

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- β_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. *Diabetes* 52:2603–2614, 2003

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, NG-monomethyl-L-arginine; NIH, National Institutes of Health; PKC, protein kinase C; RBX, ruboxistaurin; ROS, reactive oxygen species; TBS, Tris-buffered saline.

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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 2,000g 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- μ m 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% H₂O₂ 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 O₂⁻ production. The production of O₂⁻ in isolated glomeruli was measured using lucigenin chemiluminescence. This methodology has been validated to be quite specific for O₂⁻ detection and useful in studies of vascular O₂⁻ 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 CaCl₂, 1.20 MgCl₂, 1.03 K₂HPO₄, 25 NaHCO₃, 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 μ mol/l lucigenin were placed into a scintillation counter (Tri-Carb 1500; Perkin Elmer Packard 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 O₂⁻. 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 μ mol/l diphenylene iodonium (DPI; an inhibitor of flavin-containing enzymes), and 10 mmol/l Tiron (a cell-permeable O₂⁻ scavenger).

Estimation of NADPH oxidase activity. Homogenates from isolated glomeruli were prepared in 300 μ l 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 (1,000g) for 10 min to remove unbroken cells and debris. The supernatant (100 μ g protein) was then added to glass scintillation vials containing 5 μ mol/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 μ g 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 μ mol/l), NADH (100 μ mol/l), arachidonic acid (100 μ mol/l), xanthine (100 nmol/l), or succinate (5 mmol/l) was added to the incubation medium as a substrate for O₂⁻ production. Production of O₂⁻ in response to NADPH was also examined after incubation of 10 μ mol/l DPI, 10

μ mol/l indomethacin (an inhibitor of cyclooxygenase), 10 μ mol/l NG-monomethyl-L-arginine (L-NMMA; an inhibitor of nitric oxide [NO] synthase), 100 μ mol/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 μ g per lane) was electrophoretically separated on a formaldehyde 1.0% Agarose gel and transferred onto a nylon membrane (Nylon 0.45 μ m; Schleicher & Schuell, Dassel, Germany). After ultraviolet cross-linking, the membranes were prehybridized and hybridized to α 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 phosphorimage analyzer (Molecular Analysis; Bio-Rad Laboratories, 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 12,000g 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 100,000g 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 μ g protein/lane for the membrane fraction, 20–30 μ g 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- β_2 adenoviruses (Ad-PKC- β_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 × 10⁸ 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- β_2 isoform using PKC- β_2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cultured mesangial cells were infected at 1 × 10⁸ plaque-forming units/ml for 2 h with stocks of either Ad-PKC- β_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 O₂⁻. Dihydroethidium is cell permeable and reacts with O₂⁻ to form ethidium, which in turn intercalates

TABLE 1
Characteristics of rats for 4 weeks

	<i>n</i>	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-β₂ 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 rinsed 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. 1A–C). However, PKC-β inhibition by treatment with RBX revealed the loss of increased immunoreactive cells for 8-OHdG in diabetic rats (Fig. 1D).

PKC-β inhibition suppresses diabetes-induced glomerular O₂⁻ production. To further confirm the presence of oxidative stress in diabetic glomeruli, we evaluated O₂⁻ production in ex vivo conditions using a lucigenin chemiluminescence method. As shown in Fig. 2, O₂⁻ 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 O₂⁻ 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 O₂⁻ 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 O₂⁻ scavenger.

PKC-β inhibition suppresses diabetes-induced glomerular NADPH oxidase activation. To investigate the enzyme source of O₂⁻ 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. 3A, O₂⁻ 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. 3A). As

TABLE 2
Urinary 8-OHdG excretion

	<i>n</i>	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.

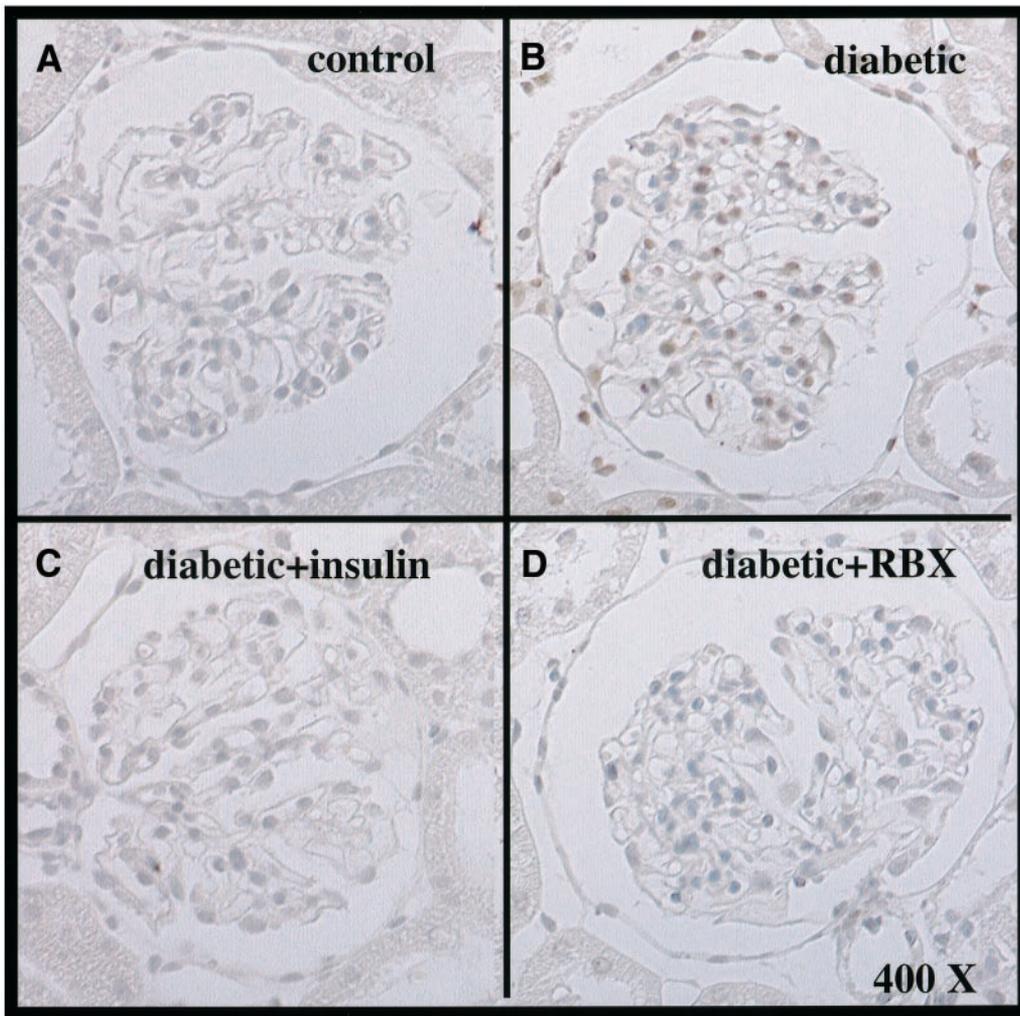


FIG. 1. PKC- β inhibition suppresses diabetes-induced glomerular 8-OHdG staining. Representative results of immunoreactive expression of renal 8-OHdG from control (A), diabetic (B), diabetic plus insulin (C), and diabetic plus RBX (RBX mesylate) (D) are shown. The data are derived from independent experiments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

shown in Fig. 3B, 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,

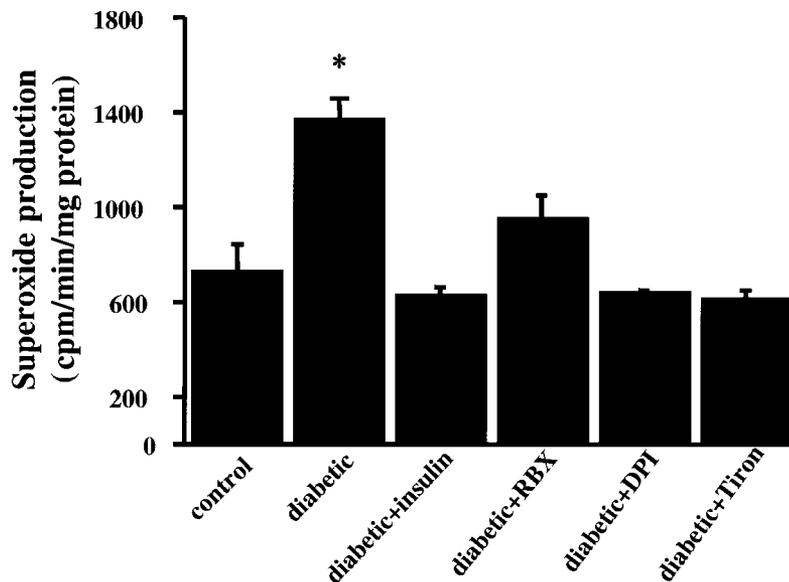


FIG. 2. PKC- β inhibition suppresses diabetes-induced glomerular O_2^- production. Production of O_2^- in isolated glomeruli from rats of each group was assessed using a lucigenin chemiluminescence assay. Some glomeruli of diabetic rats were also assessed in the absence or presence of 10 μ mol/l DPI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

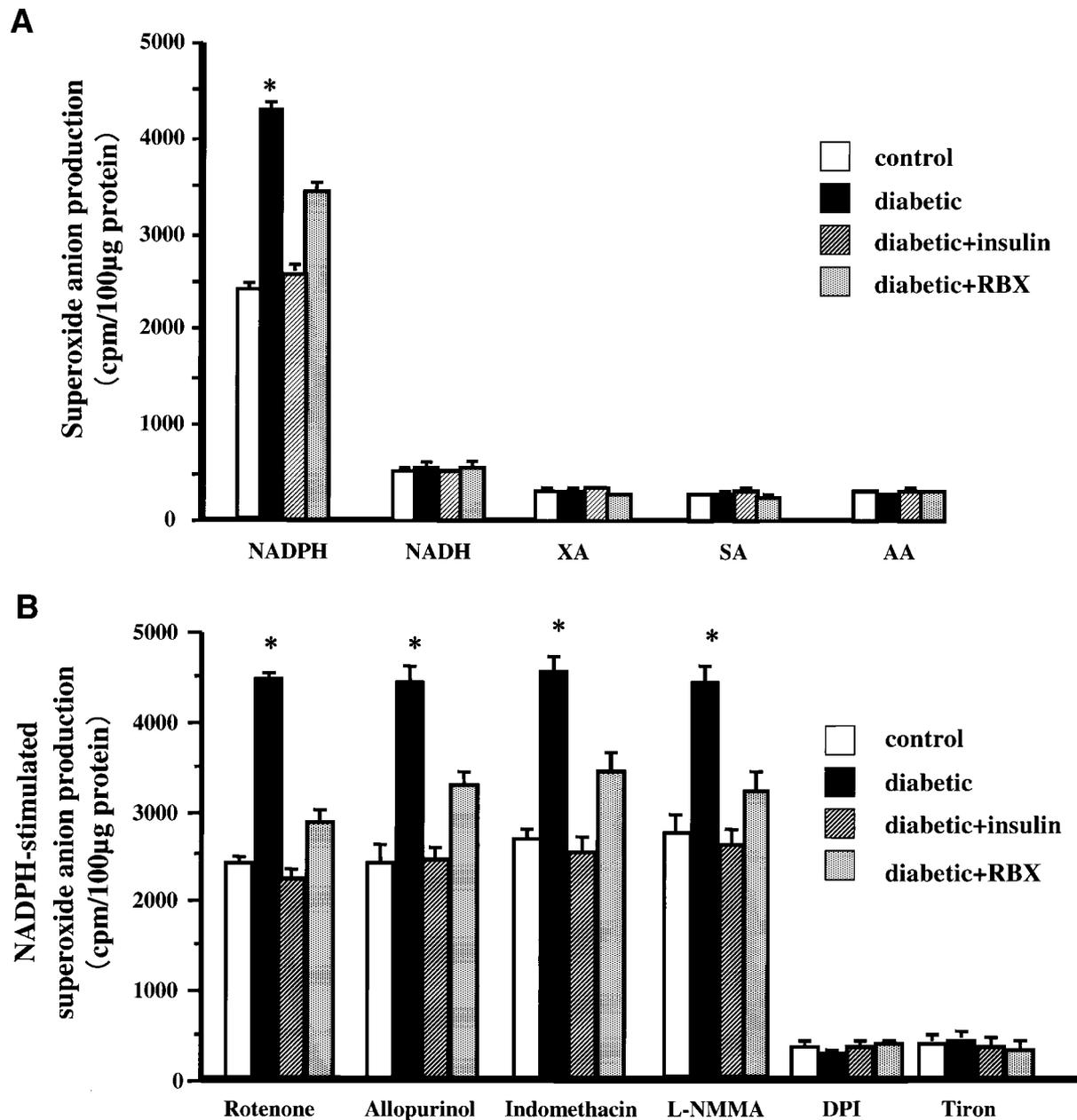


FIG. 3. Superoxide anion production. *A*: Enzyme source of O_2^- production in glomerular homogenates. Production of O_2^- in glomerular homogenates in the presence of potential substrates is measured by a lucigenin chemiluminescence method. NADPH (100 $\mu\text{mol/l}$), NADH (100 $\mu\text{mol/l}$), arachidonic acid (AA; 100 $\mu\text{mol/l}$), 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 \pm SE of five experiments in each group ($n = 5$, $*P < 0.05$ vs. other groups). *B*: Effect of various inhibitors, such as 100 $\mu\text{mol/l}$ rotenone, 100 $\mu\text{mol/l}$ allopurinol, 10 $\mu\text{mol/l}$ indomethacin, 10 $\mu\text{mol/l}$ L-NMMA, 10 $\mu\text{mol/l}$ DPI, or 10 mmol/l Tiron, on NADPH-stimulated O_2^- production of glomerular homogenates. Data are means \pm 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. 4A and B), whereas gp91phox, p22phox, and nox-1 expression was unchanged (Fig. 4D–F) and nox-4 was downregulated (Fig. 4C). The protein expression of p47phox and p67phox estimated by Western blot analysis was also upregulated in diabetic glomeruli (Fig. 4G and H), similar to the results of mRNA expression. These alterations in p47phox, p67phox, and nox-4 expression were normalized by insulin (Fig. 4A–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. 4A and B and Fig. 5A and B). However, the increased membranous translocation of p47phox and p67phox in diabetic glomeruli was

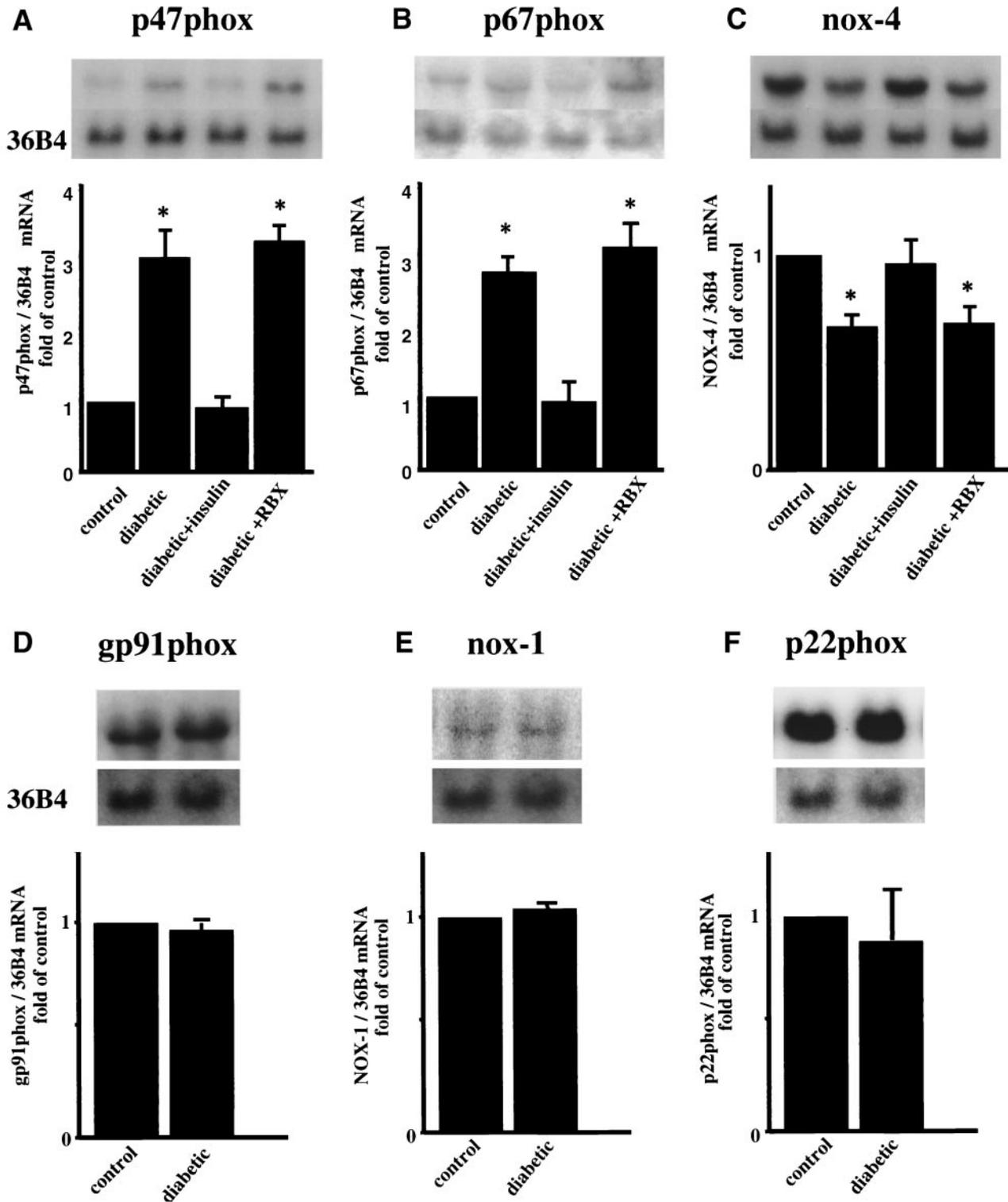


FIG. 4. The effect of diabetes on NADPH oxidase subunits in vivo. The mRNA expression of NADPH oxidase subunits consisting of p47phox (A), p67phox (B), nox-4(C), gp91phox (D), nox-1 (E), and p22phox (F) and the protein expression of p47phox (G) and p67phox (H) in glomeruli. A–F: Representative results 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. 5C and D).

The in vitro effect of PKC-β₂ on O₂⁻ production is dependent on membranous translocation of p47phox and p67phox. To delineate the underlying mechanism by

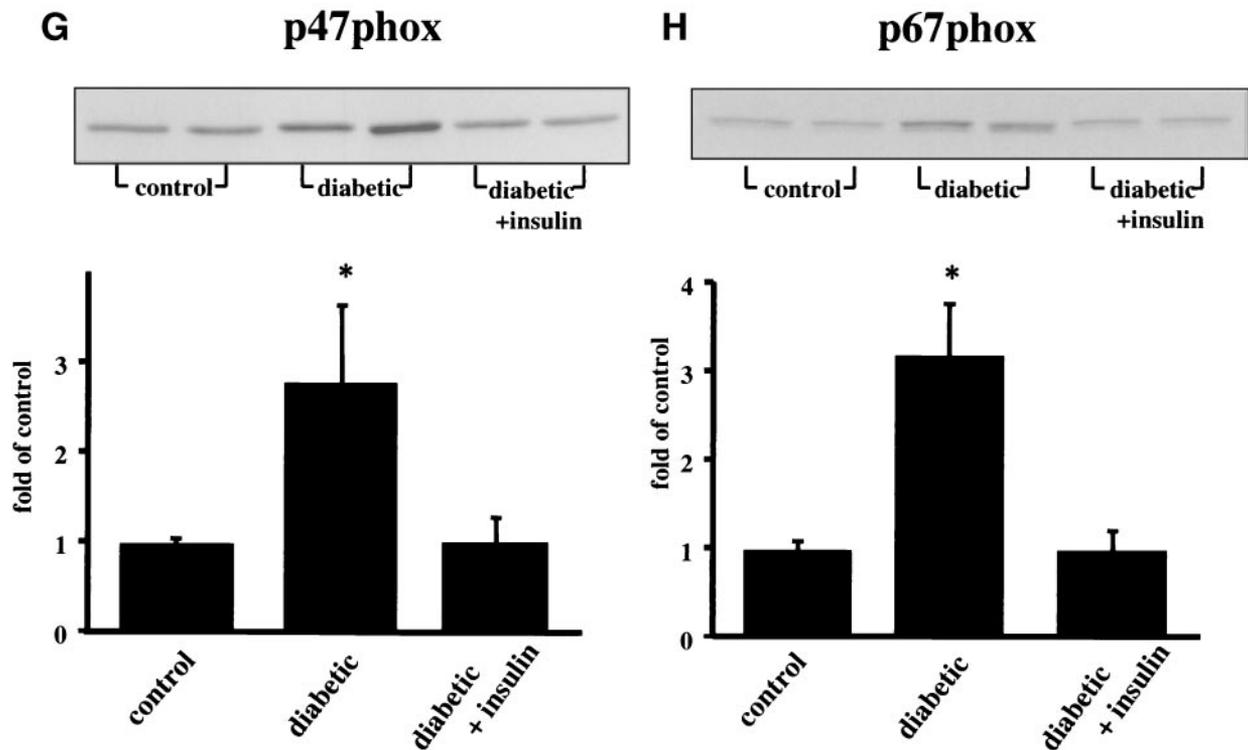


FIG. 4—Continued.

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. 6A, a PKC- β_2 recombinant adenovirus markedly enhanced PKC- β_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- β_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. 6B and C). Furthermore, we tested the role of PKC- β_2 in causing oxidative stress using dihydroethidium staining. The staining of dihydroethidium was significantly increased in cells overexpressing PKC- β_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- β_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 O₂⁻ generation occurs primarily in the isolated glomeruli using a lucigenin chemiluminescence method. Production of O₂⁻ 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 O₂⁻ production was greater in diabetic glomeruli than control rats by using their homogenates. NADH, xanthine, succinate, and arachidonic acid showed no effect on O₂⁻ production in diabetic rats. Moreover, NADPH-dependent O₂⁻ 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

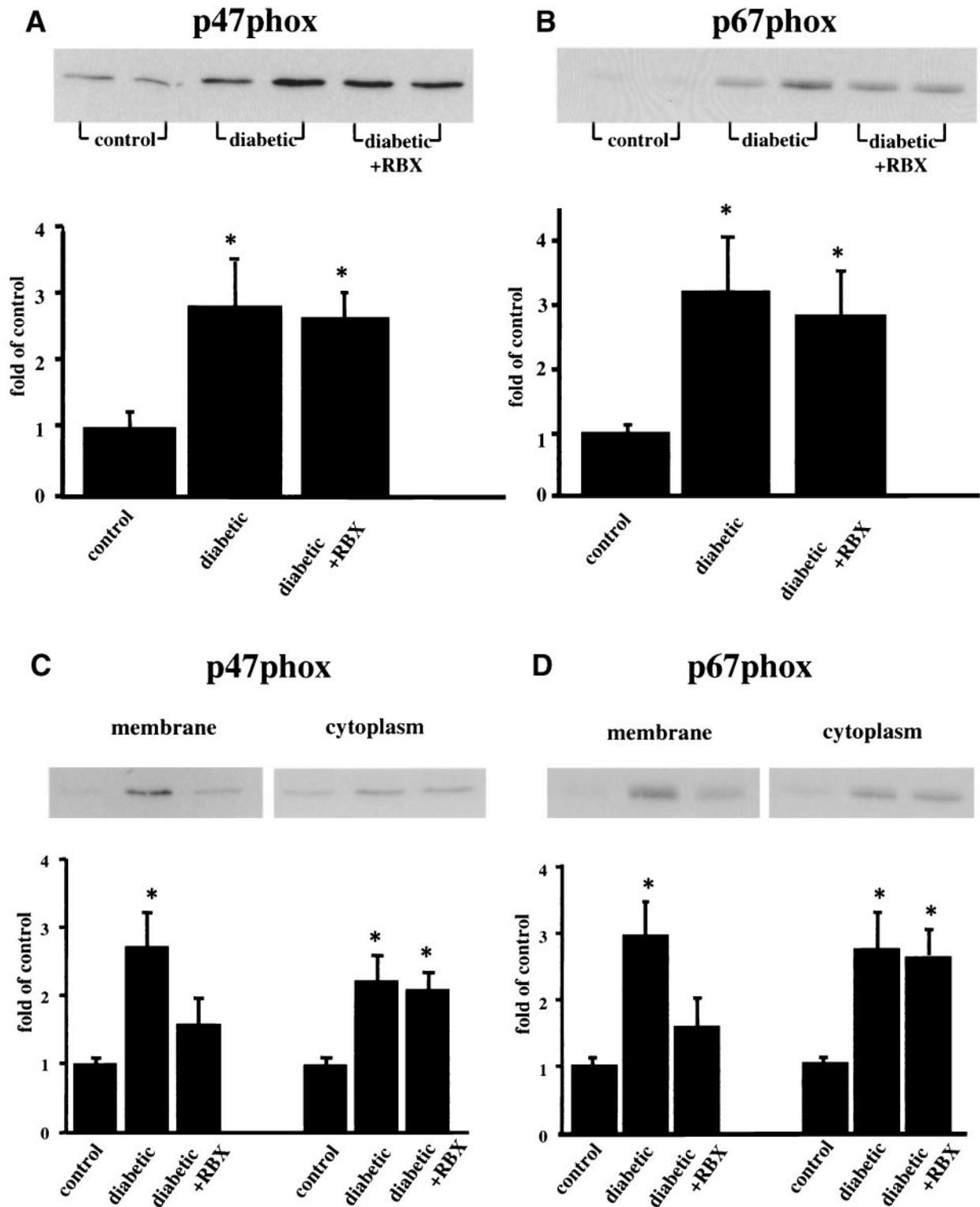


FIG. 5. Effect of PKC- β inhibition on p47phox and p67phox expression and their membranous translocation in diabetic glomeruli. *A* and *B*: Representative results of Western blot analysis for total glomerular lysates of p47phox and p67phox obtained from two independent samples are shown in the upper 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 \pm 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 \pm 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

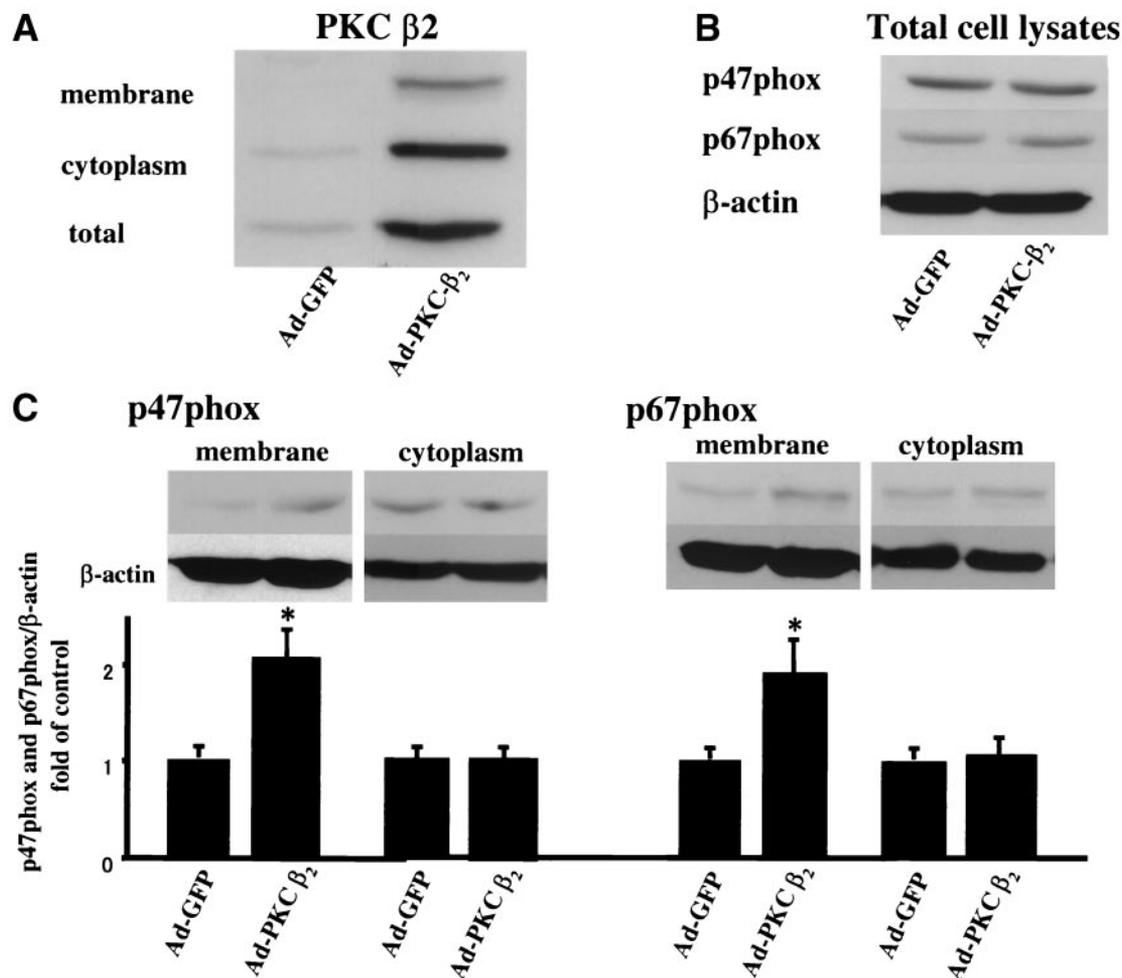


FIG. 6. Adenovirus-induced overexpression of PKC- β_2 causes the membranous translocation of p47phox and p67phox. **A:** Representative results of Western blot analysis of the membranous, cytosolic fraction, and total cell lysates of PKC- β_2 in mesangial cells overexpressing PKC- β_2 . **B and C:** 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 \pm 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 $\mu\text{mol/l}$ (39). In the present study, we used the non-redox-cycling dose of lucigenin at the concentration of 5 $\mu\text{mol/l}$, 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

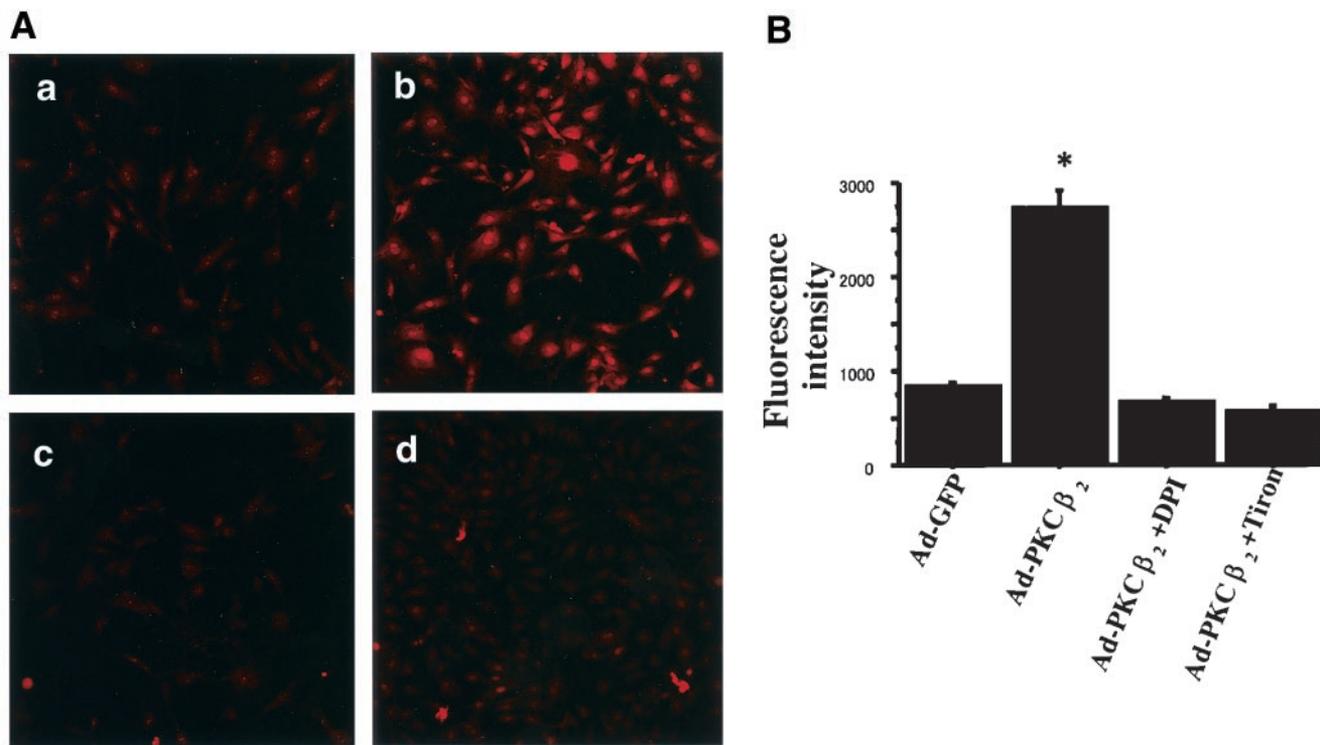


FIG. 7. Detection of O_2^- production by dihydroethidium staining in mesangial cells overexpressing PKC- β_2 . **A:** Representative results of dihydroethidium staining are shown from each independent three experiments of Ad-GFP-infected mesangial cells (*a*), Ad-PKC- β_2 -infected mesangial cells (*b*), Ad-PKC- β_2 -infected mesangial cells preincubated by 10 μ mol/l DPI for 30 min (*c*), and Ad-PKC- β_2 -infected mesangial cells preincubated by 10 mmol/l Tiron for 30 min (*d*). Photographs are 200 \times 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 μ mol/l DPI or 10 mmol/l Tiron. Data are expressed as means \pm 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- β_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 PKC β 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- β_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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REFERENCES

- Dandona P, Thusu K, Cook S, Snyder B, Makowski J, Armstrong D, Nicotera T: Oxidative damage to DNA in diabetes mellitus. *Lancet* 347: 444–445, 1996
- Leinonen J, Lehtimäki T, Toyokuni S, Okada K, Tanaka T, Hirai H, Ochi H, Laipala P, Rantalaiho V, Wirta O, Pasternack A, Alho H: New biomarker evidence of DNA damage in patients with non-insulin-dependent diabetes mellitus. *FEBS Lett* 417:150–152, 1997
- Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications. *Diabetes* 48:1–9, 1999
- Ha H, Kim C, Son Y, Chung MH, Kim KH: DNA damage in the kidneys of diabetic rats exhibiting microalbuminuria. *Free Radic Biol Med* 16:271–274, 1994
- Onozato ML, Tojo A, Goto A, Fujita T, Wilcox CS: Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effect of ACEI and ARB. *Kidney Int* 61:186–194, 2002
- Melhem MF, Craven PA, Derubertis FR: Effect of dietary supplementation of alpha-lipoic acid on early glomerular injury in diabetes mellitus. *J Am Soc Nephrol* 12:124–133, 2001
- 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
- Craven PA, DeRubertis FR, Kagan VE, Melhem M, Studer RK: Effects of supplementation with vitamin C or E on albuminuria, glomerular TGF-beta, and glomerular size in diabetes. *J Am Soc Nephrol* 8:1405–1414, 1997
- Keyse SM, Tyrrell RM: Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A* 86:99–103, 1989
- Hayashi K, Haneda M, Koya D, Maeda S, Isshiki K, Kikkawa R: Enhancement of glomerular heme oxygenase-1 expression in diabetic rats. *Diabetes Res Clin Pract* 52:85–96, 2001
- Williamson JR, Chang K, Fragos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van den Enden M, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42:801–813, 1993
- Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
- 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
- Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OTG: Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol* 271:H1626–H1634, 1996
- Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT: Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc Natl Acad Sci U S A* 94:14483–14488, 1997
- Patterson C, Ruef J, Madamanchi NR, Barry-Lane P, Hu Z, Horaist C, Ballinger CA, Brasier AR, Bode C, Runge MS: Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin: evidence that p47phox may participate in forming this oxidase in vitro and in vivo. *J Biol Chem* 274:19814–19822, 1999
- Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KG, Lambeth D: Cell transformation by the superoxide-generating oxidase Mox 1. *Nature* 401:79–82, 1999
- Jones SA, Hancock JT, Jones OTG, Neubauer A, Topley N: The expression of NADPH oxidase components in human glomerular mesangial cells: detection of protein and mRNA for p47phox, p67phox, and p22phox. *J Am Soc Nephrol* 5:1483–1491, 1995
- Greiber S, Munzel T, Kastner S, Muller B, Schollmeyer P, Pavenstadt H: NAD(P)H oxidase activity in cultured human podocytes: effects of adenosine triphosphate. *Kidney Int* 53:654–663, 1998
- Shiose A, Kurodai J, Tsuruya K, Hirai M, Hirakata H, Naito S, Hattori M, Sakai Y, Sumitomo HA: Novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem* 276:1417–1423, 2001
- Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK: Novel gp91 (phox) homologues in vascular smooth muscle cells: nox-1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 88:888–894, 2001
- Babior BM: NADPH oxidase: An update. *Blood* 93:1464–1476, 1999
- Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL: Characterization of protein kinase C β isoform activation on the gene expression of transforming growth factor- β , extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115–126, 1997
- Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKC β inhibitor. *Science* 22:728–731, 1996
- 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 PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439–447, 2000
- Isshiki K, Haneda M, Koya D, Maeda S, Sugimoto T, Kikkawa R: Thiazolidinedione compounds ameliorate glomerular dysfunction independent of insulin-sensitizing action in diabetic rats. *Diabetes* 49:1022–1032, 2000
- Eisei N, Akihida N, Koji U, Hirokazu T, Minoru O, Toshiro F, Sergey B, Michael SG: Oxidative and nitrosative stress in acute renal ischemia. *Am J Physiol* 281:F948–F957, 2001
- Kashiwagi A, Shinozaki K, Nishio Y, Maegawa H, Maeno Y, Kanazawa A, Kojima H, Haneda M, Hidaka H, Yasuda H, Kikkawa R: Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats. *Am J Physiol* 277:E976–E983, 1999
- Bayraktutan U, Blayney L, Shah AM: Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vasc Biol* 20:1903–1911, 2000
- Wassmann S, Laufs U, Muller K, Konkol C, Ahlborn K, Baumer AT, Linz W, Bohm M, Nickenig G: Cellular antioxidant effects of atorvastatin in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 22:300–305, 2002
- Schieffer B, Luchtefeld M, Braun S, Hilfiker A, Hilfiker-Kleiner D, Drexler H: Role of NAD(P)H oxidase in angiotensin II-induced JAK/STAT signaling and cytokine induction. *Circ Res* 87:1195–1201, 2000
- Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, Welch WJ, Wilcox CS: Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension* 39:269–274, 2002
- Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Ways DK, Hoit BD, Walsh RA, King GL: Targeted overexpression of protein kinase C β_2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci U S A* 94:9320–9325, 1997
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
- Kaneto H, Suzuma K, Sharma A, Bonner-Weir S, King GL, Weir GC: Involvement of protein kinase C β_2 in c-myc induction by high glucose in pancreatic β -cells. *J Biol Chem* 277:3680–3685, 2002
- Benov L, Sztajnberg L, Fridovich I: Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic Biol Med* 25:826–831, 1998
- Shigenaga MK, Gimeno CJ, Ames BN: Urinary 8-hydroxy-2-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. *Proc Natl Acad Sci U S A* 86:9697–9701, 1989
- Li Y, Zhu H, Kuppasamy P, Rouband V, Zweier JL, Trush MA: Validation of lucigenin (Bis-N-methylacridinium) as a chemiluminescent probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* 273:2015–2023, 1998
- Li JM, Shah AM: Differential NADPH- versus NADH-dependent: superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res* 52:477–486, 2001
- Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74:1141–1148, 1994
- Jaimes EA, Galceran JM, Raj L: Angiotensin II induces superoxide anion production by mesangial cells. *Kidney Int* 54:775–784, 1998
- De keulenaer GW, Alexander RW, Ushio-Fukai M, Ishizaka N, Griendling KK: Tumor necrosis factor- α activates a p22phox-based NADH oxidase in vascular smooth muscle cells. *Biochem J* 329:653–657, 1998
- Thannickal VJ, Fanburg BL: Activation of an H₂O₂ generating NADH oxidase in human lung fibroblasts by transforming growth factor β 1. *J Biol Chem* 270:30334–30338, 1995
- Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T: Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction. *Science* 270:296–299, 1995

45. Howard AB, Alexander RW, Nerem RM, Griendling KK, Taylor WR: Cyclic strain induces an oxidative stress in endothelial cells. *Am J Physiol* 272:C421–C427, 1997
46. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK: Oscillatory and laminar shear stress differentially affect human endothelial redox state. *Circ Res* 82:1094–1101, 1998
47. Fukui T, Ishizuka N, Rajagopalan S, Laursen JB, Capers QIV, Taylor WR, Harrison DG, de Leon H, Wilcox JN, Griendling KK: p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ Res* 80:45–51, 1997
48. Kim YK, Lee MS, Son SM, Kim IJ, Lee WS, Rhim BY, Hong KW, Kim CD: Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. *Diabetes* 51:522–527, 2002
49. Cifuentes ME, Rey FE, Carretero OA, Pagano PJ: Upregulation of p67phox and gp91phox in aortas from angiotensin II-infused mice. *Am J Physiol* 279:H2234–H2240, 2000
50. Dekker LV, Leitges M, Altschuler G, Mistry N, Mcdermott A, Roes J, Segal AW: Protein kinase C- β contributes to NADPH oxidase activation in neutrophils. *Biochem J* 347:285–289, 2000
51. Yamamori T, Inanami O, Nagahata H, Cui YD, Kuwabara M: Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett* 467:253–258, 2000
52. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK: Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 91:406–413, 2002
53. Fontayne A, Dang PM, Gougerot-Pocidalo MA, El-Benna J: Phosphorylation of p47phox site by PKC α , β II, δ , and ζ : effect on binding to p22phox and on NADPH oxidase activation. *Biochemistry* 41:7743–7750, 2002