Original Article

Reduction of Diabetes-Induced Oxidative Stress, Fibrotic Cytokine Expression, and Renal Dysfunction in Protein Kinase Cβ–Null Mice

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Diabetes induces the activation of several protein kinase C (PKC) isoforms in the renal glomeruli. We used PKC-β$^{-/-}$ mice to examine the action of PKC-β isoforms in diabetes-induced oxidative stress and renal injury at 8 and 24 weeks of disease. Diabetes increased PKC activity in renal cortex of wild-type mice and was significantly reduced (<50% of wild-type) in diabetic PKC-β$^{-/-}$ mice. In wild-type mice, diabetes increased the translocation of PKC-α and -β1 to the membrane, whereas only PKC-α was elevated in PKC-β$^{-/-}$ mice. Increases in urinary isoprostane and 8-hydroxydeoxyguanosine, parameters of oxidative stress, in diabetic PKC-β$^{-/-}$ mice were significantly reduced compared with diabetic wild-type mice. Diabetes increased NADPH oxidase activity and the expressions of p47^{phox}, Nox2, and Nox4 mRNA levels in the renal cortex and were unchanged in diabetic PKC-β$^{-/-}$ mice. Increased expression of endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, connective tissue growth factor (CTGF), and collagens IV and VI found in diabetic wild-type mice was attenuated in diabetic PKC-β$^{-/-}$ mice. Diabetic PKC-β$^{-/-}$ mice were protected from renal hypertrophy, glomerular enlargement, and hyperfiltration observed in diabetic wild-type mice and had less proteinuria. Lack of PKC-β can protect against diabetes-induced renal dysfunction, fibrosis, and increased expressions of Nox2 and -4, ET-1, VEGF, TGF-β, CTGF, and oxidant production. *Diabetes* 55:3112–3120, 2006

Diabetic nephropathy is characterized by glomerular hyperfiltration, extracellular matrix accumulation, glomerular enlargement, mesangial expansion, and intertubular fibrosis, resulting ultimately in diabetic glomerulosclerosis and progressive renal insufficiency (1,2). Hyperglycemia-induced metabolic and hemodynamic factors are thought to be mediators of this injury, which is associated with the diabetic state (3). The hemodynamic factors implicated in the pathogenesis of diabetic nephropathy include increased systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including the renin-angiotensin system and endothelins (3). This may interact with metabolic pathways activating signaling pathways that lead to renal injury. Multiple biochemical pathways have been proposed to explain the adverse effects of hyperglycemia. Activation of diacylglycerol (DAG)–protein kinase C (PKC) pathway (4), enhanced polyol pathway (5), increased oxidative stress (6), and overproduction of advanced glycation end products (AGEs) (7) have all been proposed as potential cellular mechanisms by which hyperglycemia induces chronic diabetes complications.

We and others (8–10) have previously reported that multiple PKC isoforms are activated in each vascular tissue of diabetic animal models, and activation of the DAG-PKC pathway is a key mediator of diabetes vascular complications. Immunoblotting studies have reported that PKC-α and -β1 isoforms were increased in vivo in membranous fractions (activated pool) of diabetic rat glomeruli and in vitro in mesangial cells exposed to elevated glucose levels (10), whereas PKC-β2 was reported to be preferentially activated in the aorta and heart of diabetic rats (8). Whiteside and Dlugosz (11) reported that PKC-β and -δ isoforms were also increased in the membrane pool in the glomeruli of diabetic rats. Treatment of diabetic animals with a selective PKC-β isoform inhibitor (LY333531 or ruboxistaurin [RBX]) was associated with normalization of hemodynamic changes, extracellular matrix, and histological features of glomerular damage in animal models of diabetes (10,12,13). Phase two clinical trial results suggested that RBX can decrease the loss of glomerular filtration rate (GFR) and proteinuria in diabetic patients already treated with inhibitors of angiotensin actions (14). Recently, activation of NADPH oxidase and increased reactive oxygen species (ROS) production have been proposed as important mediators of renal dysfuncton in diabetes (15,16). We have reported that inhibition of PKC-β by RBX can also normalize diabetes or hyperglycemia-induced oxidative stress (17). PKC-β has been noted to contribute to NADPH oxidase activations in multiple cells, including endothelial and mesangial cells (18). However, it is still unclear which of the renal abnormalities are induced by PKC-β isoforms as compared with other PKC isoforms and which of the potential downstream biochem-

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8-OHdG, 8-hydroxydeoxyguanosine; AGE, advanced glycation end product; CTGF, connective tissue growth factor; DAG, diacylglycerol; ET-1, endothelin-1; FF, filtration fraction; GFR, glomerular filtration rate; PAH, para-aminohippurate; PKC, protein kinase C; RBX, ruboxistaurin; ROS, reactive oxygen species; RPF, renal plasma flow; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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TABLE 1
General characteristics of the four experimental mouse groups

	Wild-type	Wild-type diabetic	PKC-β$^{-/-}$	PKC-β$^{-/-}$ diabetic
At 8 weeks of diabetes				
n	6	6	6	6
Body weight (g)	29.8 ± 2.1	25.2 ± 2.4*	28.3 ± 2.5	24.6 ± 2.7*
Blood glucose (mg/dl)	100 ± 10	434 ± 50†	98 ± 13	445 ± 49†‡
SBP (mmHg)	106 ± 18	108 ± 13	111 ± 18	114 ± 16
DBP (mmHg)	79 ± 17	84 ± 4	83 ± 15	84 ± 17
At 24 weeks of diabetes				
n	6	6	6	6
Body weight (g)	50.9 ± 4.8	39.4 ± 2.9†	47.7 ± 3.2	38.5 ± 4.7†‡
Blood glucose (mg/dl)	101 ± 10	412 ± 81†	99 ± 11	436 ± 42†‡
SBP (mmHg)	108 ± 8	110 ± 8	105 ± 14	105 ± 11
DBP (mmHg)	81 ± 16	76 ± 13	76 ± 13	76 ± 16

*$P < 0.05$ versus wild-type control; †$P < 0.01$ versus wild-type control; ‡$P < 0.01$ versus nondiabetic PKC-β$^{-/-}$. DBP, diastolic blood pressure; SBP, systolic blood pressure.

ical pathways are activated to cause the functional and pathological changes. In the present study, we used the PKC-β$^{-/-}$ mouse to clarify the role of PKC-β isoform activation on the various biochemical, physiological, and pathological abnormalities in the glomeruli induced by diabetes and hyperglycemia. This information could be important to evaluate the use of PKC-β inhibitors in the treatment of diabetic nephropathy by identifying the specific renal end points for determining efficacy.

RESEARCH DESIGN AND METHODS

PKC-β inhibitors were provided by Dr. Michael Leitges. The derivation of PKC-β$^{-/-}$ mice has been described previously (19). Wild-type mice with the same genetic background (129×C57BL6) as the knockout mice were used as control animals. Diabetes was induced in 6-week-old male PKC-β$^{-/-}$ and wild-type mice fasted for 12 h with intraperitoneal injections of streptozotocin in citrate buffer (70 mg/kg body wt) (Sigma Chemical, St Louis, MO) on 2 consecutive days. The diabetic state was confirmed with blood glucose levels exceeding 250 mg/dl. Control animals received an injection of sterile citrate buffer. All experiments were conducted in accord with the Joslin Diabetes Center's Animal Care and Use Committee guidelines. Blood pressure was monitored by tail-cuff plethysmography (Ueda Electric Works, Tokyo, Japan). Glucose levels from tail blood samples were measured with a Glucometer Elite XL (Bayer, Elkhart, IL). At 8 and 24 weeks after diabetes, mice were placed in metabolic cages for collection of urine. The samples were stored frozen at −80°C. Albuminuria, urinary isoprostane, and 8-hydroxydeoxyguanosine (8-OHdG) were all measured by enzyme-linked immunosorbent assay kits from Exocell (Philadelphia, PA), Oxis International (Foster City, CA), and Genox (Baltimore, MD), respectively.

Measurement of GFR. Renal clearance studies were performed at 8 weeks of diabetes as previously described, with modification for mice (20). Briefly, mice were anesthetized with an intraperitoneal injection of thiopental sodium. A catheter was inserted into the left jugular vein for continuous infusion of inulin and para-aminohippurate (PAH) solution. The urinary bladder was catheterized for urine collection. After equilibration, blood samples from mice tail and urine samples were obtained for measurement of inulin and PAH at timed intervals. The concentrations of inulin in plasma and urine were measured by cysteine-tryptophan reaction, and PAH was determined by a calorimetric technique as described previously (20). GFR was calculated from inulin clearance and renal plasma flow (RPF) from PAH clearance and filtration fraction from a standard formula, the GFR-to-RPF ratio.

Histological assessment. For morphometric analysis of glomeruli, kidneys from animals were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm. Sections were stained with hematoxylin and periodic acid Schiff. Sections were coded and read by an observer unaware of the experimental protocol applied. In each animal, glomerular area was measured in 50 consecutive glomeruli encountered in a back and forth scan between the outer and inner cortex. The images were imported into Image-Pro Plus (Media Cybernetics, Silver Spring, MD), and the glomerular tuft area was quantified for each glomerular cross-section.

Measurement of PKC activity. To evaluate PKC activity in the renal cortex, a modified in situ PKC assay was used (20). To assess activation of different isoforms of PKC, expression of PKC isoforms in the cytosolic and membranous fractions of the renal cortex were assessed by immunoblot analysis. The fractionation of the renal cortex was performed with a method involving ultracentrifugation steps as previously described (10).

The cytosolic and membranous fractions obtained were then used for immunoblotting according to methods described previously (10). Antibodies to the various PKC isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p47^{phox} antibody was supplied from Abcam (Cambridge, MA).

Gene expression. Real-time PCR was performed to evaluate the mRNA expression of transforming growth factor (TGF)-β, connective tissue growth factor (CTGF), type IV and VI collagens, endothelin-1 (ET-1), and vascular endothelial growth factor (VEGF) in the renal cortex of various groups of mice. Total RNA extraction and reverse transcription were performed with the RNeasy Mini kit (Qiagen, Valencia, CA) and the First-Strand cDNA Synthesis Kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Quantitative real-time PCR of TGF-β1, CTGF, and collagen IV and VI was performed with a TaqMan Universal PCR MasterMix and TaqMan Gene Expression assays (TGF-β1, Mm 00441724_m1; CTGF, Mm 00515790_g1; collagen IV, Mm 00802372_m1; and collagen VI, Mm 00487160_m1) (Applied Biosystems, Forest City, CA). Primer and probe sequences of NADPH oxidase subunits are listed as follows: p47^{phox} (MN 010876.2, forward 5′-ACCTTCAT TCGCCATATCGCCCT-3′, reverse 5′-TTCTGTAGACCACCTTCTCCGACA-3′, and probe 5′6FAM-CCCAGCCAGCACTATGTGTACATGTT-TAMRA3′), Nox2 (MN 007807.2, forward 5′-TGCAGCCTGCCTGAATTTCAACTG-3′, reverse 5′-AGATGTGCAATTGTGTGGATGGCG-3′, and probe 5′6FAM-TGCGTGTTGC TCGACAAGGATTCGAA-TAMRA3′), and Nox4 (NM_015760, forward 5′-TGT TGGGCCTAGGATTGTGTT-3′, reverse 5′-AAAAGGATGAGGCTGCAGTTG-3′, and probe 5′-6FAM-AAGCAGAGCATCTGCATCTGTCCTCAACC-TAMRA 3′). Primers and probes for ET-1 and VEGF were previously described (21). 18S ribosomal RNA expression was used for normalization.

Statistics. All data are presented as means ± SD. Comparisons between groups were performed using one-way ANOVA, with post hoc correction by Tukey's method. Comparisons of urinary albumin excretion between groups were made with the Mann-Whitney nonparametric test, since the values were not normally distributed. Comparisons between two groups were performed using an unpaired *t* test. $P < 0.05$ was considered significantly different.

RESULTS

General characteristics. Both diabetic wild-type and PKC-β$^{-/-}$ mice exhibited marked hyperglycemia during the experimental periods (8 and 24 weeks) (Table 1). Diabetic wild-type and PKC-β$^{-/-}$ mice had significantly lower body weight at the end of the experimental period compared with nondiabetic counterparts, regardless of genotype, but higher than at beginning of the study. There was no significant difference between wild-type and PKC-β$^{-/-}$ mice in body weight, blood glucose, or blood pressure. Similarly, there was no significant difference in these physiological parameters between diabetic wild-type and PKC-β$^{-/-}$ mice at 8 and 24 weeks.

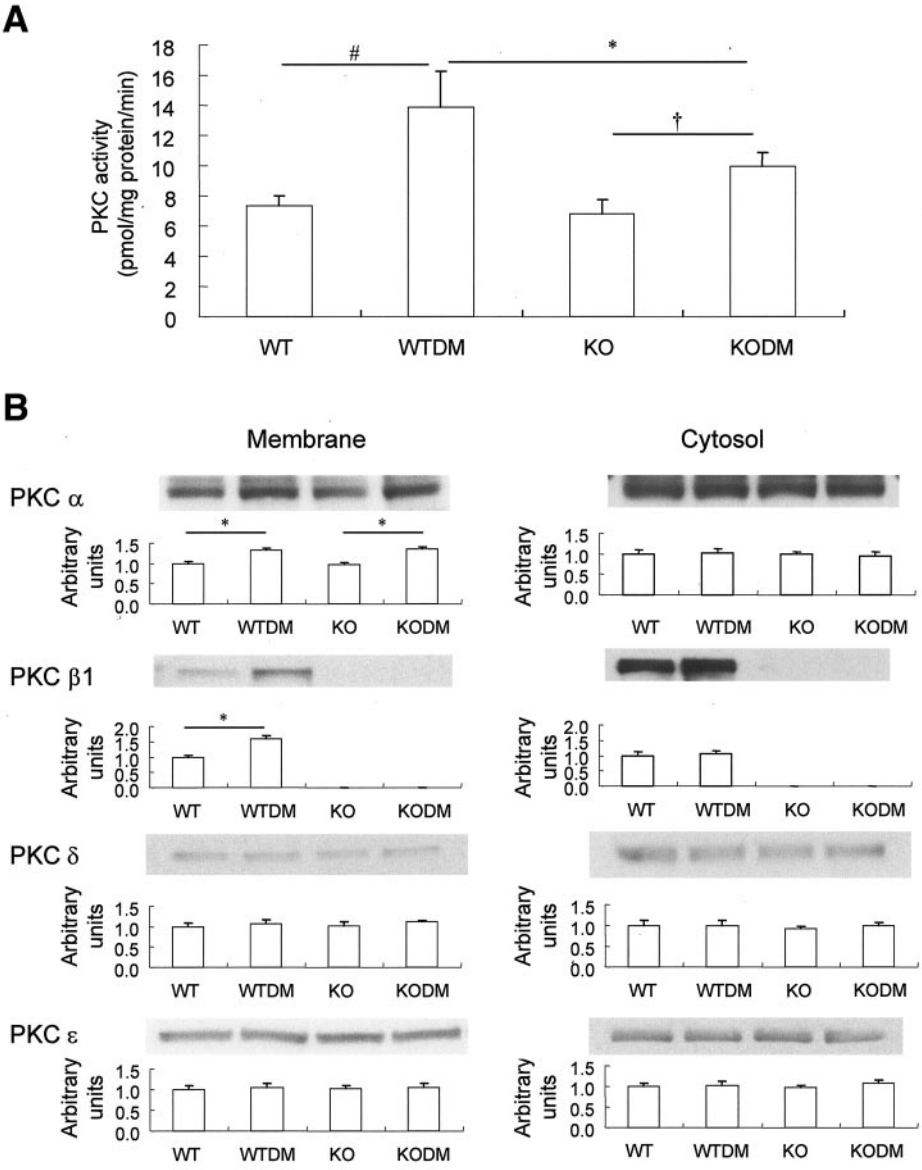

FIG. 1. ***A*****: PKC activity in renal cortex of wild-type and PKC-β$^{-/-}$ mice as measured by in situ PKC assay.** $n = 5$ **for each group.** ***B*****: Expression of various PKC isoforms in the membranous and cytosol fractions of renal cortex assessed by immunoblotting. Values for protein expression are expressed as fold change versus wild-type.** $n = 4$ **for each group (also indicates the number of mice studied). KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. †$P < 0.05$, *$P < 0.01$, #$P < 0.001$.**

Reduced PKC activation associated with diabetes in PKC-β$^{-/-}$ mice. Induction of diabetes for 8 weeks was associated with elevated PKC activity in the renal cortex in wild-type mice, from 7.35 ± 0.66 to 13.9 ± 2.39 pmol · mg protein^{-1} · min^{-1}, an increase of 89% ($P < 0.001$). When diabetes was induced in PKC-β$^{-/-}$ mice, activated PKC pool was only elevated by 46% to 9.96 ± 0.9 pmol · mg protein^{-1} · min^{-1}, which was significantly less than the increase in wild-type mice ($P < 0.01$, Fig. 1*A*). To compare the relative contribution of different isoforms of PKC to the elevated PKC activity observed in the diabetic state, expression of various isoforms of PKC in membranous and cytosolic fractions of renal cortex were examined (Fig. 1*B*). In wild-type mice, diabetes increased PKC-β1 expression by 58% ($P < 0.01$) in the membranous fraction, consistent with activation of this isoform. There was no detectable PKC-β1 in the PKC-β$^{-/-}$ mice. Diabetes increased the expression of PKC-α by 31% ($P < 0.01$) in the membranous fractions, which was observed in both wild-type and PKC-β$^{-/-}$ mice. There was no difference in expression of PKC-α, -δ, and -ε isoforms in the cytosolic fractions between nondiabetic wild-type and PKC-β$^{-/-}$ mice.

Amelioration of renal dysfunctions in diabetic PKC-β$^{-/-}$ mice. Renal function was evaluated by GFR and filtration fraction (FF) following 8 weeks of diabetes (Fig. 2). GFR and FF increased in diabetic wild-type mice by 55 and 57%, respectively, compared with nondiabetic wild-type mice ($P < 0.05$). In contrast, increases in GFR and FF were not observed in diabetic PKC-β$^{-/-}$ mice for the same duration. Furthermore, PKC-β$^{-/-}$ mice with diabetes had significantly lower GFR ($P < 0.05$) and FF ($P < 0.05$) than wild-type mice with diabetes.

After 8 weeks of diabetes, urinary albumin excretion increased 10-fold in wild-type mice (Fig. 3, $P < 0.01$). Induction of diabetes in PKC-β$^{-/-}$ mice was also associated with an increase in urinary albumin excretion by 4.4-fold, which was significantly less than that observed in

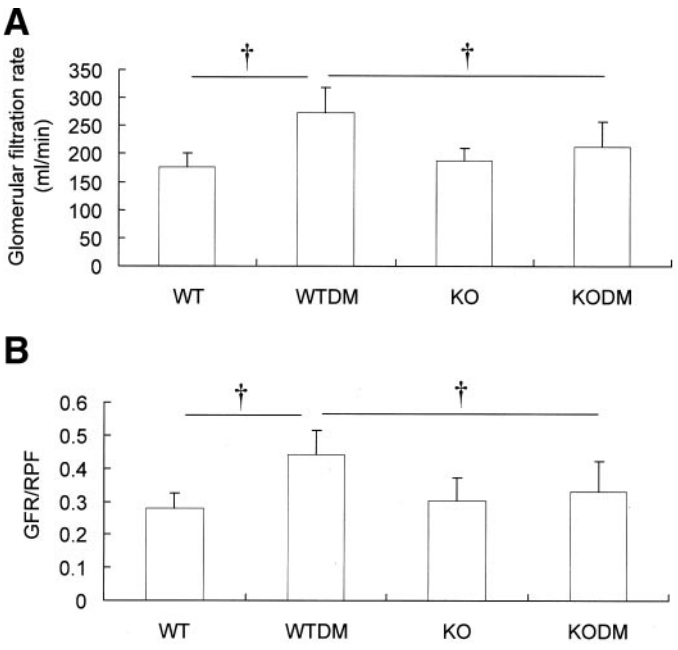

FIG. 2. GFR (*A*) and filtration fraction (GFR/RPF) (*B*) in wild-type and PKC-β$^{-/-}$ mice at 8 weeks of diabetes. KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. n = 6 for each group (also indicates the number of mice studied individually). †P < 0.05.

diabetic wild-type animals ($P < 0.01$). Similarly, following 24 weeks of diabetes, albuminuria remained significantly increased in both diabetic wild-type and PKC-β$^{-/-}$ mice. However, the amount of albuminuria in diabetic PKC-β$^{-/-}$ mice was significantly less than in wild-type mice exposed to diabetes of the same duration (Fig. 3, $P < 0.01$).

Protection against diabetes-induced oxidative stress in PKC-β$^{-/-}$ mice. Elevation in oxidative stress has been associated with diabetic nephropathy as measured by increases in the levels of several urinary oxidants (22). Thus, we measured the urinary excretions of isoprostane and 8-OHdG. After 8 weeks of diabetes, urinary isoprostane levels were increased 3.6-fold in diabetic wild-type mice as compared with nondiabetic wild-type mice ($P < 0.001$). This increase in urinary isoprostane was significantly attenuated in the diabetic PKC-β$^{-/-}$ mice (Fig. 4*A*, $P < 0.001$). Similarly, after 24 weeks of diabetes, diabetic PKC-β$^{-/-}$ mice continued to excrete significantly less isoprostane when compared with diabetic wild-type mice

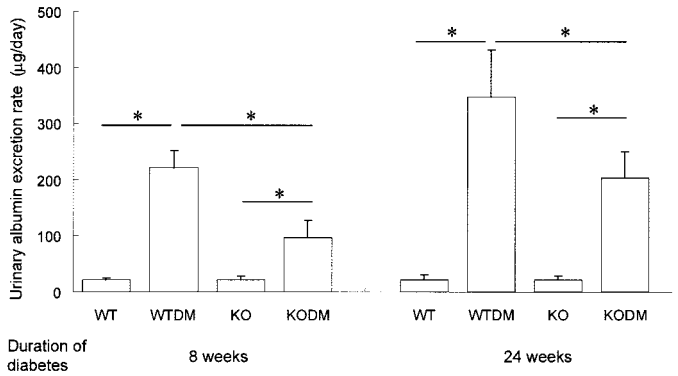

FIG. 3. Urinary albumin excretion rate in wild-type and PKC-β$^{-/-}$ mice after 8 and 24 weeks of diabetes. KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. n = 5 for each group. *P < 0.01.

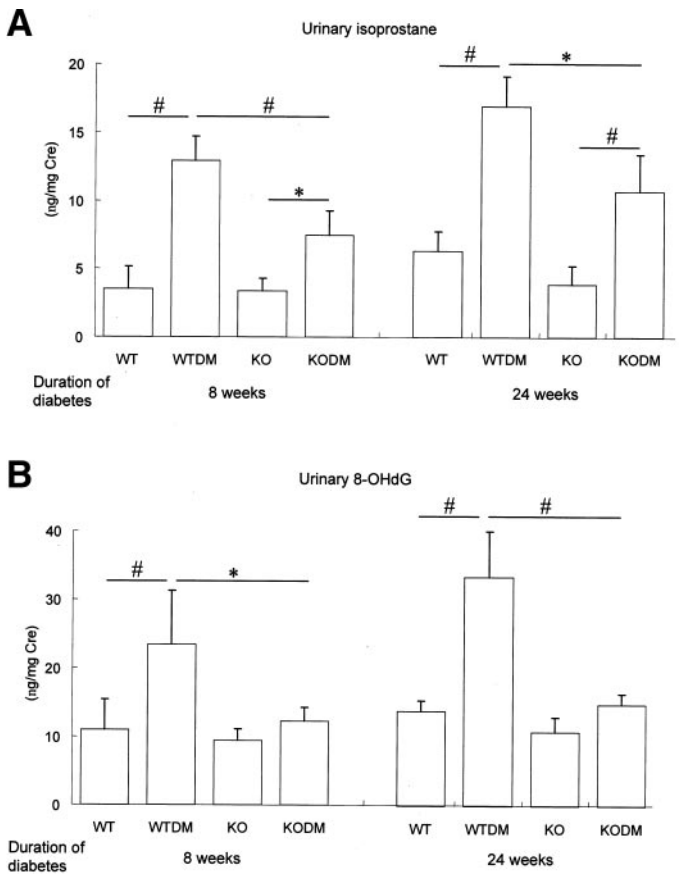

FIG. 4. Markers of oxidative stress, urinary isoprostane (*A*) and urinary 8-OHdG (*B*), in wild-type and PKC-β$^{-/-}$ mice after 8 and 24 weeks of diabetes. The levels of these two markers were normalized by urinary creatinine content. Cre, creatinine; KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. n = 5 for each group (also indicates the number of mice studied individually). *P < 0.01, #P < 0.001.

($P < 0.01$). Urinary 8-OHdG levels were not significantly increased in diabetic wild-type mice compared with nondiabetic wild-type control mice. Urinary 8-OHdG levels were significantly reduced in diabetic PKC-β$^{-/-}$ mice compared with diabetic wild-type mice ($P < 0.01$, Fig. 4*B*).

Reduced activation of NADPH oxidase in PKC-β$^{-/-}$ mice with diabetes. Since activation of NADPH oxidase has been reported to be induced by diabetes and is associated with increased oxidant production induced by hyperglycemia, we assessed changes in several components of this enzyme complex (15). As shown in Fig. 5*A*, there was increased membranous translocation of p47 subunit of NADPH oxidase by 1.73 ± 0.4-fold ($P < 0.01$) in diabetic wild-type mice compared with wild-type control. This activation was not observed in diabetic PKC-β$^{-/-}$ mice. Furthermore, diabetes also significantly increased the mRNA expressions of p47 subunit of NADPH oxidase, Nox2, and Nox4 as compared with wild-type control mice. These increases were not seen in diabetic PKC-β$^{-/-}$ mice (Fig. 5*B–D*). Expression of Nox1 mRNA level was too low to be assessed credibly (data not shown).

Altered expression of ET-1 and VEGF in PKC-β$^{-/-}$ mice with diabetes. Elevation of ET-1 in glomeruli has been postulated to be involved in diabetes-induced alteration of hemodymics and changes in extracellular matrix (23). As reported previously, expression of ET-1 mRNA in renal cortex of diabetic wild-type mice was significantly

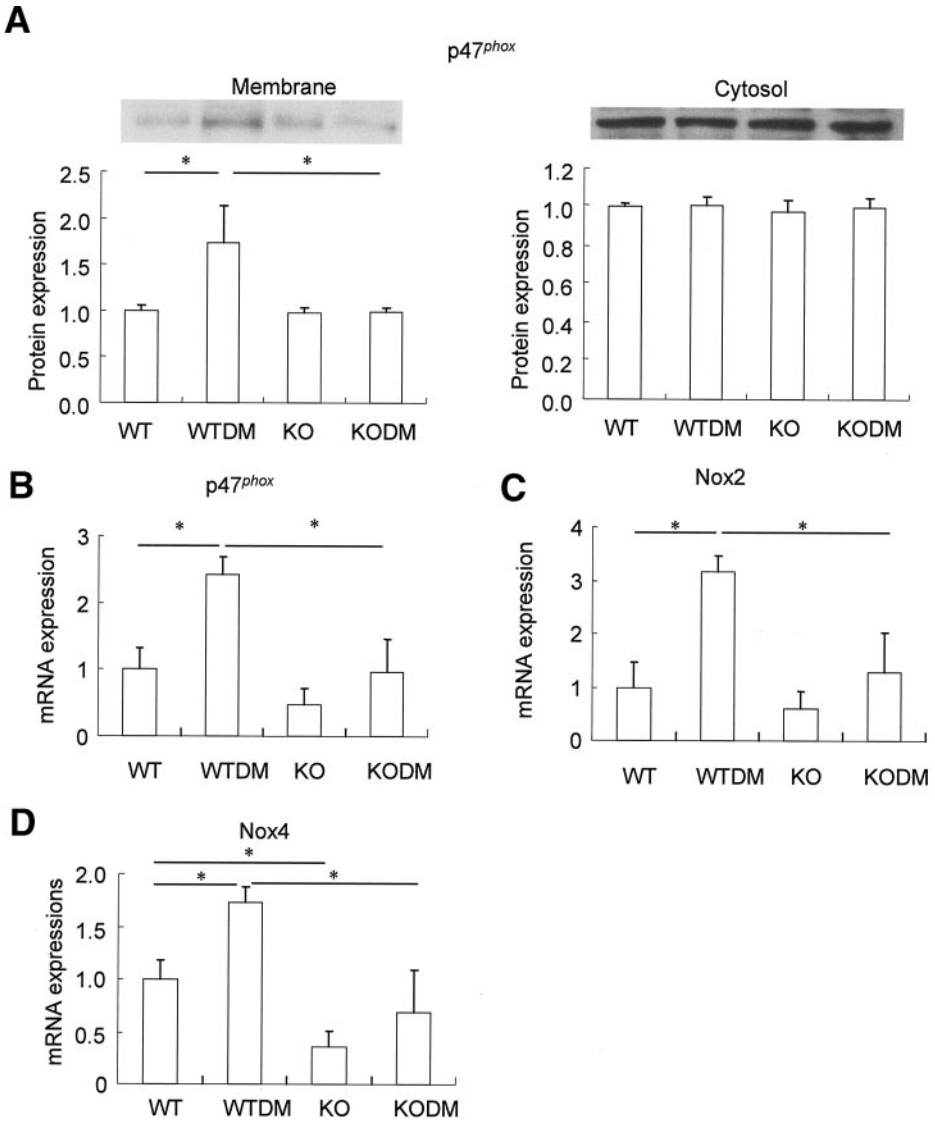

FIG. 5. Activation of NADPH oxidase by hyperglycemia in wild-type and PKC-β$^{-/-}$ mice after 8 weeks of diabetes. *A*: Immunoblot analysis of p47 subunit of NADPH oxidase in membranous and cytosol fractions of renal cortex. Values for protein expression are expressed as fold change versus wild-type. *n* = 4 for each group. Renal cortical mRNA expression of p47^{phox} (*B*), Nox2 (*C*), and Nox4 (*D*). Values for mRNA levels are expressed as fold change versus wild-type. *n* = 5 for each group (also indicates the number of mice studied). KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. *P < 0.01.

increased by twofold (1.90 ± 0.21-fold change versus nondiabetic wild-type mice, $P < 0.01$) as compared with wild-type control mice. In contrast, the mRNA level of ET-1 was not increased in diabetic PKC-β$^{-/-}$ mice (fold change compared with nondiabetic wild-type mice: nondiabetic PKC-β$^{-/-}$ 0.31 ± 0.21-fold versus diabetic PKC-β$^{-/-}$ 0.37 ± 0.16-fold, $P > 0.05$), in which expression level was significantly less compared with that observed in diabetic wild-type mice (diabetic PKC-β$^{-/-}$ 0.37 ± 0.16-fold versus diabetic wild-type 1.90 ± 0.21-fold, $P < 0.01$). The baseline expression of ET-1 in renal cortex of nondiabetic PKC-β$^{-/-}$ mice was also significantly less than that of nondiabetic wild-type mice (0.31 ± 0.21-fold of nondiabetic wild-type mice, $P < 0.01$).

Similiarily, diabetes induced a significant increase in the renal cortical expression of VEGF mRNA in diabetic wild-type mice (1.31 ± 0.16-fold of nondiabetic wild-type mice, $P < 0.05$) but not in diabetic PKC-β$^{-/-}$ mice (fold change compared with nondiabetic wild-type: nondiabetic PKC-β$^{-/-}$ 0.43 ± 0.14-fold versus diabetic PKC-β$^{-/-}$ 0.67 ± 0.28-fold, $P > 0.05$). Ablation of the *PKC-β* gene reduced the basal expression of VEGF mRNA in the renal cortex of nondiabetic PKC-β$^{-/-}$ mice as compared with nondiabetic wild-type mice (0.43 ± 0.14-fold of nondiabetic wild-type mice, $P < 0.01$).

Reduced glomerular hypertrophy and accumulation of extracellular matrix components in renal cortex of diabetic PKC-β$^{-/-}$ mice. Increases in the expressions of fibrotic factors, such as TGF-β and CTGF, and extracellular matrix proteins, such as collagens, are believed to be partly responsible for the glomerular enlargements and fibrosis (24). Wild-type mice with diabetes had increased mRNA expression of TGF-β (191%, $P < 0.01$), CTGF (63%, $P < 0.01$), collagen IV (134%, $P < 0.01$), and collagen VI (102%, $P < 0.01$), which were observed following 8 weeks of diabetes (Fig. 6). In contrast, there was no significant increase in mRNA expression of CTGF and collagen IV and VI among diabetic PKC-β$^{-/-}$ mice after 8 weeks (Fig. 6*B*–*D*). There was significant increase in expression of TGF-β after 8 weeks of diabetes in the PKC-β$^{-/-}$ mice, although

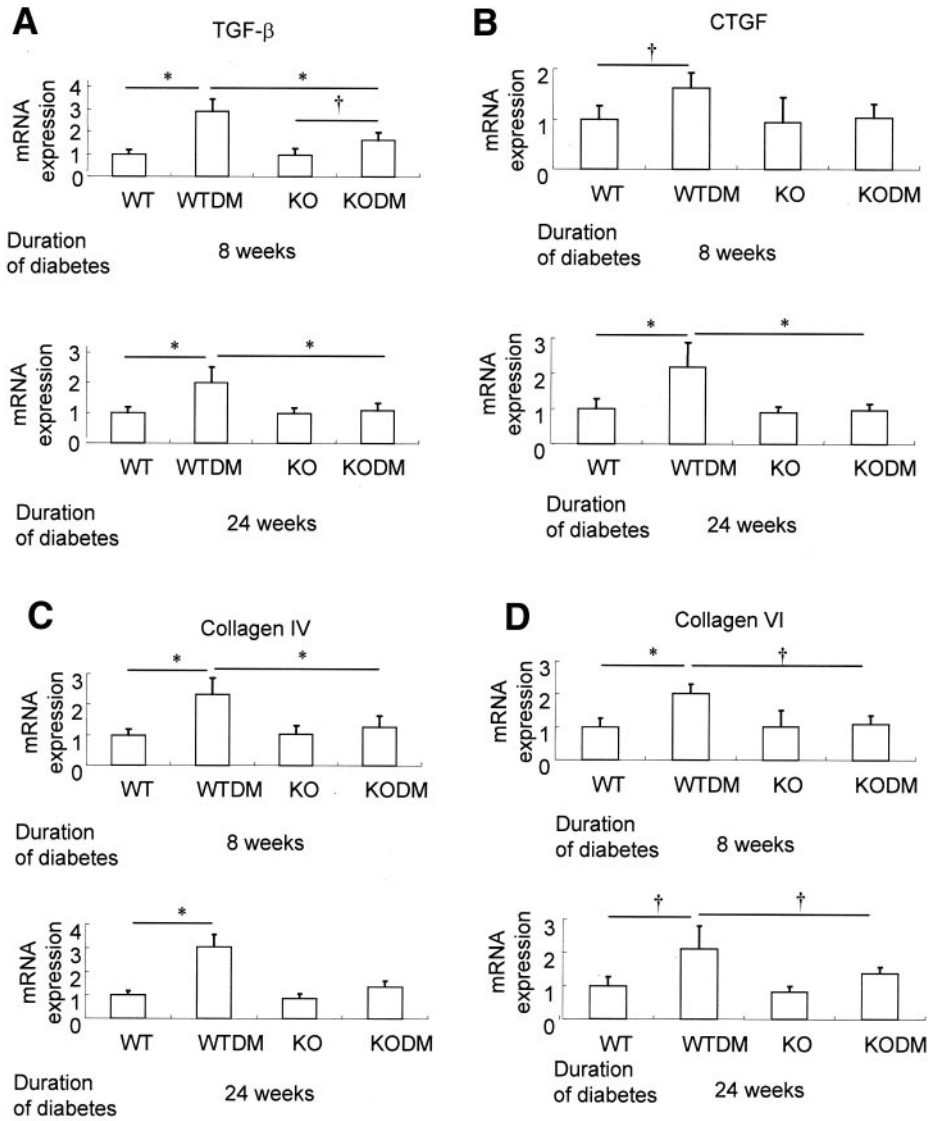

FIG. 6. Expression of genes associated with fibrosis in the renal cortex of wild-type and PKC-β$^{-/-}$ mice after 8 and 24 weeks of diabetes. Renal cortical mRNA expression of TGF-β (*A*), CTGF (*B*), collagen IV (*C*), and collagen VI (*D*). Values are expressed as fold change versus wild-type. KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. *n* = 5 for each group (also indicates the number of mice studied). **P* < 0.01, †*P* < 0.05.

this was significantly less than the increase observed in wild-type mice (Fig. 6*A*). Following 24 weeks of diabetes, there was a sustained increase in the expression of TGF-β, CTGF, and collagen IV and VI in the diabetic wild-type mice (Fig. 6). Long duration of diabetes was associated with increased expression of only collagen VI in the PKC-β$^{-/-}$ mice, although the increase was less than in the wild-type mice.

Kidney weight was significantly increased (24%, $P < 0.01$) in wild-type mice following 8 weeks of diabetes (Fig. 7*A*). This increase in kidney weight was not found in diabetic PKC-β$^{-/-}$ mice for the same duration. Likewise, while there was significant increase in kidney weight in wild-type mice after 24 weeks of diabetes, there was significantly less renal enlargement in PKC-β$^{-/-}$ mice with diabetes of the same duration (Fig. 7*A*).

Morphometric analysis showed that glomerular size was increased by 24.2% ($P < 0.05$) in wild-type mice following 24 weeks of diabetes. In contrast, there was no glomerular enlargement observed in PKC-β$^{-/-}$ mice exposed to the same duration of diabetes (Fig. 7*B*).

DISCUSSION

Previous studies have used RBX to determine which of the various renal dysfunctions and pathologies are due to the activation of PKC-β isoforms (10,12,13). However, the results are not definitive, since RBX is an isoform-selective inhibitor, which at the dose used may inhibit PKC-α activation by 10–20% (10). We showed that PKC-β$^{-/-}$ mice could be used to test the specific effects of PKC-β isoforms on renal function, since immunoblot analysis of the renal cortex demonstrated that diabetes can still activate PKC-α isoform in these mice, unlike the wild-type mice where both PKC-α and -β1 were increased. These results show for the first time that PKC-α and -β are activated independently by diabetes or hyperglycemia; several reports have shown that PKC-β activation may increase PKC-α expression (25). This suggestion of PKC-α and -β isoforms being independently activated is more than a biochemical interest because it indicates that a selective PKC-β isoform inhibitor may not completely prevent all renal pathologies of diabetes unless it can also partially inhibit the activation of PKC-α.

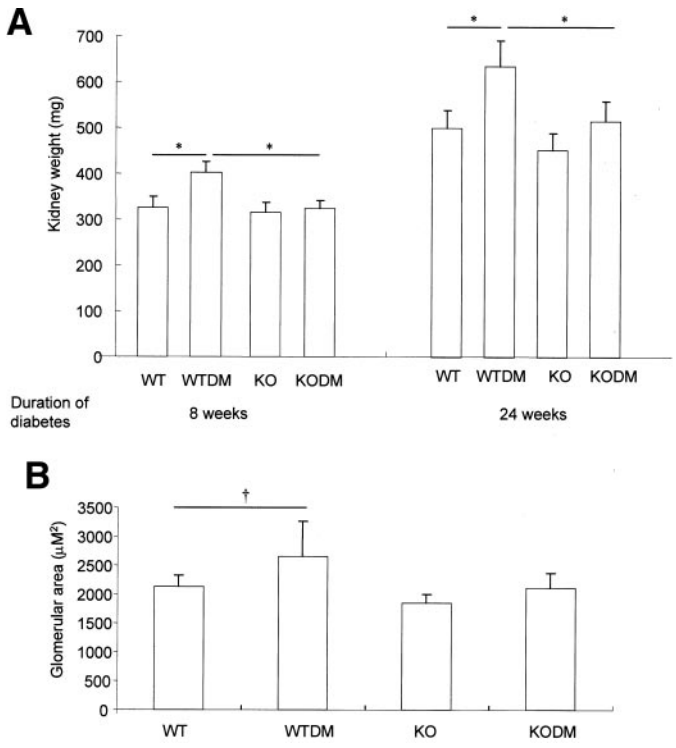

FIG. 7. ***A*: Kidney weight of wild-type and PKC-β$^{-/-}$ mice after 8 and 24 weeks of diabetes. n = 6 for each group (also indicates the number of mice studied). *B*: Glomerular area measured by morphometric analysis of glomeruli in kidneys of wild-type and PKC-β$^{-/-}$ mice after 24 weeks of diabetes. KO, PKC-β$^{-/-}$ nondiabetic mice; KODM, PKC-β$^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. *P < 0.01, †P < 0.05.**

One of the most interesting findings in this study is the protection from hyperglycemia-induced oxidative stress in mice lacking PKC-β isoforms, as shown by the significant decreases in urinary isoprostane and 8-OHdG, which were maintained even after 24 weeks of diabetes. This lowering of oxidative stress appears to be partially due to renal origin, since several subunits and isoforms of NADPH oxidase, a major producer of oxidants (26), were found to be decreased in their expression in the renal cortex of PKC-β$^{-/-}$ mice. The decreases in Nox activities appear to be due to lowering of both activation and expression, since the translocation of p47^{phox} and the expressions of Nox2 and -4 were significantly reduced in the diabetic PKC-β$^{-/-}$ mice. These data suggest that PKC-β is a major inducer of oxidative stress in diabetes, possibly via the activation of Nox complex, although confirmation by NADPH oxidase activities would be needed to support the changes in mRNA levels. This is consistent with recent work suggesting that diabetes leads to increased expression of NADPH oxidase subunits Nox4 and p22^{phox}, which may be responsible for overproduction of ROS and the renal damage seen in diabetes (15,16). Nox4-derived ROS were found to induce renal hypertrophy and increase fibronectin expression (27). In addition, chronic inhibition of NADPH oxidase was found to prevent podocyte apoptosis, albuminuria, and mesangial expansion in a mouse model of diabetes (28). Amelioration of several markers of renal damage in mice lacking PKC-β, along with absent activation of NADPH oxidase, suggests NADPH oxidase is a downstream target of PKC-β. Previous studies have demonstrated that glucose and fatty acids increase ROS production via PKC-dependent activation of NADPH oxidase (29). Recent in vitro studies using inhibitors specific for PKC-β isoforms suggest that PKC-β is the main isoform responsible for activating Nox (30). Furthermore, the increased activation of NADPH oxidase and increased urinary 8-hydroxyguanosine excretion in diabetic animals can be improved by treatment with the PKC-β inhibitor ruboxistaurin mesylate (22). Our in vivo studies confirmed the key role of PKC-β in diabetes-induced NADPH oxidase activation and the protective effect of PKC-β against ROS-induced renal damage. This is consistent with the renoprotective effect observed in both animal and human studies using RBX (10,12,14). However, the results from the present study also showed that the increases in isoprostane were not completely suppressed in diabetic PKC-β$^{-/-}$ mice, which is different from findings when diabetic animals were treated with RBX. This suggests that PKC-α can also induce oxidant production either via Nox or other pathways. Further studies are needed to determine cellular sites of oxidant production by measuring intracellular oxidative markers such as nitrosylated proteins and 4-hydroxynonenal adducts in renal tissues and cells.

The source of ROS production remains controversial, with evidence suggesting that several pathways, including enhanced formation of AGEs (6), altered polyol pathway activity (31), increased PKC activity (29), and increased superoxide release from the mitochondria (6), may be involved. Recently, there is accumulating evidence that NADPH oxidase is the most important source of ROS production in vascular tissues and may be involved in vascular damage caused by hypercholesterolemia, hypertension, and atherosclerosis (32–34). The kidney is vulnerable to oxidative damage and is known to express NADPH oxidase and generate ROS (26). Increased production of ROS within the glomeruli decreases NO bioactivity on mesangial contraction and arteriolar tone and may contribute directly to the renal hemodynamic and vascular abnormalities observed during the initiation and established phase of diabetic nephropathy (28). The beneficial effects of ACE inhibitors (35) and angiotensin receptor blockers (36) could also be related to reduction of ROS production, since angiotensin has been reported to induce or activate NADPH oxidase (32–34).

PKC-β activation can also affect hemodynamic changes by increasing the expression of ET-1 possibly via excessive oxidant production. ET-1 has been implicated in diabetic kidney injury and may contribute to glomerular injury and interstitial tubular changes in diabetes (23,37). Changes in the expression of ET-1 in PKC-β$^{-/-}$ mice are consistent with previous observations suggesting that PKC-β activation can regulate both basal and diabetes-induced ET-1 expression (38). A similar pattern of changes is noted for VEGF expression, which was increased in diabetic wild-type but not changed in the diabetic PKC-β$^{-/-}$ mice. The physiological role of VEGF overexpression in the renal cortex is not clear but will likely have effects on renal plasma flow (39). It is interesting to note that diabetes-induced ET-1 and VEGF expressions were completely suppressed in the diabetic PKC-β$^{-/-}$ mice, unlike the oxidative parameters and Nox2 and Nox4 expressions, suggesting that PKC-β activation is more important for the induction of ET-1 and VEGF than PKC-α isoform.

Renal enlargement in diabetes results especially from tubular hypertrophy and also from glomerular enlargement and accumulation of extracellular matrix (24). This hypertrophy usually precedes the development of irrevers-

ible renal changes including glomerulosclerosis and tubulointerstitial fibrosis. The present studies demonstrate that PKC-β plays a critical role in diabetes-induced tubular hypertrophy and glomerular enlargement, since diabetic PKC-$\beta^{-/-}$ mice did not show the expected increase in kidney weight or glomerular area. TGF-β and CTGF, in particular, have been identified as key cytokines mediating the changes that lead to extracellular matrix accumulation and glomerular enlargement (40,41). Whereas induction of diabetes led to marked increases in the expression of TGF-β and CTGF in wild-type mice, this increase in TGF-β expression was attenuated at 8 weeks of diabetes and was absent at 6 months of disease, whereas the increased expression of CTGF was completely absent in PKC-$\beta^{-/-}$ mice. This may account for the lack of glomerular enlargement and nephromegaly and marked attenuation in diabetes-induced increase in expression of collagen IV and VI seen in the PKC-$\beta^{-/-}$ mice. These findings are consistent with previous observations suggesting that PKC-β isoforms can mediate extracellular matrix accumulation in the diabetic kidney (10,13,42) and in other tissues (43).

In contrast to the complete lack of renal and glomerular hypertrophy in diabetic PKC-$\beta^{-/-}$ mice, diabetes in the PKC-$\beta^{-/-}$ mice was associated with only partial protection from increased urinary albumin excretion. In addition to extracellular matrix accumulation and mesangial expansion, several other factors are known to be important in determining albuminuria. This includes reduced glomerular nephrin expression, effacement of podocyte foot processes, as well as apoptosis and progressive loss of glomerular podocytes (24). Interestingly, effects of PKC activation on podocyte biology and functions are not known. Other pathways in addition to PKC-β may also mediate some of these processes. Recently, it was shown that hyperglycemia-induced downregulation of the negatively charged basement membrane heparin sulfate proteoglycan perlecan was prevented in mice lacking PKC-α, which also had a reduction in diabetes-associated albuminuria (44). Our data on PKC activity in renal cortex and activation of the α isoform in the PKC-$\beta^{-/-}$ mice would be consistent with the view that activation of PKC-α may also contribute to albuminuria observed in diabetes despite it not being involved in causing extracellular matrix accumulation and renal hypertrophy.

In summary, the results derived from using the PKC-$\beta^{-/-}$ mice have identified the specific role of PKC-β in mediating diabetes-induced increases in the expression of CTGF and TGF-β with the resultant glomerular and renal enlargement. These effects of PKC-β may be partially due to its actions on enhancing the actions or expressions of the Nox complex of oxidases. In addition, these results have also strongly suggested that PKC-α or other pathways may be partly responsible for causing diabetes-induced increases in oxidative stress and elevated albuminuria. Strategies to target the DAG-PKC pathway using isoform-specific inhibitors of PKC-β may need to take in to consideration that some inhibition of PKC-α may also be necessary to prevent most hyperglycemia-induced injury in the renal glomeruli. Inhibition of PKC-β isoforms does not appear to have significant clinical side effects. However, inhibiting both PKC-β and -α isoforms may have more side effects than inhibiting a single isoform, since PKC activation has been implicated in essential physiological functions, even for insulin secretion (45).

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REFERENCES

1. Raptis AE, Viberti G: Pathogenesis of diabetic nephropathy. *Exp Clin Endocrinol Diabetes* 109 (Suppl. 2):S424–S437, 2001
2. Mauer SM, Steffes MW, Goetz FC, Sutherland DE, Brown DM: Diabetic nephropathy: a perspective. *Diabetes* 32 (Suppl. 2):52–55, 1983
3. Cooper ME: Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy. *Diabetologia* 44:1957–1972, 2001
4. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859–866, 1998
5. Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN, Stevens MJ: Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes* 52:864–871, 2003
6. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
7. Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, Jenkins DG, Stein G, Schmidt AM, Yan SF: RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis* 185:70–77, 2006
8. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* 89:11059–11063, 1992
9. Craven PA, Studer RK, Negrete H, DeRubertis FR: Protein kinase C in diabetic nephropathy. *J Diabetes Complications* 9:241–245, 1995
10. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL: Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115–126, 1997
11. Whiteside CI, Dlugosz JA: Mesangial cell protein kinase C isozyme activation in the diabetic milieu. *Am J Physiol Renal Physiol* 282:F975–F980, 2002
12. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKCbeta inhibitor. *Science* 272:728–731, 1996
13. Koya D, Haneda M, Nakagawa H, Isshiki K, Sato H, Maeda S, Sugimoto T, Yasuda H, Kashiwagi A, Ways DK, King GL, Kikkawa R: Amelioration of accelerated diabetic mesangial expansion by treatment with a PKCbeta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439–447, 2000
14. Tuttle KR, Bakris GL, Toto RD, McGill JB, Hu K, Anderson PW: The effect of ruboxistaurin on nephropathy in type 2 diabetes. *Diabetes Care* 28:2686–2690, 2005
15. Etoh T, Inoguchi T, Kakimoto M, Sonoda N, Kobayashi K, Kuroda J, Sumimoto H, Nawata H: Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventive insulin treatment. *Diabetologia* 46:1428–1437, 2003
16. Satoh M, Fujimoto S, Haruna Y, Arakawa S, Horike H, Komai N, Sasaki T, Tsujioka K, Makino H, Kashihara N: NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 288:F1144–F1152, 2005
17. Abiko T, Abiko A, Clermont AC, Shoelson B, Horio N, Takahashi J, Adamis AP, King GL, Bursell SE: Characterization of retinal leukostasis and hemodynamics in insulin resistance and diabetes: role of oxidants and protein kinase-C activation. *Diabetes* 52:829–837, 2003
18. Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, Segal AW: Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. *Biochem J* 347:285–289, 2000
19. Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S,

Tarakhovsky A: Immunodeficiency in protein kinase cbeta-deficient mice. *Science* 273:788–791, 1996
20. Koya D, Lee IK, Ishii H, Kanoh H, King GL: Prevention of glomerular dysfunction in diabetic rats by treatment with d-alpha-tocopherol. *J Am Soc Nephrol* 8:426–435, 1997
21. Vicent D, Ilany J, Kondo T, Naruse K, Fisher SJ, Kisanuki YY, Bursell S, Yanagisawa M, King GL, Kahn CR: The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J Clin Invest* 111:1373–1380, 2003
22. Kitada M, Koya D, Sugimoto T, Isono M, Araki S, Kashiwagi A, Haneda M: Translocation of glomerular p47phox and p67phox by protein kinase C-β activation is required for oxidative stress in diabetic nephropathy. *Diabetes* 52:2603–2614, 2003
23. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579–F589, 2003
24. Wolf G, Chen S, Ziyadeh FN: From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes* 54:1626–1634, 2005
25. Collazos A, Diouf B, Guerineau NC, Quittau-Prevostel C, Peter M, Coudane F, Hollande F, Joubert D: A spatiotemporally coordinated cascade of protein kinase C activation controls isoform-selective translocation. *Mol Cell Biol* 26:2247–2261, 2006
26. Geiszt M, Kopp JB, Varnai P, Leto TL: Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97:8010–8014, 2000
27. Gorin Y, Block K, Hernandez J, Bhandari B, Wagner B, Barnes JL, Abboud HE: Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. *J Biol Chem* 280:39616–39626, 2005
28. Susztak K, Raff AC, Schiffer M, Bottinger EP: Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* 55:225–233, 2006
29. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939–1945, 2000
30. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A: Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes* 52:2795–2804, 2003
31. Chung SS, Ho EC, Lam KS, Chung SK: Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol* 14:S233–S236, 2003
32. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86:494–501, 2000
33. Li JM, Shah AM: Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res* 52:477–486, 2001
34. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK: Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105:1429–1435, 2002
35. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy: the Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
36. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
37. Mishra R, Emancipator SN, Kern TS, Simonson MS: Association between endothelin-1 and collagen deposition in db/db diabetic mouse kidneys. *Biochem Biophys Res Commun* 339:65–70, 2006
38. Yokota T, Ma RC, Park JY, Isshiki K, Sotiropoulos KB, Rauniyar RK, Bornfeldt KE, King GL: Role of protein kinase C on the expression of platelet-derived growth factor and endothelin-1 in the retina of diabetic rats and cultured retinal capillary pericytes. *Diabetes* 52:838–845, 2003
39. Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, Sugiyama H, Yamasaki Y, Eguchi K, Makino H: Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. *Diabetes* 54:2891–2903, 2005
40. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
41. Sakharova OV, Taal MW, Brenner BM: Pathogenesis of diabetic nephropathy: focus on transforming growth factor-beta and connective tissue growth factor. *Curr Opin Nephrol Hypertens* 10:727–738, 2001
42. Cohen MP, Ziyadeh FN, Lautenslager GT, Cohen JA, Shearman CW: Glycated albumin stimulation of PKC-beta activity is linked to increased collagen IV in mesangial cells. *Am J Physiol* 276:F684–F690, 1999
43. Way KJ, Isshiki K, Suzuma K, Yokota T, Zvagelsky D, Schoen FJ, Sandusky GE, Pechous PA, Vlahos CJ, Wakasaki H, King GL: Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase C β2 activation and diabetes. *Diabetes* 51:2709–2718, 2002
44. Menne J, Park JK, Boehne M, Elger M, Lindschau C, Kirsch T, Meier M, Gueler F, Fiebeler A, Bahlmann FH, Leitges M, Haller H: Diminished loss of proteoglycans and lack of albuminuria in protein kinase Cα–deficient diabetic mice. *Diabetes* 53:2101–2109, 2004
45. Sjoholm A: Glucose stimulates islet α-cell mitogenesis through GTP-binding proteins and by protein kinase C–dependent mechanisms. *Diabetes* 46:1141–1147, 1997