20 ng/mL) for 10 minutes. Phosphorylation of ERK was detected as previously described in Fig. 8.

Examination of eNOS sequence revealed presence of consensus sites for ERK phosphorylation. In bovine aortic endothelial cells treated with bradykinin, ERK phosphorylates eNOS, likely involving sites other than

ser1177; however, this results in reduction in its activity [18]. Other investigators have reported that ERK inhibitors do not affect nitric oxide generation or nitric oxide-mediated relaxation of isolated arteries [26, 27].

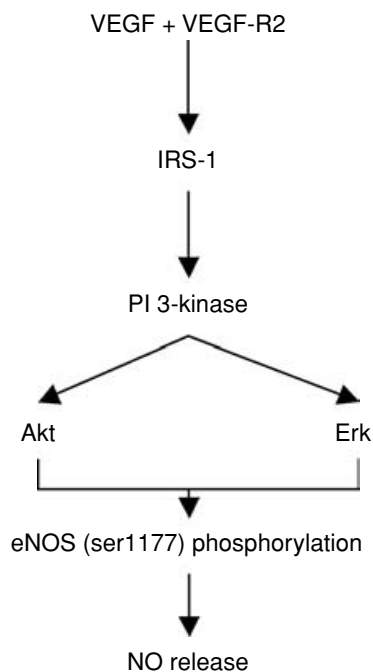


Fig. 10. A schematic showing signaling pathways that regulate endothelial growth factor (eNOS) phosphorylation on ser1177 induced by vascular endothelial growth factor (VEGF) in glomerular endothelial cells (GENCs). Abbreviations are: IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated protein kinase; NO, nitric oxide.

ERK has been implicated in eNOS activation by high-density lipoprotein (HDL) which does not seem to involve ser1177 phosphorylation of eNOS [28]. In uterine arterial endothelial cells, ERK inhibitors have been reported to partially decrease ser1177 phosphorylation of eNOS, suggesting a role for ERK in this process [29]. As these data suggested that role of ERK in eNOS phosphorylation may vary with endothelial cell origin, we examined this issue in the GENCs. VEGF robustly induced phosphorylation of ERK. PD098059, the inhibitor of MEK, an upstream activator of ERK, abolished VEGF-induced ser1177 phosphorylation of eNOS, suggesting a role for ERK. As both PI3K and ERK stimulated ser1177 phosphorylation of eNOS, we studied their interaction. ERK activation was found to be downstream of PI3K. This is similar to our previous finding in proximal tubular epithelial cells treated with insulin [30]. These observations and reports in the literature suggest that data on eNOS regulation cannot be extrapolated from one endothelial cell type to another. Recent microarray analysis supports this notion; GENCs from bovine kidney possess a distinct gene expression profile compared to nonrenal endothelial cells [31].

Since phosphorylation of eNOS on ser1177 is critical for activation of the enzyme, convergence of two signaling pathways (MAPK and PI3K) on this residue may have dual significance. First, the balance between the two sig-

nals may allow fine-tuning of eNOS activation. Additionally, the redundancy of the two signaling pathways ensures that eNOS phosphorylation and activation is maintained even in situations in which one of them is impaired. This redundancy will thus ensure access to eNOS activation and nitric oxide generation.

The functional significance of eNOS activation can be manifold. eNOS is known to regulate angiogenesis [32, 33]. Some of these effects may be involved in healing effects of VEGF reported in thrombotic microangiopathy and remnant kidney models of renal disease [34–36]. Nitric oxide generated by eNOS inhibits adhesion of leukocytes to the endothelium and its dysregulation may be involved in pathogenesis of glomerulonephritides [37]. VEGF system plays an important role in pregnancy. Excessive placental production of soluble ectodomain of VEGF-R1 leads to removal of VEGF from circulation; the resulting deficiency of VEGF has been implicated in endotheliolysis seen in preeclampsia [38]. These observations show that VEGF is needed for glomerular endothelial integrity in normal pregnancy. In contrast to the salutary effects of VEGF physiologic state of pregnancy and in inflammatory and remnant kidney models of renal disease, the growth factor may have a pathogenic role in diabetic renal disease. Increase in VEGF coincides with renal cortical growth in mice with type 1 or type 2 diabetes and *in vitro* observations suggest VEGF may contribute to renal hypertrophy by promoting protein synthesis in proximal tubular epithelial cells [10]. Administration of anti-VEGF antibodies results in amelioration of proteinuria and renal growth in animals with type 1 or type 2 diabetes [39, 40]. Additionally, podocyte-specific overexpression of VEGF results in collapsing glomerulopathy [7].

Thus, it appears that the beneficial or harmful effects of VEGF on the kidney depend on the context of renal injury. Understanding different signaling pathways employed by VEGF in activation of eNOS may permit modulation of cellular processes to benefit the kidney and assist its recovery in pathologic states.

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