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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. $VEGF_{165}$, 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 radioimmunoprecipation 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 μg/mL aprotinin, and 1 μg/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 μg 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 × 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 × 5 min in PBS containing 0.1% Tween. The plate was then scanned using the Odyssey Infrared Imaging System (169 μm 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