High glucose-induced RhoA activation requires caveolae and PKCβ1-mediated ROS generation

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Zhang Y, Peng F, Gao B, Ingram AJ, Krepinsky JC. High glucose-induced RhoA activation requires caveolae and PKCβ1-mediated ROS generation. Am J Physiol Renal Physiol 302: F159–F172, 2012. First published October 5, 2011; doi:10.1152/ajprenal.00749.2010.—Glomerular matrix accumulation is a hallmark of diabetic nephropathy. We previously showed that RhoA activation by high glucose in mesangial cells (MC) leads to matrix upregulation (Peng F, Wu D, Gao B, Ingram AJ, Zhang B, Chorneyko K, McKenzie R, Krepinsky JC. Diabetes 57: 1683–1692, 2008). Here, we study the mechanism whereby RhoA is activated. In primary rat MC, RhoA activation required glucose entry and metabolism. Broad PKC inhibitors (PMA, bisindolylmaleimide, Go6976), as well as specific PKCβ blockade with an inhibitor and small interfering RNA (siRNA), prevented RhoA activation by glucose. PKCβ inhibition also abrogated reactive oxygen species (ROS) generation by glucose. The ROS scavenger N-acetylcysteine (NAC) or NADPH oxidase inhibitors apocynin and DPI prevented glucose-induced RhoA activation. RhoA and some PKC isoforms localize to caveolae. Chemical disruption of these microdomains prevented RhoA and PKCβ1 activation by glucose. In caveolin-1 knockout cells, glucose did not induce RhoA and PKCβ1 activation; these responses were rescued by caveolin-1 reexpression. Furthermore, glucose-induced ROS generation was significantly attenuated by chemical disruption of caveolae and in knockout cells. Downstream of RhoA signaling, activator protein-1 (AP-1) activation was also inhibited by disrupting caveolae, was absent in caveolin-1 knockout MC and rescued by caveolin-1 reexpression. Finally, transforming growth factor (TGF)-β1 upregulation, mediated by AP-1, was prevented by RhoA signaling inhibition and by disruption or absence of caveolae. In conclusion, RhoA activation by glucose is dependent on PKCβ1-induced ROS generation, most likely through NADPH oxidase. The activation of PKCβ1 and its downstream effects, including upregulation of TGF-β1, requires caveolae. These microdomains are thus important mediators of the profibrogenic process associated with diabetic nephropathy.

mesangial cell: diabetic nephropathy; extracellular matrix; oxidative stress; transforming growth factor-β

THE KIDNEY IS A MAJOR SITE of diabetic microvascular complications (28). Glomerular matrix accumulation, the pathologic hallmark of diabetic nephropathy, is triggered by a complex interplay of numerous factors. Although hyperglycemia is well known to be the primary pathogenic factor (28), improved understanding of the mechanisms of matrix accumulation are required to identify new therapeutic strategies for diabetic nephropathy.

When exposed to high glucose (HG), mesangial cells (MC) synthesize the profibrotic cytokine transforming growth factor-β (TGF-β) and extracellular matrix proteins (29). Although numerous mechanisms have been implicated in this matrix elaboration, we and others have recently shown an important role for RhoA. RhoA belongs to the family of Rho GTPases, 20- to 24-kDa proteins which are essential in the regulation of diverse cellular functions. It cycles between an active GTP-bound and an inactive GDP-bound form, with its intrinsic hydrolytic activity affected by various Rho regulators. Membrane localization through posttranslational modification is a requirement for RhoA activation (7). We and others have shown that RhoA and its downstream kinase Rho-kinase mediate matrix elaboration by MC in hyperglycemic conditions and in vivo in diabetic nephropathy (9, 22, 40). The mechanism by which RhoA is activated by HG, however, is not known and is investigated herein.

Potential mediators of RhoA activation by HG include oxidative stress and PKC. The generation of reactive oxygen species (ROS), particularly by NADPH oxidase, has been recognized as an important contributor to diabetic renal disease (4, 10). Although not yet studied in HG, NADPH oxidase-generated ROS were also shown to activate RhoA (35). Furthermore, conventional PKC isoforms were not only identified as important mediators of ROS generation in diabetic kidney disease (21, 51), but PKC was also implicated in RhoA activation in other settings (6, 16, 18). Although PKC comprises a large family of kinases, the best established upstream activator of RhoA is PKCα (16, 18). The importance of conventional isoforms PKCα and -β to the pathogenesis of diabetic nephropathy was demonstrated through the study of knockout mice and use of a PKCβ inhibitor (2, 31, 32). However, PKCβ has not as yet been implicated in RhoA activation.

Caveolae are 50- to 100-nm plasma membrane omega-shaped invaginations that are important to cell signaling (44). They are found in most cell types, including MC (50), and are defined by the presence of caveolin, a 21- to 24-kDa integral membrane protein which is essential for their formation. Three isoforms of caveolin exist, with MC shown to express caveolin-1 and -2 (50). In cells lacking caveolin-1, caveolae are not present, with caveolin-1 reexpression inducing the de novo formation of caveolae (43, 44). The role of caveolin-2 is less clear, possibly functioning to stabilize caveolin-1 (45). Components of the NADPH oxidase system have recently been found in caveolae, with localization important to signal transduction and ROS production (15, 53, 59). Furthermore, we have previously shown that RhoA activation in MC by mechanical stress is dependent on caveolae (41). However, whether caveolae mediate HG-induced ROS generation and/or RhoA activation has not as yet been studied. Hence, we investigated whether HG-induced activation of RhoA in MC is dependent on PKC-mediated ROS generation, and whether this requires caveolae.
**MATERIALS AND METHODS**

**Cell culture.** Primary MC were obtained from glomeruli of rats (Sprague-Dawley) or mice (caveolin-1 knockout or their corresponding wild-type, B6129SF1/J, Jackson Laboratory, Bar Harbor, ME) by differential sieving and cultured in DMEM with 20% fetal calf serum (Invitrogen, Burlington, ON), streptomycin (100 μg/ml) and penicillin (100 U/ml). Kidneys were harvested in accordance with institutional and Canadian Council on Animal Care guidelines (Animal Utilization Protocol 04-05-25). Experiments used cells between passages 6 and 15. Unless otherwise noted, rat MC were used.

The medium contained 5.6 mmol/l D-glucose. Either 24.4 mmol/l d-glucose (final 30 mmol/l) or mannitol was added for HG or osmotic control, respectively. Where indicated, L-glucose, 2-deoxyglucose, or d-glucose was followed by incubation with 25 μM H9262 (final 30 mmol/l) or mannitol was added for HG or osmotic control.

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RESULTS

Glucose entry and metabolism are required for glucose-induced RhoA activation. We previously showed that RhoA is activated by HG in MC (40), but the mechanism of activation is unknown. We first assessed whether cellular glucose entry and its subsequent metabolism were required for RhoA activation. Cytochalasin B prevents glucose entry into cells by binding the glucose transporter glut1 in MC, thereby blocking glucose binding and uptake (60). Figure 1A shows that cytochalasin B prevented HG-induced RhoA activation. We next downregulated the major MC facilitative glucose transporter glut1 using siRNA (13). Its downregulation prevented HG-induced RhoA activation (Fig. 1B), confirming the requirement for glucose entry in RhoA activation. The inset shows the effectiveness of siRNA at decreasing glut1 protein. We then studied whether nonmetabolizable forms of glucose could activate RhoA. 3-O-methyl glucose is transported into the cell, but not metabolized, and 2-deoxy-D-glucose cannot be metabolized beyond glucose-6-phosphate (46). Neither of these activated RhoA. Similarly, the nonmetabolizable isomer l-glucose also did not activate RhoA (Fig. 1C). Thus both glucose entry and metabolism beyond glucose-6-phosphate, but not hyperosmolarity, are required for RhoA activation.

PKCβ1 is required for glucose-induced RhoA activation. PKC can function both upstream (18, 37) and downstream (38) of RhoA, with PKCα the most common isoform implicated in RhoA activation. It was recently shown in vascular smooth muscle cells (VSMC) that PKC mediated RhoA activation with prolonged HG (48 h) exposure (58). However, we have observed earlier RhoA activation in MC, within hours of HG treatment (40). We thus investigated whether PKC was involved in this early HG-induced RhoA activation. Figure 2, A and C, shows that both PKC downregulation by PMA (200 nM) and inhibition of conventional and novel PKC isoforms...
with bisindolylmaleimide I (2 μM, 30 min) prevented HG-induced RhoA activation. We then assessed the role of conventional PKC isoforms with Gö6976 (PKC-α/β inhibitor) and a PKC-β-specific inhibitor. Both blocked HG-induced RhoA activation (Fig. 2, B and C).

We next sought to confirm a role for PKC-β in RhoA activation by HG. Since PKC-β2 is not activated by glucose in MC (20), we used siRNA to downregulate PKC-β1. Figure 2D shows that PKC-β1 downregulation prevented HG-induced RhoA activation. Successful downregulation was confirmed by immunoblotting for PKC-β1. Thus PKC-β1 mediates HG-induced RhoA activation in MC.

**ROS mediate glucose-induced RhoA activation.** ROS, particularly those generated through NADPH oxidase activity, are of known importance in the pathogenesis of diabetic nephropathy (4, 10). NADPH oxidase-mediated ROS generation was also shown to mediate RhoA activation in VSMC (35). Furthermore, HG leads to both NADPH oxidase activation and ROS generation by 1–3 h in MC (25, 57), and in vivo, PKC-α/β inhibition prevented HG-induced ROS generation in diabetic glomeruli. However, whether ROS are required for HG-induced RhoA activation, and whether ROS production is mediated by PKC-β in MC, are not known. We thus first assessed the effects of the ROS scavenger NAC (4 mM, 30 min) on RhoA activation. As seen in Fig. 3A, NAC blocked RhoA activation by HG. Next, NADPH oxidase inhibitors DPI (40 μM, 30 min) and apocynin (300 μM, 30 min) were tested. Both prevented HG-induced RhoA activation (Fig. 3B). Since mitochondrial ROS are another potential major source of cellular ROS generation in response to HG (36), we next assessed the effect of the mitochondrial inhibitor CCCP (1 μM, 30 min) on RhoA activation. CCCP did not prevent HG-induced RhoA activation (Fig. 3C).

We then assessed the effects of conventional PKC inhibition on ROS (superoxide) generation, monitored in living cells loaded with DHE over 6 h as described in MATERIALS AND METHODS. Our data, shown in Fig. 3D, confirmed that the PKC-α/β inhibitor Gö6976 blocks HG-induced ROS generation, as was previously shown in MC using an alternate method to detect ROS (57). We further showed that PKC-β inhibition similarly prevented HG-induced ROS generation. Thus PKC-β1 mediates the generation of superoxide, likely through NADPH oxidase, with consequent RhoA activation.

**Caveolae are required for PKC-β activation, ROS generation, and RhoA activation in response to glucose.** We previously showed that caveolae are required for RhoA activation by mechanical stress and PKC-β in MC (41, 42). There is now increasing evidence that caveolae may be important to NADPH oxidase activation (15, 53, 59). Indeed, we recently reported that stretch-induced RhoA activation requires ROS generated by NADPH oxidase, and that depends on caveolae (61). We thus assessed whether caveolae also play a role in glucose-induced RhoA activation and ROS generation.

We first used two distinct agents to chemically disrupt caveolae, the membrane-impermeable cholesterol-binding

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**Fig. 2. PKC-β1 is upstream of glucose-induced RhoA activation.** A–C: MC were treated for 6 h with HG in the presence or absence of the broad PKC inhibitors bisindolylmaleimide I (2 μM, 30 min) and PMA (200 nM, 24 h), or the PKC-α/β inhibitor Gö6976 (2 μM, 30 min) or selective PKC-β inhibitor (100 nM, 30 min). RhoA activity was blocked by all inhibitors, suggesting that PKC-β mediated HG-induced RhoA activation (‡P < 0.001 HG vs. others; n = 3).

D: MC were transfected with control or PKC-β1 siRNA as outlined in MATERIALS AND METHODS, serum deprived, and treated with HG for 6 h. RhoA activation was prevented by PKC-β1 downregulation (†P < 0.01 vs. others; n = 3). Immunoblotting for PKC-β1 confirms its successful downregulation by siRNA.
agent cyclodextrin (10 mM, 1 h) and the membrane-permeable cholesterol-binding agent filipin III (5 μg/ml, 10 min), to perturb caveolar formation. Both are known to almost completely abolish the presence of caveolae (47, 52). Figure 4A shows that both agents prevented HG-induced RhoA activation. We then assessed their effects on HG-induced PKCθ activation. Here, the ability of immunoprecipitated PKCθ to phosphorylate the substrate MARCKS enables assessment of its activity. As seen in Fig. 4B, MARCKS phosphorylation in response to HG was also prevented by both agents.

To confirm these findings, we next used MC derived from caveolin-1 knockout mice or their wild-type counterparts.
Knockout mice lack caveolin-1 and hence caveolae in all tissues (43), as we confirmed by Western blotting (Fig. 5A). Both RhoA and PKC\(_{\beta1}\) activation, seen in wild-type MC, were absent from knockout cells (Fig. 4, C and D). To examine whether caveolin-1 reexpression could restore RhoA activation, we generated knockout cells expressing FLAG-tagged caveolin-1 (Fig. 5A). Compared with cells expressing the empty vector pLHCX, HG-induced RhoA activation occurred only in knockout cells reexpressing caveolin-1 (Fig. 5B). This demonstrates an important role for caveolin-1 and caveolae in the activation of RhoA by HG.

We then studied whether caveolae were required for glucose-induced ROS generation. As above, we monitored ROS production over 6 h after treatment with HG in the presence or absence of cyclodextrin or filipin. Figure 6A shows that both agents block HG-induced ROS generation. We next confirmed a requirement for caveolin-1/caveolae in glucose-induced ROS generation. Caveolin-1 knockout MC and their wild-type counterparts were loaded with DHE to detect superoxide generation, treated with HG, fixed, and then ROS generation was visualized by red immunofluorescence. Figure 6B demonstrates that HG led to significant ROS generation only in wild-type MC. Finally, we studied whether caveolin-1 reexpression could rescue ROS generation in knockout MC. Figure 6C shows that basal ROS generation was somewhat higher, and the HG response significantly higher, in knockout MC reexpressing caveolin-1 compared with empty vector. It should be noted, however, that knockout/pLHCX MC were still able to generate some ROS response to HG. Taken together, caveolin-1 and caveolae are necessary for HG-induced PKC\(_{\beta1}\) activation and ROS generation, both of which are required for RhoA activation.

**AP-1 activation and TGF-\(\beta1\) upregulation by glucose are dependent on caveolin-1 and caveolae.** We next studied whether this signaling pathway is important to the glucose-induced fibrogenic response in MC. The transcription factor
AP-1 is known to mediate HG-induced upregulation of the profibrogenic cytokine TGF-β1 in MC (54), and we showed that both PKCβ1 activation and RhoA signaling are required for HG-induced AP-1 activation (40, 55). Since our studies demonstrate that caveolae mediate both PKCβ1 and RhoA activation, this suggested that they should also be required for AP-1 activation. We thus first used cyclodextrin and filipin to disrupt caveolae, and showed in Fig. 7A that HG-induced AP-1 activation, as assessed by EMSA, was blocked by both inhibitors. To confirm a role for caveolae and caveolin-1, we next used knockout MC. Compared with wild-type MC, AP-1 activation by HG was absent in caveolin-1 knockout cells (Fig. 7B). This was restored by reexpressing caveolin-1 (Fig. 7C). Of note, two additional bands were consistently observed in EMSAs using nuclear protein from caveolin-1 knockout MC carrying either empty vector or caveolin-1. We were unable to identify the reason for these nonspecific bands.

Although RhoA is a well-established downstream mediator of TGF-β1 (30), its ability to regulate TGF-β expression has only recently been described in stretched airway smooth muscle cells (33). Whether RhoA signaling is required for HG-induced TGF-β1 upregulation is not known. We thus first inhibited the downstream RhoA kinase, Rho-kinase, which we previously showed mediates glucose-induced AP-1 activation (40). Two structurally unrelated Rho-kinase inhibitors, Y-27632 (10 μM, 30 min) and HA-1077 (25 μM, 30 min), prevented activation of a TGF-β1 promoter-luciferase construct by HG. The osmotic control, mannitol, did not upregulate TGF-β1 (Fig. 8A). Interestingly, Rho-kinase inhibition suppressed HG-induced responses even below control levels, while basal TGF-β promoter-luciferase activity was unaffected. We currently do not have an explanation for these findings. We then studied the effects of caveolar disruption. As seen in Fig. 8B, both cyclodextrin and filipin prevented HG-induced TGF-β1 promoter-luciferase activation. Promoter activation was also absent in caveolin-1 knockout MC compared with wild-type cells (Fig. 8C). Inhibition of promoter activation correlated with inhibited transcript upregulation as assessed by Northern blotting. Figure 8D shows that HG increased the TGF-β1 transcript in wild-type, but not caveolin-1 knockout, MC. Finally, caveolin-1 reexpression restored both HG-induced TGF-β1 promoter-luciferase activation and transcript upregulation in knockout MC (Fig. 8, E and F). Taken together, the activation of AP-1 and induction of TGF-β1 in response to HG require PKCβ1-mediated ROS generation and RhoA activation and caveolin-1/caveolae.

**DISCUSSION**

Diabetes is the leading cause of renal failure in the Western world. Although tight glycemic control and interruption of angiotensin II signaling remain the cornerstone of management, these...
measures are ineffective at completely preventing progression of diabetic nephropathy (3, 27). A greater understanding of the underlying pathogenic molecular mechanisms, enabling development of additional therapeutic strategies that specifically target diabetic renal disease, is thus needed.

We and others recently showed that RhoA/Rho-kinase signaling is required for the development of glomerular matrix accumulation and diabetic kidney disease (9, 22, 40). Very little is known, however, regarding activation of this profibrotic signaling pathway by glucose. Our work now identifies a novel central role for caveolin-1 and caveolae in glucose-induced RhoA activation. Our studies further show that these microdomains are required for the activation of PKCε and consequent ROS production, key upstream mediators of RhoA activation. These data suggest that caveolae or caveolin-1 may serve as potential modifiable targets in the treatment of diabetic renal disease.

Fig. 6. Caveolae are required for glucose-induced ROS generation in MC. A: MC were loaded with DHE and treated with HG in the presence or absence of CD or Fil to chemically disrupt caveolae. ROS production was monitored over 6 h in a fluorometer. Both agents prevented the HG-induced increase in ROS generation over time (‡P < 0.001 HG vs. others; n = 6). B: the requirement of caveolae for ROS production in response to HG was confirmed in caveolin-1 KO MC. KO and WT MC were loaded with DHE and treated for 3 or 6 h with HG. After fixation, MC were imaged. WT cells showed significant ROS production with HG (red) compared with no significant ROS generation in KO cells. Nuclei are stained with 4,6-diamidino-2-phenylindole (blue). C: caveolin-1 KO MC infected with the empty vector pLHCX or with caveolin-1 were assessed for ROS production in response to HG. Caveolin-1-reexpressing KO MC showed higher basal ROS generation over time with a further significant increase by HG compared with MC expressing pLHCX (‡P < 0.01 KO/caveolin-1 HG vs. others, †P < 0.01 KO/caveolin-1 Con vs. others; n = 8). KO/pLHCX MC were still able to generate some ROS response to HG (#P < 0.01 KO/pLHCX Con vs. HG).
Thus far, only Xie et al. (58) have examined the mechanism of RhoA activation by glucose. They demonstrated that PKC-mediated upregulation and activation of calcium-independent PLA2γ (iPLA2γ) was required for RhoA activation and VSMC contractility (58). However, RhoA activation was assessed after longer-term (48 h) glucose exposure, differing from our studies which examine early (6 h) activation of RhoA. Furthermore, the PKC isoform required for RhoA activation was not identified. Our studies show that early glucose-induced RhoA activation also depends on PKC activation, and further identified PKCβ1 as the necessary isoform.

PKCβ1 has a known important role in the pathogenesis of diabetic nephropathy. In vivo, both PKCβ inhibition and genetic deletion suppressed TGF-β1 upregulation and matrix expansion in diabetic glomeruli or kidneys (2, 31). Using a PKCβ-specific inhibitor and PKCβ1 siRNA, our data are the first to show that PKCβ is an important mediator of RhoA activation. Our results further demonstrate that PKCβ regulates...
Fig. 8. Upregulation of transforming growth factor (TGF)-β1 by glucose requires RhoA activation and caveolin-1/caveolae. A: MC transiently transfected with a TGF-β promoter-luciferase construct were treated with HG in the presence or absence of Rho-kinase inhibitors Y-27632 (Y; 10 μM) or HA-1077 (HA; 25 μM) for 24 h, and luciferase activity was assessed as described in MATERIALS AND METHODS. HG-induced TGF-β promoter activation was prevented by both inhibitors (*P < 0.05 HG vs. others; n = 6). B: TGF-β promoter-luciferase activation by HG was also blocked by disruption of caveolae with CD or Fil (*P < 0.05 HG vs. others; n = 4). C: HG also did not induce significant TGF-β promoter-luciferase activation in caveolin-1 KO MC compared with WT MC (*P < 0.05 WT HG vs. others; n = 6). D: this correlated with transcript levels. In caveolin-1 KO MC treated with HG for 24 h, no increase in the TGF-β1 transcript was seen as assessed by Northern blot analysis. In comparison, HG induced significant TGF-β1 transcript upregulation in WT cells (*P < 0.05 WT HG vs. others; n = 3). Caveolin-1 reexpression in KO cells restored both HG-induced TGF-β promoter-luciferase activation (E; *P < 0.05 KO/caveolin-1 HG vs. others; n = 6) and transcript upregulation (F; ‡P < 0.001 KO/caveolin-1 HG vs. others; n = 4).
RhoA activation through induction of ROS. This does not exclude other potential mechanisms of control, as has been shown for PKC regulation of RhoA activation. For example, PKC phosphorylated and thereby activated p115RhoGEF in response to thrombin in human umbilical vein endothelial cells (16). Conversely, in rat embryo fibroblasts, PKCα phosphorylated and thus inhibited RhoGDIα, also leading to RhoA activation (6). Thus, PKC may be involved at several levels in regulating RhoA activation. Whether PKCβ1 also mediates activation of RhoA regulators, as shown for PKCα, requires further study.

In vivo studies indicate an important role for ROS generated by NADPH oxidase in the pathogenesis of diabetic nephropathy (4, 10). In streptozotocin-induced diabetic rats, PKCβ inhibition decreased glomerular NADPH oxidase activity and ROS generation (21). In vitro, HG led to ROS generation in MC as early as 1 h. This was blocked by PKCβ inhibition, although the specific isoform involved was not identified (57). The novel PKCγ was also implicated in HG-induced NADPH oxidase activation in MC (25). Our studies show for the first time that NADPH oxidase-mediated ROS production is also required for PKCβ1 activation by HG. ROS thus appear to function both upstream and downstream of PKCβ1. We have previously shown that HG-induced PKCβ1 activation requires transactivation of the EGF receptor (EGFR) with subsequent activation of PLCγ1 and phosphatidylinositol-3-kinase (56). Our previous studies have also shown that EGFR transactivation can activate Rac1 (61). Furthermore, angiotensin II- and mechanical stress-induced EGFR transactivation and ROS generation required caveolae (61, 62). Thus EGFR signaling, possibly originating in caveolae or recruiting NADPH oxidase components to caveolae, appears to be a key upstream mediator of PKCβ1 activation by HG.

Although ROS were implicated as mediators of RhoA activation in other settings (35, 48), our work is the first to demonstrate that ROS generation mediates RhoA activation by HG. The mechanism by which this occurs is not known. Whether ROS may affect the activation of RhoA regulators

The NADPH oxidase system is a multimeric protein complex consisting of 1) cytosolic subunits including p47phox and p67phox that require membrane translocation for association with 2) a membrane-associated flavocytochrome reductase comprising p22phox and a Nox family protein, and 3) a regulatory small GTPase, predominantly Rac1 (5). The role of PKC in NADPH oxidase activation is likely to be multifactorial. PKC has been shown to phosphorylate p47phox, which induced binding to the membrane-associated component p22phox and the generation of ROS (superoxide) (8). PKCβ was also shown to enhance membrane translocation of both p47phox and p67phox (21), as well as to mediate glucose-induced activation of Rac1 (19). Our data show, however, that NADPH oxidase-mediated ROS production is also required for PKCβ1 activation by HG. ROS thus appear to function both upstream and downstream of PKCβ1. We have previously shown that HG-induced PKCβ1 activation requires transactivation of the EGF receptor (EGFR) with subsequent activation of PLCγ1 and phosphatidylinositol-3-kinase (56). Our previous studies have also shown that EGFR transactivation can activate Rac1 (61). Furthermore, angiotensin II- and mechanical stress-induced EGFR transactivation and ROS generation required caveolae (61, 62). Thus EGFR signaling, possibly originating in caveolae or recruiting NADPH oxidase components to caveolae, appears to be a key upstream mediator of PKCβ1 activation by HG.

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such as RhoGEFs or RhoGAPs is also unknown. However, it was recently shown that ROS can directly activate RhoA independently of RhoGEFs by modulating two cysteine residues located in a unique redox-sensitive motif in the phosphorylation domain of RhoA. This leads to release of bound GDP, with subsequent GTP binding and RhoA activation favored by the cystosolic excess of GTP (1, 14). The presence of a reducing agent which can enable RhoA to bind guanine nucleotides is required (14). This model was supported by a recent study in fibroblasts, in which peroxide-induced early RhoA activation and actin stress fiber formation were abolished by mutation of both cysteine residues to alanine. Interestingly, these mutations preserved RhoA activation through RhoGEFs (2). The particular mechanism of RhoA regulation by ROS in the setting of HG, however, remains to be investigated.

Recent evidence has implicated caveolae in NADPH oxidase activation. Subunits of this enzyme system, including Nox1, Nox2, and p22phox, were shown to localize to caveolae (15, 53, 59). Furthermore, some subunits, including p47phox, p67phox, and Rac1, translocated from the cytosol to caveolae on activation (53, 59). Both subunit localization and superoxide production were inhibited by cholesterol-depleting agents as we have used to disrupt caveolae (53). NADPH oxidase activity was also detected in caveolae isolated from Fas ligand-stimulated endothelial cells (59). Thus far, the role of caveolae in the pathogenesis of diabetic nephropathy has been unexplored. We previously showed these microdomains to be required for mechanical stress-induced RhoA activation (41). Our data now further demonstrate an important role for caveolae in glucose-induced RhoA/Rho-kinase signaling and PKCα activation, ROS generation, and RhoA activation. The localization of PKCα to caveolae has been addressed by others, but of the conventional isoforms only PKCα was shown to target to caveolae and associate with caveolin-1 (39). Cholesterol depletion, however, also prevented PKCβ translocation to lipid rafts, membrane microdomains related to caveolae (53). Although we have not shown direct localization of PKCβ to caveolae, our data using caveolin-1 knockout MC clearly demonstrate that PKCβ1 activation and subsequent ROS generation are dependent on caveolin-1 and caveolae.

Caveolae have been implicated in the regulation of matrix production (34). Indeed, we previously showed that mechanical stress-induced fibronectin upregulation requires caveolin-1/caveolae (41). However, no studies have yet addressed whether glucose-induced matrix upregulation is regulated by caveolae. We now show that glucose-induced TGF-β1 upregulation requires these microdomains. TGF-β1 is a well-known major mediator of matrix accumulation in diabetic kidneys and glucose-exposed MC (29). Its upregulation by HG in MC requires activity of the transcription factor AP-1 (54), which we previously showed requires RhoA/Rho-kinase signaling and PKCβ1 (40, 55). Given that both PKCβ1 and RhoA activation are dependent on caveolae, similar dependence of AP-1 on caveolae was expected and shown.

As summarized in Fig. 10, our work demonstrates that RhoA activation by glucose and downstream TGF-β1 upregulation are dependent on PKCβ1-induced ROS generation, most likely through NADPH oxidase. Caveolae are important regulators of RhoA activation and thus the fibrotic response to glucose in MC. These observations suggest that targeting caveolin-1 or caveolae may provide a new therapeutic avenue for diabetic nephropathy. It should be noted, however, that mice genetically lacking specific caveolin isoforms have demonstrated pulmonary, cardiac, or muscle disorders among others (26). Interestingly, normal renal function and structure have been found in caveolin-1 knockout mice (11, 49). Although any effects would need to be confirmed with caveolin/caveolae inhibition in normal adult rodents, developing a way to target caveolae specifically in the kidney may minimize any potential adverse systemic effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.Z., F.P., B.G., and J.C.K. performed experiments; Y.Z., A.J.I., and J.C.K. interpreted results of experiments; A.J.I. and J.C.K. edited and revised manuscript; A.J.I. and J.C.K. approved final version of manuscript; J.C.K. provided conception and design of research; J.C.K. analyzed data; J.C.K. prepared figures; J.C.K. drafted manuscript.

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