Glomerular endothelial PI3 kinase-α couples to VEGFR2, but is not required for eNOS activation

Qiu-Xia Zhang,1 Maryam Nakhaei-Nejad,1 George Haddad,1 Xuemei Wang,2 Rodger Loutzenhiser,2 and Allan G. Murray1

1Department of Medicine, University of Alberta, Edmonton; and 2Department of Pharmacology and Therapeutics, University of Calgary, Calgary, AB

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Zhang QX, Nakhaei-Nejad M, Haddad G, Wang X, Loutzenhiser R, Murray AG. Glomerular endothelial PI3 kinase-α couples to VEGFR2, but is not required for eNOS activation. Am J Physiol Renal Physiol 301: F1242–F1250, 2011. First published September 21, 2011; doi:10.1152/ajprenal.00662.2010.—Vascular endothelial growth factor (VEGF)-dependent signals are central to many endothelial cell (EC) functions, including survival and regulation of vascular tone. Akt and endothelial nitric oxide synthase (eNOS) activity are implicated to mediate these effects. Dysregulated signaling is characteristic of endothelial dysfunction that sensitizes the glomerular microvasculature to injury. Signaling intermediates that couple VEGF stimulation to eNOS activity remain unclear; hence, we examined the PI3 kinase isofoms implicated to regulate these enzymes. Using a combination of small molecule inhibitors and RNAi to study responses to VEGF in glomerular EC, we observed that the PI3 kinase p110α catalytic isoform is coupled to VEGFR2 and regulates the bulk of Akt activity. Coimmunoprecipitation experiments support a physical association of p110α with VEGFR2. Downstream, Akt-mediated FOXO1 phosphorylation in EC is regulated by p110α. The p110β isoform contributes a minor amount of VEGF-stimulated Akt activation. However, we observe no effect of p110α or p110β to regulate VEGF-stimulated eNOS activation via Akt-mediated phosphorylation on eNOS Ser1177, or NO-mediated vasodilation of the afferent arteriole ex vivo. VEGFR2-stimulated eNOS activation and NO production are inhibited by Compound C, an inhibitor of AMP-stimulated kinase, independent of PI3 kinase signaling. PI3 kinase-α/β-mediated signaling downstream of VEGFR2 activation regulates Akt-dependent survival signals, but our data suggest it is not required to activate eNOS or to elicit NO production in glomerular EC.

kidney glomerulus; endothelium vascular; vascular endothelial growth factor receptor; signal transduction; phosphatidylinositol 3 kinase; nitric oxide synthase type III

Vascular endothelial growth factor (VEGF) participates in vascular development in the embryo and maintenance of the established vasculature in the adult. Knockout mouse models, for example, demonstrate that VEGF provides a critical cue for angiogenesis, since absence of VEGF or the receptor, VEGF receptor-2 (VEGFR2), is lethal early in embryonic development (6, 7, 38). The kidney glomerular microvascular bed is notably dependent on VEGF signaling. Loss of paracrine VEGF signaling from podocytes to the glomerular endothelium aborts glomerular development and leads to early postnatal death (10). In the adult, the constitutive production of VEGF in the glomerulus is tightly regulated to maintain the specialized endothelial structure and function of the fenestrated endothelium (10). Acquired loss of VEGF signaling or responsiveness is proposed to contribute to glomerular injury in the setting of diabetes and other microvascular disease (31–33, 44).

Stimulation of endothelial cells (EC) by VEGF initiates signals through PI3 kinase activity to promote cell survival and motility required for angiogenesis (16). Considerable experimental evidence has accumulated to indicate that the VEGFR2 tyrosine kinase recruits this signaling pathway (17, 22, 43). However, an alternate receptor, VEGFR1, has also been demonstrated to mediate signals to elicit PI3 kinase activity (2).

PI3 kinases are a family of lipid kinases that include three catalytic isoforms, p110 α, β, δ, that associate with a regulatory subunit, e.g., p85α, to couple PI3 kinase activity to receptor tyrosine kinase activation (13). Recent genetic evidence implicates the p110α isoform as central to EC function, since mice lacking p110α selectively in the endothelium have defective EC motility, fail to develop a normal vasculature, and die in early gestation (18). These effects on angiogenesis, combined with characteristic PI3 kinase pathway activation among numerous cancers, prompted interest in the development of inhibitory compounds for clinical use (26). However, full understanding of the differential roles of these isoforms in the endothelium is lacking.

Akt, a serine/threonine kinase, has been identified as a major downstream effector molecule following PI3 kinase activation, and it has been implicated to couple endothelial production of nitric oxide (NO) to PI3 kinase activation by phosphorylation of endothelial NO synthase (eNOS) on Ser1177, and hence to increase eNOS responsiveness to intracellular calcium (15, 29). NO, elaborated from EC by regulated activity of the eNOS enzyme, increase eNOS responsiveness to intracellular calcium (15, 29). PARMI

Address for reprint requests and other correspondence: A. G. Murray, Rm 260F HMRC, Univ. of Alberta, Edmonton, AB, Canada T6G 2S2 (e-mail: allan.murray@ualberta.ca).
tion of VEGF-stimulated p110α activity failed to affect production of NO by human EC. p110α contributes to regulation of Akt activity in a partially redundant fashion, but it does not regulate eNOS phosphorylation. Consistent with these data, broad-spectrum inhibition of PI3 kinase activity in the intact rat kidney microcirculation does not affect VEGF-stimulated vasodilation. In contrast, treatment with Compound C, an inhibitor of AMP-stimulated kinase, blocked VEGF-stimulated eNOS phosphorylation and NO production. These data indicate that PI3 kinase-mediated Akt signaling is dispensable for VEGF-stimulated endothelial NO production in human EC, and they suggest that Compound C-sensitive kinase activity is the dominant regulator of microvascular eNOS activity and NO production.

MATERIALS AND METHODS

Reagents. M199, RPMI, HBSS, FBS, and ECGS were from Invitrogen (Burlington, ON); VEGF-A was from R&D Systems (Minneapolis, MN). The following antibodies were purchased: phospho-AKT Ser473, phospho-FOXO1 Ser319, FOXO1 (Cell Signaling Technology, Danvers, MA); VEGFR1 (R&D Systems); VEGFR2 (Cell Signaling Technology); AKT, PI3-Kinase p110α, PI3-Kinase p110β, PI3-Kinase p110δ (Santa Cruz Biotechnology); p85, tubulin-α (Militare, Temeucula, CA); PI3-Kinase p110α (BD Biosciences, San Jose, CA). The following inhibitors were used PI3 kinase-δ inhibitor IC87114 (Symansis, Auckland, New Zealand) at 10 μM for 30 min, and Compound C (EMD Chemicals, Gibbstown, NJ) at 20 μM for 30 min. DAF-2 (Cayman Chemical, Ann Arbor, MI), L-NAME, and other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described (30). Human GEC were purchased from Angio-Proteomie (Boston, MA) and subcultured twice before use in an experiment. Before stimulation with VEGF, cells were cultured in 1% FBS overnight and then without FBS during VEGF stimulation.

Flow cytometry. HUVEC and hGEC were harvested using nonenzymatic cell dissociation solution (Sigma) and then immunostained using anti-VEGFR1 or anti-VEGFR2 or species-matched control antibody. Fluorescence intensity of the cells was analyzed as previously described (30).

RNA interference. To optimally knockdown protein expression, HUVECs at ~50% confluence were transfected twice on consecutive days with specific siRNA (Qiagen, Mississauga, ON) targeting either irrelevant GFP, p110α (10 or 50 nM; 5'-CTCCGTGAGGCTACAT-3'), or KDR (50 nM; 5'-AAGCTGACATGTCCTGA3') sequences, using Hipfect according to the manufacturer's direction (Qiagen). Treatment of the EC with siRNA against either p110α sequence gave similar results; hence, the results were pooled for analysis. KDR and PI3 kinase p110α were optimally inhibited 72 h after the first transfection as tested by Western blot as described previously (34).

Western blot. EC monolayers were lysed using buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM Na2VO3, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF), with added proteinase cocktail and phosphatase inhibitor cocktail I or III (Sigma). Fifty micromolars of lysisate were resolved on SDS-PAGE and then blotted onto nitrocellulose (Bio-Rad) as described. Where indicated, immunoprecipitation was done using protein G sepharose beads (Sigma) and then washed extensively before loading on SDS gels. The membranes were immunoblotted for phosphorylated proteins overnight at 4°C in 5% BSA. TBS-Tween 20 blocking solution and then proteins were visualized using ECL (GE Life Sciences, Baie d’Urfe, PQ). The membranes were stripped using Restore buffer (Thermo Scientific, Rockford, IL) and reprobed for the total protein.

PI3 kinase assay. Confluent EC monolayers were serum starved and then stimulated with VEGF-A at 20 ng/ml for 10 min. The cells were lysed in 20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM Na2VO3, and then p85 was immunoprecipitated as above. The pellets were assayed for PI3 kinase activity in vitro by ELISA according to the manufacturer's instructions (kit K-1000s, Echelon, Salt Lake City, UT).

NO assay. NO was assayed as previously described (23). Confluent EC monolayers were preincubated with L-arginine (100 μM, 5 min) and then washed with PBS and incubated with DAF-2 (0.1 μM) and stimulated with VEGF as indicated. The supernatant fluorescence at 515 nm was quantitated by fluorimeter (DTX 880, Beckman) using the appropriate filter set. Where indicated, cells were pretreated with L-NAME (1 mM, 30 min) to inhibit eNOS activity.

Evaluation of afferent arteriolar tone. The effect of VEGF on afferent arteriolar tone was studied using the ex vivo perfused hydro-nephrotic kidney as described (41). In brief, the renal artery was cannulated in situ and the kidney was perfused with DMEM containing 30 mM bicarbonate, 5 mM glucose, 5 mM HEPES, and 10 μM ibuprofen equilibrated with 95% air-5% CO2 and then transferred to a microscope stage and maintained at 37°C. The afferent arteriole was directly visualized using a fibroptic probe, and the diameter of the arteriole was monitored by video microscopy. The afferent arteriole was preconstricted using angiotensin II (AII; 0.1 μM) and then pretreated with L-NAME (100 μM), L-NAME (10 μM), or carrier before VEGF (0.1 mM) was introduced.

Statistical evaluation. Results are tested for statistical significance (P < 0.05) by ANOVA for multiple comparisons. Pairwise comparisons were tested using paired t-test. The data are shown as means ± SE.

RESULTS

Endothelial PI3 kinase activity is elicited after VEGF stimulation; hence, we sought to define which catalytic isoform is coupled to the VEGF receptor in primary human EC. We report experiments using HUVECs and primary human microvascular glomerular EC (hGEC). The hGEC express both VEGFR1 and VEGFR2 at similar levels to HUVEC as assessed by flow cytometry (Fig. 1). The first series of experiments was to establish whether PI3 kinase activity was coupled to VEGF signaling in EC via VEGFR1 or VEGFR2. Since placental growth factor (PIGF) is known to signal through VEGFR1 (8), we assessed PIGF-stimulated effects on phosphorylation of Akt on residue Ser473, a known target of PI3-Kinase p110α (Miltenyi Biotec, Bergkamen, Germany). Treatment of the EC with siRNA against either p110α sequence gave similar results; hence, the results were pooled for analysis. KDR and PI3 kinase p110α were optimally inhibited 72 h after the first transfection as tested by Western blot as described previously (34).

Western blot. EC monolayers were lysed using buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM Na2VO3, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF), with added proteinase cocktail and phosphatase inhibitor cocktail I or III (Sigma). Fifty micromolars of lysisate were resolved on SDS-PAGE and then blotted onto nitrocellulose (Bio-Rad) as described. Where indicated, immunoprecipitation was done using protein G sepharose beads (Sigma) and then washed extensively before loading on SDS gels. The membranes were immunoblotted for phosphorylated proteins overnight at 4°C in 5% BSA. TBS-Tween 20 blocking solution and then proteins were visualized using ECL (GE Life Sciences, Baie d’Urfe, PQ). The membranes were stripped using Restore buffer (Thermo Scientific, Rockford, IL) and reprobed for the total protein.
The PI3 kinase p110α catalytic isoform has been identified to mediate signaling in EC for vascular development (18). Hence, we sought to determine whether p110α directly associates with VEGFR2. Previous work identified the association of the p85 regulatory subunit of PI3 kinase with VEGFR2 after VEGF stimulation; therefore, p85 was used as a comparator (9). We observed that p110α could be precipitated in a complex with VEGFR2 under resting conditions (Fig. 2D). Moreover, both p110α and p85 association with VEGFR2 increased within minutes of stimulation of the EC with VEGF. Furthermore, p110α was incorporated in a complex with VE-cadherin after VEGF stimulation (data not shown). Taken together, these data are consistent with physical coupling of p110α and p85 with VEGFR2 in an activation complex in EC.

To determine which catalytic isoform of PI3 kinase is functionally coupled downstream of VEGFR2, we first used

Fig. 2. VEGFR2 is functionally coupled to PI3 kinase activity in endothelial cells (EC) and physically associates with the PI3 kinase catalytic isoform, p110α. A: HUVEC or hGEC were transfected with nonsilencing siRNA, or siRNA against KDR, the VEGFR2, and then lysates were immunoblotted for VEGFR1 and VEGFR2 expression. B: si KDR-transfected HUVEC were stimulated with VEGF-A (20 ng/ml) for the indicated time, and then the cells were lysed and immunoblotted for AktS473 phosphorylation. Blots were stripped and reprobed for total Akt. C: quantitation of AktS473 phosphorylation in hGEC pretreated with control siRNA or siRNA against KDR and then stimulated with placental growth factor (PIGF) or VEGF as indicated (means ± SE; n = 4 independent experiments. *P < 0.05 vs. unstimulated hGEC; **P < 0.05 vs. VEGF-stimulated, nonsilenced hGEC). D: HUVEC were treated with VEGF-A, and then VEGFR2 was immunoprecipitated, and associated p85 and p110α were resolved by SDS-PAGE and immunoblot. D, bottom: quantitation of p85 and p110α pulled down with VEGFR2 after stimulation with VEGF for the indicated time (means ± SE; n = 3 experiments. *P < 0.05 vs. unstimulated).
RNAi to knock down p110α expression. On Western blot of whole cell EC lysates, we detected a weak band for p110α, but did not find p110β or p110δ (data not shown). In contrast, immunoprecipitation of the p85 regulatory subunit of PI3 kinase enriched for all three class IA catalytic isoforms, p110α, -β, and -δ (Fig. 3A). Knockdown of p110α expression using either of two siRNA sequences resulted in >90% depletion of p110α levels and had little effect on the expression of either the -β or -δ isoform, inducing at most a small increase in expression of each.

VEGF stimulation of EC pretreated with nonsilencing siRNA results in robust phosphorylation of Akt on Ser473 (Fig. 3B). In contrast, Akt activation was markedly blunted by knockdown of p110α expression, both in resting cells and after VEGF stimulation. We evaluated FOXO1 phosphorylation, a target of the Akt kinase, to determine the effect of p110α knockdown on Akt activity. FOXO1 phosphorylation in control EC was stimulated, like Akt, after VEGF stimulation (Fig. 3B). However, we observed little FOXO1 phosphorylation downstream of Akt in p110α-deficient EC. This indicates that the limited activation of Akt seen in p110α-deficient cells was inadequate to propagate a measurable signal downstream to FOXO1.

To directly confirm that VEGF stimulation elicits activation of p110α, we immunoprecipitated the p85 regulatory subunit in resting and VEGF-stimulated EC and assayed PI3 kinase activity. We observed that knockdown of p110α almost completely blocked VEGF-stimulated PI3 kinase activity (Fig. 4A). In contrast, we did not detect a significant contribution of p110β or p110δ to VEGF-stimulated PI3 kinase activity. Taken together, these data indicate that VEGF stimulation of VEGFR2 results in association of p85 and p110α with the receptor and increased p110α-dependent PI3 kinase activity sufficient to propagate downstream signals.

However, since p110α knockdown did not completely block either resting or VEGF-stimulated Akt phosphorylation, we sought to determine whether p110β or p110δ contributes to Akt regulation. Akt phosphorylation became evident in p110α-deficient EC stimulated with VEGF for more than 10 min; hence, we examined this time point for evidence of p110β or p110δ activity. PI3 kinase p110δ inhibition with the selective p110β inhibitor, IC87114, blunted phosphorylation of Akt (Fig. 4, C and D) but p110δ appeared to contribute much less activity than p110α. We observed no additive effect of p110β or p110δ inhibition on p110α knockdown (data not shown). This indicates that p110δ contributes a minor component to the regulation of Akt activity independent of p110α in hGEC.

PI3 kinase activity has previously been implicated as a key regulatory step in eNOS activation, because eNOS is a substrate of Akt for in vitro phosphorylation, and expression of dominant-negative Akt mutant protein inhibits NO production (15, 27). Like Akt, eNOS activity is dependent on VEGFR2 activation, since RNAi-mediated knockdown of VEGFR2 in hGEC prevented eNOS phosphorylation. Surprisingly, despite marked inhibition of VEGF-stimulated Akt and downstream FOXO1 activation by p110α knockdown, we observed no decrease in eNOS phosphorylation on Ser1177 (Fig. 5).

To evaluate signaling mediated through p110δ, we tested the effect of selective p110δ inhibition on control or p110α-deficient EC. In this series of experiments, we observed that the p110δ inhibitor modestly blunted the VEGF-stimulated phosphorylation of Akt (Fig. 5, A–C), but had no effect on eNOS phosphorylation (Fig. 5, D and E). Treatment of p110α-deficient EC with the p110δ inhibitor blocked phosphorylation of Akt similar to p110α knockdown alone, whereas eNOS displayed robust phosphorylation (data not shown). These data suggest that VEGF-stimulated PI3 kinase signaling is not required for eNOS activation in microvascular hGEC.

Other serine-threonine kinases, including AMPK-stimulated kinase (AMPK), have also been recognized to phosphorylate eNOS Ser1177 in vitro (29). As shown in Fig. 5, pretreatment of hGEC with Compound C blocked VEGF-stimulated eNOS phosphorylation more effectively than p110α deficiency with p110δ inhibition. The combination of all three interventions was no more effective than treatment of the EC with Compound C alone (data not shown).

Previous work suggested AMPK can regulate Akt activation (25, 42); hence, in these experiments we also monitored Akt

Fig. 3. p110α mediates VEGF-stimulated PI3 kinase activity. A: HUVEC were transfected with nonsilencing siRNA, or siRNA against p110α, stimulated with VEGF-A for 10 min, and then the PI3 kinase regulatory subunit p85 was immunoprecipitated and PI3 kinase p110 catalytic subunits were resolved by SDS-PAGE and immunoblotted. B: control or p110α siRNA-treated HUVEC were stimulated with VEGF-A, and then lysates were resolved and phosphorylated Akt (Thr308) and total Akt, or the Akt substrate phospho-FOXO1 Ser319 and total FOXO1, were immunoblotted. Quantification of phospho-Akt/Akt and phospho-FOXO1/FOXO1 (bottom: means ± SE; n = 4 experiments. *P < 0.05 si p110α vs. nonsilenced).
phosphorylation on Ser473. In contrast to the marked inhibitory effect of p110α deficiency, Compound C had a modest effect on Akt activation (Fig. 5, A–C). We detected no additive effect to further inhibit Akt phosphorylation when Compound C is added to blocked p110α and p110δ signaling (data not shown). Together, these observations suggest that Compound C inhibition of eNOS and Akt activation can be dissociated from the PI3 kinase pathway.

Finally, we sought to directly determine the effect of interruption of PI3 kinase signaling on glomerular microvascular endothelial NO production. Consistent with the lack of an effect on eNOS phosphorylation, we observed no inhibition of endothelial NO production by p110α-deficient EC. The p110δ inhibitor, used alone or in combination with p110α knockdown, did not reproducibly affect NO production in either HUVEC or microvascular hGEC, consistent with the effect on eNOS phosphorylation. In contrast, Compound C-mediated serine-threonine kinase inhibition blocked VEGF-stimulated NO production, consistent with Compound C-mediated inhibition of eNOS phosphorylation.

To extend these observations to the intact kidney microcirculation, we tested the effect of PI3 kinase inhibition on VEGF-mediated vasodilation of the glomerular afferent arteriole. ALL-mediated vasoconstriction of the afferent arteriole was completely reversed by VEGF stimulation in a NO-dependent fashion. Pretreatment of hGEC in vitro or the renal microvasculature ex vivo with the broad-spectrum PI3 kinase inhibitor, LY294002, to block PI3 kinase-α, -β, and -δ isoforms, blunted ALL-stimulated vasoconstriction by ~30% (Fig. 6C), but failed to inhibit VEGF-stimulated eNOS phosphorylation or arteriolar vasodilation (Fig. 6). These data suggest that regulation of renal microvascular tone by VEGF is not critically dependent on PI3 kinase activity.

**DISCUSSION**

VEGF stimulation of endothelial cells cues both endothelial survival and NO production. In the glomerular microvasculature, the endothelium receives signals from constitutive VEGF production by the podocyte. The development of new pharmaceuticals such as VEGF receptor inhibitors and isoform-selective PI3 kinase inhibitors for the management of cancer suggests the kidney microvasculature may be particularly vulnerable to interruption of these signaling pathways from the use of these agents (26). Conversely, since VEGF-stimulated NO production appears to be critical to defend the glomerular microcirculation against injury, modulation of PI3 kinase-dependent signaling may present therapeutic opportunities to augment glomerular endothelial function in diseases such as diabetic nephropathy. Hence, we sought to better characterize the signal transduction events that couple VEGF stimulation to NO production in GEC.

VEGFR2 signaling is sufficient to drive mitogenesis, but the contribution of the VEGFR1 to the VEGF receptor complex responsible for signal transduction in native EC is not well-defined. VEGFR1 and VEGFR2 are known to heterodimerize after VEGF stimulation and cross-phosphorylate cytoplasmic domain tyrosine residues (3). Previous work shows selective VEGFR1 ligation can stimulate PI3 kinase activation and eNOS phosphorylation when expressed in nonendothelial cell types (2, 20), whereas in a similar heterologous cell reconstitution system, Blanes et al. (4) demonstrate phosphorylation of Y801 on VEGFR2 couples VEGF stimulation to Akt and eNOS. We observed little Akt phosphorylation after stimulation with the VEGFR1-selective agonist PlGF, suggesting VEGFR2 mediates signaling on this pathway in HUVEC and
primary microvascular GEC. Furthermore, we observe that p110α is recruited to a complex with VEGFR2. Finally, knockdown of VEGFR2 expression blocked VEGF-stimulated Akt phosphorylation and eNOS activation. Since this intervention had no effect on VEGFR1 expression or phosphorylation, we conclude that in EC expressing endogenous levels of VEGFR1, this receptor is dispensable for signaling to the Akt pathway.

The current studies identify the PI3 kinase isoform coupled to VEGFR2 to regulate Akt activation and highlight complexity in downstream control of NO production. The p110α, p110β, and p110δ catalytic isoforms are associated with the p85 regulatory subunit in resting EC, but the p110α isoform of PI3 kinase is the principal mediator of a survival signal, through Akt to forkhead transcription factors upon VEGF stimulation. Knockdown of p110α lowered the resting level of phosphorylated Akt and markedly blunted VEGF-stimulated Akt phosphorylation. This is consistent with observations from the endothelial-restricted p110α knockout mouse model that was lethal at day 11 in embryonic development from vascular anomalies attributed to disordered EC migration (18, 24). Our data indicate that these consequences of p110α-mediated signaling in the EC are distinct from the role of endothelial NO production, which also participates in angiogenesis (14). The p110α knockout mice died before the kidney microcirculation formed from angioblasts in the metanephric mesenchyme; hence, there are no direct observations of the effect of p110α deficiency on glomerular development in vivo. However, our data predict that p110α will mediate VEGF defense of the renal microvasculature against apoptosis during development and in disease of the adult (1). The p110δ isoform appears to contri-
ute a minor redundant signal to Akt phosphorylation, but the functional role of this is unclear.

The activity of eNOS, while dependent on intracellular calcium (36), is thought to be largely regulated by eNOS phosphorylation (15). Previous work that implicates PI3 kinases in the regulation of eNOS phosphorylation relied on the use of small molecule inhibitors (15, 42) that lack specificity (21) or overexpression of dominant-negative Akt protein (15). This is the first report of genetic PI3 kinase isoform loss-of-function experiments to determine the contribution of this pathway to eNOS regulation. Surprisingly, when we examined eNOS activation after VEGF stimulation, we found that neither p110α- nor p110β-dependent signaling accounted for eNOS Ser1177 phosphorylation. Furthermore, we observed that neither p110α nor p110β activity was required to generate NO in either VEGF-stimulated GEC or HuVEC, using two independent target sequences for the siRNA design, and a well-characterized isoform-specific inhibitor (21). PI3K p110α knockdown does not appear to have measurably altered the remaining isoforms. Therefore, PI3 kinase-dependent signaling to Akt does not appear to contribute to regulation of eNOS in glomerular endothelial cells, and it suggests VEGF regulates the renal microcirculation independent of endothelial PI3 kinase activity.

We directly tested this prediction in the ex vivo perfused rat kidney. We observed that VEGF mediates potent NO-dependent vasodilation of the glomerular afferent arteriole and is able to completely overcome vasoconstriction driven by high physiologic concentrations of AII (35). Blockade of PI3 kinase activity failed to inhibit the VEGF-mediated vasodilation. Taken together, we conclude the adult renal microvascular endothelium, although dependent on VEGF for survival cues mediated by PI3 kinase-α and perhaps -β, is not critically dependent on this same signal transduction pathway for NO production and regulation of microvascular tone.

Several serine threonine kinases have been identified to mediate eNOS Ser1177 phosphorylation in vitro, including Akt, protein kinase A, protein kinase G, and calmodulin kinase II (5, 12, 28). Several papers implicate AMP-activated kinase as a critical signaling element to regulate eNOS phosphorylation and endothelial NO production (29, 42). However, Stahmann et al. (40) recently used small molecule inhibitors and RNAi approaches to establish that neither calmodulin kinase II nor the α1 or α2 alternative catalytic subunits of AMP-stimulated kinase regulate eNOS phosphorylation or endothelial NO production. Compound C, a commonly used inhibitor of AMP-stimulated kinase, blocked both eNOS phosphorylation and NO production in microvascular GEC, indicating a kinase targeted by this inhibitor lies downstream of VEGFR2 to regulate eNOS activity. Our data suggest that this pathway is not dependent on VEGF-stimulated PI3 kinase signaling.

The kinase inhibited by Compound C may be acting directly on eNOS, or indirectly upstream of the eNOS kinase. We observed a modest inhibitory effect of Compound C on Akt Ser473 phosphorylation and eNOS Ser1177 in EC. The mTORC2 has been identified to phosphorylate Akt on Ser473 (37, 39), and in turn regulate Akt activity (19). Hence, Compound C appears to partially inhibit mTORC2, but there is no direct evidence that mTORC2 regulates eNOS. These observations are at odds with an earlier report studying GECs derived from the SV40 T antigen transgenic mouse (11). The author’s conclusion that eNOS activation was dependent on PI3 kinase activity was based on inhibition of eNOS phosphorylation by expression of a dominant-negative Akt mutant. This observation is compatible with a model of mTORC2-regulated, Akt-dependent eNOS phosphorylation, but it may be confounded by the effect of SV40 T antigen on this signal transduction pathway if the thermolabile variant used was not silenced.
In summary, the current data demonstrate that both PI3 kinase-α and -δ are coupled downstream of VEGFR2 stimulation in glomerular microvascular EC. Inhibition of either isoform alone allows residual activation of Akt and potentially transduction of a survival signal to the glomerular endothelium, although the p110α isoform mediates most of this activity. Interruption of VEGF-stimulated PI3 kinase and downstream Akt activity revealed PI3 kinase-independent regulation of eNOS phosphorylation in glomerular EC. The observations draw attention to investigation of PI3 kinase-independent approaches to reverse deficient endothelial NO production that contributes to microvascular glomerular pathology. Conversely, these observations imply that selective inhibition of PI3 kinase-α, as a component of adjuvant chemotherapy, for example, is likely to reduce EC resistance to noxious injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


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