# Translocation of Glomerular p47phox and p67phox by Protein Kinase C-β Activation Is Required for Oxidative Stress in Diabetic Nephropathy

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**Oxidative stress is implicated to play an important role in the development of diabetic vascular complications, including diabetic nephropathy. It is unclear whether oxidative stress is primarily enhanced in the diabetic glomeruli or whether it is merely a consequence of diabetes-induced glomerular injury. To address this issue, we examined diabetic glomeruli to determine whether oxidative stress is enhanced, as well as examined the role of protein kinase C (PKC)-β activation in modulating NADPH oxidase activity. Urinary 8-hydroxydeoxyguanosine excretion and its intense immune-reactive staining in the glomeruli were markedly higher in diabetic than in control rats, and these alterations were ameliorated by a treatment with a selective PKC-β inhibitor, ruboxistaurin (RBX; LY333531) mesylate, without affecting glycemia. NADPH oxidase activity, which was significantly enhanced in diabetic glomeruli and the source of reactive oxygen species (ROS) generation, was also improved by RBX treatment by preventing the membranous translocation of p47phox and p67phox from cytoplasmic fraction without affecting their protein levels. Adenoviral-mediated PKC-$\beta_2$ overexpression enhanced ROS generation by modulating the membranous translocation of p47phox and p67phox in cultured mesangial cells. We now demonstrate that oxidative stress is primarily enhanced in the diabetic glomeruli due to a PKC-β–dependent activation of NADPH oxidase resulting in ROS generation.**
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Increased oxidative stress has been shown in patients with diabetes (1,2) and has been implicated in the development and progression of diabetic microvascular complications, including diabetic nephropathy (3). Indeed, enhanced oxidative stress has been recently shown in the kidney of diabetic rats (4,5), and the treatment with antioxidants was shown to improve diabetes-induced renal injury (6–8). We have also reported that the expression of *HEMOX1* (heme oxygenase-1), a redox-sensitive and -inducible gene (9), is enhanced in the glomeruli of rats with streptozotocin-induced diabetes (10). This enhanced expression is inhibited by treatment with vitamin E (10). These results suggest that oxidative stress plays an important role in initiating diabetic nephropathy, although it remains unknown whether oxidative stress in diabetic glomeruli is primarily enhanced or whether it is a secondary indicator of diabetes-induced renal damage.

In diabetes, reactive oxygen species (ROS) is thought to be generated through the increased formation of advanced-glycation end products (3), increased polyol pathway (11), mitochondrial dysfunction (12), and protein kinase C (PKC) activation (13). Numerous reports have recently shown that NADPH oxidase, which is primarily found in phagocytic cells, is the main source of ROS in nonphagocytic cells such as endothelial cells (14), adventitial cells (15), smooth muscle cells (16,17), mesangial cells (18), podocytes (19), and fibroblasts (15). NADPH oxidase consists of several membrane-bound subunits (gp91phox, nox, and p22phox) and cytosolic subunits (p47phox and p67phox). It has become evident that there are other gp91phox homologues: gp91phox is expressed in endothelial cells and adventitial cells, nox-1 is expressed in vascular smooth muscle cells (17), and nox-4 is expressed in vascular smooth muscle cells and in renal cortex (20,21). Upon activation, some subunits are phosphorylated and translocated to the membrane by several kinases, including PKC, and form the catalytically active oxidase (22). PKC is one of the regulators of NADPH oxidase activation in neutrophils (22); however, its molecular mechanism in enhancing NADPH oxidase activity in nonphagocytic cells remains unknown. Among at least 12 isoforms of PKC, we and others have previously reported that PKC-β is activated in diabetic glomeruli and that treatment with a specific inhibitor of PKC-β improved not only diabetes-induced renal dysfunction, including glomerular hyperfiltration and albuminuria (23,24), but also diabetic glomerulosclerosis (25).

The aims of the present study were to determine whether oxidative stress is primarily enhanced in the diabetic glomeruli and to determine the role of NADPH oxidase in the generation of ROS. We also examined the underlying molecular mechanism by which PKC-β activation might modulate NADPH oxidase in diabetic rats and in glomerular mesangial cells.

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8-OHdG, 8-hydroxydeoxyguanosine; DPI, diphenylene iodonium; GFP, green fluorescent protein; L-NMMA, *N*G-monomethyl-L-arginine; NIH, National Institutes of Health; PKC, protein kinase C; RBX, ruboxistaurin; ROS, reactive oxygen species; TBS, Tris-buffered saline.

## RESEARCH DESIGN AND METHODS

**Animals.** Male Sprague-Dawley rats weighing 180–200 g were made diabetic by a single intravenous injection of streptozotocin (50 mg/kg body wt) in 0.1 mol/l citrate buffer (pH 4.5). Rats receiving an injection of citrate buffer were used as controls. Glycemia was determined 2 days after the injection, and rats with blood glucose levels >16.7 mmol/l were considered to have diabetes. Rats were divided into the following groups: control rats, diabetic rats, diabetic rats treated with implantation of insulin pellets (Linshin, Scarborough, ON, Canada) subcutaneously, and diabetic rats treated with a selective PKC-β inhibitor, ruboxistaurin (RBX; LY333531) mesylate (10 mg · kg body $wt^{-1}$ · $day^{-1}$). Four weeks after the induction of diabetes, body weight and blood glucose levels were measured and the following in vivo studies were performed. After rats were anesthetized with pentobarbital (50 mg/kg i.p.), the cervical artery was cannulated and the kidneys perfused with 50 ml ice-cold PBS. Renal glomeruli were isolated by sieving with stainless steel and nylon meshes, as previously described (26), for mRNA and/or protein expression of NADPH oxidase subunits (p47phox, p67phox, p22phox, gp91phox, nox-1, and nox-4), measurement of NADPH oxidase activity. The Shiga University of Medical Science Animal Care Committees approved all experiments.

**Measurement of urinary 8-hydroxydeoxyguanosine.** Twenty-four-hour urine samples were collected from each group of rats using metabolic cages. The samples were stored frozen at −80°C until analyzed. Urine samples were centrifuged at 2,000*g* for 20 min, and the supernatant was used for the determination of 8-hydroxydeoxyguanosine (8-OHdG) by a competitive enzyme-linked immunosorbent assay kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan). The urinary 8-OHdG excretion was expressed as total amounts excreted in 24 h.

**Immunohistochemical analysis.** Immunohistochemical staining of 3-μm paraffin sections was performed with a Histofine Simple Stain mouse MAX PO (MULTI) kit (Nichirei, Tokyo, Japan), according to the manufacturer's instruction. In brief, the sections were deparaffinized and rehydrated by incubating at room temperature in xylene three times for 3 min, in 100% ethanol twice for 3 min, in 95% ethanol twice for 3 min, and once in Tris-buffered saline (TBS; 0.1 mol/l Tris-HCl, pH 7.4, 0.15 mol/l NaCl) for 5 min. The sections were immersed in 3% $H_2O_2$ absolute methanol solution at room temperature for 15 min. After washing with TBS, sections were incubated overnight at 4°C with primary monoclonal antibodies raised against 8-OHdG (1:10; Nihon-Yushi, Tokyo, Japan), followed by amino acid polymers that are conjugated to multiple molecules of peroxidase and anti-mouse IgG and washed with TBS (27). The sections were stained with ACE solution for 20 min at room temperature and washed with distilled water, followed by counterstaining with hematoxyline.

**Measurements of $O_2^-$ production.** The production of $O_2^-$ in isolated glomeruli was measured using lucigenin chemiluminenescence. This methodology has been validated to be quite specific for $O_2^-$ detection and useful in studies of vascular $O_2^-$ production (28). Isolated glomeruli were placed in a modified Krebs-HEPES buffer (pH 7.4) containing (in mmol/l) 99.0 NaCl, 4.69 KCl, 1.87 $CaCl_2$, 1.20 $MgCl_2$, 1.03 $K_2HPO_4$, 25 $NaHCO_3$, 20 Na-HEPES, and 11.1 glucose and then allowed to equilibrate for 30 min at 37°C. After 5 min dark adaptation, scintillation vials containing 2 ml Krebs-HEPES buffer with 5 μmol/l lucigenin were placed into a scintillation counter (Tri-Carb 1500; Perkin Elmer Packerd Instruments, Boston, MA) switched to the out-of-coincidence mode. Chemiluminescence values were obtained at 30-s intervals over 15 min, and readings in each of the last 10 min were averaged. Isolated glomeruli were resuspended to a modified Krebs-HEPES buffer, and the suspension was used for measurement of $O_2^-$. Lucigenin count was expressed as counts per minute per milligram protein content. Background counts were determined by glomeruli-free incubations and subtracted from glomerular suspension. Some glomeruli were incubated for 30 min with either 10 μmol/l diphenylene iodonium (DPI; an inhibitor of flavin-containing enzymes), and 10 mmol/l Tiron (a cell-permeable $O_2^-$ scavenger).

**Estimation of NADPH oxidase activity.** Homogenates from isolated glomeruli were prepared in 300 μl homogenizing buffer (pH 7.8; consisting of 50 mmol/l phosphate buffer and 0.01 mmol/l EDTA) using a glass homogenizer. Homogenates were subjected to low-speed centrifugation (1,000*g*) for 10 min to remove unbroken cells and debris. The supernatant (100 μg protein) was then added to glass scintillation vials containing 5 μmol/l lucigenin in a 2-ml homogenizing buffer. The chemiluminescence value was recorded at 30-s intervals over 15 min, and readings in each of the last 5 min were averaged. Lucigenin count was expressed as counts · $min^{-1}$ · 100 μg protein $content^{-1}$. The background was determined by measurement in the absence of homogenate and then subtracted from the reading obtained in the presence of homogenate. NADPH (100 μmol/l), NADH (100 μmol/l), arachidonic acid (100 μmol/l), xanthine (100 nmol/l), or succinate (5 mmol/l) was added to the incubation medium as a substrate for $O_2^-$ production. Production of $O_2^-$ in response to NADPH was also examined after incubation of 10 μmol/l DPI, 10 μmol/l indomethacin (an inhibitor of cyclooxygenase), 10 μmol/l *N*G-monomethyl-L-arginine (L-NMMA; an inhibitor of nitric oxide [NO] synthase), 100 μmol/l allopurinol (an inhibitor of xanthine oxidase), and 10 mmol/l Tiron.

**The mRNA expression of NADPH oxidase subunit.** For Northern blot analysis of NADPH oxidase subunit consisting of p47phox, p67phox, p22phox, gp91phox, nox-1, and nox-4, total RNA was extracted from isolated glomeruli using a commercial preparation based on guanidinium and phenol extraction (TRIzol Reagent; Gibco BRL, Grand Island, NY). Total RNA (12 μg per lane) was electrophoretically separated on a formaldehyde 1.0% Agarose gel and transferred onto a nylon membrane (Nyron 0.45 μm; Schleicher & Schuell, Dassel, Germany). After ultraviolet cross-linking, the membranes were prehybridized and hybridized to $\alpha^{32}$-P CTP (New England Nuclear, Boston, MA)-labeled p47phox, p67phox, p22phox, gp91phox, nox-1, and nox-4 cDNA in a buffer (Perfecthybri; Toyobo, Osaka, Japan) for 16 h at 68°C. Radioactivity of corresponding bands was measured quantitatively by a phosphoimage analyzer (Molecular Analysis; Bio-Rad Labolatories, Hercules, CA). After radioactive probes were stripped from the membrane, it was rehybridized with a radioactive probe of acidic ribosomal phosphoprotein PO (36B4) as an internal standard (23). The cDNAs for p47phox, p67phox, p22phox, gp91phox, nox-1, and nox-4 were cloned by RT-PCR of total RNA isolated from cultured rat mesangial cells, rat smooth muscle cells, and rat spleen (17,29–32).

**The protein expression of p47phox and p67phox in glomeruli.** The protein expression of p47phox and p67phox was further examined by Western blot analysis, as previously described (26). Glomeruli isolated from rats were homogenized in 0.3 ml ice-cold buffer A (30 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EGTA, 5 mmol/l EDTA, 1 mmol/l dithiothreitol, and 250 mmol/l sucrose) with a Dounce homogenizer (60 strokes). The homogenates were centrifuged at 12,000*g* for 10 min at 4°C, and the supernatants were used as total glomerular lysates. To obtain membranous and cytoplasmic fractions, total glomerular lysates were then centrifuged at 100,000*g* for 60 min at 4°C. The resulting pellets were resuspended with buffer B (buffer A without sucrose), solubilized with 1% Triton X-100 (33), and used for the membrane fraction. The supernatants of postultracentrifugation were used for the cytoplasmic fraction. After boiling for 5 min, samples (50–70 μg protein/lane for the membrane fraction, 20–30 μg protein/lane for the cytoplasmic fraction and total lysates) were electrophoresed on 15% SDS-polyacrylamide gels, as described by Laemmli (34), and transferred to a polyvinylidene difluoride filter (Immobilon; Millipore, Bedford, MA) for 5 h at 100 V using the Mini-Transblot Cell apparatus (Bio-Rad). For blocking, the filter was incubated in 5% nonfat milk in a buffer containing TBS and 0.1% Tween-20 for 1 h at room temperature. The filter was incubated with anti-mouse p47phox antibody (1:500; Transduction Laboratories, Lexington, KY), anti-mouse p67phox antibody (1:500; Transduction Laboratories) in the buffer (TBS containing 5% BSA and 0.1% Tween-20) overnight at 4°C. The filter was washed three times with TBS containing 0.1% Tween-20 and then incubated with a horseradish peroxidase–conjugated anti-mouse IgG second antibody for 1 h at room temperature. The bands were detected using the enhanced chemiluminescence system (Amersham, Buckinghamshire, U.K.). The density of the corresponding bands was measured quantitatively using National Institutes of Health (NIH) Image software (http://rsb.info.nih.gov/nih-image). Protein content was measured using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

**Essential cell culture and recombinant adenoviruses.** Mesangial cells were obtained from a culture of glomeruli isolated from male Sprague-Dawley rats weighing 100–150 g in DMEM medium containing 20% fetal bovine serum and antibiotics. Cultured cells were identified as mesangial cells as previously described (26). Subconfluent cells from the fourth to ninth passages were used for in vitro experiments.

PKC-$\beta_2$ adenoviruses (Ad-PKC-$\beta_2$) and control adenoviruses expressing green fluorescent protein (GFP) (Ad-GFP) were kindly provided by Dr. George L. King (Joslin Diabetes Center, Boston, MA) (35). The adenoviruses were applied at a concentration of $1 \times 10^8$ plaque-forming units/ml. Infection efficiency was monitored by fluorescence intensity, which showed expression in >80% of cells. Expression of recombinant protein was confirmed by Western blot analysis of PKC-$\beta_2$ isoform using PKC-$\beta_2$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cultured mesangial cells were infected at $1 \times 10^8$ plaque-forming units/ml for 2 h with stocks of either Ad-PKC-$\beta_2$ or Ad-GFP. Infected cells were further incubated for 48 h at 37°C in DMEM with 2% fetal bovine serum and used for Western blot analysis and dihydroethidium staining.

The protein expression of p47phox and p67phox in mesangial cells was estimated by Western blot analysis. We obtained the samples for p47phox and p67phox expression with the same methods described in the protein expression of p47phox and p67phox in glomeruli.

**Dihydroethidium staining.** The oxidative fluorescent dihydroethidium was used to evaluate intracellular production of $O_2^-$. Dihydroethidium is cell permeable and reacts with $O_2^-$ to form ethidium, which in turn intercalates

TABLE 1
Characteristics of rats for 4 weeks

| | *n* | Body weight (g) | Blood glucose (mmol/l) | Kidney mass (g/100 g body wt) |
|---|---|---|---|---|
| Control | 20 | 351.0 ± 5.2 | 6.01 ± 0.1 | 0.75 ± 0.01 |
| Diabetic | 20 | 245.9 ± 9.3* | 24.92 ± 0.47* | 1.11 ± 0.01* |
| Diabetic plus insulin | 15 | 351.9 ± 5.1† | 6.01 ± 0.12† | 0.76 ± 0.01† |
| Diabetic plus RBX | 17 | 239.6 ± 2.1* | 24.68 ± 0.52* | 1.11 ± 0.01* |

Data are means ± SE. *$P < 0.001$ vs. control; †$P < 0.001$ vs. diabetic. RBX, RBX mesylate.

with DNA, providing nuclear fluorescence at an excitation wavelength of 520 nm and emission wavelength of 610 nm (36). Mesangial cells were plated in a glass-bottom dish. When cells were 80% confluent, they were infected with Ad-PKC-$\beta_2$ and Ad-GFP. The cells were then incubated in phenol red-free Hanks containing dihydroethidium (10 μmol/l) at 37°C. After 15 min, cells were linsed in phenol red-free Hanks, and images were obtained with a laser scanning confocal microscope system (LSM510 Meta; Carl Zeiss, Jena, Germany) equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. The averages of fluorescence intensity values from each 30 cells of three different examinations were calculated using LSM software. Some cells were preincubated for 30 min with either 10 μmol/l DPI or 10 mmol/l Tiron.

**Statistical analysis.** Results were expressed as mean ± SE. Comparison among each group was performed by one-way ANOVA, followed by the Scheffe's test. *P* values <0.05 were defined as statistically significant.

## RESULTS

**Characteristics of experimental rats.** As shown in Tables 1 and 2, blood glucose levels were significantly higher in diabetic than in control rats 4 weeks after streptozotocin injection. In rats treated with insulin, blood glucose was normalized. As anticipated, the PKC-β inhibitor RBX had no effect on glycemia. Body weight was significantly lower and kidney weight greater in diabetic compared with control rats, and insulin treatment again normalized these alterations. RBX had no effect on body or kidney weight.

To evaluate whether oxidative stress is enhanced in diabetic rats, we measured the urinary excretion of 8-OHdG, a sensitive marker of oxidative DNA damage. The 24-h urinary 8-OHdG excretion was significantly higher in diabetic than in control rats (Table 2). The increased urinary 8-OHdG excretion in diabetic rats was completely normalized by treatment with insulin and significantly ameliorated by RBX (Table 2).

**PKC-β inhibition suppresses diabetes-induced glomerular 8-OHdG staining.** Urinary 8-OHdG is supposed to be the marker of total systemic oxidative stress in vivo. Therefore, to determine the evidence of oxidative stress in diabetic glomeruli, we examined the immunoreactive expression of 8-OHdG. The staining of 8-OHdG was markedly increased in the nuclei of diabetic glomerular cells such as mesangial cell, endothelial cell, and podocyte compared with those from control and diabetic rats treated with insulin (Fig. 1*A*– *C*). However, PKC-β inhibition by treatment with RBX revealed the loss of increased immunoreactive cells for 8-OHdG in diabetic rats (Fig. 1*D*).

**PKC-β inhibition suppresses diabetes-induced glomerular $O_2^-$ production.** To further confirm the presence of oxidative stress in diabetic glomeruli, we evaluated $O_2^-$ production in ex vivo conditions using a lucigenin chemiluminescence method. As shown in Fig. 2, $O_2^-$ production by isolated glomeruli from diabetic rats was significantly increased compared with that from control rats (1,384.5 ± 79.4 vs. 724.3 ± 113.0 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 4$, $P < 0.05$). Normalization of glycemia by insulin completely normalized increased $O_2^-$ production (630.9 ± 28.3 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 4$, NS vs. control). PKC-β inhibition with RBX also suppressed the increased $O_2^-$ production in diabetic glomeruli without affecting glycemia (947.7 ± 85.5 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 4$, $P < 0.05$ vs. diabetic), consistent with the finding in glomerular 8-OHdG staining. In addition, the enhanced lucigenin signal in diabetic glomeruli was significantly inhibited by treating with 10 μmol/l DPI, a specific inhibitor of NADPH oxidase, and 10 mmol/l Tiron, an $O_2^-$ scavenger.

**PKC-β inhibition suppresses diabetes-induced glomerular NADPH oxidase activation.** To investigate the enzyme source of $O_2^-$ production, oxidase activity in response to the addition of a variety of substrates was examined in glomerular homogenates using a lucigenin chemiluminescence method. As shown in Fig. 3*A*, $O_2^-$ production derived from NADPH oxidase was greater than that from other potential substrates, including NADH, xanthine, succinate or arachidonic acid. NADPH oxidase activity was significantly enhanced in the diabetic glomerular homogenates compared with those from controls (4,296.0 ± 75.8 vs. 2,399.4 ± 79.8 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 5$, $P < 0.05$). This enhanced activation of NADPH oxidase was normalized by insulin (2,574.6 ± 54.7 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 5$, $P < 0.05$ vs. diabetic). PKC-β inhibition with RBX significantly suppressed NADPH oxidase activation (3,432.6 ± 152.2 cpm · $min^{-1}$ · mg $protein^{-1}$, $n = 5$, $P < 0.05$ vs. diabetic) (Fig. 3*A*). As

TABLE 2
Urinary 8-OHdG excretion

| | *n* | Body weight (g) | Blood glucose (mmol/l) | Kidney mass (g/100 g body wt) | Urinary 8-OhdG (μg/day) |
|---|---|---|---|---|---|
| Control | 7 | 342.1 ± 6.3 | 5.49 ± 0.07 | 0.74 ± 0.01 | 0.65 ± 0.04 |
| Diabetic | 7 | 235.3 ± 3.3* | 25.39 ± 0.87* | 1.14 ± 0.01* | 1.80 ± 0.03* |
| Diabetic plus insulin | 7 | 337.0 ± 4.5† | 5.79 ± 0.11† | 0.74 ± 0.01† | 0.62 ± 0.07† |
| Diabetic plus RBX | 5 | 233.0 ± 3.7* | 24.09 ± 0.82* | 1.12 ± 0.01* | 1.20 ± 0.18† |

Data are means ± SE. *$P < 0.001$ vs. control; †$P < 0.001$ vs. diabetic. RBX, RBX mesylate.

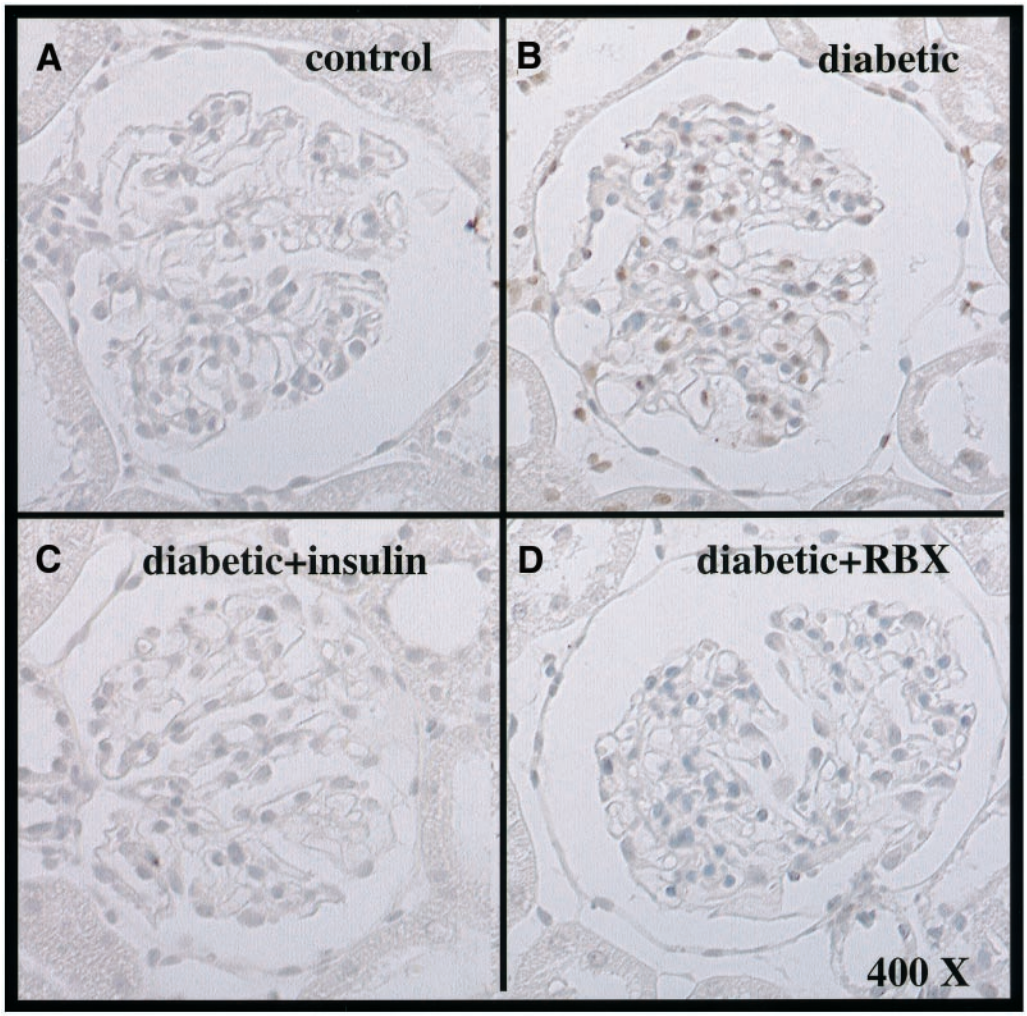


**FIG. 1. PKC-β inhibition suppresses diabetes-induced glomerular 8-OHdG staining. Representative results of immunoreactive expression of renal 8-OHdG from control (*A*), diabetic (*B*), diabetic plus insulin (*C*), and diabetic plus RBX (RBX mesylate) (*D*) are shown. The data are derived from independent experiments in each group of three rats. Photographs are 400× magnification.**

shown in Fig. 3*B*, NADPH oxidase activity was completely blocked by DPI and Tiron, whereas rotenone (an inhibitor of the electron transport chain), allopurinol (an inhibitor of xanthine oxidase), indomethacin (a cyclooxygenase inhibitor), and L-NMMA (a NO synthase inhibitor) had no effect. This appears to indicate that the main enzyme source for production of $O_2^-$ in all the glomeruli from control and diabetic rats is derived from NADPH oxidase activity.

**The effect of diabetes on NADPH oxidase subunits in vivo.** The mRNA expression of NADPH oxidase subunits consisting of gp91phox, nox-1, nox-4, p22phox, p47phox,

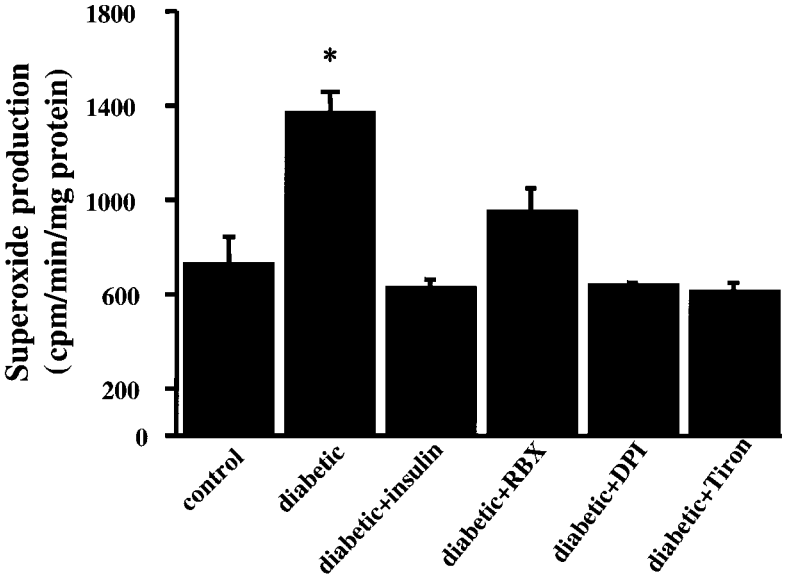


**FIG. 2. PKC-β inhibition suppresses diabetes-induced glomerular $O_2^-$ production. Production of $O_2^-$ in isolated glomeruli from rats of each group was assessed using a lucigenin clemiluminescence assay. Some glomeruli of diabetic rats were also assessed in the absence or presence of 10 μmol/l DPI and 10 mmol/l Tiron. Data are expressed as counts per minute per milligram glomerular protein. Data are means ± SE ($n$ = 4, *$P$ < 0.05 vs. other groups). RBX, RBX mesylate.**

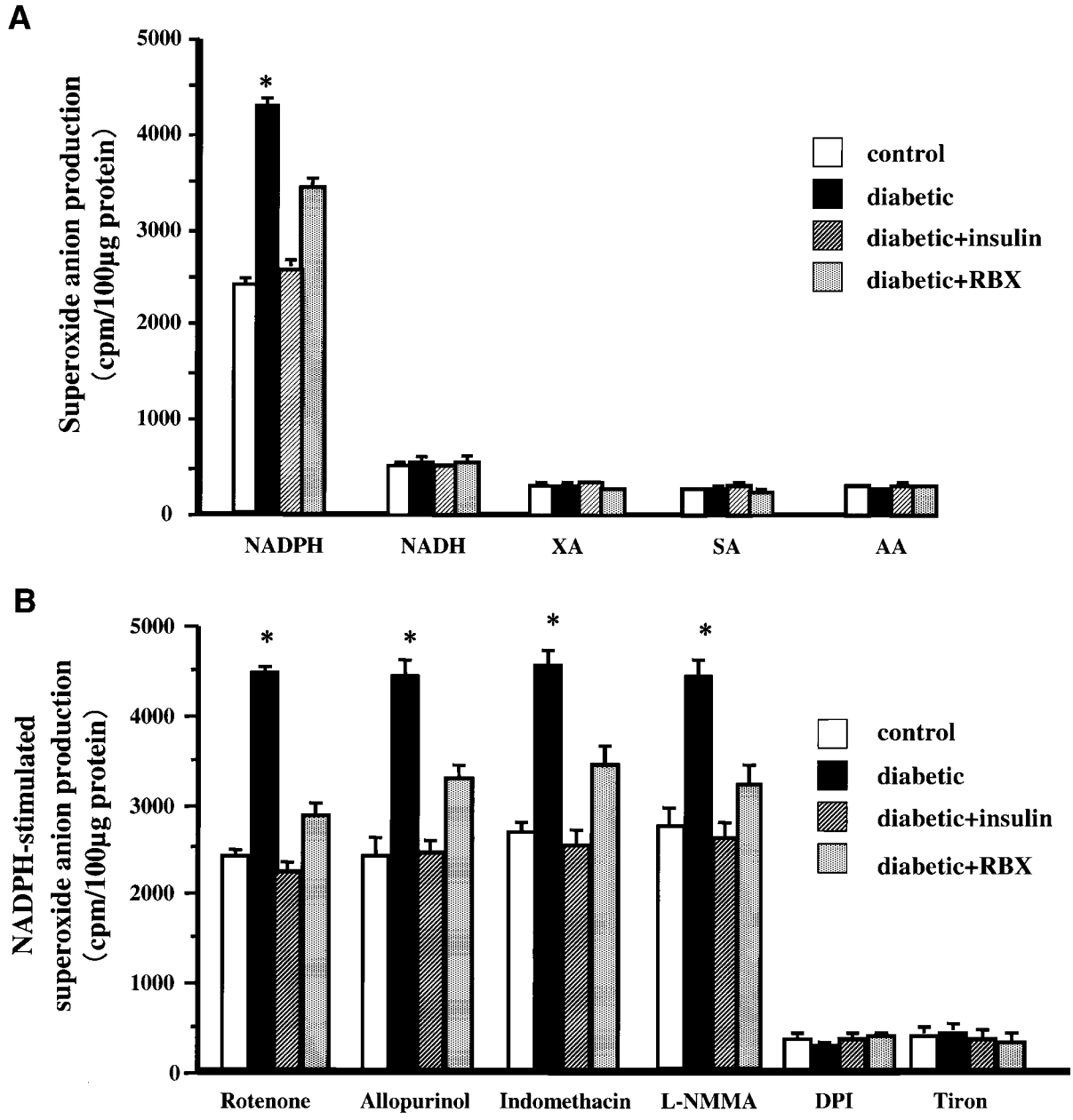


**FIG. 3. Superoxide anion production.** *A*: Enzyme source of $O_2^-$ production in glomerular homogenates. Production of $O_2^-$ in glomerular homogenates in the presence of potential substrates is measured by a lucigenin chemiluminescence method. NADPH (100 μmol/l), NADH (100 μmol/l), arachidonic acid (AA; 100 μmol/l), succinate (SA; 5 mmol/l), or xanthine (XA; 100 nmol/l) is added to glomerular homogenates from control, diabetic, diabetic plus insulin, or diabetic plus RBX (RBX mesylate). Data are means ± SE of five experiments in each group ($n = 5$, $^*P < 0.05$ vs. other groups). *B*: Effect of various inhibitors, such as 100 μmol/l rotenone, 100 μmol/l allopurinol, 10 μmol/l indomethacin, 10 μmol/l L-NMMA, 10 μmol/l DPI, or 10 mmol/l Tiron, on NADPH-stimulated $O_2^-$ production of glomerular homogenates. Data are means ± SE of three experiments in each group ($n = 3$, $^*P < 0.05$ vs. other groups).

and p67phox in glomeruli was evaluated by Northern blot analysis. Among these subunits, the mRNA expression of p47phox and p67phox was significantly upregulated in diabetic glomeruli (Fig. 4*A* and *B*), whereas gp91phox, p22phox, and nox-1 expression was unchanged (Fig. 4*D–F*) and nox-4 was downregulated (Fig. 4*C*). The protein expression of p47phox and p67phox estimated by Western blot analysis was also upregulated in diabetic glomeruli (Fig. 4*G* and *H*), similar to the results of mRNA expression. These alterations in p47phox, p67phox, and nox-4 expression were normalized by insulin (Fig. 4*A– C*, *G*, and *H*), and the mRNA expression of gp91phox, p22phox, and nox-1 was unaltered by insulin (data not shown).

**Effect of PKC-β inhibition on mRNA and protein expression of p47phox and p67phox components and on membranous translocation of p47phox and p67phox in vivo.** To determine whether the PKC-β, which has been shown to be activated in diabetic glomeruli (23,24), contributes to the NADPH oxidase activation, we examined the effect of PKC-β inhibition with RBX on p47phox and p67phox overexpression in diabetic glomeruli. PKC-β inhibition with RBX failed to normalize the mRNA and protein expression of p47phox and p67phox protein using total cell lysates (Fig. 4*A* and *B* and Fig. 5*A* and *B*). However, the increased membranous translocation of p47phox and p67phox in diabetic glomeruli was

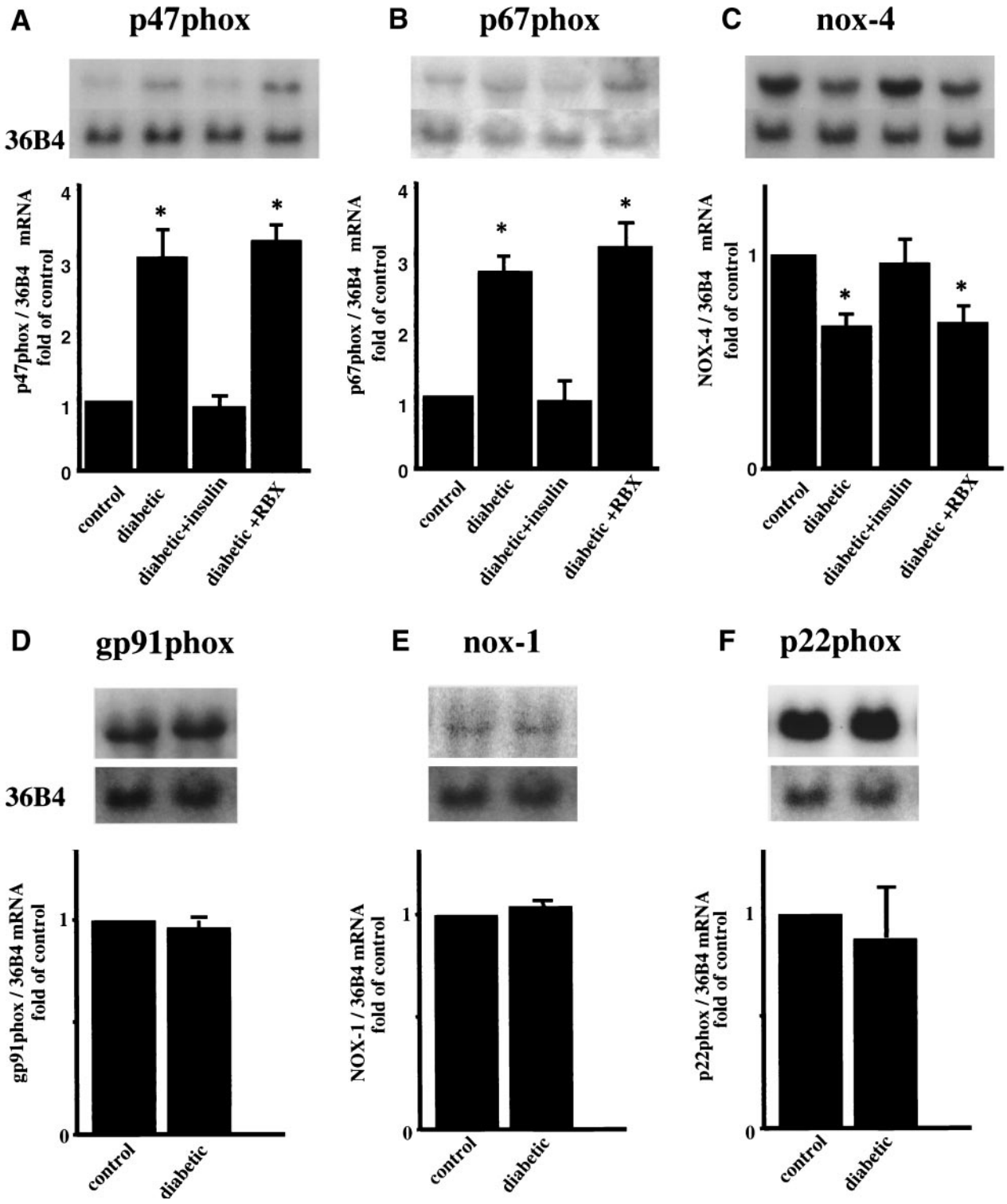


**FIG. 4. The effect of diabetes on NADPH oxidase subunits in vivo. The mRNA expression of NADPH oxidase subunits consisting of p47phox (*A*), p67phox (*B*), nox-4(*C*), gp91phox (*D*), nox-1 (*E*), and p22phox (*F*) and the protein expression of p47phox (*G*) and p67phox (*H*) in glomeruli. *A–F*: Representative results of Northern blot analysis of NADPH oxidase subunits and 36B4 are shown in the upper panel. Radioactivity of the corresponding bands is measured quantitatively by a phosphoimage analyzer, and the ratio to 36B4 mRNA is shown in the lower panel. Data are expressed as means ± SE ($n = 4$, * $P < 0.05$ vs. control and diabetic plus insulin groups). *G* and *H*: Representative results of Western blot analysis for total glomerular lysates of p47phox and p67phox obtained from two independent samples are shown in the upper panel. Densitometric quantification of the corresponding bands is performed using NIH Image software, and the ratio to control is shown in the lower panel. Data are expressed as means ± SE ($n = 5$, *$P < 0.05$ vs. other groups).**

significantly suppressed by PKC-β inhibition with RBX without affecting their cytoplasmic contents (Fig. 5*C* and *D*).

**The in vitro effect of PKC-$\beta_2$ on $O_2^-$ production is dependent on membranous translocation of p47phox and p67phox.** To delineate the underlying mechanism by

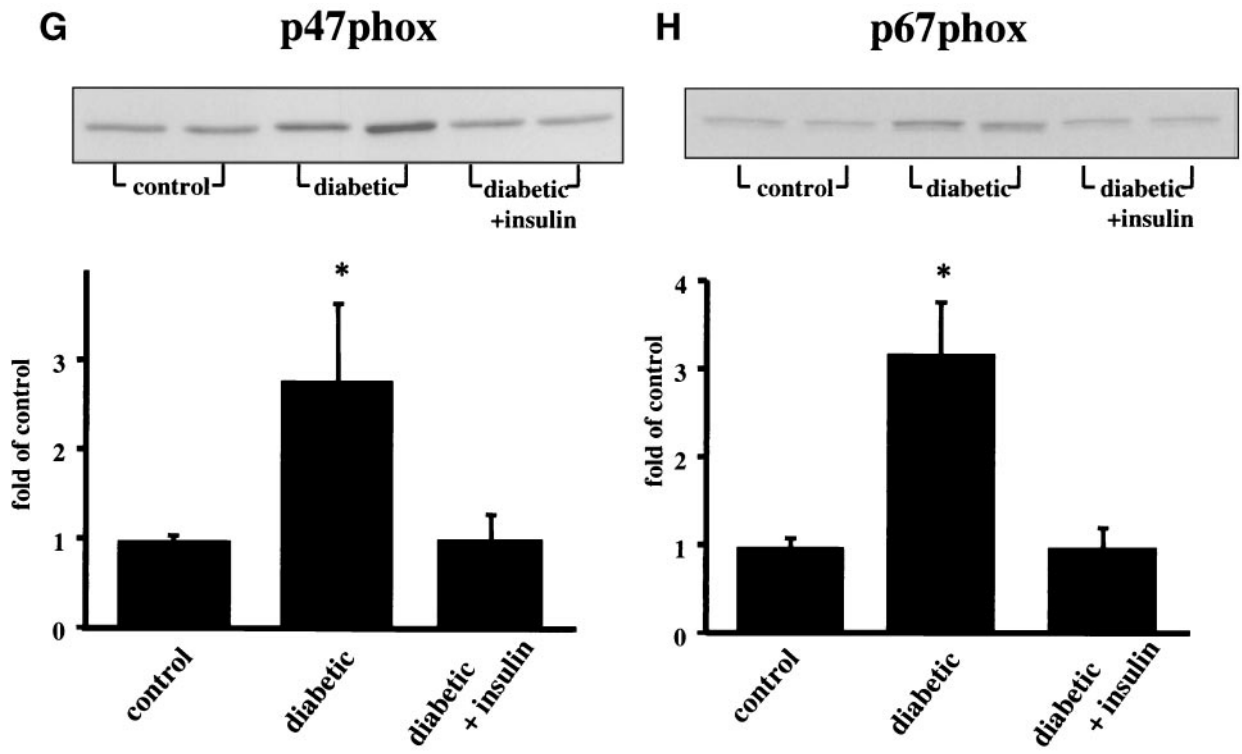


**FIG. 4—*Continued*.**

which PKC-β could modulate NADPH oxidase, we used an in vitro system to evaluate membranous translocation of p47phox and p67phox in rat mesangial cells. As shown Fig. 6*A*, a PKC-$\beta_2$ recombinant adenovirus markedly enhanced PKC-$\beta_2$ protein content in the membranous fraction, the cytoplasmic fraction, and total cell lysates, whereas in GFP-infected cell, it was hard to detect. In a similar system, the membranous translocation of p47phox and p67phox was significantly enhanced by PKC-$\beta_2$ overexpression compared with GFP overexpression, whereas the expression of p47phox and p67phox in the cytoplasmic fraction and total cell lysates did not change (Fig. 6*B* and *C*). Furthermore, we tested the role of PKC-$\beta_2$ in causing oxidative stress using dihydroethidium staining. The staining of dihydroethidium was significantly increased in cells overexpressing PKC-$\beta_2$, and this increased staining was reversed by treatment with either DPI or Tiron (Fig. 7).

## DISCUSSION

Our data show that not only overexpression of p47phox and p67phox but also their translocation to the membrane are correlated with the overall increased NADPH oxidase activity, resulting in oxidative stress in diabetic glomeruli. Furthermore, we demonstrated that membranous translocation of p47phox and p67phox was dependent on the PKC-β activation. This was shown by different methods, including PKC-β inhibition with RBX in vivo and adenoviral-mediated PKC-$\beta_2$ overexpression that allowed the detection of ROS generation in vitro.

Oxidative stress has been implicated in the pathogenesis of diabetic vascular complications, including diabetic nephropathy. We first proved that oxidative stress is enhanced in diabetic glomeruli by the estimation of urinary 8-OHdG excretion by enzyme-linked immunosorbent assay and by the immunoreactive expression of 8-OHdG immunohistochemically, since it is a product of the oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine and is excreted in the urine. Urinary 8-OHdG is a well-known biomarker of the total systemic oxidative stress in vivo (37). Leinonen et al. (2) showed that urinary 8-OHdG excretion was significantly increased in patients with type 2 diabetes, in association with high $HbA_{1c}$. In the present study, we also confirmed that urinary 8-OHdG excretion was significantly increased in diabetic compared with nondiabetic rats. However, the relative contribution of oxidative stress in glomeruli to urinary 8-OHdG excretion remains unclear. Our study shows that the immunoreactive expression of 8-OHdG was enhanced in diabetic glomerular cells consisting of mesangial cells, podocytes, and endothelial cells and provides evidence for the first time that oxidative stress is primarily enhanced in diabetic glomeruli.

We further investigated whether $O_2^-$ generation occurs primarily in the isolated glomeruli using a lucigenin chemiluminescence method. Production of $O_2^-$ was significantly increased in the diabetic glomeruli, and this enhancement was inhibited by DPI, a NADPH oxidase inhibitor. However, DPI can inhibit other flavoprotein-containing enzymes such as xanthine oxidase, NO synthase, cyclooxygenase, and mitochondrial electron transport chain. Therefore, we confirmed that NADPH-dependent $O_2^-$ production was greater in diabetic glomeruli than control rats by using their homogenates. NADH, xanthine, succinate, and arachidonic acid showed no effect on $O_2^-$ production in diabetic rats. Moreover, NADPH-dependent $O_2^-$ production was almost completely inhibited by DPI and Tiron, whereas rotenone, allopurinol, indomethacin, and L-NMMA were not effective. Therefore, it is likely that the

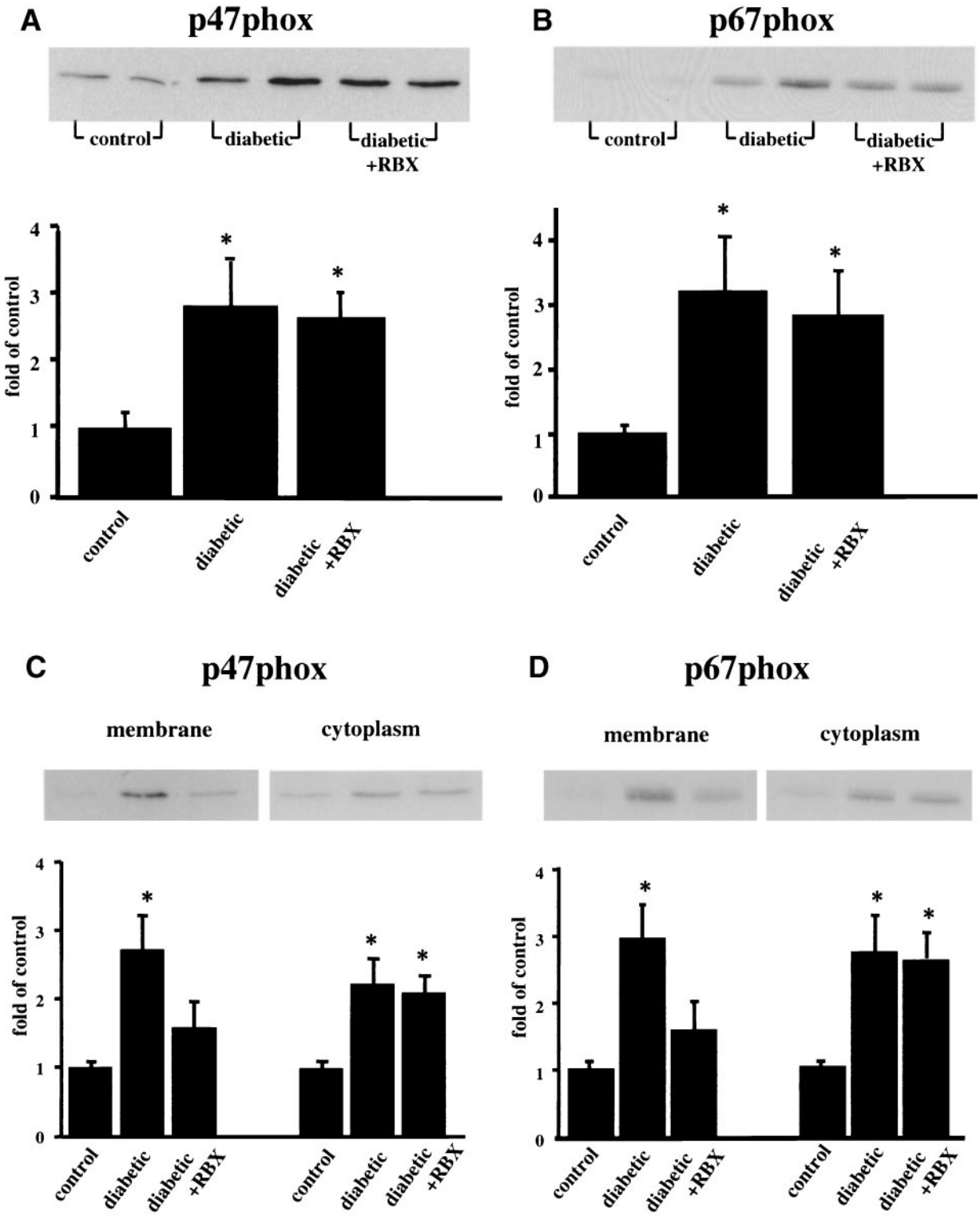


**FIG. 5. Effect of PKC-β inhibition on p47phox and p67phox expression and their membranous translocation in diabetic glomeruli. *A* and *B*: Representative results of Western blot analysis for total glomerular lysates of p47phox and p67phox obtained from two independent samples are shown in the upper panel. Densitometric quantification of the corresponding bands is performed using NIH Image software, and the ratio to control is shown in the lower panel. Data are expressed as means ± SE ($n = 5$, *$P < 0.05$ vs. control). RBX, RBX mesylate. *C* and *D*: Representative results of Western blot analysis for the membranous and cytosolic fraction of p47phox and p67phox are shown in the upper panel. Densitometric quantification of the corresponding bands is performed using NIH Image software, and the ratio to control is shown in the lower panel. Data are expressed as means ± SE ($n = 5$, *$P < 0.05$ vs. other groups). RBX, RBX mesylate.**

production of $O_2^-$ was enhanced through NADPH-dependent oxidase activation in diabetic glomeruli. Although the lucigenin chemiluminescence method is useful for the measurement of $O_2^-$, recent work has emphasized that the lucigenin dose itself is an important experimental variable and that in certain settings, high lucigenin concentrations

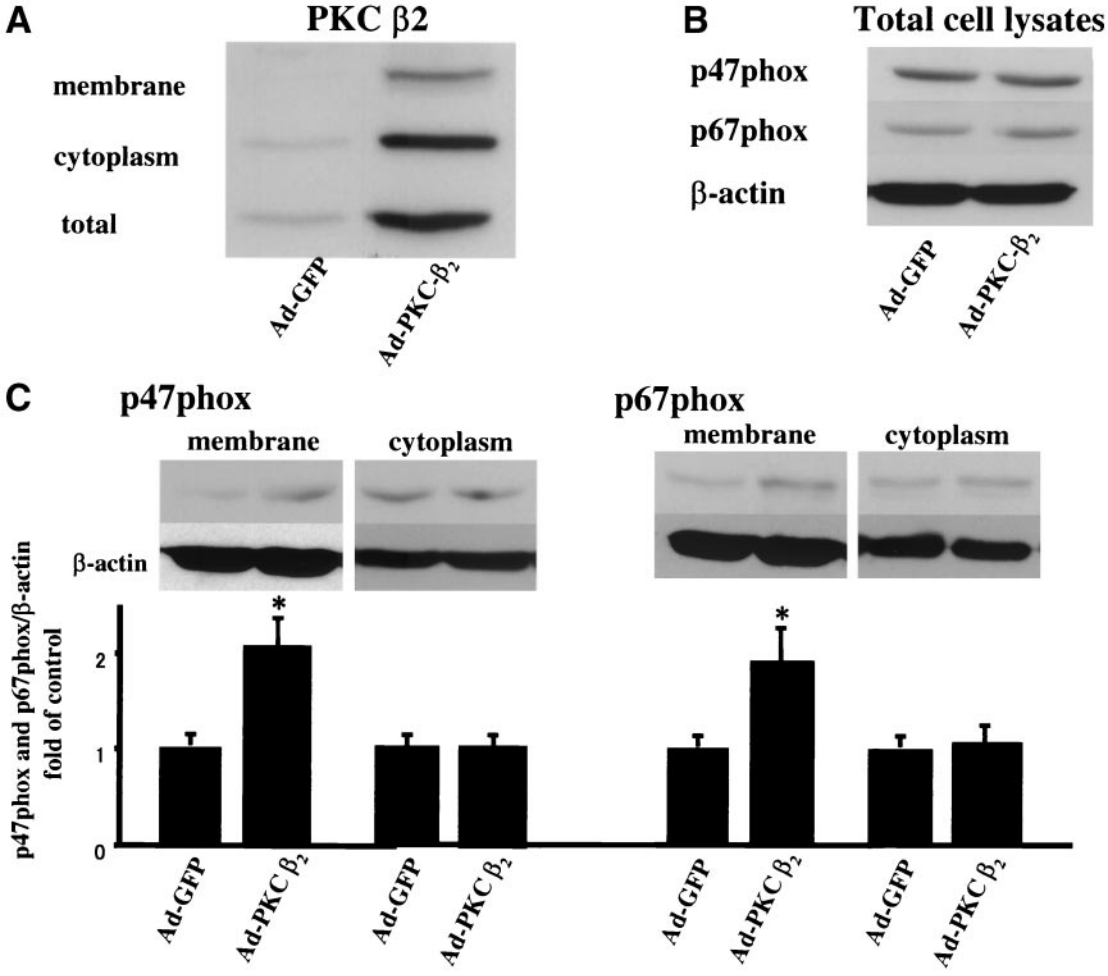


**FIG. 6. Adenovirus-induced overexpression of PKC-$\beta_2$ causes the membranous translocation of p47phox and p67phox. *A*: Representative results of Western blot analysis of the membranous, cytosolic fraction, and total cell lysates of PKC-$\beta_2$ in mesangial cells overexpressing PKC-$\beta_2$. *B* and *C*: Representative results of Western blot analysis for the membranous, cytosolic fraction, and total cell lysates of p47phox and p67phox are shown in the upper panel. Densitometric quantification of the corresponding bands is performed using NIH Image software, and the ratio to β-actin is shown in the lower panel. Data are expressed as means ± SE ($n$ = 5, *$P$ < 0.05 vs. other groups).**

can lead to artifactual $O_2^-$ production based on its redox cycling of the probe (38) when NADH-dependent $O_2^-$ production is measured by using lucigenin dose >50 μmol/l (39). In the present study, we used the non–redox-cycling dose of lucigenin at the concentration of 5 μmol/l, suggesting that the observed $O_2^-$ production in glomerular homogenates correlated specifically to NADPH oxidase activity.

The nonphagocytic NADPH oxidase, which is constitutively active, normally produces relatively low levels of ROS under basal conditions. It generates higher levels of ROS in response to hormones (40,41), growth factors, cytokines (42–44), or hemodynamic forces (45,46). It is proposed that regulation of oxidase activity in nonphagocytic cells could occur at at least two levels. One is overexpression of NADPH oxidase subunits, including gp91phox, gp91phox homologues (nox-1 and nox-4), gp22phox, gp47phox, and gp67phox. Recently, the expression of NADPH oxidase subunits (p22phox, p67phox, gp91phox, etc.) has been shown to be upregulated in several tissues in cardiovascular diseases (32,47–49). In diabetic rats, renal expression of p47phox, when evaluated by Western blot analysis and immunohistochemistry, has been shown to be increased in the kidney (5). In the present study, we extended the previous findings by using isolated glomeruli and demonstrating that the expression of p47phox and p67phox was enhanced in the glomeruli of diabetic rats. Other subunits, such as gp91phox, nox-1, and p22phox, did not differ between control and diabetic rats, and nox-4 was downregulated in diabetic rats. The physiological relevance of increased p47phox and p67phox expression in the induction of oxidative stress is supported by our observation of intense staining of 8-OHdG and of enhanced oxidase activity in the glomeruli of diabetic rats. By contrast, it has been reported that the overexpression of nox-4 in cultured cells such as HEK293 and COS7 leads to increased ROS production (20), suggesting a novel role of nox-4 as a ROS source in kidney. To understand the ability of diabetes and/or hyperglycemia to regulate the gene expression of NADPH oxidase subunits and their action requires further studies. We also demonstrated that the enhanced expression of p47phox and p67phox is normalized by insulin, but not by RBX. These results indicate that the increased p47phox and p67phox expression was mediated by hyperglycemia per se rather than by hyperglycemia-induced PKC-β activation, which

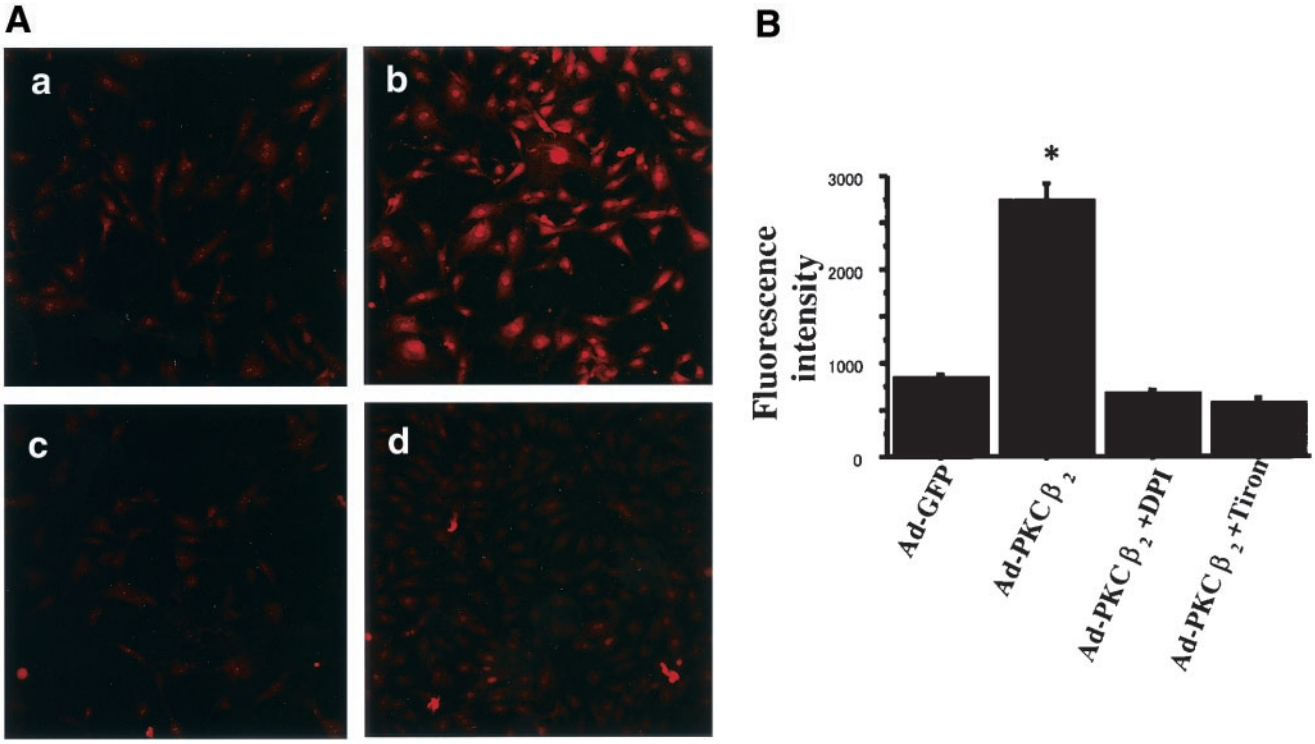


**FIG. 7. Detection of $O_2^-$ production by dihydroethidium staining in mesangial cells overexpressing PKC-$\beta_2$. *A*: Representative results of dihydroethidium staining are shown from each independent three experiments of Ad-GFP–infected mesangial cells (*a*), Ad-PKC-$\beta_2$–infected mesangial cells (*b*), Ad-PKC-$\beta_2$–infected mesangial cells preincubated by 10 μmol/l DPI for 30 min (*c*), and Ad-PKC-$\beta_2$–infected mesangial cells preincubated by 10 mmol/l Tiron for 30 min (*d*). Photographs are 200× magnification. *B*: The averages of fluorescence intensity values from each 30 cells of three different examinations were calculated using LSM software. Some cells were preincubated for 30 min with either 10 μmol/l DPI or 10 mmol/l Tiron. Data are expressed as means ± SE ($n$ = 3, *$P$ < 0.001 vs. other groups).**

ourselves and others have identified as the crucial biochemical signaling pathway in mediating the development and progression of diabetic nephropathy (23).

Another regulation of oxidase activation is posttranslational modification, including phosphorylation and membranous translocation by the kinases, such as PKC (22). Although PKC has been shown to be one of the regulators of NADPH oxidase activation in phagocytic cells, there are few reports in nonphagocytic cells. Recently, Inoguchi et al. (13) have shown that high glucose level stimulates ROS production through the PKC-dependent activation of DPI-inhibitable oxidase in cultured endothelial cells and smooth vascular cells using a general PKC inhibitor. However, they failed to measure oxidase activity or to investigate the expression of NADPH oxidase subunits. Our present data clearly suggest that the membranous translocation of p47phox and p67phox via PKC-β activation in diabetic glomeruli has a crucial role in causing oxidative stress because PKC-β inhibition with RBX prevented their translocation from the cytoplasm to the membrane. This is consistent with the amelioration in 8-OHdG staining and in oxidase activity. Furthermore, expression of PKC-$\beta_2$ recombinant adenovirus in glomerular mesangial cells caused the membranous translocation of p47phox and p67phox without affecting their protein contents and resulted in increased $O_2^-$ production, as judged by dihydroethidium staining. It should be noted that the link between diabetes, PKC-β activation, NADPH oxidase activity, and oxidative stress, to the best of our knowledge, has not been previously reported in vascular tissues and cells. To support our data, Dekker et al. (50) have reported that PKC-β–deficient neutrophils show a 50% reduction of oxidase response to phorbol myristic acid, a stimulator of PKC, compared with wild-type neutrophils. However, PKC-β inhibition with RBX did not completely inhibit diabetes-induced oxidative stress. Similarly, the membranous translocation of p47phox and p67phox was only partially, although statistically significantly, inhibited. The membranous translocation can be regulated by not only PKCβ but also by other PKC isoforms: protein kinase A, mitogen-activated kinases, and phosphatidylinositol-3 kinase (22,51,52). Indeed, Fontayne et al. (53) have also shown that PKC-α, -δ, and -ζ can also phosphorylate p47phox and induce its membranous translocation. In addition to PKC-$\beta_2$, it also seems possible that other PKC isoforms and kinases might be associated with the membranous translocation of p47phox and p67phox, resulting in oxidative stress.

In conclusion, the present study provides evidence that oxidative stress in the diabetic glomeruli in the early course of diabetes is mediated by NADPH oxidase activation, at least in part through PKC-β–dependent p47phox and p67phox membranous translocation. Also, our data suggest that PKC-β inhibition with RBX could potentially become a therapeutic approach to treating diabetes-induced oxidative stress.

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