Renal hypertrophy and extracellular matrix accumulation are early features of diabetic nephropathy (DN) (1–4). Whole kidneys, glomeruli, and tubules undergo hypertrophy by increase in cell size and accumulation of extracellular matrix (3, 4). Hypertrophy of the glomerular and tubular compartments precedes the development of irreversible renal changes in diabetes including glomerulosclerosis and tubulointerstitial fibrosis (3, 5). Data from animal models as well as cultured renal cells indicate that hyperglycemia and high glucose induce hypertrophy and extracellular matrix expansion (3, 6, 7).

Oxidative stress has emerged as a critical pathogenic factor in the development of DN (8–11). Diabetes is accompanied by increased generation of reactive oxygen species (ROS) in tissues including the kidney (12–15). However, the results of treatment with antioxidants have been inconclusive (16). Although multiple pathways may result in ROS generation, recent studies indicate that a multicomponent phagocyte-like NAD(P)H oxidase is a major source of ROS in many nonphagocytic cells, including renal cells such as tubular epithelial cells and glomerular mesangial cells (MCs) (17–19). Under physiologic conditions, NAD(P)H oxidases have a very low constitutive activity that can be up-regulated in response to various stimuli (15, 17, 20–22). For instance, it has been reported that enhanced NAD(P)H oxidase activity is associated with oxidative damage to DNA in diabetic glomeruli (23, 24). These NAD(P)H oxidases are isoforms of the neutrophil oxidase, in which the catalytic subunits, termed Nox proteins, correspond to homologues of gp91phox (or Nox2), the catalytic moiety found in phagocytes (17, 20). In this family, Nox4, which appears to share the same overall structure with gp91phox/Nox2, is abundant in the vascular system, kidney cortex, and MCs (17, 20, 25–27). However, the biological role(s) of Nox4 is not well understood at present. It has been proposed that Nox4, a major source of ROS in the vasculature and the kidney, could function under pathologic conditions (20, 22, 24). We have reported previously that Nox4-derived ROS mediate angiotensin II (Ang II)-induced signaling and protein synthesis in mesangial cells (27, 28), suggesting its potential involvement in kidney hypertrophy under pathologic conditions.

In this study, we determined whether Nox4 mediates ROS generation induced by diabetes in vivo and by high glucose in cultured cells. Antisense oligonucleotides for Nox4 were administered to a rat model of streptozotocin-induced type 1 diabetes and to cultured cells in vitro, and their effects on oxidative stress, Akt/protein kinase B (PKB) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation, renal hypertrophy, and fibronectin expression were investigated.

**EXPERIMENTAL PROCEDURES**

**Animals and Treatments**

Male Sprague-Dawley rats weighing between 200 and 225 g were divided into four groups of 10 rats/group. Group 2 was injected intravenously via the tail vein with 55 mg/kg body weight streptozotocin (STZ) in sodium citrate buffer (0.01 M, pH 4.5) to induce diabetes. Group 1 was injected with an equivalent amount of sodium citrate.
buffer alone. Rats in groups 3 and 4 were injected with STZ followed by either phosphorothioated sense or antisense (AS) oligonucleotides for Nox4 (90 ng/g body weight/day) administered subcutaneously by an ALZET osmotic pump for 14 days (ALZA, Palo Alto, CA). Oligonucleotides were administered 72 h after STZ injection for 14 days. Blood glucose concentration (LifeScan One Touch glucometer (Johnson & Johnson)) was monitored 24 h later and periodically thereafter. Three additional groups of control, diabetic, and diabetic rats treated with insulin were also studied. Twenty-four h after STZ injection, diabetic rats were treated daily with 4–6 units of NPH insulin supplemented with regular insulin (Novo Nordisk Pharmaceuticals Inc., Princeton, NJ) subcutaneously. All rats had unrestricted access to food and water and were maintained in accordance with Institutional Animal Care and Use Committee procedures.

At day 14, all rats were euthanized, and both kidneys were removed and weighed. A slice of kidney cortex at the pole was embedded in paraffin or flash-frozen in liquid nitrogen for light microscopy and image analyses. In addition, cortical tissue was used for isolation of glomeruli by differential sieving as described (29), and samples of cortical tissue was used for isolation of paraffin or flash-frozen in liquid nitrogen for light microscopy and image analyses. In addition, cortical tissue was used for isolation of glomeruli by differential sieving as described (29), and samples of cortical tissue were frozen for biochemical analyses. NAD(P)H oxidase activity measurements were performed on freshly obtained tissue.

Antisense oligonucleotides were designed near the 5'-ATG start codon of rat Nox4 (5'-AGTCTCTCCAGGGACAGCGCC-3') (27, 28). Antisense and the corresponding sense oligonucleotides were synthesized as phosphorothioated oligonucleotides and purified by high performance liquid chromatography (Advanced Nucleic Acid Core Facility, University of Texas Health Science Center at San Antonio).

Cell Culture and Transfections

Rat glomerular MCs were isolated and characterized as described (30). These cells were used between the 15th and 30th passages. Selected experiments were performed in primary and early passaged MCs to confirm the data obtained with late passages. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with antibiotic/antifungal solution and 17% fetal bovine serum. Transient transfection of antisense and sense oligonucleotides for Nox4 was performed by electroporation or with Lipofectamine as described previously (27, 28).

**NADPH Oxidase Assay**

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method.

Kidney Cortex and Glomeruli—Homogenates from renal cortex or isolated glomeruli were prepared in 1 ml and 500 µl, respectively, of lysis buffer (20 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml apotinin, and 0.5 µg/ml leupeptin) by using a Dounce homogenizer (100 strokes on ice). Homogenates were subjected to a low speed centrifugation at 800 × g, 4 °C, for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100 µl of homogenates were added to 900 µl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin, and 100 µM NADPH. Photon emission in terms of relative light units was measured every 20 or 30 s for 10 min in a luminometer. There was no measurable activity in the absence of NADPH. A buffer blank (less than 5% of the cell signal) was subtracted from each reading. Superoxide production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent.

**Cultured Mesangial Cells—NADPH oxidase activity in cells was measured as described previously (27). Briefly, MCs grown in serum-free medium containing 5 or 25 mM glucose were washed five times in ice-cold phosphate-buffered saline and were scraped from the plate in the same solution followed by centrifugation at 800 × 4 °C, for 10 min. The cell pellets were resuspended in lysis buffer. Cell suspensions were homogenized with 100 strokes in a Dounce homogenizer on ice. Aliquots of the homogenates were used immediately to measure NADPH-dependent superoxide generation as above.

**Measurement of Superoxide Anion Production in Intact Isolated Glomeruli**

Measurement of superoxide anion released by isolated glomeruli was performed by detection of superoxide dismutase-inhibitable ferricytochrome c reduction (27, 31). Isolated glomeruli were incubated in Hanks’ balanced salt solution without phenol red containing 80 µM cytochrome c with or without superoxide dismutase (50 µg/ml) for 6 h at 37 °C. At the end of the incubation, glomeruli were centrifuged for 2 min at 10,000 × g at 4 °C. The optical density of the supernatant was measured by spectrophotometry at 550 nm and converted to nmol of cytochrome c reduced using the extinction coefficient Δε₅₅₀ = 21.0 × 10³ M⁻¹ cm⁻¹. The reduction of cytochrome c that was inhibitable by pretreatment with superoxide dismutase represents superoxide release.

**Detection of Intracellular ROS**

The peroxide-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes) was used to assess the generation of intracellular ROS as described previously (27, 32). This compound is converted by intracellular esterases to 2',7'-dichlorodihydrofluorescein, which is then oxidized by hydrogen peroxide to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). Differential interference contrast images were obtained simultaneously using an Olympus inverted microscope with ×40 Aplanfluor objective and an Olympus fluoview confocal laser-scanning attachment. The DCF fluorescence was measured with an excitation wavelength of 488 nm of light, and its emission was detected using a 510–550-nm bandpass filter.
Western Blotting Analysis

In Vivo Experiments—Homogenates from renal cortex were prepared in 500 μl of radioimmune precipitation assay buffer (20 mmol/liter Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1% Nonidet P-40) using a Dounce homogenizer. Homogenates were incubated for 1 h at 4 °C and centrifuged at 10,000 × g for 30 min at 4 °C. Isolated glomeruli were suspended in radioimmune precipitation assay buffer and incubated for 1 h at 4 °C. After centrifugation at 10,000 × g for 30 min at 4 °C, protein in the supernatant was determined using the Bio-Rad method.

In Vitro Experiments—MCs grown to near confluence were made quiescent by serum deprivation overnight and were exposed to serum-free Dulbecco’s modified Eagle’s medium containing 5 mM D-glucose,
FIGURE 2. Effects of AS Nox4 treatment on diabetes-induced ROS generation. A, NADPH oxidase activity in cortical homogenates. Superoxide anion generation was determined by photomission every 30 s for 5–10 min. The initial rate of enzyme activity was calculated over the first 30–120 s of exposure to NADPH. NADPH-driven superoxide production was expressed as RLU/min/mg protein. Values are the means ± S.E. of the activities from glomeruli of six animals for each group. **, p < 0.01 versus control rats; #, p < 0.01 versus diabetes. B, NADPH oxidase activity in glomerular homogenates from control (C), diabetes (●), diabetes + sense Nox4 (□), and diabetes + AS Nox4 (▲) groups. (S, sense). NADPH-dependent superoxide generation was measured by lucigenin-enhanced chemiluminescence as in A and expressed as RLU/mg protein. Data represent the means ± S.E. of the activities from glomeruli of six animals for each group. **, p < 0.01 versus control rats; #, p < 0.01 versus diabetes.

Immunohistochemistry

Localization of cellular fibronectin was assessed by immunoperoxidase histochemistry using polyclonal Nox4 antibodies or mouse monoclonal antibodies specific for the alternatively spliced extra domain (EIIIA) (clones 3E2, Sigma and IST-9, Serotec, Harlan Bioproducts for Science, Indianapolis, IN) as described previously. Frozen cortical sections (6 μm thick) were fixed in acetone and permeabilized in PBS-0.1% BSA before blocking with the appropriate IgG. Primary antibodies were added at dilutions of 1:100 for 45 min at room temperature following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). The sections were then dehydrated and mounted with Permount (Sigma) and viewed by bright-field microscopy.

Determination of Glomerular Surface Area

Light microscopy of hematoxylin and eosin-stained sections from the different treatment groups was used for morphometric studies. The surface area (μm²) of a minimum of 50 glomerular sections from each animal was determined in digital images using the Image-Pro Plus 4.5 software (Media Cybernetics). Glomerular surface area was measured in captured digital images by tracing around the perimeter of the glomerular capillary tuft using the polygram tool. The analysis software was calibrated to a stage micrometer.

Immunofluorescence

Six-μm-thick frozen sections were mounted on glass slides and then fixed in aceton. Sections were rehydrated in PBS-0.1% BSA before blocking with the appropriate IgG. Primary antibodies were added at concentrations of 10 μg/ml for 1 h at room temperature. After incubation with primary antibodies, sections were washed three times for 5 min in PBS-0.1% BSA. Fluorescence-conjugated secondary antibodies were added at dilutions of 1:100 for 45 min at room temperature followed by washing in PBS-0.1% BSA. Sections were mounted with Crys-
tal Mount (Dako) and allowed to dry before viewing with fluorescence microscopy. α-Smooth muscle actin was used as a marker for MCs within the glomerulus.

**Statistical Analysis**

Results are expressed as mean ± S.E. Statistical significance was assessed by Student’s unpaired t test. Significance was determined as probability (p) less than 0.05.

**RESULTS**

*Nox4 Expression*—TABLE ONE displays the blood glucose levels and body and kidney weights after 2 weeks of diabetes in the different groups of rats. Untreated diabetic rats and diabetic rats treated with either AS or the corresponding sense Nox4 had equivalently elevated blood glucose concentration at the end of the study period compared with the control rats. Body weight was similarly reduced in the diabetic rats treated with either sense or AS oligonucleotides. Total kidney weight and kidney weight to body weight ratio significantly increased in diabetic rats and sense Nox4-treated diabetic rats compared with non-diabetic control animals. In contrast, total kidney weight in AS Nox4-treated diabetic rats was significantly reduced compared with that observed for the diabetic or sense Nox4-treated diabetic groups (TABLE ONE).

To test whether the oligonucleotides were effectively delivered to the kidney and to assess the effect of diabetes on Nox4 expression, we examined the protein levels of Nox4 in renal cortex from the different groups. Western blot analysis using a mouse polyclonal Nox4 antibody directed against recombinant glutathione S-transferase-mouse Nox4-(299–515) showed that a predominant 70-kDa band corresponding to Nox4 was increased in diabetic kidney cortex compared with that in control non-
diabetic rats. AS Nox4 but not sense Nox4 administration reversed diabetes-induced Nox4 protein expression and significantly reduced Nox4 levels in kidney cortex from diabetic animals (Fig. 1). To confirm the specificity of action of the AS treatment toward Nox4, we also examined the protein expression of another Nox isoform, gp91phox/Nox2. The levels of gp91phox/Nox2 were also increased in diabetic animals. More importantly, administration of AS Nox4 had no effect on gp91phox/Nox2 expression (Fig. 1A). Immunoperoxidase staining showed that Nox4 protein expression is significantly increased in diabetic glomeruli. AS but not sense Nox4 administration markedly reduced diabetes-induced Nox4 protein expression (Fig. 1B). Double immunofluorescence staining revealed the colocalization of Nox4 (green) and α-smooth muscle actin (red) in the mesangial area of diabetic glomeruli (Fig. 1C). These observations demonstrate that Nox4 expression is consistent with mesangial distribution. These data indicate that mesangial expression of Nox4 is increased in diabetes and that subcutaneous administration of AS oligonucleotides effectively and specifically inhibits Nox4 NAD(P)H oxidase expression.

**ROS Generation**—NADPH-dependent superoxide production was significantly increased in renal cortical and glomerular homogenates of diabetic animals compared with controls as measured by lucigenin-enhanced chemiluminescence (Fig. 2, A and B). AS Nox4 but not sense Nox4 treatment suppressed diabetes-induced NADPH oxidase activation in cortical and glomerular homogenates (Fig. 2, A and B). Preincubation of homogenates with diphenyleneiodonium, an inhibitor of flavin-containing oxidases, completely blocked NADPH oxidase activity. In addition, superoxide dismutase (50 μg/ml) also inhibited the photoemission, thereby confirming identity of the product as superoxide (data not shown). The correlation between the inhibitions of NADPH-dependent ROS generation and the decrease in Nox4 expression following AS Nox4 administration in the diabetic rats suggest that Nox4 is the enzyme responsible for the increase in NADPH oxidase activity in diabetes.

To further confirm the inhibitory effect of AS Nox4 on diabetes-induced oxidative stress in glomeruli, superoxide generation was evaluated ex vivo in isolated glomeruli incubated in the presence of cytochrome c. As shown in Fig. 2C, superoxide generation by isolated glomeruli from diabetic rats was markedly increased compared with control rats. AS Nox4 treatment significantly inhibited the increase in superoxide anion production in diabetic glomeruli. Conversely, superoxide release was not affected by sense Nox4 treatment (Fig. 2C).
Effects of Insulin Treatment—To determine whether the increased expression of Nox4 and ROS generation were because of the diabetic state and not because of a toxic effect of STZ, diabetic rats were treated with insulin. Tight glycemic control was achieved in the diabetic rats treated with insulin (mean plasma glucose concentrations on the last day were 102.6 mg/dl ± 3.9 in control rats, 433.5 mg/dl ± 25.8 in diabetic rats, and 103.7 mg/dl ± 32.9 in diabetic rats treated with insulin). Western blot and immunochemical analysis showed that the increased protein levels of Nox4 in diabetic rat kidneys were completely prevented in the rats treated with insulin (Fig. 3, A and B). In addition, the increase in NADPH oxidase activity in cortical homogenates from diabetic animals was also prevented in the diabetic rats treated with insulin (Fig. 3C).

Renal Hypertrophy—As expected, diabetic animals exhibited significantly greater kidney weight to body weight ratio (whole kidney hypertrophy) as compared with nondiabetic rats (TABLE ONE). Treatment with AS Nox4 but not sense Nox4 resulted in a significant decrease in kidney weight (TABLE ONE). These data suggest that Nox4 is involved in renal hypertrophy.

The decrease in whole kidney hypertrophy is accompanied by a decrease in glomerular hypertrophy. Glomerular surface area was
examined and quantified in histological sections of kidneys removed from control, diabetic, AS Nox4-treated, and sense Nox4-treated rats. Fig. 4, A and B, show that glomeruli of diabetic animals are significantly larger compared with controls. AS Nox4 treatment resulted in a decrease in glomerular size. In contrast, glomeruli from sense Nox4-treated animals are not different from glomeruli of diabetic animals.

Collectively, these results demonstrate that AS Nox4 treatment reduces whole kidney and glomerular hypertrophy in diabetic animals suggesting that Nox4 is positioned distal to hyperglycemia in the pathway that leads to both whole kidney and glomerular hypertrophy.

**Fibronectin Expression**—The effect of AS Nox4 administration on the accumulation of the extracellular matrix protein fibronectin was determined in whole cortex and isolated glomeruli by Western blot. As depicted in Fig. 5, A and B, expression of fibronectin protein was significantly increased in both cortex and isolated glomeruli of diabetic animals as compared with controls. The increased expression of fibronectin was markedly reduced in the renal cortex and glomeruli of diabetic rats treated with AS Nox4 (Fig. 5, A and B). Sense Nox4 treatment had no effect on diabetes-induced fibronectin expression.

These observations were confirmed by immunohistochemical analysis of fibronectin expression. As shown in Fig. 5C, the amount of fibronectin was increased in the diabetic group and treatment with AS Nox4 decreased the increased expression of fibronectin induced by diabetes. Sense Nox4 treatment did not alter the enhanced immunoreactivity of fibronectin observed in diabetic rats. Quantitative analysis revealed a significant inhibitory effect of AS Nox4 on glomerular expression of fibronectin induced by diabetes as compared with the control group (Fig. 5D).

**Akt/PKB and ERK1/2 Phosphorylation**—The serine-threonine kinase Akt/PKB and the mitogen-activated protein kinase family members, ERK1 and -2, are activated by phosphorylation. Both kinases play a critical role in cell growth and hypertrophy as well as matrix expansion (3, 27, 28, 32–39). To assess the potential role of these kinases during early DN, the phosphorylation of Akt/PKB and ERK1/2 was examined using phospho-specific antibodies. As illustrated in Fig. 6, phosphorylation of both Akt/PKB and ERK1/2 was markedly increased in the diabetic kidney cortex, and treatment of diabetic animals with AS Nox4 but not sense Nox4 almost abolished this effect. These findings demonstrate that Nox4 is required for diabetes-induced Akt/PKB and ERK1/2 phosphorylation and that the two kinases are positioned downstream of Nox4 in the signaling pathway(s) activated in diabetes.

**Effect of High Glucose on ROS Generation and Fibronectin Expression in Cultured Mesangial Cells**—We also assessed the effects of AS Nox4 on ROS production and fibronectin accumulation in cultured rat MCs exposed to high glucose (HG) concentration. The effect of the oligonucleotides in MCs was confirmed by the observation that AS Nox4 but not sense Nox4 significantly decreased Nox4 protein expression (Fig. 7A).

AS Nox4- or sense Nox4-transfected MCs were incubated for 24 h in serum-free medium containing either normal glucose concentration (NG, 5 mm D-glucose), HG (25 mm D-glucose), or 5 mm D-glucose + 20 mm L-glucose, and NADPH oxidase activity was measured in crude homogenates using lucigenin-enhanced chemiluminescence. HG caused a robust increase in NADPH-dependent superoxide generation (Fig. 7B). Transient transfection of MCs with AS Nox4 (1 μM) but not sense Nox4 (1 μM) markedly decreased the activation of NADPH oxidase by HG. To confirm these results, additional studies were undertaken using DCF fluorescence. As shown in Fig. 7C, HG-induced intracellular ROS production was significantly blocked in MCs transfected with AS Nox4. Conversely, fluorescence was not affected by transfection of MCs with sense Nox4.

The effect of HG on fibronectin expression was measured by Western blot analysis in total cell lysates. As anticipated, expression of fibronectin over 24 h was significantly higher in MC cultures that contained 25 mm D-glucose compared with 5 mm D-glucose (Fig. 7D). Similar to ROS generation, transfection of MCs with AS Nox4 significantly decreased HG-mediated increase in fibronectin expression (Fig. 7D). In contrast, sense Nox4 did not alter the increase in fibronectin accumulation in MCs exposed to HG. These effects are not observed in cells cultured with osmotic control. These findings demonstrate that Nox4 NAD(P)H oxidase is involved in HG-stimulated extracellular matrix protein fibronectin production in MCs.

**DISCUSSION**

Oxidative stress has been implicated in the pathogenesis of diabetic complications (8–15, 21, 22). However, the mechanisms of ROS generation in diabetes are not fully understood. In this study, we demonstrate that Nox4 is a major source of ROS overproduction in diabetes and that Nox4-derived ROS mediate Akt/PKB and ERK1/2 activation, kidney hypertrophy, and fibronectin expression.

Elevated ROS levels contribute to the development of diabetic vascular complications, such as atherosclerosis and DN (8–15, 20–22). In the vasculature, the most important enzyme responsible for ROS production is NAD(P)H oxidase. This oxidase is involved in vascular pathology caused by hypercholesterolemia or hypertension (20–22). NAD(P)H oxidase is originally found in neutrophils and is composed of the catalytic subunit gp91<sub>phox</sub> together with the regulatory subunits p22<sub>phox</sub>, p47<sub>phox</sub>, and p67<sub>phox</sub> and the small GTPase Rac (17, 20, 40). Electrons from NAD(P)H are transferred through the enzyme to molecular oxygen to generate superoxide and subsequently other ROS such as hydrogen peroxide. Gp91<sub>phox</sub> is only one member of a family of homologous proteins termed Nox (17, 20). The kidney is known to express NAD(P)H oxidase and generate ROS (12, 15, 17–19, 21, 22). The isoform Nox4 was cloned from the kidney and found to be highly expressed in this organ (17, 20, 25–27). Nox4 is nearly identical in size and structure to gp91<sub>phox</sub> (also known as Nox2). However, the requirement for Nox4 activity of other components of the gp91<sub>phox</sub>/Nox2 complex is not known. Nox4 is a 578-amino acid protein that exhibits 39% identity to gp91<sub>phox</sub>/Nox2 with special conservation in the membrane-spanning regions and binding sites for NADPH, FAD, and heme, the electron transfer centers that pass electrons from NAD(P)H to oxygen to form superoxide (17, 20, 25, 26). We show increased expression of Nox4 protein in the kidney of STZ-induced diabetic rats that is associated with an increase in NADPH-dependent ROS generation in kidney cortex and isolated glomeruli. Immunostaining analysis reveals that diabetes up-regulates Nox4 pro-
FIGURE 7. Effects of AS Nox4 on high glucose-induced ROS generation and fibronectin expression in MCs. A, MCs were not (Con) or were transfected by electroporation with sense Nox4 (1 μM) or AS Nox4 (1 μM), and Nox4 protein expression was determined by direct immunoblotting with mouse polyclonal Nox4 antibodies. S, sense. B, the left panel shows representative kinetics of NADPH-dependent superoxide generation measured in homogenates of MCs transfected by sense Nox4 or AS Nox4 and exposed to NG (5 mM D-glucose), HG (25 mM D-glucose), or 5 mM D-glucose + 20 mM L-glucose (L-G) for 24 h. Superoxide generation was determined as described in Fig. 2. Right panel, the initial rate of enzyme activity was calculated over the first 30–120 s of exposure to NADPH and expressed as RLU/min/mg protein. Values are the mean ± S.E. of three independent experiments. **, p < 0.01 versus control; ##, p < 0.01 versus diabetes. C, representative photomicrographs of DCF fluorescence imaged with a confocal laser-scanning fluorescence microscope in untransfected MCs after exposure to NG, HG, or 5 mM D-glucose + 20 mM L-glucose for 24 h with or without AS and sense Nox4. Lower panel, DCF fluorescence, reflecting the relative levels of ROS (arbitrary units), was semi-quantified using the Image-Pro Plus 4.5 software. Values are the means ± S.E. from three independent experiments. **, p < 0.01 versus control (NG); ##, p < 0.01 versus HG. D, MCs were transfected with sense Nox4 or AS Nox4 by using Lipofectamine and exposed to NG, HG, or 5 mM D-glucose + 20 mM L-glucose for 24 h. Fibronectin protein expression was determined by direct immunoblotting of cell lysates. Each histogram in the bottom panel represents the ratio of the intensity of fibronectin bands quantified by densitometry factored by the densitometric measurement of the actin band. The data are expressed as percent of control where the ratio in the control (NG) was defined as 100%. Values are the means ± S.E. from three independent experiments. **, p < 0.01 versus control (NG); ##, p < 0.01 versus HG.
tein levels in a pattern consistent with mesangial cell distribution. The administration of AS Nox4 markedly inhibited diabetes-induced NADPH oxidase activity concomitantly with the down-regulation of Nox4 protein expression. The increases in Nox4 protein expression and ROS generation were prevented by insulin treatment, suggesting that these changes were caused by the diabetic state and were not a direct toxic effect of STZ. Interestingly, gp91<sup>pHox</sup>/Nox2 was also up-regulated in the diabetic kidney. This is in agreement with previous reports (20, 41–43) showing that diabetes enhanced expression of gp91<sup>pHox</sup>/Nox2 in the kidney and vasculature. However, no attenuation of gp91<sup>pHox</sup>/Nox2 expression was seen in AS Nox4-treated rats, indicating that the decrease in ROS generation is related to inhibition of Nox4. The lack of correlation between ROS generation and increased gp91<sup>pHox</sup>/Nox2 expression in the AS Nox4-treated animals is somewhat surprising. However, unlike the requirement for Nox4 activation, activation of gp91<sup>pHox</sup>/Nox2 is dependent on a number of cytosolic and membrane subunits that form the active enzyme complex, and these may not be readily available or not regulated by diabetes. Indeed, there is emerging evidence that in contrast to gp91<sup>pHox</sup>/Nox2, Nox4 functions independently of the presence of the cytosolic subunits (44, 45). Therefore, it is tempting to speculate that Nox4 activity depends primarily on the expression of the catalytic unit itself. A more complete explanation will await a better understanding of the mechanism of Nox4 activation and its precise subunit requirement. In addition, the function of Nox4 as a source of ROS in diabetes is supported by the <i>in vitro</i> observation that transfection of MCs with AS Nox4 markedly reduced high glucose-induced NADPH oxidase activation. The potent effect of AS oligonucleotides is most likely because of their uptake and sequestration in kidney tissue and cells (46).

The nature of the enzymatic sources of oxidative stress in diabetes or upon exposure of cells to high glucose is not precisely defined. Mitochondrial oxidation is an important source of ROS in diabetes (47–50). Nishikawa et al. (48) emphasized the pivotal role of the increase in production of ROS from the mitochondrial electron transport chain in diabetes. ROS derived from a mitochondrial source were shown to be predominant in various cell types including MCs after exposure to high glucose (48, 51–54). However, high glucose-induced ROS generation also occurs through activation of a p47<sup>phox</sup>-containing NAD(P)H oxidase in cultured cells (55) and <i>in vivo</i> in the aorta of a rat model of type 2 diabetes, characterized by an up-regulation of p22<sup>phox</sup> and gp91<sup>pHox</sup>/Nox2 (43). Likewise, in STZ-induced diabetic rats expression of certain components of the oxidase is augmented in the kidney (13, 23, 24). It is possible that both enzymatic pathways play a role in diabetes- and high glucose-induced ROS generation. For example, ROS generated by membrane-bound NAD(P)H oxidase may enhance generation of superoxide by mitochondria. The recent findings that mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling support this contention (56). Moreover, Kimura et al. (57) recently reported that Ang II-induced ROS generation via NAD(P)H oxidase triggered mitochondrial ROS release in cardiac myocytes. Conversely, mitochondrial ROS generation may lead to NAD(P)H oxidase activation (58). Such interactions are compatible with the concept of ROS-triggered ROS generation.

Our work not only demonstrated that Nox4-derived ROS contribute to oxidative stress during the initial stages of diabetes but also provided evidence that Nox4-dependent ROS generation mediates renal hypertrophy and fibronectin expression. Inhibition of Nox4 oxidase by administration of AS Nox4 reduced whole kidney hypertrophy and glomerular hypertrophy as well as fibronectin accumulation in diabetic cortex and glomeruli. Furthermore, transfection of cultured MCs with AS Nox4 significantly reduced high glucose-induced accumulation of fibronectin. The mechanisms by which diabetes and high glucose concentrations activate the oxidase remain speculative. They may exert a direct effect on Nox4 to stimulate hypertrophy and fibronectin expression or indirectly via the release of other mediators such as Ang II and/or transforming growth factor-β. Oxidants may also alter matrix-degrading enzymes. We have shown previously that Ang II induces protein synthesis and hypertrophy via Nox4 in MCs (27, 28). Thus, the renin-angiotensin system may contribute to the stimulation of Nox4-based NAD(P)H oxidase activity. This hypothesis is supported by the observation that ROS generation and NAD(P)H oxidase subunit p47<sup>phox</sup> protein expression were increased in glomeruli of rats with type 1 diabetes, effects that were inhibited by treatment with angiotensin-converting enzyme inhibitor or Ang II type 1 (AT<sub>1</sub>) receptor blocker (13). High glucose concentration enhances Ang II generation via up-regulation of angiotensinogen, angiotensin-converting enzyme, or renin in renal cells including MCs (59).

We further dissected the involvement of Nox4 by identifying the downstream targets of the oxidase in the signaling cascade linking diabetes and high glucose to cell hypertrophy and fibronectin expression. There is evidence that ERK1/2 mediates hypertrophy and extracellular matrix accumulation both in animal models of diabetes and in cultured renal cells (3, 7, 36, 38, 39, 60). <i>In vitro</i> studies suggested that activation of the Akt/PKB pathway is involved in renal cell hypertrophy or matrix accumulation (27, 32, 33, 37, 61, 62). We now demonstrate that both ERK1/2 and Akt/PKB are activated <i>in vivo</i> and that inhibition of Nox4 function with AS Nox4 nearly abrogates diabetes-induced activation of ERK1/2 and Akt/PKB, suggesting that Nox4 functions as an upstream activator of the two kinases not only <i>in vitro</i> but also <i>in vivo</i>. It is tempting to speculate that Nox4 is a pivotal signal transducer commonly shared by both hypertrophic and fibrotic pathways triggered by the diabetic milieu in the kidney.

In conclusion, this study establishes that activation of the NAD(P)H oxidase Nox4 plays a critical role in diabetes-induced oxidative stress, kidney hypertrophy, and fibronectin expression. It would be important to confirm the efficacy of anti-Nox4 therapy in chronic progressive DN. Specific inhibition of this enzyme may selectively target several important biological responses to prevent or reverse pathophysiologic manifestations of diabetes.

Acknowledgments—We thank Sergio Garcia for help with the cell culture and Fredyne Springer for the immunohistochemistry.

REFERENCES
1. Bilous, R. W., Mauer, S. M., Sutherland, D. E., and Steffes, M. W. (1989) Diabetes 38, 1142–1147
2. Ziyadeh, F. N., Hoffman, B. B., Han, D. C., Iglesias-De La Cruz, M. C., Hong, S. W., Isono, M., Chen, S., McGowan, T. A., and Sharma, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8015–8020
3. Wolf, G., and Ziyadeh, F. N. (1999) Kidney Int. 56, 393–405
4. Ziyadeh, F. N. (1993) <i>Am. J. Kidney Dis.</i> 22, 736–744
5. Phillips, A. O. (2003) <i>Curr. Diab. Rep.</i> 3, 491–496
6. Abboud, H. E. (1997) <i>Kidney Int.</i> 60, 53–56
7. Wolf, G. (2003) <i>Curr. Diab. Rep.</i> 3, 485–490
8. Baynes, J. W. (1991) <i>Diabetes</i> 40, 405–412
9. Hinokio, Y., Suzuki, S., Hirai, M., Chiba, M., Hirai, A., and Toyota, T. (1999) <i>Diabetes</i> 48, 995–998
10. Sano, T., Umeda, F., Hashimoto, T., Nawata, H., and Utsumi, H. (1998) <i>Diabetologia</i> 41, 1355–1360
11. Schnackenberg, C. G. (2002) <i>Curr. Opin. Pharmacol.</i> 2, 121–125
12. Ha, H., Kim, C., Son, Y., Chung, M. H., and Kim, K. H. (1994) <i>Free Radic. Biol. Med.</i> 16, 271–274
13. Onozato, M. L., Tojo, A., Goto, A., Fujita, T., and Wilcox, C. S. (2002) <i>Kidney Int.</i> 61, 186–194
