EGFR-PLCγ1 signaling mediates high glucose-induced PKCβ1-Akt activation and collagen I upregulation in mesangial cells

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Wu D, Peng F, Zhang B, Ingram AJ, Kelly DJ, Gilbert RE, Gao B, Kumar S, Krepsinsky JC. EGFR-PLCγ1 signaling mediates high glucose-induced PKCβ1-Akt activation and collagen I upregulation in mesangial cells. Am J Physiol Renal Physiol 297: F822–F834, 2009. First published July 15, 2009; doi:10.1152/ajprenal.00054.2009—Glomerular matrix accumulation is a hallmark of diabetic nephropathy. We have recently shown that epidermal growth factor receptor (EGFR) transactivation mediates high glucose (HG)-induced collagen I upregulation through PI3K-PKCβ1-Akt signaling in mesangial cells (MC). Phospholipase Cγ1 (PLCγ1) interacts with activated growth factor receptors and activates classic PKC isoforms. We thus studied its role in HG-induced collagen I upregulation in MC. Primary rat MC were treated with HG (30 mM) or mannitol as osmotic control. Protein kinase activation was assessed by Western blotting and collagen I upregulation by Northern blotting. Diabetes was induced in rats by streptozotocin. HG treatment for 1 h led to PLCγ1 membrane translocation and Y783 phosphorylation, both indicative of its activation. Mannitol was without effect. PLCγ1 Y783 phosphorylation was also seen in cortex and glomeruli of diabetic rats. HG induced a physical association between EGFR and PLCγ1 as identified by coimmunoprecipitation. PLCγ1 activation required EGFR kinase activity since it was prevented by the EGFR inhibitor AG1478 or overexpression of kinase-inactive EGFR (K721A). Phosphoinositide-3-OH kinase inhibition also prevented PLCγ1 activation. HG-induced Akt S473 phosphorylation, effected by PKCβ1, was inhibited by the PLCγ1 inhibitor U73122. PLCγ1 inhibition or downregulation by small interference RNA also prevented HG-induced collagen I upregulation. Our results indicate that EGFR-PLCγ1 signaling mediates HG-induced PKCβ1-Akt activation and subsequent collagen I upregulation in MC. Inhibition of EGFR or PLCγ1 may provide attractive therapeutic targets for the treatment of diabetic nephropathy.

diabetic nephropathy; extracellular matrix; epidermal growth factor receptor

DIABETES IS THE LEADING CAUSE of end-stage renal disease, accounting for over 50% of patients new to dialysis in developed countries (31). Hyperglycemia is an indispensable prerequisite to the pathogenesis of diabetic renal disease (4). Elevated glucose concentrations affect numerous pathways thought to contribute to the pathogenesis of diabetic kidney injury. These include activation of protein kinase C (PKC), increased production of advanced glycation end products (AGEs), and increased flux through the polyol and hexosamine pathways (5). Landmark clinical studies have shown that intensive glucose control reduces the risk of the development and progression of diabetic nephropathy in type 1 and type 2 diabetes. However, rigid glucose control at best only delays diabetic renal disease (1, 2). Therefore, novel therapeutic strategies for diabetic nephropathy are required.

Glomerular accumulation of matrix proteins is the pathological hallmark of diabetic nephropathy (22). When exposed to high glucose (HG), mesangial cells (MC) synthesize extracellular matrix proteins including collagens I and IV, the production of which is also increased in diabetic glomeruli in humans and animal models (22, 23, 31). Although numerous mechanisms have been implicated in the HG induction of MC matrix elaboration, our recent work indicates an important role for epidermal growth factor receptor (EGFR) transactivation and downstream activation of Akt in HG-induced collagen I upregulation (35). The molecular mechanism linking these, however, has not been fully elucidated.

We have shown that phosphoinositide-3-OH kinase (PI3K) is required for the activation of Akt in response to HG in MC. PI3K generates phosphorylated lipid second messengers, which recruit proteins with pleckstrin homology domains such as Akt to the membrane (6). Here, Akt is activated by phosphorylation at both T308 and S473. The former is effected by phosphoinositide-dependent protein kinase 1 (PDK1) (6). Both phosphorylation events are required for activation, although that of S473 (pS473) is known to parallel full PI3K/Akt phosphorylation at and stabilization at the membrane, although PI3K activation is phospholipase Cγ1 downstream PI3K/Akt activation (30). Several S473 kinases have been proposed, with identity likely determined by cell and stimulus (9, 13). In response to HG in MC, we have shown PKCβ1 to be important in the activation of Akt, functioning as an S473 kinase. This function of PKCβ1 was dependent on both EGFR and PI3K activity (36).

One potential candidate linking EGFR transactivation to downstream PI3K and PKCβ1 activation is phospholipase C γ (PLCγ). Four PLCγ subfamilies have been identified, with PLCγ1 the only isoform known to be downstream of receptor tyrosine kinases (RTKs). PLCγ1 itself has two isoforms: PLCγ1 is ubiquitous, and PLCγ2 is expressed mainly in hematopoietic cells (16, 29). Activation of PLCγ1 requires recruitment to the membrane and association, through its SH2 domain, with activated RTKs such as the EGFR (33). This results in the phosphorylation of PLCγ1 on tyrosine residues, particularly Y783, and an increase in its enzymatic activity (16, 29). PI3K, through the generation of phosphatidylinositol 3,4,5-triphosphate (PIP3), has been shown to facilitate PLCγ1 recruitment to and stabilization at the membrane, although PLCγ1 activation may also occur independently of PI3K (21, 29, 33). PLCγ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), generating inositol 1,4,5-triphosphate.
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Given our observations of the importance of EGFR-PI3K-PKCβ1-Akt signaling in HG-induced collagen I upregulation in MC (35, 36), and the known interactions between PLCγ1 and the EGFR, we asked whether PLCγ1 may be a potential candidate linking HG-induced EGFR transactivation with downstream PKCβ1/Akt-mediated collagen I upregulation. Our studies demonstrate for the first time that in response to HG, PLCγ1 both associates with the EGFR and mediates downstream PKCβ1-Akt activation and collagen I upregulation in MC. Thus inhibition of EGFR or PLCγ1 represents potential alternate therapeutic targets for the treatment of diabetic nephropathy.

**RESEARCH DESIGN AND METHODS**

**Cell culture.** Sprague-Dawley primary rat MC (passages 6–18) were cultured in DMEM supplemented with 20% fetal calf serum (Invitrogen, Burlington, ON), streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in 95% air-5% CO2. Standard medium contained 5.6 mM glucose. Either 24.4 mM glucose (yielding a final concentration of 30 mM) or mannitol was added for HG experimental conditions or osmotic control, respectively. MC were made quiescent with FBS-free DMEM containing (in mM) 20 Tris-HCl (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 2 DTT, 1 sodium vanadate, and 1 phenylmethylsulfonfluryl fluoride as well as 1% Triton X-100, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. Lysates were centrifuged at 4°C, 14,000 rpm for 10 min to pellet cell debris. The supernatant (50 μg) was separated on 10% SDS-PAGE, and Western blotting was performed as described elsewhere (35). Antibodies used included polyclonal phospho-Akt S473 (1:1,000), polyclonal Akt (1:1,000), polyclonal EGFR (1:1,000, all Cell Signaling, Boston, MA), monoclonal β-actin (1:5,000, Sigma), polyclonal phospho-PLCγ1 Y783 (1:1,000, Cell Signaling), polyclonal PLCγ1 (1:2,000, R&D Systems, Burlington, ON), monoclonal collagen I (1:1,000, Sigma), and monoclonal FLAG (10 μg/ml, M2, Sigma).

For immunoprecipitation, MC were lysed with buffer containing 60 mM N-octyl-glucopyranoside, clarified, and equal amounts of lysate were incubated overnight with 1 μg of polyclonal EGFR antibody or polyclonal PLCγ1 antibody (goat, Santa Cruz Biotechnology, Santa Cruz, CA), rotating at 4°C. Subsequently, 25 μl of protein G-agarose slurry was added for 1.5 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, resuspended in 2× sample buffer, boiled, and analyzed by Western blotting as above.

Membrane fractions were obtained as described previously (19). Briefly, cells were harvested in hypotonic lysis buffer, homogenized by passage through a 25-g needle, and centrifuged at 100,000 g for 60 min. The pellet was resuspended in regular lysis buffer with 60 mM N-octyl-glucopyranoside, centrifuged again at 100,000 g for 60 min, and the supernatant was collected as the membrane fraction.

**Animal studies.** Experiments were conducted in accordance with guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes and the Canadian Council on Animal Care guidelines. Diabetes was induced in 200-g male Sprague-Dawley rats (Charles River, Montreal, Quebec) or 8-wk-old female, homozygous (mRen-2)27 rats with 55 mg/kg streptozotocin (STZ; Sigma) by tail-vein injection. Control rats received 0.1 M citrate buffer, pH 4.5. Rats were monitored weekly for weight and blood glucose and monthly for blood pressure by tail-cuff plethysmography for 2 wk or 6 mo (Sprague-Dawley) or 3 mo [(mRen-2)27] (14). Only rats with blood glucose >15 mmol/l were considered diabetic. At study termination, glomeruli from the kidney cortex were harvested by differential sieving for Sprague-Dawley rats, or cortex was snap-frozen in liquid nitrogen for (mRen-2)27 rats. Protein was extracted as described above, except that tissue was also passed through a dounce homogenizer after lysis. For immunohistochemistry, cortical sections stored in OCT were processed as previously described (35). Primary antisera used were polyclonal phospho-PLCγ1 Y783 (1:25, Calbiochem), monoclonal Thy1.1 (1:200, BD Transduction), monoclonal collagen I (1:50, Sigma), and sheep polyclonal von Willebrand factor (VWF, 1:100, Cedarlane).

**Northern blot analysis.** Total RNA (10 μg), extracted using TRIzol (Invitrogen), was separated on a formaldehyde-agarose gel and transferred to a nylon membrane (Hybond, Amersham Biosciences, Baie d’Urie, Quebec). Hybridization was performed with random primed digoxigenin-11-dUTP-labeled cDNA probes prepared from collagen Iα1 cDNA amplified by PCR. Hybridized probes were detected using alkaline phosphatase-labeled anti-digoxigenin antibodies and CDP-star as a substrate. Kits and reagents were from Roche Applied Science (Mississauga, ON). Intensity of the 28S band was used as a loading control.

**PKCβ1 phosphorylation of Akt.** PKCβ1 was immunoprecipitated using 1 μg PKCβ1 antibody (Santa Cruz Biotechnology) from 500 μg membrane protein obtained as described above. After washing the immunoprecipitated PKCβ1, kinase reactions were carried out for 10 min at 30°C in kinase buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 40 μM ATP, 100 mM PMA (Sigma), 15 μg phosphatidylinositol-4-phosphate (Sigma), 0.1 μg GST-Akt (Cell Signaling) and protease/phosphatase inhibitor cocktail per reaction. Beads were resuspended in 5× reducing sample buffer, boiled for 5 min, and the resulting supernatant was resolved on 10% SDS-PAGE. Membrones were probed for phospho-Akt S473 and total Akt to ensure equal substrate presence.

**MC infection.** Epitope-tagged dominant-negative EGFR (dnEGFR, K721A, kindly provided by S. Parsons, Charlottesville, VA) was cloned into the empty vector pLHCX (Clontech, Mountain View, CA) for retroviral infection, and MC were infected as previously described (35). A competent virus capable of a single infection was generated using the vesicular stomatitis virus system (Stratagene, La Jolla, CA), and MC, passages 5–12, were exposed to the virus concentrated by centrifugation for 90 min at 50,000 g, 4°C in the presence of polybrene. Seventy-two hours after infection, a 2-μl antibiotic selection period was begun. Experiments were performed using a pooled, stably infected MC.

**RNA interference.** Rat PLCγ1 Silencer? Select small interference (si) RNA and control non-targeting siRNA were purchased from Ambion (Stratechville, ON). MC were transfected with 100 nM GeneEraser siRNA reagent (Stratagene) at 60% confluence. After the infection period was begun. Experiments were performed using a population of pooled, stably infected MC.
PLCγ1 is activated by HG in MC and in diabetic kidneys. PKC activation plays an important role in the glomerular matrix accumulation characteristic of diabetic nephropathy (34). We have recently shown that EGFR-P13K-Akt signaling mediates HG-induced collagen I upregulation in MC (35) and that PKCβ1 functions as an Akt S473 kinase in this pathway (36). PLCγ1 interacts with an activated EGFR, is phosphorylated on tyrosine 783, and can activate classic PKC isozymes including PKCβ1 (25, 29, 32). In studying PLCγ1 as a potential candidate linking HG-induced EGFR transactivation with downstream PKCβ1/Akt activation and collagen I upregulation, we first sought to assess whether HG could activate PLCγ1 in MC. PLCγ1 activation by HG has only previously been shown in vascular smooth muscle cells (27). PLCγ1 Y783 phosphorylation, as assessed by immunoblotting, was significantly elevated by 30 min and sustained to 1 h in MC exposed to 30 mM glucose (Fig. 1A). This increase was dose dependent, seen with 15 mM or higher glucose concentrations (Fig. 1B). As a second measure of PLCγ1 activation, we assessed its translocation to the membrane after HG exposure. As expected, HG induced an obvious increase in PLCγ1 membrane translocation with similar kinetics (Fig. 1C). PLCγ1 at the membrane was also seen to be phosphorylated at Y783 by HG. Maximum PLCγ1 phosphorylation and translocation were observed at 30 min to 1 h of HG; 1 h was thus used for subsequent experiments. The observed increase in PLCγ1 activation was not osmotically mediated as mannitol was without effect (Fig. 1, A and C). Furthermore, PLCγ1 activation as assessed by both phosphorylation at Y783 and its translocation was also seen with prolonged glucose exposure to 24–48 h (Fig. 1, D and E).

We next sought to confirm that PLCγ1 activation also occurred in vivo. Type 1 diabetes was induced in either Sprague-Dawley rats or homozygous (mRen-2)27 rats by STZ injection. Ren-2 rats overexpress the mouse renin gene, which leads to hypertension, and have been shown to develop renal lesions similar to those observed in human diabetic nephropathy as early as 3 mo after STZ injection (15). We have previously shown Akt phosphorylation at S473 in both of these models (35, 36). After 2 wk or 6 mo, Sprague-Dawley diabetic rats had an average plasma glucose of 19.5 ± 1.68 mmol/l (26), and PLCγ1 Y783 phosphorylation was significantly increased in their glomeruli (Fig. 2). We have previously shown glomerular sclerosis after 6 mo of diabetes in this model (26). In the Ren-2 rats, after 3 mo there was no difference between groups in systolic blood pressure (204 ± 14 control vs. 208 ± 10 mmHg diabetic). Diabetic rats had an average plasma glucose level of 25.7 ± 1.94 mmol/l (14), and a significant increase in PLCγ1 Y783 phosphorylation was seen in the renal cortex (Fig. 2). A greater increase in PLCγ1 phosphorylation was observed in Ren-2 diabetic rats compared with Sprague-Dawley diabetic rats, possibly as a result of elevated angiotensin II signaling and/or their significant degree of hypertension. These data demonstrate that the PLCγ1 activation by HG observed in vitro in MC also occurs in vivo in diabetic kidneys.

PLCγ1 interacts with and is activated by the EGFR in HG. Since PLCγ1 is known to interact with an activated EGFR (16, 29), and EGFR is transactivated by HG in MC (35), we examined the effects of EGFR inhibition on HG-induced PLCγ1 activation. EGFR inhibition with AG1478 (1 μM) effectively blocked HG-induced PLCγ1 Y783 phosphorylation and membrane translocation (Fig. 3, A and B). To further confirm that EGFR activity is required for HG-induced PLCγ1 activation, we studied responses in cells overexpressing the dominant-negative (kinase inactive) mutant EGFR K721A (dnEGFR). We have previously demonstrated stable overexpression of this construct in MC (35). MC with empty vector pHLCX showed the expected increase in PLCγ1 Y783 phosphorylation (Fig. 3C) and membrane translocation (Fig. 3D) in response to HG. This increase was prevented in cells overexpressing dnEGFR.

We next assessed whether HG induces a physical association between EGFR and PLCγ1 in MC. Coimmunoprecipitation experiments were performed on MC lysate after 1 h of HG exposure. HG clearly induced PLCγ1 association with EGFR (Fig. 4, A and B); mannitol was without effect. This physical association was completely prevented by the EGFR inhibitor AG1478 or overexpression of dnEGFR, indicating the necessity of EGFR activation for this association (Fig. 4, C and D).

PLCγ1 mediates HG-induced PKCβ1/Akt activation. We have previously demonstrated that downstream of EGFR transactivation, PKCβ1 functions as a glucose-induced Akt S473 kinase in MC (36). How EGFR transduces this signal in HG, however, is not known. Having shown that PLCγ1 is activated by HG downstream of the EGFR, coupled with the known role of PLCγ1 in activating conventional PKC isozymes through DAG production (25), we sought to determine whether PLCγ1 is also involved in HG-induced PKCβ1-Akt activation. MC were incubated with HG in the presence or absence of the PLCγ1 inhibitor U73122 (1 μM). PKCβ1 was immunoprecipitated from HG-treated MC and assessed for its ability to phosphorylate exogenous GST-Akt on S473. Figure 5A shows that PKCβ1 activity as an Akt S473 kinase was effectively prevented by PLCγ1 inhibition with U73122. As expected, assessment of total cell lysate for HG-induced Akt S473 showed this to be similarly prevented by U73122 (Fig. 5B). To further establish a specific role for PLCγ1 in HG-induced Akt activation, we used siRNA to downregulate PLCγ1. Figure 5C, top, demonstrates effective downregulation of PLCγ1 by RNAi. HG-induced Akt S473 phosphorylation was prevented in RNAi-treated MC compared with untransfected MC (control) and MC transfected with off-target control siRNA (Fig. 5C).

P13K mediates PLCγ1 activation by HG. The lipid mediator produced by P13K activity, PIP3, has been shown to facilitate PLCγ1 membrane recruitment and stabilization, thereby enhancing its activation (28, 29). However, PLCγ1 membrane translocation and activation may also occur independently of P13K, as occurs in response to EGF and platelet-derived growth factor (21, 33). We have previously shown that P13K is required for PKCβ1-induced phosphorylation of Akt at S473 and thus Akt activation, by HG (36). We thus assessed whether P13K was also required for PLCγ1 activation. Figure 6 shows that two inhibitors of P13K, wortmannin (100 nM) and LY294002 (10 μM), completely prevented HG-induced PLCγ1 phosphorylation at Y783. Thus P13K is necessary for PLCγ1 phosphor-
PLCy1 is activated by high glucose (HG) in mesangial cells (MC). A: MC were treated for the indicated times with 30 mM HG, or for 60 min with equimolar mannitol. Immunoblot analysis shows a time-dependent increase in PLCy1 phosphorylation at Y783 in response to glucose (*P < 0.05 vs. control, n = 6). B: treatment of MC for 60 min with increasing concentrations of HG shows a dose-dependent increase in PLCy1 phosphorylation at Y783 as assessed by immunoblotting (*P < 0.05, n = 6). C: Membrane-bound (active) PLCy1 is increased by glucose with similar kinetics (*P < 0.05, n = 4). Phosphorylation at Y783 is also observed in membrane-localized PLCy1 (*P < 0.05, n = 3). Mannitol (Man) is without effect on PLCy1 activation. Actin is used as the cytosolic loading control. D and E: prolonged treatment with HG, for 24–48 h, also increased both phosphorylation of PLCy1 at Y783 (*P < 0.05, n = 4, D) and its membrane translocation (n = 2, E).
ylation and thus activation in response to EGFR transactivation by HG.

HG-induced collagen I upregulation is dependent on PLCγ1 activity. Since our previous data have shown that Akt activation mediates HG-induced collagen I upregulation in MC (35), we studied the role of PLCγ1 in upregulation of this matrix protein. MC were exposed to HG for 3 days in the presence or absence of the PLCγ inhibitor U73122 (1 μM). HG-induced collagen IA1 transcript upregulation, assessed by Northern blot analysis, was abrogated by PLCγ inhibition (Fig. 7A). Colla-
Fig. 3. PLCγ1 activation by HG is dependent on the EGF receptor (EGFR). MC were treated with HG for 60 min. A: pretreatment with the EGFR inhibitor AG1478 (1 μM) blocks HG-induced PLCγ1 phosphorylation at Y783 (#P < 0.01, n = 4). B: PLCγ1 membrane translocation is also prevented by EGFR inhibition (*P < 0.05, n = 4). C: PLCγ1 phosphorylation at Y783 is absent in MC stably overexpressing dominant-negative (dn) EGFR compared with empty vector pLHCX (#P < 0.01 vs. all others, n = 3). D: HG-induced PLCγ1 membrane translocation is similarly prevented (#P < 0.01 vs. all others, n = 5).
Fig. 4. HG-induced PLCγ1 association with the EGFR is dependent on EGFR activity. A: EGFR immunoprecipitates derived from 60-min HG-treated MC were immunoblotted with PLCγ1 antisera, showing increased interaction between the 2 in response to HG. Mannitol is without effect (*P < 0.05 HG vs. others, n = 6). B: reverse immunoprecipitation with PLCγ1 and immunoblotting with EGFR antisera confirm an HG-induced association between the 2 proteins (#P < 0.01 vs. others, n = 4). C: treatment with the EGFR inhibitor AG1478 (1 μM, 30 min) before 60-min HG prevents this interaction (#P < 0.01 vs. all others, n = 2). D: overexpression of dominant-negative EGFR in MC significantly decreases the HG-induced EGFR association with PLCγ1 in MC compared with empty vector pLHCX (*P < 0.05 vs. all others, n = 3).
Fig. 5. PLCγ1 mediates HG-induced Akt S473 phosphorylation in MC. A: MC were treated with the PLCγ inhibitor U73122 (U; 1 μM) for 30 min before treatment with HG for 60 min. PKCβ1 activity as an Akt S473 kinase, performed as described in RESEARCH DESIGN AND METHODS, is prevented by PLCγ inhibition (*P < 0.05 vs. all others, n = 5). B: Akt S473 phosphorylation in total cell lysate induced by 60-min HG is blocked by PLCγ inhibition with U73122 (#P < 0.01 vs. all others, n = 5). C: MC were transfected with control target-specific small interference RNA (RNAi; ConRNAi) or PLC1 RNAi (RNAi) as indicated. RNAi significantly attenuates PLC1 protein expression and prevents AktS473 phosphorylation in response to HG for 60 min (#P < 0.01 vs. all others, n = 3).
Fig. 6. Phosphoinositide-3-OH kinase (PI3K) mediates PLCγ1 activation by HG. MC were treated with the PI3K inhibitors wortmannin (100 nM, 60 min) or LY294002 (10 μM, 30 min) before HG for 60 min. Both inhibitors prevent PLCγ1 phosphorylation at Y783 as assessed by immunoblotting (#P < 0.01 vs. all others, n = 4).

As the prevalence of diabetes increases worldwide, the number of patients with diabetic nephropathy is also expected to rise. Although tight glycemic control remains the cornerstone of the management of diabetic patients, it is ineffective at completely preventing the pathogenic progression of diabetic nephropathy (1, 2). Therapeutic interventions tailored to inhibit specific pathways have to date proved largely disappointing. Additional therapeutic strategies that specifically target diabetic renal disease, based on greater understanding of the molecular mechanisms underlying the development and progression of diabetic nephropathy, are thus urgently needed.

The data presented herein indicate that PLCγ1 signaling is an important novel mediator of collagen I upregulation by MC exposed to HG. To our knowledge, PLCγ1 has not previously been shown to regulate matrix production in any setting. Its activation in response to HG has only recently been demonstrated in vascular smooth muscle cells (27), also occurring at early time points as we have observed in MC. In vivo, PLCγ1 activation has thus far only been observed in lenses of type 1 diabetic rats (20). Our data now show that PLCγ1 activation also occurs in diabetic kidneys, suggesting a more prevalent role for this phospholipase in diabetic pathophysiology.

In vascular smooth muscle cells, aldose reductase was shown to be required for PLCγ1 activation, possibly by inducing generation of reactive oxygen species (ROS). However, the effect of ROS inhibitors on PLCγ1 activation was not directly assessed (27). Our studies now demonstrate that the EGFR, transactivated by HG, is a critical early mediator of PLCγ1 activation in MC. The activation of PLCγ by RTKs in response to their ligands has been well described (16, 29). Indeed, of the four PLC subtypes known, PLCγ is the only one known to be activated downstream of RTKs, with interaction enabled through its SH2 domains (16). Complex formation with an activated RTK such as the EGFR leads to phosphorylation of PLCγ1 on Y783 and an increase in its enzymatic activity (28, 29). Our studies now describe, for the first time, that HG elicits both EGFR-PLCγ1 association and PLCγ1 activation in MC.

The EGFR is a 170-kDa cell surface receptor with intrinsic tyrosine kinase activity. Two primary mechanisms of activation have been identified. Upon activation by distinct ligands such as epidermal growth factor, the EGFR undergoes autophosphorylation, enabling it to interact with numerous intermediary effector molecules (12). Transactivation refers to the observation that stimuli other than direct ligand binding may lead to rapid EGFR activation. The events leading to EGFR transactivation by HG are unknown. In vascular smooth muscle cells, the EGFR was shown to be N-glycosylated in a hyperglycemic environment, increasing its responsiveness to transactivation by angiotensin II through its receptor (17). While receptor glycosylation may contribute to longer-term responses to HG such as matrix elaboration, this is unlikely to account for the relatively rapid transactivation of the EGFR observed in our studies. Rapid release by metalloproteinases of membrane-bound EGF ligands such as transforming growth factor-α and heparin-binding EGF (11, 24) are also possible, although this has not to date been demonstrated to occur in HG. Finally, the generation of ROS, which may inactivate phosphatases and lead to enhanced receptor phosphorylation and hence activation, is also a potential mechanism for EGFR transactivation by HG (39). Indeed, in MC, early (1 h) generation of ROS has...
been demonstrated (37). Further studies are required to identify the mechanism whereby HG leads to EGFR transactivation.

Our studies have identified that EGFR-PLCγ1 signaling mediates HG-induced Akt activation. This extends our previous work in which we demonstrated that the PI3K/Akt signaling pathway is a mediator of collagen I production by MC exposed to HG and that Akt is activated in the glomeruli of insulin-deficient diabetic rats (35). The EGFR has been
shown to directly activate both PLCγ1 and PI3K (12). PI3K has also been shown, in some contexts, to facilitate the recruitment and activation of PLCγ1 to the membrane through generation of the lipid mediator PIP3 (21, 32).

Indeed, our studies using two distinct PI3K inhibitors have shown a requirement for PI3K in the activation of PLCγ1. It is of interest that direct activation of the EGFR by its ligand EGF occurred independently of PI3K (33), suggesting dif-

Fig. 8. Collagen I levels and PLCγ1 phosphorylation are increased in diabetic glomeruli. A: frozen cortical sections from Ren-2 diabetic rats demonstrate an increase in PLCγ1 Y783 phosphorylation (green) in glomeruli, marked by the MC marker Thy1.1 (red). Areas of overlap, demonstrating colocalization, appear in yellow. B: immunofluorescence also demonstrates a significant increase in collagen I (green) in the cortex of diabetic rats. Upregulation is seen both within glomeruli, identified by the endothelial cell marker von Willebrand factor (VWF; red), as well as surrounding both tubules and glomeruli.
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ferential requirement for this phospholipid kinase in receptor transactivation compared with direct ligand binding.

Downstream of PI3K, Akt requires both T308 and S473 phosphorylation for full activation, with that of S473 corresponding to Akt activity (30). Although PDK1 has been well established as the T308 kinase, the identity of the S473 kinase has remained more elusive and is likely cell and context specific (6, 9, 13). Recently, we have identified the classic PKC isofrom PKCβ1 as a HG-induced Akt S473 kinase in MC (36). Our current data demonstrate a requirement for EGFR-PLCγ1 signaling in HG-induced PKCβ1 activation as an S473 kinase.

Activated PLCγ1 hydrolyzes the phosphatidylinositol (PI) PIP2 to produce the second messengers IP3 and DAG, with DAG required for activation of both classic and novel PKC isozymes (16, 25). DAG upregulation observed in response to HG and in diabetic animals has been thought to result primarily from longer-term (days) HG exposure through de novo synthesis (18). Short-term exposure to HG such as that used in our study was also shown to generate DAG in MC and in vascular smooth muscle cells (3, 27). In the latter, DAG formation was dependent on PLC activity and correlated with activation of the PLCγ1 isoform (27). However, in MC, glomeruli and vascular smooth muscle cells, the source for the PLC-mediated DAG synthesis does not appear to be PI (3, 8, 27). Since PLCγ1 is a PI-specific phospholipase (28), the mechanism whereby it activates PKCβ1/Akt remains to be identified. Phospholipase-independent functions of PLCγ1 have, however, been described. For example, the mitogenic effects of PLCγ1 could still be induced by a mutant lacking phospholipase activity (16). PLCγ1 has also been shown to act as a guanine nucleotide exchange factor for several proteins including dynamin-1 and PIKE, functions which are independent of its phospholipase activity but require its SH3 domain (7, 38). Further studies are thus required to identify how PLCγ1 leads to PKCβ1 and Akt activation and its downstream effects on matrix production in the context of HG.

In conclusion, our studies show a new role for PLCγ1, downstream of EGFR transactivation, in HG-induced PKCβ1/Akt activation and collagen I upregulation in MC. Given that current treatment options are not effective in fully preventing the progression of diabetic nephropathy, our data support the study of agents which target the EGFR or PLCγ1 as potential new therapeutic candidates. Indeed, the availability of orally administered EGFR inhibitors developed for the treatment of malignancies characterized by EGFR hyperactivation (10) will enable their assessment as therapeutic options for diabetic nephropathy.

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