High Glucose Forces a Positive Feedback Loop Connecting Akt Kinase and FoxO1 Transcription Factor to Activate mTORC1 Kinase for Mesangial Cell Hypertrophy and Matrix Protein Expression

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Background: Hyperglycemia contributes to renal hypertrophy and fibrosis.
Results: Inactivation of FoxO1 is required for high glucose-induced sustained activation of Akt and mTORC1 for renal pathology.
Conclusion: A positive feedback loop involving catalase exists between Akt and FoxO1.
Significance: FoxO1-mediated catalase expression may alleviate renal glomerular hypertrophy and fibrosis.

High glucose-induced Akt acts as a signaling hub for mesangial cell hypertrophy and matrix expansion, which are recognized as cardinal signatures for the development of diabetic nephropathy. How mesangial cells sustain the activated state of Akt is not clearly understood. Here we show Akt-dependent phosphorylation of the transcription factor FoxO1 by high glucose. Phosphorylation-deficient, constitutively active FoxO1 inhibited the high glucose-induced phosphorylation of Akt to suppress the phosphorylation/inactivation of PRAS40 and mTORC1 activity. In contrast, dominant negative FoxO1 increased the phosphorylation of Akt, resulting in increased mTORC1 activity similar to high glucose treatment. Notably, FoxO1 regulates high glucose-induced protein synthesis, hypertrophy, and expression of fibronectin and PAI-1. High glucose paves the way for complications of diabetic nephropathy through the production of reactive oxygen species (ROS). We considered whether the FoxO1 target antioxidant enzyme catalase contributes to sustained activation of Akt. High glucose-inactivated FoxO1 decreases the expression of catalase to increase the production of ROS. Moreover, we show that catalase blocks high glucose-stimulated Akt phosphorylation to attenuate the inactivation of FoxO1 and PRAS40, resulting in the inhibition of mTORC1 and mesangial cell hypertrophy and fibronectin and PAI-1 expression. Finally, using kidney cortices from type 1 diabetic OVE26 mice, we show that increased FoxO1 phosphorylation is associated with decreased catalase expression and increased fibronectin and PAI-1 expression. Together, our results provide the first evidence for the presence of a positive feedback loop for the sustained activation of Akt involving inactivated FoxO1 and a decrease in catalase expression, leading to increased ROS and mesangial cell hypertrophy and matrix protein expression.

Chronic kidney disease resulting from diabetes involves changes in the glomerular compartment with altered hemodynamics and matrix expansion (1, 2). The earliest changes include glomerular hypertrophy followed by a high glomerular filtration rate, which leads to microalbuminuria (3). These changes are also associated with a subsequent thickening of the basement membrane, progressive mesangial dysfunction, and glomerulosclerosis, which result from the accumulation of the extracellular matrix proteins collagen, fibronectin, and laminin (2, 4). The accumulation of mesangial matrix clearly correlates with the progression of the disease, indicating a central role of mesangial cells in diabetic glomerular injury (2).

Hyperglycemia as well as hyperglycemia-induced growth factors and cytokines present in the diabetic milieu activate multiple signal transduction pathways, including PI3 3 kinase/Akt kinase. We and others have shown a significant role of this kinase cascade in inducing renal hypertrophy and matrix expansion in diabetic animals and in cultured mesangial and proximal tubular epithelial cells (5–10). Our recent observation that hyperglycemia reduces the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10), which acts as a lipid phosphatase to dephosphorylate the PI 3,4,5-trisphosphate, also contributes to the hyperactivation of Akt in diabetic renal glomeruli and in mesangial cells, therefore providing an additional mechanism of Akt activation in diabetic kidney disease (7, 11). Subsequently, the phosphor-

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5 The abbreviations used are: PI, phosphatidylinositol; ROS, reactive oxygen species; LG, low glucose; HG, high glucose; DN, dominant negative; Ad, adenovirus.
ylation of key substrates such as PRAS40 and tuberin by Akt activates the mechanistic target of rapamycin complex 1 (mTORC1) to induce hypertrophy and matrix protein expression, two pathological features of diabetic nephropathy (5, 8, 11).

Forkhead box (Fox) proteins represent a group of transcription factors with a winged helix DNA binding domain that regulate cell fate decision, including proliferation, differentiation, and metabolism (12). In mammals, four FoxO proteins (FoxO1, FoxO3, FoxO4, and FoxO6) have been identified. Although FoxO6 is restricted to neurons, the other three subtypes are ubiquitous in distribution, including high levels of expression in kidney (13). FoxO proteins are substrates of Akt kinase. Activated nuclear Akt phosphorylates FoxO at three conserved residues. The phosphorylated FoxO translocates to the cytoplasm, resulting in inhibition of transcription of its target genes (12). Reactive oxygen species generated by the mitochondrial respiratory chain and by NADPH oxidases in the diabetic renal tissues contribute significantly to the pathology of nephropathy (2, 14). However, the levels of ROS are also known to be regulated by antioxidant enzymes such as SOD2 and peroxiredoxin 3. Interestingly, the expression of these antioxidant enzymes is transcriptionally regulated by FoxO3 downstream of Akt kinase (15, 16). In this study, we demonstrate a role of FoxO1 in high glucose-induced mesangial cell hypertrophy and matrix protein expression. We find that high glucose maintains a positive feedback loop involving FoxO1 and Akt kinase, which inactivates PRAS40 to increase the mTORC1 kinase activity necessary for mesangial cell hypertrophy. Furthermore, we show that down-regulation of the antioxidant enzyme catalase as a target of FoxO1 in response to high glucose serves as a mechanism for the feedback loop. Finally, in the kidneys of mice with diabetes, we demonstrate the suppression of catalase expression as a result of FoxO1 inactivation and a concomitant increase in matrix protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phenylmethylsulfonyl fluoride, Nonidet P-40, Na$_2$VO$_4$, d-glucose, d-mannitol, TRI reagent, protease inhibitor mixture, and anti-fibronectin and anti-FLAG antibodies were purchased from Sigma. Phospho-FoxO1 (Thr-24), FoxO1, phospho-Akt (Ser-473), phospho-Akt (Thr-308), Akt, phospho-S6 kinase (Thr-24), S6 kinase, phospho-4EBP-1 (Thr-37/46), phospho-4EBP-1 (Ser-65), 4EBP-1, phospho-PRAS40 (Thr-246), and PRAS40 antibodies were obtained from Cell Signaling Technology (Boston, MA). PTEN and PAI-1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). The catalase antibody was obtained from Research Diag-nostic (Boston, MA). Anti-HA antibody was purchased from Covance (Princeton, NJ). FuGENE HD transfection reagent was purchased from Promega (Madison, WI). The adenovirus vector expressing constitutively active mutant FKHR;AAA (FoxO1/A3) with all three Akt phosphorylation sites changed to alanine was provided by Dr. William R. Sellers (Dana-Farber Cancer Institute). The adenovirus vector containing the dominant negative FoxO1 with deletion of the transactivation domain was provided by Dr. D. Accili (College of Physicians and Surgeons of Columbia University). Ad CMV catalase (Ad Catalase) was purchased from the Gene Transfer Vector Core (University of Iowa). Adenovirus vectors expressing dominant negative PI 3 kinase, PTEN, and HA-tagged dominant negative Akt have been described previously (7, 17). The luciferase reporter plasmid 8xFKTK-Luc containing eight copies of the FoxO binding element has been described previously (18).

**Cell Culture and Adenovirus Infection**—Normal rat glomerularmesangial cells were grown in DMEM with low glucose containing 17% fetal bovine serum in the presence of penicillin and streptomycin, as described previously (17). At confluence, the cells were washed with PBS, and serum-free medium was added for 24 h. The cells were then incubated with DMEM with 25 mm glucose for the indicated times. For osmotic control, DMEM with 5 mm glucose plus 20 mm mannitol was used. When necessary, the cells were infected with adenovirus vectors at a multiplicity of infection of 50 for 24 h, essentially as described previously (17). Adenovirus vectors containing green fluorescence protein (Ad GFP) or β-galactosidase (Ad β-Gal) were used as controls.

**Animals**—The pancreatic β cell-targeted calmodulin transgenic OVE26 mice and their control littermate FVB mice were purchased from The Jackson Laboratories. The type 1 diabetic OVE26 mice develop significant renal as well as glomerular hypertrophy and albuminuria at 2 months of age (19, 20). The animals were maintained in the University of Health Science Center animal facility and had free access to food and water. At 3 months of age, both control FVB and OVE26 mice were euthanized, and both kidneys were removed. Renal cortical sections from each mouse were pooled and frozen as described previously (11, 21). The animal protocol was approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio.

**Cell Lysis, Preparation of Renal Cortical Lysates, Immunoblotting, and Immunoprecipitation**—After incubation, the cells were washed with PBS and harvested in radioimmuno precipitation assay buffer (20 mm Tris-HCl (pH 7.5), 5 mm EDTA, 150 NaCl, 1 mm Na$_2$VO$_4$, 1 mm PMSF, 0.1% protease inhibitor mixture, and 1% Nonidet P-40). Similarly, renal cortices from control and diabetic mice were harvested in the same radioimmuno precipitation assay buffer. These cells and the renal cortices were lysed at 4 °C for 30 min as described previously (11, 17, 22). The crude cell extracts were centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was collected, and protein concentration was determined. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a PVDF membrane. Immunoblotting was performed using the indicated antibodies, and the major bands were developed with HRP-conjugated secondary antibody using ECL reagent as described previously (5, 11, 17). For immunoprecipitation, equal amounts of proteins were immunoprecipitated with FoxO1 antibody as described (17, 22). The immunobeads were suspended in sample buffer followed by electrophoresis in SDS-polyacrylamide gel. The separated proteins were immunoblotted with phospho-FoxO1 (Thr-24) antibody as described above.

**RNA Isolation and Real-time RT-PCR**—Total RNA was prepared from mesangial cells and renal cortices using TRI reagent. cDNAs were prepared by q-script cDNA synthesis kit. The cDNAs were amplified using catalase primers (for-
ward, 5'-CCTCCTCGTTCAAGATGTGGTTTTC-3'; reverse, 5'-CGTGGGTGACCTCAAAGTATCCAA-3'). Data analyses were done by comparative Ct method as described previously (22).

**Transient Transfection**—Glomerular mesangial cells were transfected with the 8xFKTK-Luc reporter plasmid using FuGENE according to the protocol of the manufacturer (23). The luciferase activity in the cell lysates was determined using an assay kit according to the instructions of the vendor (22, 23).

**Protein Synthesis**—Glomerular mesangial cells were serum-starved and treated with 25 mM glucose for 24 h as described above. [35S] Methionine incorporation was used to determine protein synthesis as described (5, 7, 17).

**Hypertrophy**—At the end of the incubation period, the cells were trypsinized and counted using a hemocytometer. The cells were pelleted by centrifugation at 4000 × g for 5 min at 4 °C. The cells were washed with PBS and lysed in radioimmune precipitation assay buffer as described above. Total protein concentration was determined in the lysate. Hypertrophy was determined as a ratio of total protein content to cell number, as described previously (5, 7).

**Flow Cytometry**—The cells were trypsinized and resuspended in PBS. 1 μg/ml propidium iodide was added before flow cytometry. Cytometry was performed in a LSR II four laser system (BD Biosciences). The cell size was analyzed with FlowJo v7.6 software.

2′,7′-Dichlorodihydrofluorescin Assay—Cell-permeable 2′,7′-dichlorodihydrofluorescin diacetate was used. Glomerular mesangial cells were grown in chamber slides and serum-starved. The cells were washed with Hanks’ balanced salt solution, loaded with 10 mM 2′,7′-dichlorodihydrofluorescin diacetate, and incubated for 30 min at 37 °C. High glucose was added for 24 h, and differential interference contrast images were obtained using a confocal laser microscope (Olympus Fluoview 500) (24).

**Statistics**—Data were analyzed by paired Student’s t test or analysis of variance, followed by Student-Newman-Keuls analysis where necessary (11, 22). A p value of less than 0.05 was considered significant.

**RESULTS**

High Glucose-induced FoxO1 Phosphorylation Regulates Akt Activation—We have shown previously that high glucose rapidly increases PI 3 kinase activity in mesangial cells, resulting in Akt activation (5). The transcription factor FoxO1 is a direct substrate of Akt and undergoes inactivating phosphorylation at Thr-24 and at Ser-256/319 (12, 13, 25). In insulin signaling, FoxO1 is known to regulate metabolism (26). However, the phosphorylation of FoxO1 and its function downstream of Akt activation in response to high glucose has not been examined. We have reported previously that high glucose-stimulated Akt kinase contributes to mesangial cell pathology (7, 11, 17, 23). We tested the effect of high glucose on the phosphorylation of FoxO1 in mesangial cells. Because the phosphorylation sites are highly conserved among FoxO family members, to specifically determine the phosphorylation of FoxO1, we immunoprecipitated FoxO1 from high glucose-treated mesangial cells. The immunoprecipitates were immunoblotted with anti-phospho-FoxO-specific antibody. As shown in Fig. 1A, high glucose rapidly increased the phosphorylation of FoxO1 in a time-dependent manner. Prolonged incubation of mesangial cells with high glucose showed a sustained increase in phosphorylation of this transcription factor (Fig. 1, B and C). Both these early and late phosphorylation events were concomitant with an increase in phosphorylation of Akt at Ser-473 and Thr-308, which are required for its full activation (Fig. 1, D–F). Furthermore, expression of dominant negative Akt kinase blocked the high glucose-stimulated phosphorylation of FoxO1 (data not shown).

The mechanism by which Akt undergoes sustained phosphorylation/activation by high glucose is not known. Phosphorylation of FoxO1 by Akt is known to induce its translocation from the nucleus to the cytoplasm, inhibiting the transcription of its target genes. We hypothesized that FoxO1, an Akt substrate, regulates Akt phosphorylation. To test this hypothesis, we used an adenovirus containing mutant FoxO1/A3 in which all three phosphorylation sites are changed to alanine. Infection of mesangial cells showed the expression of this mutant (Fig. 1G). This mutant acts as a constitutively active transcription factor, as evidenced by the increase in a reporter activity regulated by the FoxO1 DNA binding element (Fig. 1H). Expression of this constitutively active mutant inhibited the high glucose-stimulated phosphorylation of Akt at both activating sites (Fig. 1I). To further substantiate our results, we employed a loss-of-function strategy. We used an adenovirus vector expressing a dominant negative FoxO1 that inhibits FoxO1-dependent transcription (Fig. 1, J and K). Expression of dominant negative FoxO1 increased the phosphorylation of Akt similar to treatment with high glucose (Fig. 1L, compare the third lane with the second lane). Incubation of cells with high glucose along with dominant negative FoxO1 expression did not further increase Akt phosphorylation (Fig. 1L). These results provide evidence for the presence of a positive feedback loop involving the high glucose-stimulated activation of Akt and the phosphorylation/inactivation of FoxO1 in mesangial cells.

FoxO1 Regulates High Glucose-induced mTORC1 Activation—We have shown recently that high glucose activates mTORC1 in an Akt kinase-dependent manner (5, 8, 11, 27). This activation of mTORC1 occurs via Akt-dependent phosphorylation of PRAS40, which is a component and negative regulator of mTORC1. Phosphorylation of PRAS40 induces its dissociation from mTORC1, resulting in activation of mTORC1 kinase activity (28). Our results above show that FoxO1 regulates Akt kinase activity (Fig. 1, I and L). Therefore, we examined the involvement of FoxO1 in the phosphorylation of the Akt substrate PRAS40. High glucose increased the phosphorylation of PRAS40. The expression of constitutively active FoxO1 inhibited high glucose-induced PRAS40 phosphorylation (Fig. 2A). In contrast, the expression of dominant negative FoxO1 increased the phosphorylation of PRAS40 in cells incubated with low glucose similar to high glucose treatment (Fig. 2B).
These results suggest that inactivated FoxO1-mediated phosphorylation and inactivation of PRAS40 would activate mTORC1 activity. We examined high glucose-induced mTORC1 activity by measuring the phosphorylation of S6 kinase at Thr-389, which is the direct substrate for mTORC1 (28). The expression of constitutively active FoxO1 suppressed high glucose-stimulated mTORC1 activity (Fig. 2C). On the other hand, dominant negative FoxO1 augmented mTORC1 activity similar to high glucose (Fig. 2D). To corroborate our results, we used a second substrate of mTORC1, the translation initiation
The expression of FoxO1/A3 inhibited the phosphorylation of 4EBP-1 at Thr-37/46 and Ser-65, indicative sites for mTORC1-mediated phosphorylation (Fig. 2E). In contrast to this observation, dominant negative FoxO1 increased the phosphorylation of 4EBP-1 analogous to high glucose (Fig. 2F). These results indicate that FoxO1 regulates high glucose-induced mTORC1 activity.

FoxO1 Regulates High Glucose-stimulated Mesangial Cell Hypertrophy and Matrix Protein Expression—Early changes during the progression of diabetic nephropathy involve mesangial hypertrophy and matrix expansion (2, 3). We have shown previously that mTORC1 contributes to renal hypertrophy, especially mesangial cell hypertrophy (5, 11, 27). Because FoxO1 regulates mTORC1 activity by high glucose, we tested its involvement in mesangial cell hypertrophy. An increase in protein synthesis stimulated by high glucose is necessary for hypertrophy (22). Therefore, we examined the role of FoxO1 in high glucose-induced protein synthesis. The expression of phosphorylation-deficient constitutively active FoxO1/A3 significantly inhibited high glucose-induced protein synthesis in mesangial cells (Fig. 3A). On the contrary, dominant negative FoxO1 markedly increased protein synthesis similar to that obtained with high glucose treatment (Fig. 3B). The addition of high glucose to cells expressing dominant negative FoxO1 did not further increase protein synthesis (Fig. 3B). We also determined mesangial cell hypertrophy by the ratio of protein content to cell number. FoxO1/A3 significantly inhibited high glucose-induced mesangial cell hypertrophy (Fig. 3C). On the other hand, expression of dominant negative FoxO1 induced mesangial cell hypertrophy in low glucose-incubated cells, similar to high glucose treatment (Fig. 3D). Next, we determined the increase in cell size by flow cytometry using forward scatter as the parameter of cell size. High glucose increased the mesangial cell size (Fig. 3, E and F). Expression of constitutively active FoxO1 inhibited a high glucose-induced increase in cell size (Fig. 3E). In contrast, expression of dominant negative FoxO1 increased the mesangial cell size similar to that treated with high glucose (Fig. 3F). These results indicate that FoxO1 contributes to mesangial cell hypertrophy induced by high glucose.

Along with hypertrophy, accumulation of matrix proteins, including fibronectin, represents a major pathologic feature of diabetic nephropathy (2). The levels of many matrix proteins are controlled at the levels of degradation. Plasmin degrades matrix proteins such as collagen, laminin, and fibronectin as well as promatrix metalloproteinases. PAI-1 (plasminogen activator inhibitor 1) blocks the production of plasmin from plasminogen and, thus, induces the accumulation of matrix proteins (29). The expression of PAI-1 is augmented in the
glomeruli of patients with diabetic nephropathy (29). Many cells, including mesangial cells, express PAI-1, and its levels are increased in high glucose-treated mesangial cells (23, 29). Therefore, we considered the expression of fibronectin and PAI-1 as candidates of high glucose-induced fibrotic protein expression. We have shown previously that expression of both fibronectin and PAI-1 is regulated by Akt kinase (7, 23, 30). Because FoxO1 regulates Akt kinase (Fig. 1, I and L), we examined the role of constitutively active FoxO1/A3 on both fibronectin and PAI-1 protein levels. The expression of FoxO1/A3 inhibited high glucose-induced fibronectin and PAI-1 expression (Fig. 3, G and H). On the other hand, the expression of dominant negative FoxO1 increased the expression of fibronectin and PAI-1 similar to that found with high glucose treatment (Fig. 3, I and J). These results show that, similar to the results found with mesangial cell hypertrophy,

**FIGURE 3. FoxO1 regulates mesangial cell hypertrophy and matrix protein expression.** A–D, mesangial cells were infected with Ad GFP and Ad FoxO1/A3 (A and C) or Ad DN FoxO1 (B and D), followed by incubation with LG or HG as described in Fig. 1, I and L. A and B, protein synthesis was determined as a measure of [35S]methionine incorporation as described under “Experimental Procedures.” C and D, hypertrophy of mesangial cells was determined as a ratio of the total amount of protein to cell number. The mean ± S.E. of triplicate measurements is shown. A and C, *, p < 0.001 versus LG; **, p < 0.001 versus HG. B and D, *, p < 0.001 versus LG. Bottom panels, expression of FLAG- and HA-tagged FoxO1/A3 and DN FoxO1, respectively. E and F, mesangial cells were infected with Ad FoxO1/A3 (E) or Ad DN FoxO1 (F), followed by incubation with LG or HG. The size distribution of cells was determined by flow cytometry using the forward scatter parameter (FSC). G–J, serum-starved mesangial cells were infected with the indicated adenovirus expression vectors. The cells were incubated with LG or HG medium as indicated. The cell lysates were immunoblotted with fibronectin (G and I) and PAI-1 (H and J). Immunoblots with FLAG, HA, and actin antibodies are shown.
FoxO1 contributes to high glucose-induced matrix protein expression in mesangial cells.

High Glucose Decreases Catalase Expression in a PI 3 Kinase/Akt-dependent Manner—Our data above demonstrate FoxO1-mediated Akt activation by high glucose and subsequent mesangial cell hypertrophy and matrix protein expression. Because FoxO1 is a transcription factor, we hypothesized that it regulates target gene expression, which contributes to Akt activation. FoxO1 regulates the expression of many antioxidant genes (12). We considered the antioxidant enzyme catalase, a target of the FoxO transcription factor (31). Incubation of mesangial cells with high glucose time-dependently decreased catalase mRNA expression in a sustained manner (Fig. 4, A and B). Similarly, the expression of catalase protein was suppressed by high glucose (Fig. 4, C and D).

FoxO1 is phosphorylated by PI 3 kinase-dependent Akt. Furthermore, phosphorylated FoxO1 is translocated to the cytoplasm, therefore resulting in the suppression of its target gene expression (25). We first examined the effect of the PI 3 kinase inhibitor on catalase expression. Incubation of mesangial cells with Ly294002 reversed the high glucose-induced suppression of catalase expression (Fig. 4E).

To confirm this observation, we used a vector containing a dominant negative p85 regulatory subunit that blocks the enzymatic activity of the catalytic subunit of PI 3 kinase (32). The expression of dominant negative PI 3 kinase also prevented the suppression of catalase expression.
by high glucose (Fig. 4F). PI 3 kinase produces the second mes- senger PI 3,4,5-trisphosphate, which activates Akt kinase (33). The lipid phosphatase PTEN dephosphorylates PI 3,4,5-tris- phosphate, therefore inhibiting PI 3 kinase-dependent signal- ing (33). We used an adenovirus vector expressing PTEN. The expression of PTEN in mesangial cells abolished high glucose- induced decrease in catalase expression (Fig. 4G). Next, we determined the involvement of Akt kinase downstream of PI 3 kinase. The expression of dominant negative Akt reversed the high glucose-mediated down-regulation of catalase (Fig. 4H). These results demonstrate that the high glucose-induced repression of catalase requires PI 3 kinase/Akt signaling.

**FoxO1-controlled Catalase Expression Contributes to High Glucose-induced Reactive Oxygen Species (ROS)—** Catalase is a FoxO target gene (31). Akt phosphorylates FoxO1, resulting in its cytosolic localization and inactivation as a transcription fac- tor (12, 25). Treatment of mesangial cells with high glucose also induced the translocation of FoxO1 to the cytoplasm (data not shown). Our data above show that the expression of catalase is down-regulated by high glucose (Fig. 4, A–D). We examined the involvement of the FoxO1 transcription factor in catalase mRNA expression. As predicted, high glucose significantly reduced catalase mRNA expression (Fig. 5A). The expression of the constitutively active FoxO1/A3 reversed the high glucose- induced suppression of catalase mRNA expression (Fig. 5A).

Similarly, FoxO1/A3 prevented the decrease in catalase protein expression induced by high glucose (Fig. 5B). In contrast to these results, the expression of dominant negative FoxO1 alone in cells grown in low glucose was sufficient to decrease catalase mRNA and protein expression similar to that observed with high glucose treatment (Fig. 5, C and D). Both dominant negative FoxO1 and high glucose together inhibited catalase expression to the same extent as high glucose alone (Fig. 5, C and D).

We and others have shown previously that high glucose increases the production of ROS in renal cells, including mesangial cells (1, 14, 21). Our results above show decreased catalase expression in response to high glucose-induced inactiva- tion of FoxO1. Therefore, we tested the role of FoxO1 on high glucose-stimulated ROS production. As expected, high glucose elevated the levels of ROS, as determined by the fluorescence-based assay using peroxide-sensitive 2,7’-dichlorodihydrofluorescin diacetate and confocal microscopy, which essentially measures hydrogen peroxide (14). The expression of constitu- tively active FoxO1/A3 abrogated high glucose-stimulated ROS production (Fig. 5E, compare d with b). In contrast, the expres- sion of dominant negative FoxO1 itself increased ROS produc- tion (Fig. 5F, compare c with a). However, dominant negative FoxO1 in the presence of high glucose increased the production of ROS similar to high glucose (Fig. 5F, compare d with b). These results indicate that the high glucose-stimulated phos- phorylation of FoxO1 and, hence, its inactivation regulates ROS production in mesangial cells.

**Catalase Regulates High Glucose-induced Akt Signal Trans- duction—** Because our results demonstrate that high glucose- inactivated FoxO1 regulates Akt activation and its downstream signaling to induce pathology in mesangial cells, we hypothe- size that FoxO1-regulated catalase contributes to Akt signal transduction and, hence, to mesangial cell pathology. We

examined the effect of catalase on the phosphorylation of Akt. The expression of catalase inhibited the high glucose-induced phosphorylation of Akt at both sites (Fig. 6A). Because Akt phosphorylates FoxO1, we tested its phosphorylation. The expression of catalase suppressed the high glucose-stimulated phosphorylation of FoxO1 (Fig. 6B). Also, Akt phosphorylates the mTORC1 component PRAS40 at Thr-246 (28). Catalase blocked this phosphorylation in response to high glucose (Fig. 6C). Because phosphorylation and, hence, inactivation of PRAS40 controls mTORC1 activity, the expression of catalase inhibited mTORC1 activity, as judged by the phosphorylation of S6 kinase and 4EBP-1 (Fig. 6, D and E). These results show that catalase regulates high glucose-induced Akt/mTORC1 signal transduction in mesangial cells.

Because FoxO1 regulates mesangial cell hypertrophy and matrix protein expression (Fig. 3) and FoxO1 controls catalase expression (Fig. 5, A–D), we examined the role of this antioxi- dant enzyme on these features of mesangial cell pathology. As shown in Fig. 7A, the expression of catalase significantly inhib- ited high glucose-stimulated protein synthesis. Similarly, cata- lace blocked hypertrophy of mesangial cells induced by high glucose (Fig. 7B). Furthermore, expression of catalase reduced the increase in mesangial cell size by high glucose, as measured by forward scatter (Fig. 7C). Catalase also suppressed the expression of both fibronectin and PAI-1 in mesangial cells (Fig. 7, D and E). These results suggest that a high glucose- induced decrease in catalase contributes to increased Akt sig- naling, leading to mesangial cell hypertrophy and matrix protein expansion.

**Reduced Expression of Catalase in Mouse Kidneys with Diabetes—** To investigate the in vivo relevance of our findings above, we used the transgenic OVE26 mouse model of type 1 diabetes. These mice are hyperglycemic within 3 days of birth (19). They develop diabetic nephropathy showing pathologic features, including an increase in mesangial volume and matrix protein expression (20). The phosphorylation of FoxO1 was examined in the renal cortices from 3-month-old diabetic mice and compared with control FVB non-diabetic mice. Fig. 8, A and B, shows a significant increase in phosphorylation of FoxO1 in diabetic renal tissues. This increase in FoxO1 phos- phorylation was associated with phosphorylation of Akt at both Thr-308 and Ser-473 (Fig. 8, C and D). Next, we determined the expression of catalase. The levels of catalase protein and mRNA in the diabetic mice were reduced significantly compared with the control non-diabetic mice (Fig. 8, E–G). Finally, concom-itant with the decrease in catalase expression, we found a significantly increased expression of fibronectin and PAI-1 (Fig. 8, H–K). These results show a reciprocal correlation between catalase expression and Akt/FoxO1 phosphoryla- tion and fibronectin/PAI-1 expression in renal tissues of mice with type 1 diabetes.

**DISCUSSION**

We show that inactivated FoxO1 controls a positive feedback loop for sustained Akt activation in response to high glucose. Our results, for the first time, demonstrate that FoxO1 regu-lates high glucose-stimulated mTORC1 activity, mesangial cell hypertrophy, and matrix protein expression. These biological
functions are controlled by the FoxO1-target gene catalase, which contributes to sustained levels of ROS necessary for mesangial cell pathology (Fig. 9). Furthermore, our results demonstrate the presence of an antagonistic relationship between catalase expression and matrix protein abundance in the kidneys of diabetic mice.

Three members of FoxO (1, 3, and 4) appear to regulate the common target genes (12, 26). Using a liver-specific deletion strategy, it has been shown that FoxO1, and not FoxO3/4, reduced blood glucose concentration both in normal and diabetic mice (34). Furthermore, the expression of FoxO1 in different tissues caused insulin resistance and glucose intolerance.

**FIGURE 5. FoxO1 regulates catalase expression and ROS production.** A–D, mesangial cells were infected with Ad FoxO1/A3 (A and B) or Ad DN FoxO1 (C and D) and Ad GFP for 24 h prior to incubation with LG or HG glucose for 24 h. A and C, total RNAs were prepared and used to detect catalase mRNA. The mean ± S.E. of triplicate measurements is shown. A, *, p < 0.001 versus LG; **, p < 0.001 versus HG. B and D, cell lysates were immunoblotted with catalase, FLAG, HA, and actin antibodies as indicated. E and F, FoxO1 regulates high glucose-stimulated ROS production. Serum-starved mesangial cells were infected with Ad FoxO1/A3 (E) or Ad DN FoxO1 (F) and Ad β-Gal for 24 h. The cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescin diacetate for 30 min before incubation with LG and HG for 24 h. 2′,7′-dichlorodihydrofluorescin fluorescence was measured using confocal microscopy as described under “Experimental Procedures.” In parallel samples, the expression of FLAG- and HA-tagged FoxO1/A3 and DN FoxO1 was examined (data not shown).
These results suggest a significant role of FoxO1 in diabetic complications. FoxO transcription factors undergo posttranslational modifications, including phosphorylation at different Ser/Thr residues by different kinases. The phosphorylation of FoxOs has been shown to be either activating or inactivating, depending upon the kinase that phosphorylates the transcription factor (12). In this study, we show the phosphorylation of FoxO1 by high glucose-stimulated Akt kinase (Fig. 1, A–D, and data not shown). To carry out the biological function of high glucose in mesangial cells, such as hypertrophy and matrix expansion, sustained activation of Akt is required. In fact we found sustained phosphorylation of Akt and FoxO1 (Fig. 1, A–F).

Recently, it has been shown that FoxO1 activates Akt in cardiomyocytes, fibroblasts, and various cancer cells (37, 38). The mechanism of Akt activation involves phosphorylation at both Thr-308 and Ser-473 (39, 40). PP2A/B dephosphorylates Akt, therefore inhibiting its activity (41). FoxO1 binds to these phosphatases and disrupts their complexes with Akt, resulting in the activation of Akt (37). In contrast to these results, FoxO1 inhibited high glucose-stimulated Akt phosphorylation in mesangial cells (Fig. 1I). To corroborate these results, we also found increased phosphorylation of Akt by the expression of dominant negative FoxO1 (Fig. 1I). Therefore, our results demonstrate a new positive feedback mechanism of Akt activation in mesangial cells in which activated Akt by high glucose phosphorylates and inactivates FoxO1, which, in turn, promotes the phosphorylation of Akt in a sustained manner (Fig. 9).

In mesangial cells, high glucose-induced pathology is mediated by the Akt-dependent sustained activation of mTOR, which exists in two complexes (mTORC1 and mTORC2) (5, 11, 28). mTORC1 is activated by Akt-dependent phosphorylation and, hence, inactivation of the tumor suppressor protein tuberin, which blocks mTORC1 kinase activity (28). An additional mechanism involves phosphorylation and inactivation of PRAS40, a negative regulatory subunit of mTORC1 (28). Recently, Chen et al. (38) showed that FoxO1 increased the expression of sestrin3, which negatively regulates mTORC1 activity in fibroblasts and cancer cells. In addition, these authors demonstrated an increased expression of rictor, which negatively regulates mTORC1 activity in fibroblasts and cancer cells. In contrast to these observations, we found decreased activation of high glucose-induced mTORC2 in the presence of FoxO1, resulting in the inhibition of Akt phosphorylation (Fig. 1I). In line with this observation, we found that high glucose-induced phosphorylation of the Akt substrate PRAS40 was inhibited by active FoxO1 (Fig. 2A). Therefore, we propose that a lack of phosphorylation/inactivation of PRAS40 by active FoxO1 contributes to the sustained suppression of mTORC1 activity (Fig. 2, C and E). In fact, this observation is...
confirmed by increased mTORC1 activity by dominant negative FoxO1 (Fig. 2, D and F).

mTORC1-mediated phosphorylation of 4EBP-1 induces its inactivation, therefore relieving its suppressive effect on translation initiation, leading to the increased protein synthesis necessary for cellular hypertrophy (5, 17, 42). In addition, activation of S6 kinase phosphorylates the ribosomal protein s6 to increase the translation efficiency of the attached mRNAs. Our results show that FoxO1 regulates high glucose-stimulated mTORC1 activation (Fig. 2). Now we provide evidence that FoxO1 contributes to the inactivation and activation of 4EBP-1 and S6 kinase, respectively, resulting in high glucose-stimulated protein synthesis and mesangial cell hypertrophy (Fig. 3, A–F).

Expansion of matrix proteins is a significant pathologic feature of diabetic kidney disease. Activated mesangial cells in response to high glucose produce matrix proteins such as fibronectin. Also, the levels of matrix proteins are controlled by a regulatory mechanism, which involves high glucose-stimulated expression of PAI-1 (29). In the present study, we for the first time demonstrate that high glucose-induced inactivation of FoxO1 contributes to both fibronectin and PAI-1 expression (Figs. 3G – 3J). These results suggest that the role of FoxO1 in high glucose condition does not involve its direct transcriptional effect on the expression of these genes. Rather we propose that FoxO1 induces expression of other gene(s), which may contribute to sustained signaling events involving Akt/mTORC1 to regulate mesangial cell hypertrophy and expression of fibronectin and PAI-1 (see below).

To this end, we considered the involvement of the signaling function of hydrogen peroxide as an alternative mechanism of Akt activation via FoxO1 (43). In mesangial cells, high glucose...
readily produces hydrogen peroxide by dismutation of superoxide (14). Therefore, hydrogen peroxide may induce the inactivation of phosphatases such as PTEN or the activation of kinases, including Akt (44, 45). Multiple enzymes, including glutathione peroxidases, peroxiredoxins, and catalase, remove hydrogen peroxide from cells (43). Therefore, repression of expression of these enzymes could contribute to the sustained levels of hydrogen peroxide and, hence, its signaling capacity.

Patients with homozygous mutations in the catalase gene possess a remaining 10% of catalase activity. Therefore, they display hypocatalasemia (46). These patients are more prone to type 2 diabetes (46). In diabetic mice, catalase has been found to be significantly down-regulated (47). Moreover, catalase is one of 20 susceptible genes in type 1 diabetic patients with nephropathy (48). Patients with nephropathy showed a lowered expression of catalase when compared with those without complications (49). In line with these observations, in this study, we found that high glucose decreased the expression of both catalase mRNA and protein in mesangial cells (Fig. 4, A–D). These results provide a mechanism for sustained hydrogen peroxide levels in response to high glucose in mesangial cells (Fig. 9). In breast tumor cells, it has been reported that the FoxO transcription factor does not regulate the expression of catalase gene (50). In contrast to these results, our data demonstrate that the inactivation of FoxO1 contributes to a high glucose- and hyperglycemia-induced decrease in catalase protein and mRNA expression, indicating a transcriptional mechanism of regulation (Fig. 5, C and D). Furthermore, Akt signal transduction is required for a decrease in catalase expression (Fig. 4H). Importantly, our data, for the first time, demonstrate that the production of hydrogen peroxide by high glucose in mesangial cells is controlled by the Akt-mediated phosphorylation/inactivation of FoxO1 (Fig. 5, E and F). Therefore, our results pro-

**FIGURE 8. Phosphorylation of FoxO1 is associated with inhibition of catalase expression and fibronectin and PAI-1 expression in OVE26 mice renal cortices.** A, renal cortical lysates from 3-month-old control FVB and OVE26 type 1 diabetic mice were immunoprecipitated with FoxO1 antibody, followed by immunoblotting with phospho-FoxO1 (Thr-24) and FoxO1 antibodies. Total lysates were immunoblotted with actin antibody. B, quantification of phospho-FoxO1 with mean ± S.E. of three animals, *, p = 0.001 versus control. C, control; D, diabetes. C, the renal cortical lysates were immunoblotted with phospho-Akt (Ser-473), phospho-Akt (Thr-308), and Akt antibodies. D, quantification of phospho-Akt (Ser-473) (left panel) and phospho-Akt (Thr-308) (right panel) with mean ± S.E. of three animals is shown, *, p = 0.002 versus control. E, renal cortical lysates from FVB and OVE26 mice were immunoblotted with catalase and actin antibodies. F, quantification of catalase protein expression with mean ± S.E. of three animals, *, p = 0.001 versus control. G, total RNAs from FVB and OVE26 mice were used to detect catalase mRNA as described under “Experimental Procedures.” The mean ± S.E. of four animals is shown, *, p = 0.01 versus control animals. H and J, renal cortical lysates were immunoblotted with fibronectin (H) and PAI-1 (J) and actin antibodies. I and K, quantification of fibronectin (I) and PAI-1 (K) with mean ± S.E. of three animals, *, p = 0.005 and 0.006 versus control for J and K, respectively.
provide a mechanism for abundance of increased hydrogen peroxide in high glucose-treated mesangial cells by down-regulation of catalase (Fig. 5). This conclusion is further supported by our observation that the expression of catalase inhibited high glucose-stimulated Akt phosphorylation, resulting in attenuation of mesangial cell hypertrophy and matrix protein expression, resulting in diabetic nephropathy.

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