

# Activation of Phosphoinositide 3-kinase in Response to High Glucose Leads to Regulation of Reactive Oxygen Species-Related Nuclear Factor- $\kappa$ B Activation and Cyclooxygenase-2 Expression in Mesangial Cells

Meei Ling Sheu, Feng Ming Ho, Kuo Fang Chao, Ming Liang Kuo, and Shing Hwa Liu

*Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan (M.L.S., K.F.C., M.L.K., S.H.L.); and Department of Nursing, Chung-Tai Institute of Health Sciences and Technology, Taichung, Taiwan (F.M.H.)*

Received September 4, 2003; accepted April 14, 2004

This article is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

Hyperglycemia causes glomerular mesangial cell proliferation and increases matrix synthesis, contributing to early diabetic glomerulopathy. Immunohistochemical and functional correlations of renal cyclooxygenase-2 in experimental diabetes have been identified. However, the role of cyclooxygenase-2 in early diabetes-induced mesangial cell proliferation remains unknown. The authors tested the hypothesis that hyperglycemia modulates an intrarenal cyclooxygenase-2 expression, which might mediate the mesangial cell proliferation via a possible phosphoinositide 3-kinase/Akt pathway. Expression of cyclooxygenase-2, but not cyclooxygenase-1, could be induced in mesangial cells cultured under high glucose. Antioxidants (pyrrolidine dithiocarbamate and *N*-acetyl-L-cysteine) and phosphoinositide 3-kinase inhibitors [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002) and wortmannin] effectively inhibited this high glucose-induced response. Moreover, high glucose markedly triggered the activation of phosphoinositide 3-kinase and Akt in mesangial cells, suggest-

ing that a phosphoinositide 3-kinase/Akt pathway is involved in the high glucose-induced responses. Phosphoinositide 3-kinase inhibitors could also effectively attenuate the high glucose-triggered intracellular reactive oxygen species generation and nuclear factor- $\kappa$ B activation. Likewise, blocking the phosphoinositide 3-kinase or Akt activity with the dominant-negative vectors DN-p85 or DN-Akt, respectively, also greatly diminished the high glucose-triggered reactive oxygen species generation and nuclear factor- $\kappa$ B activation. Treatment of mesangial cells with LY294002 and cyclooxygenase-2 inhibitors [*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide (NS398) and aspirin] effectively inhibited the high glucose-induced mesangial cell proliferation. These results suggest that high glucose may trigger the reactive oxygen species-regulated nuclear factor- $\kappa$ B activation and cyclooxygenase-2 expression and cell proliferation in mesangial cells through a phosphoinositide 3-kinase-dependent pathway.

Hyperglycemia is a well recognized pathogenic factor of long-term complications in diabetes mellitus. Altered growth of renal cells is one of the early abnormalities detected after the onset of diabetes (Shankland and Wolf, 2000). Cell culture studies whereby renal cells are exposed to high glucose concentrations have provided a considerable amount of insight into mechanisms of altered growth. Early streptozotocin (STZ) diabetes studies showed that rats exhibit a 15% increase of whole kidney weight within 72 h of induction with STZ (Osterby and Gundersen, 1975; Phillips et al., 1999).

This work was supported by research grant from National Science Council of the Republic of China (NSC90-2315-B-002-009).

More detailed studies showed that glomerular enlargement was accompanied by glomerular cell proliferation, which predominantly involved the mesangial cells, in the early phase of diabetes (Flyvbjerg et al., 1995; Young et al., 1995). However, the specific factor(s) and the natural mechanism(s) that initiate the progression of diabetic glomerulopathy remains poorly defined.

Cyclooxygenase (COX) is the key enzyme that mediates the production of prostaglandins from arachidonic acid. Two COX isoforms have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by a number of inflammatory, mitogenic, and physical

**ABBREVIATIONS:** STZ, streptozotocin; COX, cyclooxygenase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; ROS, reactive oxygen species; HG, high glucose; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTS, 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction; DTT, dithiothreitol; DN, dominant negative; DCF, 2',7'-dichlorofluorescein; HM, high mannitol; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetyl-L-cysteine; DPI, diphenylene iodonium; HA, hemagglutinin; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; NS398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide; MMC, MES-13 cells; RMC, rat mesangial cells.

stimuli (Vane et al., 1998). In a number of cell and animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis and enhance cell motility and adhesion (Cao and Prescott, 2002). It has also been shown that prostaglandin E2 could enhance the increased [ $^3\text{H}$ ]thymidine uptake in glomerular core preparations enriched in mesangial cells from STZ-induced diabetic rats (Mahadevan et al., 1996). Moreover, changes in glomerular eicosanoid production have been implicated in the development of diabetes-induced glomerular hyperfiltration. Glomerular mesangial cells were major eicosanoid-producing cells within the glomerulus (Williams and Schrier, 1993). Komers et al. (2001) documented an increase in renal cortical COX-2 protein expression associated with a different renal hemodynamic response to selective systemic COX-2 inhibition in diabetic animals compared with control animals. Nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) is a transcriptional regulator of inducible expression of genes, including COX-2, regulating cell proliferation (Lim et al., 2001). Activation of NF- $\kappa\text{B}$  can be induced by various molecules, such as cytokines and reactive oxygen species (ROS) (Li and Stark, 2002). ROS has been demonstrated to act as glucose signaling molecules in mesangial cells cultured under high glucose (Ha and Lee, 2000). It has recently been shown that high media glucoses (HG) rapidly activated NF- $\kappa\text{B}$  activation in mesangial cells through protein kinase C and ROS (Ha et al., 2002). However, the relationship among ROS generation, NF- $\kappa\text{B}$  activation, and COX-2 expression in early diabetes-induced renal mesangial cell proliferation remains unknown.

Phosphoinositide 3-kinase (PI3K) phosphorylates the 3'-OH position of the inositol ring of inositol phospholipids. There are multiple isoforms of PI3K in mammalian cells. These are subdivided into three classes: I (A and B), II, and III (Fruman and Cantley, 2002; Foster et al., 2003). A class IA PI3K consists of a catalytic subunit (p110 family) and a tightly associated regulatory subunit (p85 family); class IA was the first to be identified and cloned and thus is best understood. PI3K plays a central role in a diverse range of cellular responses including cell growth, survival, and malignant transformation (Toker, 2000; Fruman and Cantley, 2002; Foster et al., 2003). Among the downstream targets of PI3K are serine/threonine kinase Akt and the protein kinase C. Recent studies have shown that the activation of PI3K might play a role in regulating a COX-2 expression or a COX-2-related survival mechanism in some cultured cells (Lin et al., 2001; Tang et al., 2001; Weaver et al., 2001). Much evidence has also indicated the importance of PI3K in the oxidative burst (Koyasu, 2003). The authors hypothesized that elevated glucose levels may trigger ROS-related NF- $\kappa\text{B}$  activation and COX-2 expression via a possible PI3K/Akt pathway in mesangial cells. To address this issue, we explored the changes of ROS generation, NF- $\kappa\text{B}$  activation, and PI3K/Akt activities in HG-treated mesangial cells and their relationship to the HG-triggered COX-2 expression. Further experiments examined the role of the PI3K/Akt pathway in mesangial cell proliferation.

## Materials and Methods

**Mesangial Cell Culture.** Dulbecco's modified Eagle's medium (DMEM) containing 5.6 mM glucose was used to culture mesangial cells, unless otherwise stated. Murine glomerular mesangial cells (MES-13) were obtained from American Type Culture Collection

(Manassas, VA); these transformed cells have been shown to exhibit characteristics similar to those of primary cultures of murine mesangial cells. Primary rat mesangial cells were also used to confirm the responses obtained with MES-13. Primary rat mesangial cells were obtained by culturing glomeruli isolated from kidneys of 100- to 150-g male Sprague-Dawley rats by conventional sieving methods as described previously and characterized (Ha et al., 2002). Cells were cultured in DMEM containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 44 mM  $\text{NaHCO}_3$ , and 14 mM HEPES. For MES-13 cells, DMEM contained 5% FBS, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 14 mM HEPES. Cells were routinely passaged by trypsinization after they reached 80% confluence using 10-cm culture dishes and incubating them at 37°C in a humidified chamber with a 5%  $\text{CO}_2/95\%$  air mixture. Subculturing mesangial cells in 10-cm culture dishes were used in electrophoretic mobility shift assay and intracellular ROS measurement. In the PI3K and Akt activities assay, cells were cultured in six-well culture dishes. For immunocytochemistry or immunofluorescence staining, cells were cultured on cover glass coated with 1 N HCl.

## Cell Proliferation Assay

**MTS Assay.** Cell proliferation was measured using a nonradioactive cell proliferation assay kit (CellTiter 96 AQueous; Promega, Madison, WI). The assay was composed of a solution of tetrazolium compound [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron coupling reagent (phenazine methosulfate). The assay was based on the cellular conversion of the colorimetric reagent MTS into soluble formazan by dehydrogenase enzymes found only in metabolically active cells. Mesangial cells ( $1 \times 10^5/\text{ml}$ ) in 96-well cell culture dishes were incubated for 24 h with or without HG (33 mM) in the presence or absence of inhibitors. 20  $\mu\text{l}/\text{well}$  of combined MTS/phenazine methosulfate solution was then added. After 1 h of incubation at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere, absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay microplate reader. All MTS assays were at least repeated three times in duplicate.

**[ $^3\text{H}$ ]Thymidine Incorporation.** DNA synthesis was measured by incorporation of [ $^3\text{H}$ ]thymidine into cellular DNA. Cells were seeded in 96-well microtiterplates in a density of  $5 \times 10^3$  cells/ml in DMEM containing 5% FBS. After attachment of the cells overnight, they were washed three times with phosphate-buffered saline (PBS) and kept in resting medium (DMEM with 0.5% FBS) for a further 3 days. Control cells received an appropriate volume of PBS (serum-starved cells). DNA synthesis was measured by a 24-h pulse of [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{ml}$ ) from 24 h after stimulation. At the end of the labeling period, the cells were washed twice with PBS, trypsinized, and harvested onto glass filters with an automated 96-well glass fiber harvester (PerkinElmer Life and Analytical Sciences, Boston, MA). Finally, the radioactivity retained on the filter was measured in a PerkinElmer scintillation  $\beta$ -counter.

## PI3K Activity Assay

PI3K activities were assayed as described previously (Lin et al., 2001) with some modifications. In brief, cells in six-well culture dishes were serum-deprived for 24 h. Cells ( $2 \times 10^5$ ) received different treatments and were washed twice with ice-cold phosphate-buffered saline and lysed with 1 mM lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 1% Nonidet P-40, 10% glycerol, 1 mg/ml bovine serum albumin, 20 mM Tris, pH 8.0, and 2 mM orthovanadate). Cell extracts were incubated with 2  $\mu\text{g}$  of anti-p85 antibody overnight at 4°C. The immunocomplex was precipitated with 50  $\mu\text{l}$  of protein A-Sepharose for 1 h at 4°C and washed three times with lysis buffer, twice with LiCl buffer (0.5 M LiCl, 100 mM Tris, pH 7.6, and twice with TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, and 1 mM EDTA). The immunocomplex was preincubated with 10  $\mu\text{l}$  of 20 mM HEPES, pH 7.4, containing 2 mg/ml phosphatidylinositol 4-monophosphate (Sigma) on ice for 10 min. Kinase

reaction was performed by adding 40  $\mu$ l of reaction buffer (10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP, 20 mM HEPES, pH 7.4, 20  $\mu$ M ATP, and 5 mM  $MgCl_2$ ) at room temperature for 15 min. Adding 100  $\mu$ l of 1 M HCl extracted with 200  $\mu$ l of a 1:1 mixture of chloroform and methanol stopped the reaction. The radiolabeled lipids were separated by thin-layer chromatography and visualized by filmless autoradiographic analysis.

### Immunoblotting

A 100- $\mu$ g sample of each cell lysate or nuclear extract was subjected to electrophoresis on 10% SDS-polyacrylamide gels. The samples were then electroblotted on polyvinylidene difluoride membranes. After blocking, blots were incubated with anti-COX-1, anti-COX-2 (BD Transduction Laboratories, Lexington, KY), anti-p65, anti-Akt, and anti-phospho-Akt (New England BioLabs, Beverly, MA) antibodies in PBS within 0.1% Tween 20 for 1 h followed by two 15-min washes in PBS within 0.1% Tween 20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ) were employed to depict the protein bands on membranes.

### RT-PCR for COX-2 Expression

The expression of COX-2 was determined by the reverse transcription-polymerase chain reaction (RT-PCR) analysis technique. In brief, approximately  $5 \times 10^5$  cells were homogenized with 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA). The total RNA was isolated according to the manufacturer's protocols. The first standard cDNA was synthesized by the extension of (dT) primers with 200 units of SuperScript II reverse transcriptase (Invitrogen) in a mixture containing 1  $\mu$ g of total RNA digested by RNase-free DNase (2 units/ $\mu$ g of RNA) for 15 min at 37°C. Then the cDNA served as a template in a PCR using the PerkinElmer DNA Thermal Cycler (model 480). The primers (5' or 3') used for COX-2 were sense primer, CATTCTTTGCCAGCACTTCAC and antisense primer, GACCAGGCACCAAGA CCAAAGAC at a concentration of 0.4  $\mu$ M. The amplification cycles included 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s. Then the PCR products were subjected to electrophoresis on a 2% agarose gel after 30 cycles. The electrophoresis products were visualized by ethidium bromide staining. The mRNA of  $\beta$ -actin served as control for the sample integrity and loading.

### Preparation of Nuclear Extracts

At the end of the culture, approximately  $2 \times 10^6$  were harvested and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride for 10 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000g for 20 s. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was suspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12,000g for 20 min and stored at -70°C. Protein concentration was determined using a colorimetric assay by a Bio-Rad assay kit (Bio-Rad, Hercules, CA).

### Transient Transfection with Dominant-Negative Vectors-DN-P85 and DN-Akt

Mesangial cells in a 10-cm plate were seeded in complete medium 16 h before transfection. Cells were transfected with 2  $\mu$ g of plasmids containing the DN-p85 ( $\delta$ -p85) or DN-Akt (Akt K179A) (Kuo et al., 2001; Lin et al., 2001), kindly provided by Dr. R. H. Chen (Institute of Molecular Medicine, National Taiwan University, Taiwan), or pcDNA3 control vector using the Effectene transfection reagent (QIAGEN, Valencia, CA). Transfections were performed in triplicate. Twenty-four hours after transfection, the cell medium was replaced with a fresh serum-free medium for 12 h and then exposed to HG. The efficiency of transfection (about 80%) was determined using an

equal amount of a plasmid encoding the green fluorescent protein under the cytomegalovirus promoter.

### Electrophoretic Mobility Shift Assays

The following oligonucleotide with the NF- $\kappa$ B consensus binding sequence was used for electrophoretic mobility shift assay: 5'-GATC-CAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3' (Invitrogen). The consensus oligonucleotide probes were end-labeled with [ $\gamma$ - $^{32}$ P]ATP according to the manufacturer's description. For the binding reaction, 2 ng of the labeled oligonucleotide (approximately 20,000 cpm) and 2  $\mu$ g of poly(dIdC) (Amersham Biosciences) carrier was incubated with 20  $\mu$ g of nuclear protein in a binding buffer (10 mM HEPES, 60 mM KCl, 1 mM DTT, 1 mM EDTA, and 7% glycerol, pH 7.6) for 30 min at room temperature. Protein-DNA complexes were separated by electrophoresis on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography. For competition experiments, a 100-fold excess of the unlabeled NF- $\kappa$ B oligonucleotides were added 15 min before incubation of nuclear extracts with the end-labeled oligonucleotides. For the supershift assay, 2  $\mu$ l of affinity-purified rabbit polyclonal antisera specific for p65, p50, and p52 (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extract for 30 min at room temperature before binding reaction.

### Immunofluorescence Staining for NF- $\kappa$ B

To measure NF- $\kappa$ B expression in situ, 80% confluent mesangial cells were seeded on the cover glass coated with 1 N HCl (control cells or cells treated with HG in the absence or presence of the different factors). The cells were exposed for 60 min, then fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min at 4°C, washed with PBS, blocked for 1 h at room temperature with 5% BSA in PBS, then reacted overnight at 4°C temperature with anti-mouse monoclonal NF- $\kappa$ B p65 antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology). After washes, the slides were incubated for 1 h at room temperature with mouse-immunoglobulin/RPE and then viewed on a fluorescent microscope.

### Detection of Intracellular ROS

Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe [2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR)]. In brief, cells ( $2 \times 10^5$ ) were coincubated with 50  $\mu$ M DCFH-DA in the absence or presence of HG or other drugs at 37°C for varying lengths of time. DCFH-DA is converted by intracellular esterases to DCFH. In the presence of a proper oxidant, DCFH is oxidized into the highly fluorescent 2',7'-dichlorofluorescein (DCF). After incubation, cells were resuspended in ice-cold PBS and placed on ice in darkness for flow cytometry analysis. The increase of fluorescence in each treatment was calculated by the relative fluorescence of each treatment compared with the control untreated cells normalized.

### Statistical Analyses

The values given in this article are presented as mean  $\pm$  S.E.M. All analyses were performed by analysis of variance followed by a Fisher's least significant difference test. A *P* value of less than 0.05 was viewed as statistically significant.

## Results

**Effects of HG on the Expression of COX-2 and the Involvement of PI3K/Akt.** D-Glucose (33 mM; HG) significantly activated COX-2 protein expression in MES-13 and primary rat mesangial cells. There was a significant initial increase after 6 h, and peaking after 24 h (Fig. 1, A and D). Gradually it decreased but remained higher compared with the control for up to 48 h. HG did not affect the expression of COX-1 protein in mesangial cells (Fig. 1, B and D). Further-

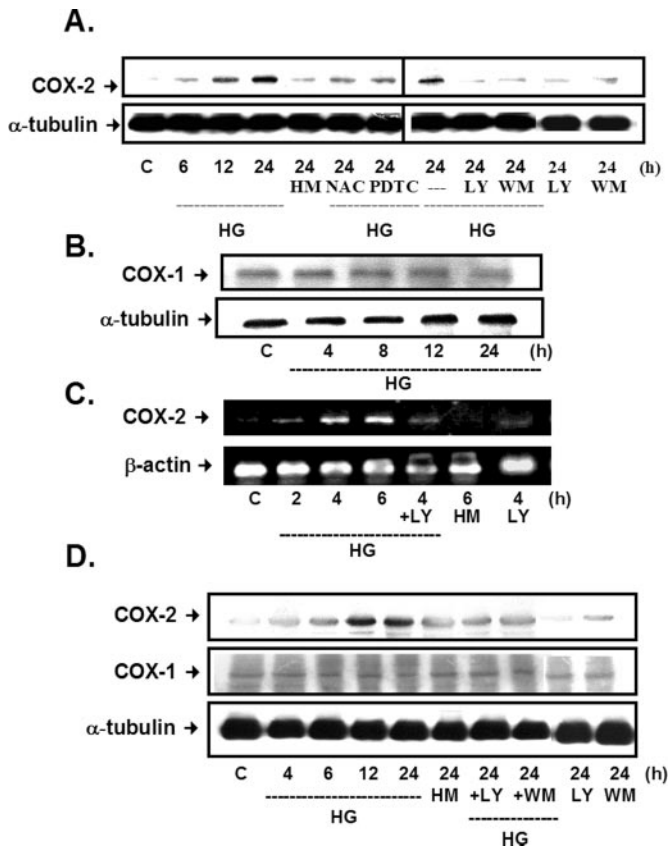
more, HG significantly induced the expression of COX-2 mRNA in mesangial cells (Fig. 1C). Unlike HG, the addition of 33 mM mannitol (HM) to the media did not affect the expression of COX-2 in mesangial cells compared with the control (Fig. 1), suggesting that the HG-triggered COX-2 expression is not the result of high osmolality within the media.

To test the possible signaling pathways involved in the HG-triggered COX-2 expression in mesangial cells, the cells were treated with PI3K inhibitors LY294002 (10  $\mu$ M) and wortmannin (100 nM) and the antioxidants pyrrolidine dithiocarbamate (PDTC; 10  $\mu$ M) and *N*-acetyl-L-cysteine (NAC; 7.5 mM) for 24 h. The results showed that both PI3K inhibitors and antioxidants caused a marked reduction in the COX-2 expression when induced by HG (Fig. 1). LY294002 and wortmannin by themselves did not affect the expression of COX-2 (Fig. 1).

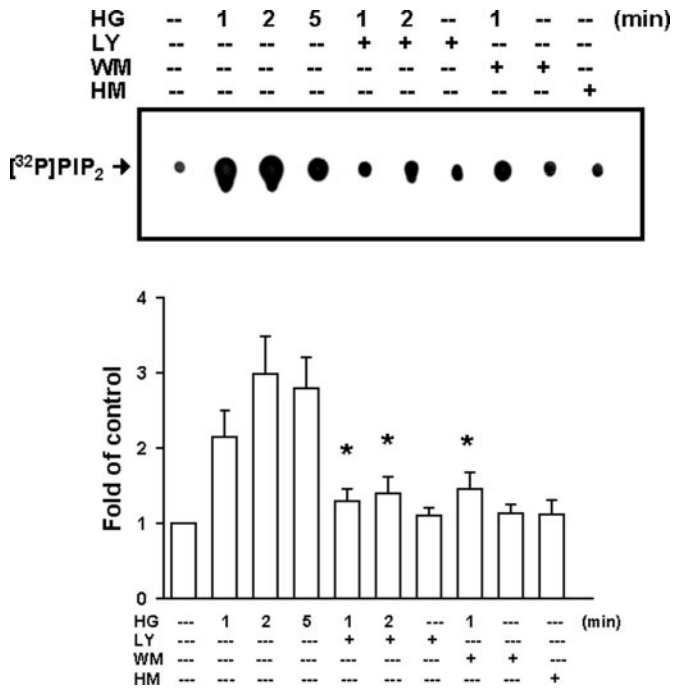
Furthermore, the authors investigated the change of PI3K activity in HG-treated mesangial cells. The authentic PI3K activity of mesangial cells after treatment with HG was determined. Immunoprecipitates with the anti-p85 antibody revealed a substantial increase in PI3K activity 1 to 5 min after the exposure of HG. LY294002 (10  $\mu$ M) and wortman-

nin (100 nM) could completely inhibit this increase (Fig. 2). To further evaluate the involvement of Akt in HG-triggered response, the Akt activity in MES-13 and primary rat mesangial cells was examined by determining the serine-phosphorylated status of Akt, employing an anti-phospho-Akt antibody. The Akt activity correlates well with the phosphorylation of Akt molecules on serine 473. In a time-dependent manner, a significant activation of Akt was shown 5 to 20 min after the treatment of HG (Fig. 3, A and B). LY294002 (10  $\mu$ M) markedly reduced the phosphorylation of Akt induced by HG in mesangial cells (Fig. 3, A and B). This indicates that PI3K functions upstream of Akt in response to HG.

**The HG-induced Generation of Reactive Oxygen Species Requires the Activity of PI3K/Akt.** Reactive oxygen species as the glucose signaling molecules have been identified in mesangial cells under HG (Catherwood et al., 2002). The authors next investigated the HG-induced generation of dichlorofluorescein (DCF)-sensitive ROS in mesangial cells as early as 15 min and gradually increased up to 2 h (Fig. 4). HM did not activate ROS generation in mesangial cells compared with the control. Antioxidants PDTC, NAC, diphenyleneiodonium (DPI), and rotenone, abolished HG-induced DCF-sensitive ROS generation (Fig. 4C). These antioxidants entirely abolished ROS production in mesangial cells under HG (Fig. 4C). Moreover, treatment of mesangial



**Fig. 1.** Effects of HG on COX-1 and -2 expressions in MES-13 cells (MMC; A–C) and rat mesangial cells (RMC; D). Cells were treated with HG (33 mM) or HM (33 mM) for various time intervals. In some experiments, cells that were stimulated with HG for 24 h and pretreated with antioxidants PDTC (10  $\mu$ M) and NAC (7.5 mM) or PI3K inhibitors LY294002 (LY; 10  $\mu$ M) and wortmannin (WM; 100 nM) for 30 min in the dark. For measurement of COX-1 protein (B, D) or COX-2 protein (A, D) expression, Western blot analysis was carried out using anti-COX-1 or -2 antibodies. Moreover, the COX-2 mRNA levels were detected using the method of RT-PCR (C). Results shown are representative of five independent experiments.

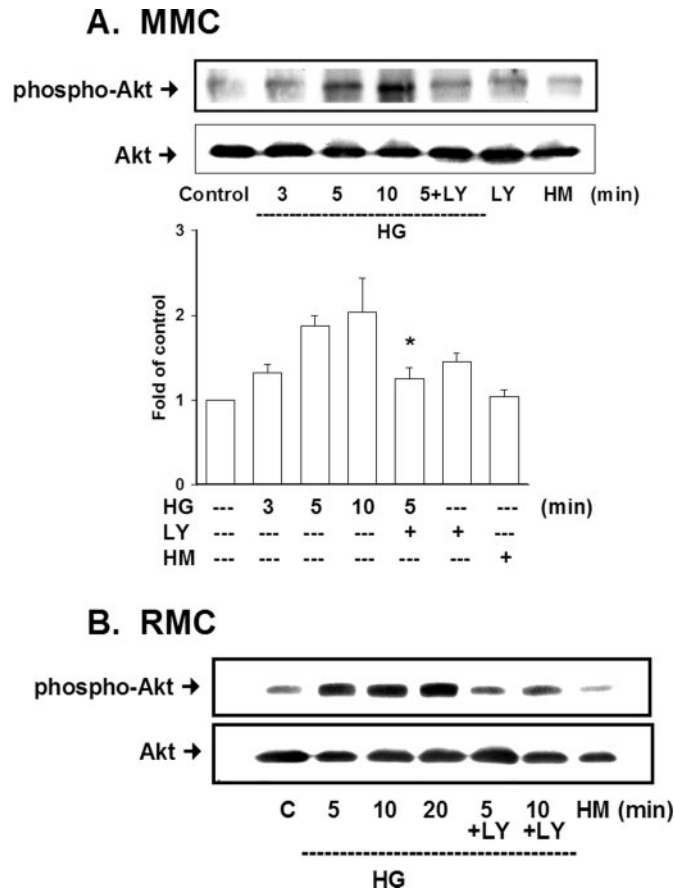


**Fig. 2.** HG activated the PI3K in mesangial cells. MES-13 cells were treated with HG in the absence or presence of LY294002 (LY; 10  $\mu$ M) and wortmannin (WM; 100 nM) for the indicated times. Cell lysates with equal amounts of protein were subjected to immunoprecipitations with anti-p85 PI3K subunit antibody. The immunocomplex was employed for PI3K activity assays as described under *Materials and Methods* using phosphatidylinositol 4-monophosphate as substrate, and the product, phosphatidylinositol bisphosphate (PIP<sub>2</sub>), was resolved by thin-layer chromatography. Incorporation of <sup>32</sup>P into PIP<sub>2</sub> was quantitated using filmless autoradiographic analysis. Quantification of the activity of PI3K was performed by densitometric analysis. Data are presented as mean  $\pm$  S.E.M. from three to five independent experiments. \*, *P* < 0.05 compared with HG without any other drugs.

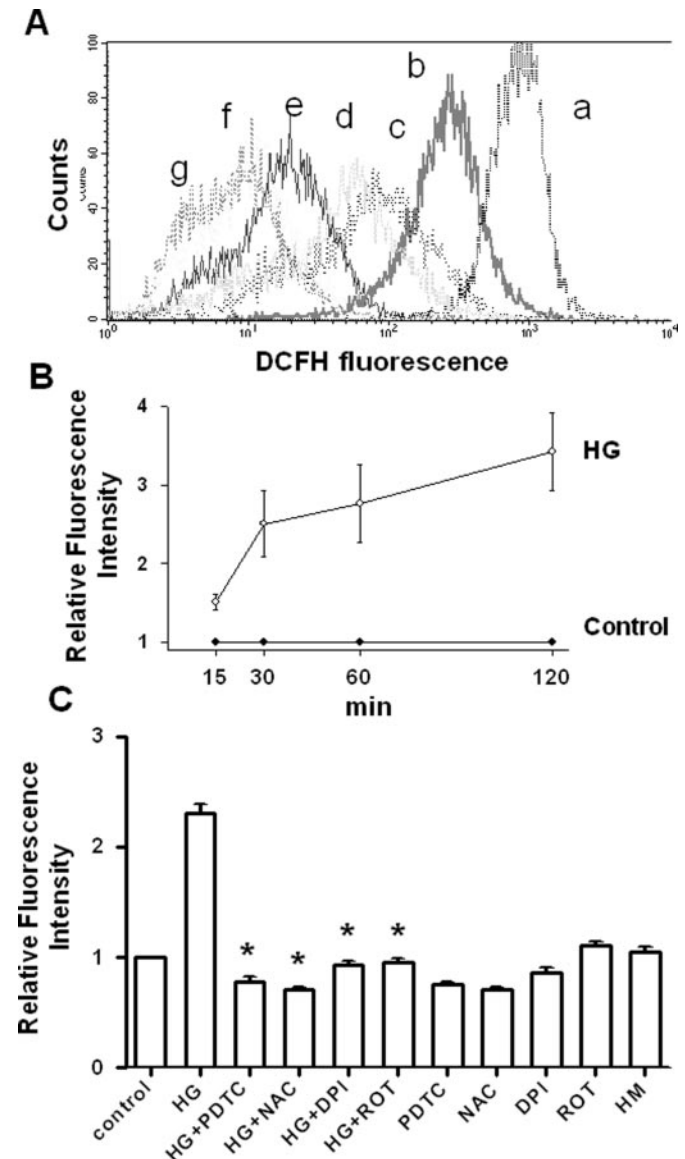
cells with LY294002 (10  $\mu$ M) showed significantly decreased DCF fluorescence induced by HG (Fig. 5B, a).

The next aim of the investigation was to ascertain whether PI3K or Akt activity inhibition might affect the HG-induced ROS generation in mesangial cells. To address this issue, cells were transfected with a dominant-negative form of the regulatory subunit of PI3K, DN-p85, or a dominant-negative form of the PI3K downstream effector Akt, DN-Akt. To confirm the successful expression of the dominant negative mutants, cell lysates from mesangial cells, transfected with hemagglutinin (HA) epitope-tagged DN-p85 or DN-Akt or control pcDNA3 vector, were immunoprecipitated with anti-HA antibody and followed by Western blotting with anti-p85 or anti-Akt antibodies to detect the expression of mutant proteins (Fig. 5A). The serine phosphorylation of Akt and Akt protein levels were also detected (Fig. 5A). The expression levels of Akt protein in DN-Akt-transfected cells were increased compared with nontransfected cells (Control,  $2.45 \pm 0.24$ ; HG,  $2.56 \pm 0.21$ ; DN-Akt,  $5.28 \pm 0.36$ ; HG+DN-Akt,  $5.88 \pm 0.32$  optical density/ $\mu$ g of protein). Blocking the PI3K and Akt activity with the dominant negative vectors DN-p85

and DN-Akt greatly diminished the HG-triggered ROS generation (Fig. 5B, b), suggesting an important role for a PI3K/Akt pathway in signal transduction by HG. Moreover, to further confirm the relationship between Akt and ROS in mesangial cells cultured under HG, the effect of antioxidants on the phosphorylation of Akt was investigated. As shown in Fig. 5C, NAC (7.5 mM), PDTC (10  $\mu$ M), DPI (20  $\mu$ M; an inhibitor in the production of superoxide by mitochondrial respiration and NAD(P)H oxidase activities), and rotenone



**Fig. 3.** High glucose activated the Akt in mesangial cells. Cells were treated with HG in the absence or presence of LY294002 (LY; 10  $\mu$ M) for the indicated times, and then each cellular lysates was prepared to perform electrophoresis and immunoblotting as described under *Materials and Methods* employing a specific anti-phospho-Akt antibody or anti-Akt antibody. Phosphorylation of Akt at Ser<sup>473</sup> was increased in MMC (A) and RMC (B) treated with HG, but not HM. Quantification of the phosphorylated Akt protein expression was performed by densitometric analysis. Data are presented as mean  $\pm$  S.E.M. from three to five independent experiments. \*,  $P < 0.01$  compared with HG without any other drugs. Results in RMC (B) shown are representative of five independent experiments.

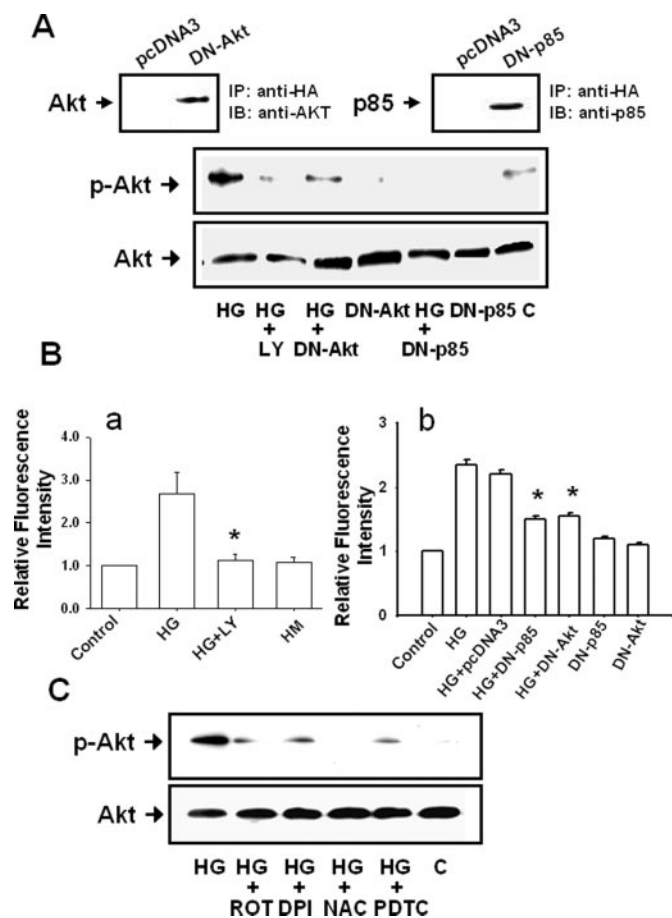


**Fig. 4.** HG induced the DCF-sensitive ROS generation in mesangial cells. Intracellular ROS generation was determined by fluorescence of DCFH-DA as described under *Materials and Methods*. A, relative fluorescence intensity in MES-13 cultured under HG for 1 h with or without antioxidants was quantitated by flow cytometry. Peaks: a, H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M, positive control); b, HG; c, HG+PDTC (10  $\mu$ M); d, HG+NAC (7.5 mM); e, solvent control; f, PDTC alone; and g, NAC alone. B, MMC were incubated with HG for various time intervals as indicated, and then the ROS generation within cells was detected. C, cells were pretreated with antioxidants PDTC (10  $\mu$ M), NAC (7.5 mM), DPI (20  $\mu$ M), rotenone (ROT, 20  $\mu$ M) for 30 min before the addition of HG. Cells were then exposed to HG for 1 h, and the intracellular DCF-sensitive ROS was detected. Data are presented as mean  $\pm$  S.E.M. from three to five experiments performed in duplicate. \*,  $P < 0.01$  compared with HG without any other drugs.

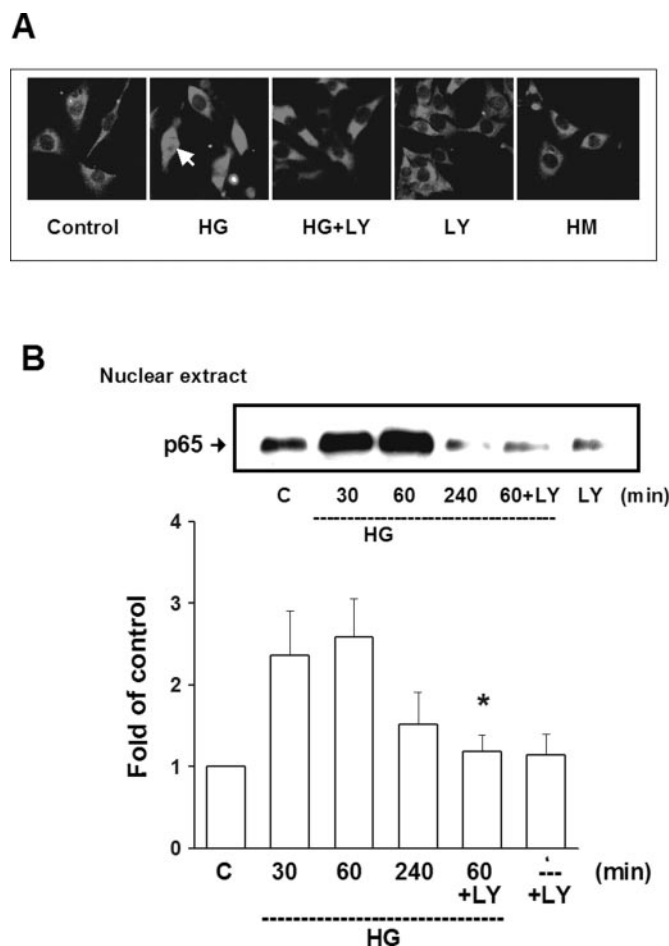
(20  $\mu$ M; an inhibitor of reactive oxygen intermediate production by mitochondrial electron transport system) could inhibit the phosphorylation of Akt induced by HG.

**ROS-Related NF- $\kappa$ B Activation in Mesangial Cells Cultured under High Glucose and the Involvement of PI3K/Akt.** To determine the involvement of NF- $\kappa$ B activation in the response of HG, the levels of NF- $\kappa$ B p65 protein in the nuclei of HG-treated mesangial cells was investigated with immunofluorescence and Western blotting. HG-stimulated mesangial cells showed a marked NF- $\kappa$ B p65 staining (Figs. 6A and 7A) and p65 protein expression (Fig. 6B) in the nuclei. HG-induced NF- $\kappa$ B nuclear translocation was seen at

30 min and was maximal at 1 h (Fig. 6B). In subsequent experiments, we determined NF- $\kappa$ B binding activity in MES-13 cells and primary rat mesangial cells at 30 min and 1 h after HG treatment using electrophoretic mobility shift assay. As shown in Fig. 8, basal NF- $\kappa$ B activation was observed in mesangial cells. HG significantly increased the NF- $\kappa$ B activation. HM did not trigger NF- $\kappa$ B nuclear translocation and DNA binding activation in mesangial cells compared with the control (Figs. 6A and 8). Addition of unlabeled consensus NF- $\kappa$ B oligonucleotide (100-fold in excess) completely abolished the mobility shift band (Fig. 8A, a), demonstrating the specificity of the protein/DNA interaction. Supershift analysis has shown that the induced NF- $\kappa$ B was a p65/p50 heterodimer (data not shown); this is consistent with the previous reports (Ha et al., 2002). These results provided the specificity of the NF- $\kappa$ B binding activity in mesangial cells.



**Fig. 5.** PI3K inhibitors DN-p85 and DN-Akt blocked HG-induced ROS generation. A, expression of DN-p85 and DN-Akt in mesangial cells. Cell lysates from mesangial cells, transfected with HA epitope-tagged DN-p85, DN-Akt, or control pcDNA3 vector, were immunoprecipitated with anti-HA antibody and followed by Western blotting with anti-p85 or anti-Akt antibodies to detect the expression of mutant proteins. In some experiments, cells were treated with HG in the absence or presence of LY294002 (LY; 10  $\mu$ M) or DN mutants (DN-p85 and DN-Akt). Cellular lysates were prepared to perform electrophoresis and immunoblotting as described under *Materials and Methods* employing a specific anti-phospho-Akt antibody or anti-Akt antibody. Results shown are representative of four independent experiments. B, intracellular ROS generation was determined by fluorescence of DCFH-DA as described under *Materials and Methods*. Relative fluorescence intensity in MMC cultured under HG for 1 h with or without LY294002 (LY; 10  $\mu$ M) or DN mutants was quantitated by flow cytometry. Data are presented as mean  $\pm$  S.E.M. from three to five experiments performed in duplicate. \*,  $P < 0.05$  compared with HG without any other drugs. C, effects of antioxidants on the HG-induced Akt phosphorylation. Cells were treated with HG in the absence or presence of antioxidants DPI (20  $\mu$ M), rotenone (ROT; 20  $\mu$ M), PDTC (10  $\mu$ M), and NAC (7.5 mM). Results shown are representative of five independent experiments.

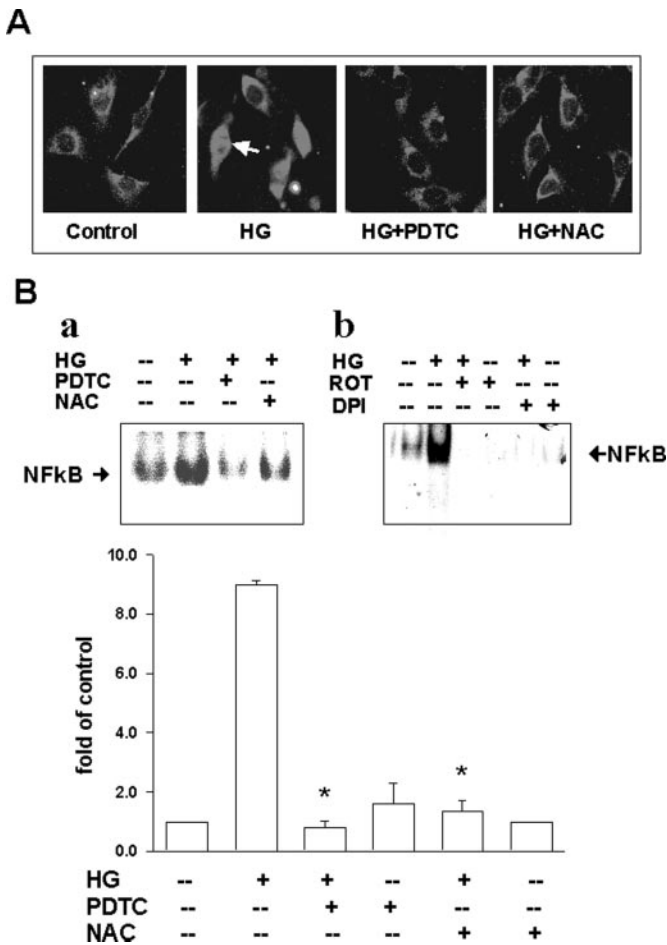


**Fig. 6.** Effect of high glucose on NF- $\kappa$ B activation in mesangial cells. A, MES-13 cells grown on cover slides were exposed to HG and were then fixed and incubated with monoclonal antibodies directed against the p65 subunit of NF- $\kappa$ B. Immunofluorescence staining for NF- $\kappa$ B was examined as described under *Materials and Methods*. Most of the nuclei in mesangial cells were positively stained (arrow) at 1 h after the treatment of HG. LY294002 (LY) could significantly reduce this HG-induced response. Results shown are representative of at least three experiments. B, cells were exposed to HG with or without LY (10  $\mu$ M) for 30 to 60 min. At the indicated time points, nuclear extracts were prepared and analyzed by Western blotting. Quantification of the nuclear NF- $\kappa$ B p65 subunit protein expression was performed by densitometric analysis. Data are presented as mean  $\pm$  S.E.M. from three-five independent experiments. \*,  $P < 0.01$  compared with HG without any other drugs.

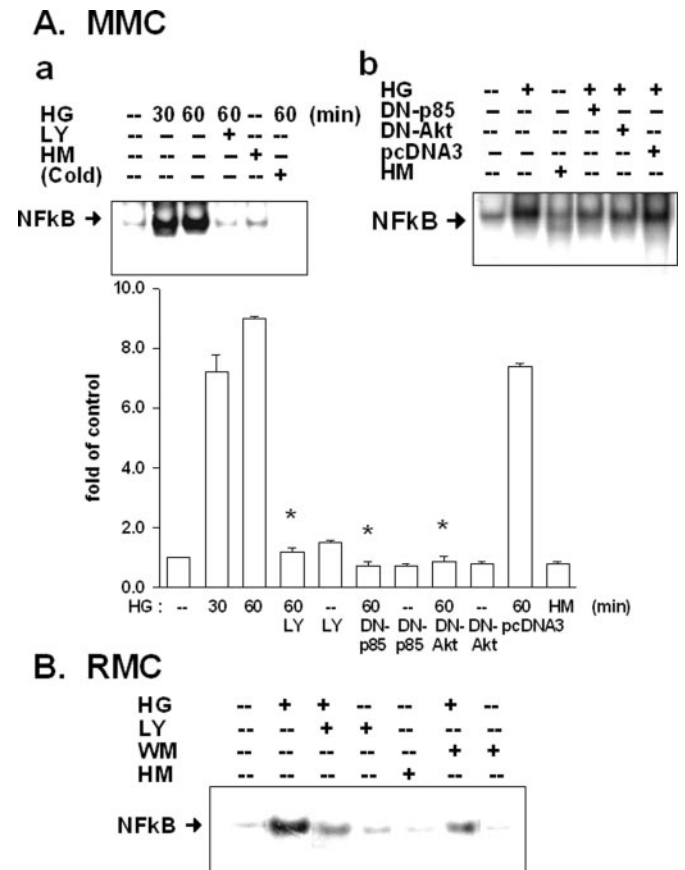
To investigate whether ROS was involved in the regulation of HG-triggered NF- $\kappa$ B activation, the authors studied the effects of antioxidants on this HG-induced response by immunocytochemistry and electrophoretic mobility shift assay. As shown in Fig. 7A, HG-stimulated mesangial cells showed marked NF- $\kappa$ B p65 staining in the nuclei, whereas cells treated with antioxidants (10  $\mu$ M PDTC and 7.5 mM NAC) showed an effective reduction in nuclear NF- $\kappa$ B expression. NF- $\kappa$ B nuclear translocation was observed at 60 min after the treatment of HG, when nearly all mesangial cells nuclei were stained positively for p65. Likewise, PDTC and NAC could block the HG-induced NF- $\kappa$ B binding activity in mesangial cells (Fig. 7B, a). Other antioxidants, DPI (20  $\mu$ M) and

rotenone (20  $\mu$ M), could also abolish the HG-induced NF- $\kappa$ B activation in mesangial cells (Fig. 7B, b).

On the other hand, LY294002 (10  $\mu$ M) completely abolished this HG-triggered NF- $\kappa$ B p65 staining and p65 protein expression in the nuclei (Fig. 6, A and B). LY 294002 (10  $\mu$ M) could also abolish the NF- $\kappa$ B DNA binding activity in mesangial cells cultured under HG (Fig. 8, A and B), suggesting that a PI3K pathway may mediate the effect of HG on NF- $\kappa$ B activation in mesangial cells. The next aim of the investigation was to ascertain whether PI3K or Akt activity inhibition might affect the HG-induced NF- $\kappa$ B activation in mesangial cells. To address this issue, cells were transfected with DN-p85, a dominant-negative form of the regulatory subunit of PI3K, or DN-Akt, a dominant-negative form of the PI3K downstream effector Akt. The transfection results revealed that the DN-p85 and DN-Akt transfection, but not the control pcDNA3, significantly inhibited HG-induced NF- $\kappa$ B activation in mesangial cells as determined by the electrophoretic



**Fig. 7.** Role of ROS in the HG-induced NF- $\kappa$ B activation in mesangial cells. **A**, MES-13 cells grown on cover slides were preincubated with or without antioxidants PDTC (10  $\mu$ M) and NAC (7.5 mM) for 30 min before the treatment of HG. Cells were then fixed and incubated with monoclonal antibodies directed against the p65 subunit of NF- $\kappa$ B. Immunofluorescence staining for NF- $\kappa$ B was examined as described under *Materials and Methods*. Most of the nuclei in cells were positively stained (arrow) at 1 h after the treatment of HG. PDTC and NAC could significantly reduce this HG-induced response. **B**, cells were pretreated with PDTC (10  $\mu$ M) and NAC (7.5 mM) and other antioxidants DPI (20  $\mu$ M) and rotenone (ROT, 20  $\mu$ M) for 30 min before the addition of HG. Cells then were exposed to HG for 1 h, and nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay as described under *Materials and Methods*. Preincubation of cells with the antioxidants before the treatment of HG, resulted in a reduction of NF- $\kappa$ B activation compared with HG alone. Quantification of the nuclear NF- $\kappa$ B binding activity was performed by densitometric analysis. Data are presented as mean  $\pm$  S.E.M. from five independent experiments. \*,  $P < 0.01$  compared with HG without any other drugs.



**Fig. 8.** High glucose-induced activation of NF- $\kappa$ B in mesangial cells requires the activation of a PI3K/Akt pathway. Nuclear extracts were prepared and subjected to an electrophoretic mobility shift assay as described under *Materials and Methods*. MMC (**A**-a) and RMC (**B**) were treated with HG with or without LY294002 (LY, 10  $\mu$ M) for 30 to 60 min. Addition of unlabeled consensus NF- $\kappa$ B oligonucleotide (100-fold in excess) completely abolished mobility shift band (lane 6), demonstrating the specificity of the protein/DNA interaction. Moreover, MMC were transiently transfected with DN-p85 or DN-Akt or control pcDNA3 vector (**A**-b). After 24 h of transfection, the cell medium was replaced with a fresh serum-free medium for 12 h, and then cells were exposed to HG. Cells were harvested 60 min after the HG treatment. Quantification of the nuclear NF- $\kappa$ B binding activity was performed by densitometric analysis. Data are presented as mean  $\pm$  S.E.M. from five independent experiments. Results in RMC (**B**) shown are representative of five independent experiments.

mobility shift assay (Fig. 8A-b). These results suggest that PI3K is necessary and plays an important role in HG-induced NF- $\kappa$ B DNA binding activity in mesangial cells.

**Effect of HG on Mesangial Cell Proliferation and the Involvement of PI3K/Akt.** HG was also capable of inducing mesangial cells proliferation as determined by MTS assay (Fig. 9A) and [ $^3$ H]thymidine incorporation (Fig. 9B) after 24 h in culture. Treatment of a selective COX-2 inhibitor, NS398 (20 and 40  $\mu$ M), or nonselective inhibitor, aspirin (1 and 2 mM), resulted in a suppression of cell proliferation induced by HG (Fig. 9, A and B). NS398 (20  $\mu$ M) or aspirin (2 mM) treatment without HG had no effect on the basal proliferation of cells (Fig. 9). Unlike HG, the addition of 33 mM mannitol to the media did not affect the cell proliferation in mesangial cells compared with the control (Fig. 9, A and B),

suggesting that the HG-triggered cell proliferation is not the result of high osmolality within the media.

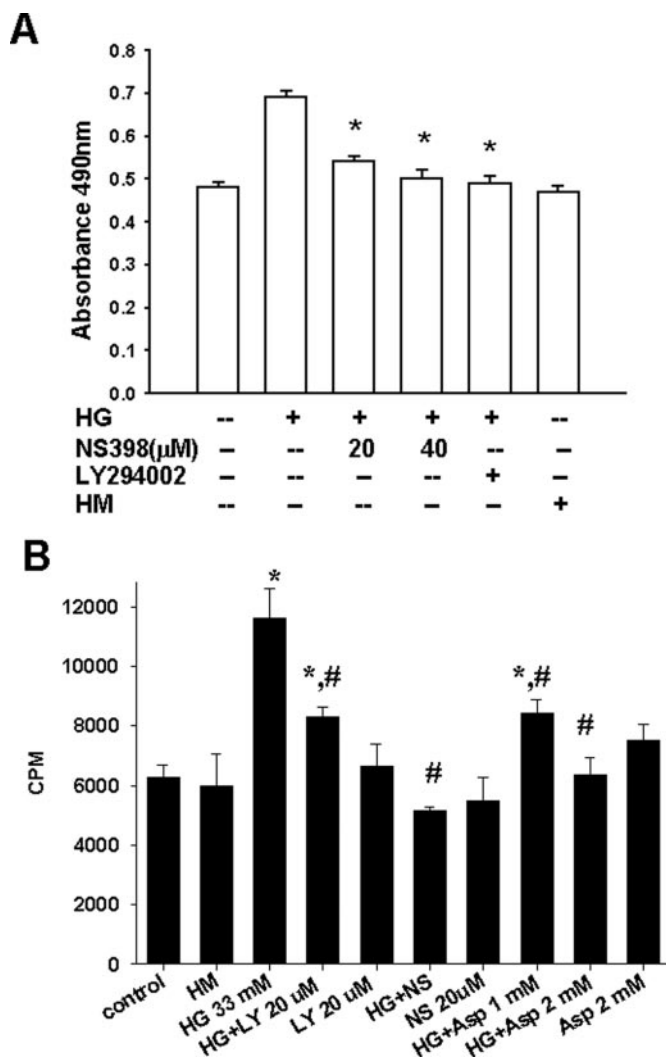
To investigate whether a PI3K pathway was involved in the regulation of mesangial cells proliferation, the authors first studied the effect of PI3K inhibitors LY294002 or wortmannin on HG-induced mesangial cells proliferation. LY294002 significantly inhibited the HG-triggered cell proliferation using the MTS assay and [ $^3$ H]thymidine incorporation (Fig. 9, A and B). LY294002 (20  $\mu$ M) treatment without HG had no effect on the basal proliferation of cells (Fig. 9, A and B).

## Discussion

There are several major findings in the present study. First, HG triggers the COX-2 protein expression in mesangial cells. This may be related to the HG-induced cell proliferation, because the selective COX-2 inhibitor NS398 can effectively inhibit the HG-induced cell proliferation. Furthermore, HG can rapidly increase PI3K/Akt activities, inducing ROS generation and subsequently activates NF- $\kappa$ B in mesangial cells. Supporting this conclusion are the observations that PI3K inhibitors and blocking the activities of PI3K and its downstream effector Akt with the dominant-negative vectors DN-p85 and DN-Akt, respectively, greatly diminished the HG-evoked ROS generation and NF- $\kappa$ B activation. Antioxidants can also inhibit the HG-triggered NF- $\kappa$ B activation, indicating that NF- $\kappa$ B was the downstream effector responsible for HG-induced ROS generation.

COX-2 was constitutively expressed in occasional renal cells of the thick ascending loop of Henle (Harris et al., 1994) and in the region of the macula densa of the rat kidney (Vio et al., 1997). Exposure in vivo or in vitro to elevated glucose could increase the production of vasoactive prostaglandins by glomeruli and mesangial cells (Schambelan et al., 1985; Williams and Schrier, 1993). A recent study has shown that immunoreactive COX-2 was increased in the renal cortex of diabetic rats compared with control rats and normalized by improved glycemic control (Komers et al., 2001). An augmented COX-2 expression was also observed in rat mesangial cells under HG culture as well as in diabetic rat glomeruli (Makino et al., 2002). These diabetes-related prostaglandins derived from COX-2 might play a role in pathological renal hemodynamic changes in diabetes. Oxidant stress has been demonstrated to be a specific and important inducer of COX-2 gene expression in rat mesangial cells (Feng et al., 1995). However, there is little literature about the mechanism of HG-triggered COX-2 expression and the role of COX-2 in early diabetes-induced renal mesangial cell proliferation. We observed augmented COX-2 mRNA and protein expression in HG-treated mesangial cells, and such induction seemed to be attenuated by PI3K inhibitors and antioxidants. HG did not affect the protein expression of COX-1. Treatment of mesangial cells with COX-2 inhibitors NS398 and aspirin resulted in a suppression of cell proliferation induced by HG. These results imply that a signaling pathway with PI3K and ROS might be involved in the HG-induced COX-2 expression in mesangial cells, which might be related to the HG-triggered cell proliferation.

NF- $\kappa$ B is a transcriptional regulator of inducible gene expression, including COX-2, regulating cell proliferation (Lim et al., 2001). The antioxidants could inhibit NF- $\kappa$ B activation



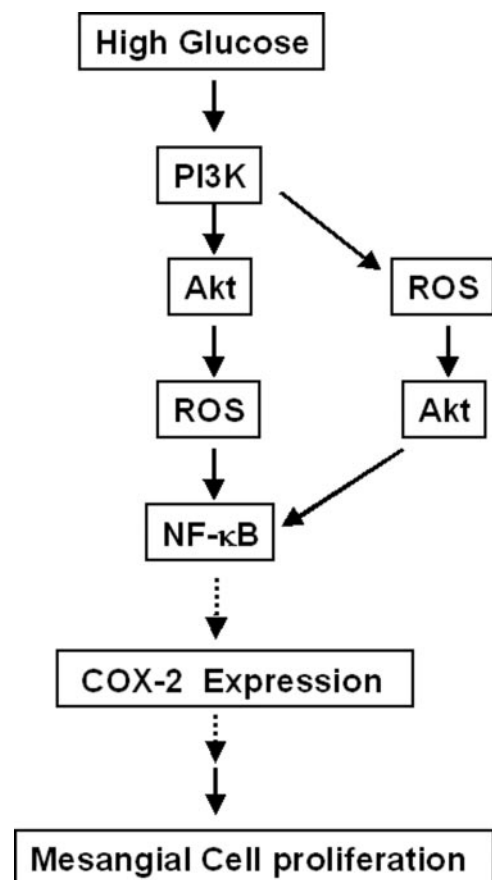
**Fig. 9.** Effects of high glucose on cell proliferation in mesangial cells. MES-13 cells were treated with HG (33 mM) or HM (33 mM) for various time intervals. In some experiments, cells were pretreated with PI3K inhibitor LY294002 (LY; 10  $\mu$ M), or COX-2 inhibitors NS398 (NS; 20 and 40  $\mu$ M) and aspirin (Asp; 1 and 2 mM) for 1 h, and then incubated under HG for 24 h. For determination of the cell proliferation, cells were subjected to the MTS assay (A) and [ $^3$ H]thymidine incorporation (B) as described under *Materials and Methods*. Data are presented as mean  $\pm$  S.E.M. from three to five experiments performed in duplicate. The conversion of MTS was measured by the amount of absorbance in 490 nm. \*,  $P < 0.05$  compared with HG (A) or control (B) without any other drugs. #,  $P < 0.05$  compared with HG without any other drugs (B).

in a wide variety of cells, possibly by suppressing the production of intracellular ROS (Rangan et al., 1999). The observations that antioxidants suppressed HG-induced extracellular matrix protein synthesis in mesangial cells (Trachtman, 1994) and in experimental diabetic animals (Koya et al., 1997), and that vitamin E normalized glomerular hyperfiltration in human diabetes (Bursell et al., 1999), revealed a pathogenic role of ROS in diabetes. A recent report showed that ROS generation by glucose metabolism might act as integral signaling molecules in mesangial cells cultured under HG (Ha and Lee, 2000). It was further documented that HG rapidly activated NF- $\kappa$ B in mesangial cells through protein kinase C and ROS, and it was suggested that HG-induced NF- $\kappa$ B activation in mesangial cells might play a role in diabetic renal injury (Ha et al., 2002). However, it is still unclear whether NF- $\kappa$ B is involved in the HG-induced COX-2 expression and the role of ROS in this signaling pathway in mesangial cells. Consistent with above observations, the authors found that HG could rapidly activate ROS generation and NF- $\kappa$ B activation in mesangial cells. Our further results showed that antioxidants NAC and PDTC could effectively inhibit NF- $\kappa$ B activation and COX-2 expression in mesangial cells cultured under HG, suggesting that ROS may act as signaling molecules of HG-induced NF- $\kappa$ B activation and COX-2 expression in mesangial cells. Moreover, the other antioxidants DPI, an inhibitor of the production of superoxide by mitochondrial respiration and NAD(P)H oxidase activities, and rotenone, an inhibitor of reactive oxygen intermediate production by mitochondrial electron transport system (Chen et al., 2003), could also abolish the DCF-sensitive ROS generation induced by HG (Fig. 4C) and the HG-induced NF- $\kappa$ B activation in mesangial cells (Fig. 7B, b). These results further confirm the involvement of ROS in a HG-related signaling pathway in mesangial cells. However, the real mechanism(s) of ROS generation induced by HG in mesangial cells remains unclear. Therefore, the next aim of the present study was to investigate whether a PI3K/Akt pathway was involved in the mechanism(s) of ROS generation induced by HG in mesangial cells.

The PI3K pathway is implicated in human diseases, including diabetes and cancer (Cantley, 2002). One of the downstream effectors of PI3K is the serine/threonine kinase Akt. Akt is involved in promotion of cell survival and possibly plays a role in PI3K-mediated cell proliferation (Toker, 2000; Koyasu, 2003). Activation of a PI3K/Akt pathway in response to cytokines leads to phosphorylation and activation of the NF- $\kappa$ B p65/RelA subunit (Burow et al., 2000). It has been shown that the addition of p40(phox) to the minimal core complex allows phosphatidylinositol 3-phosphate, a lipid product of PI3K, to stimulate specifically the formation of ROS in neutrophil (Ellson et al., 2001). Some recent reports have also shown that the PI3K-dependent pathway might regulate toxic levels of ROS generated by oxidative stress (Goldshmit et al., 2001), and that platelet-derived growth factor-induced  $H_2O_2$  production required the activation of PI3K (Bae et al., 2000). These findings indicate that PI3K may play an important role in the regulation of ROS and NF- $\kappa$ B signaling pathways. Because it remains unclear which signaling pathway is activated by HG and thereby leads to ROS generation in mesangial cells, the authors examined whether the PI3K/Akt pathway is involved in this HG-induced response. The present results showed that HG

rapidly activated PI3K activity within a few minutes in mesangial cells, which could be completely blocked by PI3K inhibitors. PI3K inhibitors and the dominant-negative vectors DN-p85 and DN-Akt effectively attenuated HG-mediated ROS generation and NF- $\kappa$ B activation in mesangial cells. PI3K inhibitors could also block the expression of COX-2 protein and cell proliferation in mesangial cells cultured under HG. These findings strongly support a role for PI3K/Akt signaling as an upstream regulator in the sequence of events leading to ROS generation and NF- $\kappa$ B activation and subsequent induction of a COX-2 expression and cell proliferation in mesangial cells. Nevertheless, it cannot exclude the possibility that other pathways, such as the MAPK pathway, are involved in regulation of HG-induced mesangial cells proliferation. This issue requires further investigation in the future. On the other hand, some studies have shown that ROS could regulate the activation of Akt in mesangial cells and some other cultured cells (Esposito et al., 2003; Gorin et al., 2003). Therefore, to further confirm the relationship between Akt and ROS in mesangial cells cultured under HG, the effect of antioxidants on the phosphorylation of Akt was investigated. The results showed that antioxidants could inhibit the phosphorylation of Akt induced by HG, indicating that ROS generation by HG may have ability to activate Akt in mesangial cells.

In conclusion, as indicated in Fig. 10, our study indicates



**Fig. 10.** Schematic representation of proposed intracellular signaling leading to high glucose-induced COX-2 expression and cell proliferation in mesangial cells. This overview is based on the present findings as well as those previously published by other investigators and does not exclude participation of other signaling pathways or complementary signals within the presented diagram.

that the activation of a PI3K signaling is required for HG-induced ROS generation and subsequent activation of NF- $\kappa$ B in mesangial cells. Moreover, it seems to exist on a mutual regulation between ROS generation and Akt activation. It does not exclude participation of other signaling pathways or complementary signals within the presented diagram. This PI3K-related signal pathway may be one of the mechanisms involved in the early diabetes-related mesangial cells proliferation and progression of diabetic glomerulopathy through up-regulation of COX-2.

## Acknowledgments

We thank professor R. H. Chen for providing dominant-negative Akt and p85, and professor Y. J. Sung for helpful instructions on electrophoretic mobility shift assay.

## References

- Bae YS, Sung JY, Kim OS, Kim YJ, Hur KC, Kazlauskas A, and Rhee SG (2000) Platelet-derived growth factor-induced  $H_2O_2$  production requires the activation of phosphatidylinositol 3-kinase. *J Biol Chem* **275**:10527–10531.
- Burow ME, Weldon CB, Melnik LL, Duong BN, Collins-Burow BM, Beckman BS, and McLachlan JA (2000) PI3-K/AKT regulation of NF-kappaB signaling events in suppression of TNF-induced apoptosis. *Biochem Biophys Res Commun* **271**:342–345.
- Bursell SE, Clermont AC, Aiello LP, Aiello LM, Schlossman DK, Feener EP, Laffel L, and King GL (1999) High-dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes. *Diabetes Care* **22**:1245–1251.
- Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science (Wash DC)* **296**:1655–1657.
- Cao Y and Prescott SM (2002) Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. *J Cell Physiol* **190**:279–286.
- Catherwood MA, Powell LA, Anderson P, McMaster D, Sharpe PC, and Trimble ER (2002) Glucose-induced oxidative stress in mesangial cells. *Kidney Int* **61**:599–608.
- Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, and Lesnfsky EJ (2003) Production of reactive oxygen species by mitochondria. *J Biol Chem* **278**:36027–36031.
- Ellison CD, Gobert-Gosse S, Anderson KE, Davidson K, Erdjument-Bromage H, Tempst P, Thuring JW, Cooper MA, Lim ZY, Holmes AB, et al. (2001) PtdIns<sub>3</sub>P regulates the neutrophil oxidase complex by binding to the PX domain of P40(Phox). *Nat Cell Biol* **3**:679–682.
- Esposito F, Chirico G, Gesualdi NM, Posadas I, Ammendola R, Russo T, Cirino G, and Cimino F (2003) Protein kinase B activation by reactive oxygen species is independent of tyrosine kinase receptor phosphorylation and requires src activity. *J Biol Chem* **278**:20828–20834.
- Feng L, Xia Y, Garcia GE, Hwang D, and Wilson CB (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha and lipopolysaccharide. *J Clin Invest* **95**:1669–1675.
- Flyvbjerg A, Landau D, Domene H, Hernandez L, Gronbaek H, and LeRoith D (1995) The role of growth hormone, insulin-like growth factors (IGFs) and IGF-binding proteins in experimental diabetic kidney disease. *Metabolism* **44**:67–71.
- Foster FM, Traer CJ, Abraham SM, and Fry MJ (2003) The phosphoinositide (PI) 3-kinase family. *J Cell Sci* **116**:3037–3040.
- Fruman DA and Cantley LC (2002) Phosphoinositide 3-kinase in immunological systems. *Semin Immunol* **14**:7–18.
- Goldshmit Y, Erlich S, and Pinkas-Kramarski R (2001) Neuregulin rescues PC12-ErbB4 cells from cell death induced by  $H_2O_2$ . Regulation of reactive oxygen species levels by phosphatidylinositol 3-kinase. *J Biol Chem* **276**:46379–46385.
- Gorin Y, Ricono JM, Kim NH, Bhandari B, Choudhury GG, and Abboud HE (2003) Nox4 mediates angiotensin II-induced activation of AKT/protein kinase B in mesangial cells. *Am J Physiol* **285**:F219–F229.
- Ha H and Lee HB (2000) Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. *Kidney Int Suppl* **77**:S19–S25.
- Ha H, Yu MR, Choi YJ, Kitamura M, and Lee HB (2002) Role of high glucose-induced nuclear factor-kappaB activation in monocyte chemoattractant protein-1 expression by mesangial cells. *J Am Soc Nephrol* **13**:894–902.
- Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, and Breyer MD (1994) Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J Clin Invest* **94**:2504–2510.
- Komers R, Lindsley JN, Oyama TT, Schutzer WE, Reed JF, Mader SL, and Anderson S (2001) Immunohistochemical and functional correlations of renal cyclooxygenase-2 in experimental diabetes. *J Clin Invest* **107**:889–898.
- Koya D, Lee IK, Ishii H, Kanoh H, and King GL (1997) Prevention of glomerular dysfunction in diabetic rats by treatment with D-alpha-tocopherol. *J Am Soc Nephrol* **8**:426–435.
- Koyasu S (2003) The role of PI3K in immune cells. *Nat Immunol* **4**:313–319.
- Kuo ML, Chuang SE, Lin MT, and Yang SY (2001) The involvement of PI3-K/Akt-dependent up-regulation of Mcl-1 in the prevention of apoptosis of Hep3B cells by interleukin-6. *Oncogene* **20**:677–685.
- Li X and Stark GR (2002) NFkappaB-dependent signaling pathways. *Exp Hematol* **30**:285–296.
- Lim JW, Kim H, and Kim KH (2001) Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. *Lab Invest* **81**:349–360.
- Lin MT, Lee RC, Yang PC, Ho FM, and Kuo ML (2001) Cyclooxygenase-2 inducing Mcl-1-dependent survival mechanism in human lung adenocarcinoma CL1.0 cells. Involvement of phosphatidylinositol 3-kinase/akt pathway. *J Biol Chem* **276**:48997–49002.
- Mahadevan P, Larkins RG, Fraser JR, and Dunlop ME (1996) Effect of prostaglandin E2 and hyaluronan on mesangial cell proliferation. A potential contribution to glomerular hypercellularity in diabetes. *Diabetes* **45**:44–50.
- Makino H, Tanaka I, Mukoyama M, Sugawara A, Mori K, Muro S, Suganami T, Yahata K, Ishibashi R, Ohuchida S, et al. (2002) Prevention of diabetic nephropathy in rats by prostaglandin E receptor EP1-selective antagonist. *J Am Soc Nephrol* **13**:1757–1765.
- Osterby R and Gundersen HJ (1975) Glomerular size and structure in diabetes mellitus. I. Early abnormalities. *Diabetologia* **11**:225–229.
- Phillips A, Janssen U, and Floege J (1999) Progression of diabetic nephropathy. Insights from cell culture studies and animal models. *Kidney Blood Press Res* **22**:81–97.
- Rangan GK, Wang Y, Tay YC, and Harris DC (1999) Inhibition of NFkappaB activation with antioxidants is correlated with reduced cytokine transcription in PTC. *Am J Physiol* **277**:F779–F789.
- Schambelan M, Blake S, Sraer J, Bens M, Nivez MP, and Wahbe F (1985) Increased prostaglandin production by glomeruli isolated from rats with streptozotocin-induced diabetes mellitus. *J Clin Invest* **75**:404–412.
- Shankland SJ and Wolf G (2000) Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation and apoptosis. *Am J Physiol* **278**:F515–F529.
- Tang Q, Gonzales M, Inoue H, and Bowden GT (2001) Roles of AKT and glycogen synthase kinase 3beta in the ultraviolet b induction of cyclooxygenase-2 transcription in human keratinocytes. *Cancer Res* **61**:4329–4332.
- Toker A (2000) Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol Pharmacol* **57**:652–658.
- Trachtman H (1994) Vitamin E prevents glucose-induced lipid peroxidation and increased collagen production in cultured rat mesangial cells. *Microvasc Res* **47**:232–239.
- Vane JR, Bakhle YS, and Botting RM (1998) Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* **38**:97–120.
- Vio CP, Cespedes C, Gallardo P, and Masferrer JL (1997) Renal identification of cyclooxygenase-2 in a subset of thick ascending limb cells. *Hypertension* **30**:687–692.
- Weaver SA, Russo MP, Wright KL, Kolios G, Jobin C, Robertson DA, and Ward SG (2001) Regulatory role of phosphatidylinositol 3-kinase on TNF-alpha-induced cyclooxygenase 2 expression in colonic epithelial cells. *Gastroenterology* **120**:1117–1127.
- Williams B and Schrier RW (1993) Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells. *J Clin Invest* **92**:2889–2896.
- Young BA, Johnson RJ, Alpers CE, Eng E, Gordon K, Floege J, Couser WG, and Seidel K (1995) Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* **47**:935–944.

**Address correspondence to:** Dr. Shing-Hwa Liu, Institute of Toxicology, College of Medicine, National Taiwan University, 1, Section 1, Jen-Ai Road, Taipei, 10043, Taiwan. E-mail: shliu@ha.mc.ntu.edu.tw