Role of High Glucose–Induced Nuclear Factor–κB Activation in Monocyte Chemoattractant Protein-1 Expression by Mesangial Cells

HUNJOO HA,* MI RA YU,* YOON JIN CHOI,* MASANORI KITAMURA,† and HI BAHL LEE*‡
*Hyonam Kidney Laboratory, Soon Chun Hyang University, Seoul, Korea; and †University College London Medical School, The Rayne Institute, London, United Kingdom.

Abstract. Although high glucose (HG) has been shown to induce nuclear factor–κB (NF-κB) activation in vascular cells, the upstream regulation and the biologic significance of NF-κB activation in diabetic renal injury are not clear. It was, therefore, examined if HG-induced generation of reactive oxygen species (ROS) and protein kinase C (PKC) activation are involved in NF-κB activation in mesangial cells (MC), and the role of NF-κB activation in HG-induced monocyte chemoattractant protein-1 (MCP-1) expression by MC was further investigated. Recent observations suggest that MCP-1 may play a role in the development and progression of diabetic nephropathy. HG rapidly induced NF-κB activation in MC as estimated by electrophoretic mobility shift assay. Supershift assay suggests that most of the binding activity arose from p50/p50 and p50/p65 dimers. Antioxidants, pyrrolidine dithiocarbamate, N-acetyl-l-cysteine, and trolox effectively inhibited HG-induced NF-κB activation in MC. HG rapidly generated dichlorofluorescin-sensitive intracellular ROS in MC as measured by laser-scanning confocal microscopy. HG also activated PKC rapidly in MC. Inhibition of PKC effectively blocked HG-induced intracellular ROS generation and NF-κB activation in MC. HG increased MCP-1 mRNA expression by 1.9-fold and protein secretion by 1.6-fold that of control glucose in MC transfected with control vector but not in MC transfected with dominant negative mutant inhibitor of NF-κB IκBaM. Inhibition of either PKC or ROS effectively blocked HG-induced, but not basal, MCP-1 protein secretion by MC transfected with control vector. Thus this study demonstrates that HG rapidly activates NF-κB in MC through PKC and ROS and suggests that HG-induced NF-κB activation in MC may play a role in diabetic renal injury through upregulation of MCP-1 mRNA and protein expression.

Nuclear factor–κB (NF-κB) is a transcription factor widely distributed in most cell types, including glomerular mesangial cells (MC) (1,2). It mainly consists of dimers of the two subunits p50 and p65 (Rel A) and exists in inactive form in the cytoplasm associated with an inhibitory protein called IκB (3). Activation of NF-κB results from the release of the inhibitory IκB subunit from the heterotrimeric complex followed by translocation of dimer to the nucleus, where it initiates transcription (3). Activation of NF-κB can be induced by various molecules, such as cytokines, lipopolysaccharide (LPS), phorbol ester, and reactive oxygen species (ROS), and controls the transcription of several genes involved in immune and inflammatory responses, cell growth, and adhesion (3,4). Increasing evidences suggest that NF-κB may play an important role in MC activation leading to renal injury (1,2).

High glucose (HG) is the main determinant of the development and progression of diabetic nephropathy in both type 1 (5) and type 2 diabetes (6). As in other diabetic vascular complications (7,8), we and others have proposed that oxidative stress generated by hyperglycemia is one of the major mediators of diabetic nephropathy (9–12). Recent studies suggest that the activation of NF-κB is one possible mechanism for oxidative stress-induced vascular complications in diabetes. NF-κB activation in isolated peripheral blood mononuclear cells of patients with poorly controlled type 1 diabetes was significantly prevented by an antioxidant thiocotic acid (13). Cardiac tissues of diabetic rats also show increased NF-κB activation associated with oxidative stress (14). In addition, HG can induce NF-κB activation in endothelial (15–17) and vascular smooth muscle cells (18).

A recent report (19) demonstrating higher renal expression of NF-κB system in experimental diabetic rats than in control animals suggests possible involvement of NF-κB activation in diabetic renal injury as well. However, it is not known if HG activates NF-κB in MC or if HG-induced NF-κB activation plays a role in diabetic renal injury. Thus, the following were the objectives of our present study: (1) to determine if HG can activate NF-κB in MC; (2) to examine if HG-induced ROS and protein kinase C (PKC) are involved in NF-κB activation to understand the upstream regulation of HG-induced NF-κB activation in MC; and (3) to evaluate the role of NF-κB
activation in monocyte chemoattractant protein–1 (MCP-1) mRNA and protein expression in MC cultured under HG to address the biologic significance of NF-κB activation. MCP-1 is a chemokine induced by NF-κB (20–23). MCP-1 promoter and enhancer regions contain NF-κB binding sequences (20). Increased MCP-1 expression in MC cultured under HG (24) and glycated albumin (25) and in diabetic kidney (25) suggest that MCP-1 may be involved in the development and progression of diabetic renal injury. Rat MC that have been stably transfected with the dominant negative mutant inhibitor of NF-κB (26) were used to demonstrate the role of HG-induced NF-κB activation in MCP-1 mRNA and protein expression in MC.

Materials and Methods

All chemicals and tissue culture plates were obtained from Sigma Chemical Company (St. Louis, MO) and Becton Dickinson Labware (Lincoln Park, NJ), unless otherwise stated.

Mesangial Cell Culture

Dulbecco’s modified Eagle’s medium (DMEM) containing 5.6 mM glucose was used to culture MC, unless otherwise stated. A murine MC line (MES-13, cloned from mice transgenic for the early region of SV-40 virus, passage 25) was obtained from American Type Culture Collection (ATCC, Rockville, MD), these transformed cells have been shown to exhibit similar characteristics to those of primary cultures of murine MC (27). Primary mouse and rat MC were also used to confirm the responses obtained with MES-13. Primary MC were obtained by culturing glomeruli isolated from kidneys of 100- to 150-g male Sprague-Dawley rats or 15- to 20-g male BALB/c mice by conventional sieving methods as described previously and characterized (28). Cells were cultured in DMEM containing 20% fetal bovine serum (FBS; Life Technologies BRL, Gaitherburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 44 mM NaHCO3, and 14 mM N-hydroxy-ethylpiperazine-N’-2-ethane sulfonic acid (HEPES). For MES-13, DMEM containing 5% FBS was used. Cells were cultured in 100-mm culture dish for electrophoretic mobility shift assay (EMSA) and PKC measurement, on cover glass coated with polylysine for intracellular ROS measurement, and 6-well culture plate for enzyme-linked immunosorbent assay (ELISA). Near-confluent MC were incubated with serum-free medium for 48 h to arrest and synchronize the cell growth. After this time period, the media were changed to fresh serum-free DMEM containing 5.6 (control) or 30 mM (high) glucose for up to 48 h. Incubation of cells in control as well as HG in serum-free condition for up to 96 h did not have significant effect on cell viability as determined by lactate dehydrogenase (LDH) release. In some experiments, cells were pretreated with 0.2 μM cytochalasin B, 100 nM calphostin C, 24 nM 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GFX 109203X, a bisindolylmaleimide PKC inhibitor; Calbiochem-Novac homer Corporation, San Diego, CA), 100 μM pyrrolidine dithiocarbamate (PDTC), 5 mM N-α-acyetyl-l-cysteine (NAC), 500 μM trolox (Aldrich Chemical Co., Milwaukee, WI) for 1 h and incubated with control and HG media for a given period. Cytochalasin B was used to inhibit cellular uptake of glucose. Preliminary study demonstrated that cytochalasin B up to 2 μM did not cause cytotoxicity as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The effective concentrations were decided on the basis of published data and our own preliminary study. In the study using depletion of cellular PKC, MC were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Cells were processed for nuclear protein extraction, measurement of ROS and PKC activity, and MCP-1 mRNA and protein expression at the end of the incubation period as described below. Experiments using primary rat and mouse MC were performed using cells between the 6th and 8th passages.

Establishment of Mesangial Cells Stably Transfected with IkBoM

Establishment of MC stably transfected with IkBoM has been previously described and well characterized (26). In brief, overexpression of IkBoM were created in MC obtained from a male Harlan Sprague-Dawley rat. pLkBoMMSN (a gift from Dr. Inder Verma, Salk Institute, La Jolla, CA) comprising IkBoM cDNA and a neomycin phosphotransferase gene (neo) were transfected into a helper-free ecotopic packaging line ØE. Stable transfecants were selected in the presence of the neomycin analogue G418 (500 μg/ml). Conditioned media of the transfecnts were used as sources of the IkBoM retrovirus. In the presence of 10 μg/ml of polybren, MC were exposed to a diluted retrovirus, stable infectants were selected in the presence of G418 (750 μg/ml), and the IkBoM transfecnts were established. As control cells, mock-transfected MC that express neo alone were created. Both IkBoM- and mock-transfected cells have been shown to retain most phenotypic characteristics of MC and IkBoM transfecnts are resistant to both basal and interleukin-1β (IL-1β) and HG-induced NF-κB activation as shown in Figure 9A.

Preparation of Nuclear Extracts

Nuclear extracts were prepared according to the method described by Lee et al. (29) with modifications (30). In brief, cells were washed twice with ice-cold phosphate-buffered saline after incubations and suspended in one packed cell volume (PCV) of buffer A containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin and aprotinin, pH 7.9, on ice for 10 min and then lysed by passing the cell suspension through a 27-gauge needle 5 times. Crude nuclei were washed twice with buffer A to prevent cytotoxic contamination, and the nuclear proteins were extracted with two-thirds PCV of ice-cold buffer B containing 20 mM HEPES, 420 mM KCl, 1.5 mM MgCl2, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, and 25% glycerol, pH 7.9. A two-thirds PCV of ice-cold buffer C (20 mM HEPES, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethysulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, 20% glycerol, pH 7.9) was added. The mixture was then centrifuged at 14,000 rpm at 4°C for 15 min. The nuclear proteins were transferred to new tubes as aliquots and stored at −70°C until use. The protein concentration was determined by a Bio-Rad assay (Bio-Rad, Richmond, CA).

Electrophoretic Mobility Shift Assay

For EMSA, the following oligonucleotide with the NF-κB consensus binding sequence was used: 5′-AGTTGAGGCCTGAGGGAACCCAGGC-3′ (Santa Cruz Biotechnology, Santa Cruz, CA). A mutant motif with G to C substitution (5′-AGTTGAGGCCTTG-CCAGGC-3′, Santa Cruz Biotechnology) served as a control. The consensus oligonucleotide was labeled with [γ-32P]ATP (DuPont NEN, Boston, MA) according to the manufacturer's description. The binding reaction was performed in a final volume of 20 μl containing binding buffer (10 mM HEPES, 60 mM KCl, 1 mM DTT, 1 mM EDTA, 7% glycerol, pH 7.6), 0.0175 pmol of labeled probe (>10,000 cpm), 20 μg of nuclear protein, and 2 μg of poly dIdC (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Reactions were started by
addition of nuclear extracts and allowed for 30 min at room temperature. Samples were loaded on 6% polyacrylamide nondenaturing gel and electrophoresed for 2 h at 180 V. The dried gel was exposed to Kodak XR5 film (Eastman Kodak, Rochester, NY) on intensifying screen for 10 to 20 h at −70°C. For competition assay, an unlabeled NF-κB oligonucleotide was added in 100-fold excess. For supershift assay, 1 μl of anti-p65 or anti-p50 antibody (Santa Cruz Biotechnology) was incubated with nuclear extract for 30 min at room temperature before binding reaction.

Assay of Intracellular ROS
Intracellular ROS production was measured by the method of Bass et al. (31), as modified for confocal microscopy (32). In brief, coverslips of confluent cells at various times after stimulation with HG were washed with Dulbecco’s phosphate-buffered saline and incubated in the dark for 5 min in Krebs-Ringer solution containing 5 mM 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes Inc., Eugene, OR). CM-H2DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2',7'-dichlorofluorescin (DCF) and thereby trapped within the cells (31). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Culture dishes were transferred to a Leica DM IRB/E inverted microscope (Leica, Wetzlar, Germany) equipped with a ×20 Fluotar objective and Leica TCS NT confocal attachment, and the ROS generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission, 515 to 540 nm). The effect of DCFH photooxidation was minimized by collecting the fluorescence image with a single rapid scan (line average, 4 s; total scan time, 5.2 s) under identical conditions, such as contrast and brightness, for all samples. The cells were then imaged by differential interface contrast microscopy. Preliminary study demonstrated that HG induced intracellular ROS in MC as early as 15 min and gradually increased up to 4 h.

Assay of PKC Activity
Membrane and cytosol fractions were obtained according to the method previously described (33), and PKC activity was measured by a PepTag nonradioactive detection kit (Promega Corporation, Madison, WI) using fluorescence peptide (PLSRTLSVAAK), which is a highly specific substrate for PKC, according to the manufacturer’s descriptions. According to manufacturer, <10 ng of kinase can be detected by this assay. Phosphorylation of the substrate by PKC alters the peptide’s net charge from +1 to −1. The phosphorylated substrate was separated by an agarose gel at pH 8.0. Negatively charged bands from the gel were removed and heated at 95°C until the gel slice melted. Fluorescence of the solubilized solution was measured by a microplate spectrofluorometer (Luminescence spectrometer LS50B; Perkin Elmer, Norwalk, CT). Specific PKC activity was reported as relative PKC activity per mg of total protein.

Isolation of Total RNA and Northern Blot Analyses
Standard Northern blot analyses were performed as described previously (10,28) after isolation of total RNA using the method of Chomczynski and Sacchi (34). Partial cDNAs for rat MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cloned after reverse transcription–PCR and confirmed by DNA sequencing were used for the respective DNA probe. In brief, 20 μg of total RNA was denatured, separated by electrophoresis through a 1.2% agarose gel containing 2.2 M formaldehyde, transferred onto nylon membranes using a capillary transfer, and covalently crosslinked to the membrane with ultraviolet light using a gene-linker (Bio-Rad). The membranes were hybridized with 32P-labeled cDNA probes for MCP-1 and then rehybridized with a probe for GAPDH as an internal control to assess RNA quantity and integrity. Autoradiography was performed by exposing the blots to Kodak x-ray film with intensifying screens at −70°C for 1 to 5 d. Quantitation of mRNA signals was performed by densitometry using MCID (Imaging Research Inc., St. Catharines, Ontario, Canada) and normalized with GAPDH mRNA signals.

Measurements of MCP-1 by ELISA
To quantitate the level of MCP-1 protein under different experimental conditions, MCP-1 was measured in MC culture supernatant using a commercial solid phase quantitative sandwich ELISA kit for MCP-1 (Biosource International, Inc, Camarillo, CA) according to the manufacturer’s descriptions. According to the manufacturer, the ELISA kit for MCP-1 is specific for rat MCP-1 and sensitive to 8 pg MCP-1/ml.

Statistical Analyses
All results are expressed as mean ± SE. ANOVA was used to assess the differences between multiple groups. If the F statistic was significant, the mean values obtained from each group were then compared by Fisher’s least significant difference method. P < 0.05 was used as the criterion for a statistically significant difference.

Results
HG-Induced NF-κB Activation in Mesangial Cells
As presented in Figure 1A, basal NF-κB activation was observed in MC maintained in serum-free DMEM for 48 h. NF-κB activation increased significantly at 30 mM glucose and remained elevated at 50 and 100 mM glucose. Thus 30 mM D-glucose was used as HG for this study. The density of the upper two bands increased in parallel under HG, and the difference in optical density between control and different glucose concentrations remained unchanged whether each band alone or both were analyzed. Thus, the sum of optical density of two bands is shown in Figure 1B. Unlike D-glucose, the addition of 25 mM L-glucose to the media containing 5.6 mM D-glucose did not activate NF-κB activation in MC compared with control (Figure 1C), suggesting that HG-induced NF-κB activation is not the result of high osmolality per se.

To ensure that the mobility shift bands under the present experimental condition were the NF-κB/oligonucleotide complex, we used an NF-κB mutant oligonucleotide with ‘G’ to ‘C’ substitution in the NF-κB binding motif as a control. Addition of this cold NF-κB mutant oligonucleotide (100-fold in excess) did not affect the bands (p50/p65 and p50/p50) that were associated with the labeled NF-κB oligonucleotide probe, whereas the cold consensus NF-κB oligonucleotide (100-fold in excess) completely abolished these bands (Figure 2A). AP-1 or Oct-1 consensus oligonucleotide probe also failed to displace the labeled NF-κB oligonucleotide probe (Figure 2A). These results provided the specificity of the NF-κB binding activity. Incubation of nuclear extracts with antisera against NF-κB p50 or p65 subunit caused a supershift of the corresponding NF-κB/oligonucleotide band (Figure 2B). Antibody to p50 interacted and retarded both of upper two bands. Antibody to p65 retarded the slowest migrating band, leaving most
of the lower part of upper two bands unaffected. These results suggest that most of the binding activity arose from p50/p50 and p50/p65 dimers. The molecular weight of other members of NF-κB family are more than 50 kDa; therefore, the band between p50/p50 homodimer and unbound probe was considered to be nonspecific. Both primary rat and primary mouse MC exhibited similar pattern of NF-κB activation in response to HG (data not shown).

In a time-response study, HG significantly activated NF-κB activity at 30 min (Figure 3, A and B). HG-induced NF-κB activation was maximal at 1 h and gradually decreased but remained increased compared with control for up to 48 h. In subsequent experiments, we determined NF-κB binding activity at 1 h after HG treatment.

Role of ROS in HG-Induced NF-κB Activation in Mesangial Cells

Exogenous H$_2$O$_2$ induced mesangial NF-κB activation in a dose-dependent manner and was significant at 100 μM (Figure 4A). Structurally different cell-permeable antioxidants, PDTC, NAC, and trolox, abolished HG-induced, but not basal, NF-κB activation in MC cultured under HG (Figure 4, B and C), suggesting that the effect of HG on NF-κB activation in MC is mediated by ROS. In addition, as summarized in Figure 5, HG induced intracellular ROS in MC at 1 h. Unlike D-glucose, the addition of 25 mM of either L-glucose or 3-O-methyl-D-glucose to the media containing 5.6 mM D-glucose failed to induce intracellular ROS in MC (Figure 5). A glucose transporter inhibitor, cytochalasin B, effectively inhibited HG-induced intracellular ROS (Figure 5, inset picture a). These results suggest that glucose metabolism, but not glucose autooxidation, is necessary for HG-induced ROS generation in MC, because 3-O-methyl-D-glucose is an autooxidizable glucose analog that enters into the cell but is not metabolized. ROS visualized by confocal microscopy is often restricted in the cytoplasm as presented in Figure 5 (inset picture b). Our preliminary study (35) demonstrated that structurally different antioxidants and ROS scavenger PDTC, trolox, dimethylthiourea, and catalase abolished HG-induced ROS generation in MC.
Role of PKC in HG-Induced NF-κB Activation and Intracellular ROS Generation in Mesangial Cells

Blockade of PKC either by a PKC inhibitor calphostin C 100 nM or by depletion of PKC by preincubating MC with 100 nM PMA for 24 h effectively inhibited HG-induced, but not basal, NF-κB binding activity (Figure 6, A and B) and ROS generation (Figure 7) in MC. PMA at 100 nM increased ROS generation fourfold and NF-κB activity threefold (data not shown). Both pretreatment with calphostin C and PKC depletion effectively inhibited PMA-induced ROS generation and NF-κB activation in MC. Structurally different PKC inhibitor GFX 109203X at 24 nM also effectively blocked PMA- and HG-induced intracellular ROS generation in MC without significant effect on basal ROS generation (data not shown). In addition, as summarized in Figure 8, HG induced PKC translocation from cytosol to membrane in MC as early as 15 min and continuously up to 48 h. PKC translocation at 48 h was significantly higher than that at 24 h. Total PKC activity was also increased after 24 h (data not shown).

Role of HG-Induced NF-κB Activation in MCP-1 mRNA and Protein Expression by Mesangial Cells

As summarized in Figure 9A, 1kBaM-transfected MC did not exhibit NF-κB nuclear translocation at basal as well as in response to HG. IL-1β–induced NF-κB activation was im-

---

Figure 4. Role of reactive oxygen species (ROS) in high glucose (HG)–induced NF-κB activation in mesangial cells. (A) Synchronized quiescent MES-13 were incubated with H2O2 for 1 h. NF-κB activity was assessed by EMSA. (B) Synchronized quiescent MES-13 were pretreated with 100 μM pyrrolidine dithiocarbamate (PDTC), 5 mM N-acetyl-l-cysteine (NAC), and 500 μM trolox for 1 h and incubated under 5.6 mM control or 30 mM HG for 1 h. (C) Bar graph showing the mean ± SE from five to seven experiments. *P < 0.05 compared with control glucose without antioxidant. †P < 0.05 compared with HG without antioxidant.

Figure 5. Specificity of 2′,7′-dichlorofluorescein (DCF)–sensitive ROS in mesangial cells cultured under HG. Synchronized quiescent MES-13, primary mouse, or rat MC were incubated under control glucose and after the addition of 25 mM (for total of 30 mM) of D-glucose, 3-O-methyl-D-glucose, or L-glucose for 1 h, and intracellular DCF-sensitive ROS was visualized by a confocal microscopy. (Inset image a) To confirm the role of glucose uptake in HG-induced ROS generation, MC were pretreated with 0.2 μM cytochalasin B for 1 h before the addition of 25 mM D-glucose. (Inset image b) Intracellular ROS is distributed in cytoplasm but not in the nuclei (arrowhead).
paired in IκBαM-transfected MC as previously demonstrated (26). HG increased MCP-1 protein secretion by 1.6-fold that of control in MC transfected with control vector at 48 h but not in MC transfected with dominant negative mutant of NF-κB (Figure 9B). MCP-1 mRNA expression was significantly upregulated (1.9-fold that of control) in mock-transfected, but not in IκBαM-transfected, MC cultured under HG for 24 h (Figure 9C), suggesting that HG-induced NF-κB activation is responsible for MCP-1 mRNA expression and protein secretion in MC.

Effects of PKC Inhibition and Antioxidants on HG-Induced MCP-1 Expression by Mesangial Cells

As summarized in Figure 10, HG increased MCP-1 protein secretion by 1.6-fold that of control glucose in MC transfected with control vector at 48 h. Blockade of PKC either by a PKC inhibitor GFX 109203X at 24 nM or by depletion of PKC by preincubating MC with 100 nM PMA for 24 h effectively inhibited HG-induced MCP-1 protein secretion. PDTC, NAC, and trolox at doses inhibiting NF-κB activation abolished HG-induced MCP-1 protein secretion in MC cultured under HG. PKC inhibition or antioxidants did not affect basal MCP-1 protein secretion (data not shown).

Discussion

This study has clearly shown that HG rapidly increases NF-κB activity in MC as in other vascular cells (15–18), that ROS generated from glucose metabolism play an important role in HG-induced NF-κB activation in MC, and that PKC also has a role in HG-induced ROS generation and NF-κB activation in MC. In addition, our data demonstrated that NF-κB regulates HG-induced MCP-1 mRNA expression and protein secretion in MC.

We observed that NF-κB–specific protein DNA complex induced in MC by HG consisted mainly of p50/p50 and p50/p65 dimers. The p65 subunit has been shown to be the key component of NF-κB complex in vascular smooth muscle cells cultured under HG (18) and peripheral blood mononuclear cells from uncontrolled type 1 diabetes (13). P50/p65 heterodimer has been demonstrated as a classical transactivator of NF-κB family in which p50 functions to improve the DNA binding of p65 and helps p65 act as a more effective transactivator (36,37). However, p50 may actively inhibit the transactivation potential of p65 (38–40). Thus, it is now postulated that the relative importance of p50 and p65 subunits may determine HG-induced gene regulation in MC.
We visualized intracellular ROS in MC by confocal microscopy. CM-H$_2$DCFDA enters into cells and makes DCFH, which fluoresces when it reacts with hydroperoxides or H$_2$O$_2$ (41,42). HG generates DCF-sensitive ROS in MC within 15 min (data not shown), and it depends on glucose uptake and metabolism. In this study, 3-O-methyl-D-glucose failed to induce intracellular ROS generation in MC. In contrast, 3-O-methyl-D-glucose significantly induced ROS in endothelial cells (17), suggesting that different mechanisms may operate in HG-induced ROS generation in different cell types under different conditions. Our observation that NF-κB is activated through ROS in MC cultured under HG provides evidence that ROS can act as signaling molecules of HG as in other membrane receptor signaling (43,44).

Our observation that H$_2$O$_2$ activates NF-κB in a dose-dependent manner is different from previous studies showing lack of effect in murine and human MC (45,46). This difference is possibly due to difference in time frame of observation given the instability of H$_2$O$_2$ and transient nature of H$_2$O$_2$-induced NF-κB activation.

Increased PKC activity has been implicated in HG-induced vascular complications, including diabetic nephropathy (47). This study demonstrated that a PKC inhibitor and PKC depletion can significantly suppress HG-induced ROS generation as well as NF-κB activation, suggesting that PKC plays a role in HG-induced ROS generation leading to NF-κB activation. Our data agree with a recent report demonstrating PKC-dependent ROS generation in vascular cells cultured under HG (48). On the other hand, ROS can regulate the activation of PKC through redox changes in sulfhydryl groups of cysteine-rich regions of PKC or through activation of phospholipase D leading to production of diacylglycerol. Studer et al. (49) have demonstrated that antioxidants effectively inhibit PKC activation in MC cultured under HG. A recent study by Nishikawa et al. (8) also suggests the role of ROS in PKC activation in endothelial cells cultured under HG.

In conclusion, HG rapidly activates NF-κB in HG-induced MCP-1 expression in mesangial cells. Synchronized quiescent rat MC transfected with control vector or IxBαM were incubated under control (CG), HG, or 0.1 ng/ml interleukin-1β (IL-1β). (A) After 1 h, nuclear proteins were prepared and NF-κB activity was assessed by EMSA. A representative data from five experiments. (B) After 48 h, MCP-1 secreted were measured by enzyme-linked immunosorbent assay (ELISA). Values are expressed as mean ± SE of five experiments. (C) After 24 h, total RNA was isolated and subjected to Northern blot analyses for MCP-1 and GAPDH mRNA. Values are expressed as mean ± SE of four experiments.

**Figure 9.** Role of NF-κB in HG-induced MCP-1 expression in mesangial cells. Synchronized quiescent rat MC transfected with control vector or IxBαM were incubated under control (CG), HG, or 0.1 ng/ml interleukin-1β (IL-1β). (A) After 1 h, nuclear proteins were prepared and NF-κB activity was assessed by EMSA. A representative data from five experiments. (B) After 48 h, MCP-1 secreted were measured by enzyme-linked immunosorbent assay (ELISA). Values are expressed as mean ± SE of five experiments. (C) After 24 h, total RNA was isolated and subjected to Northern blot analyses for MCP-1 and GAPDH mRNA. Values are expressed as mean ± SE of four experiments.

**Figure 10.** Role of PKC and ROS in HG-induced MCP-1 secretion by mesangial cells. Synchronized quiescent MC transfected with control vector were incubated for 48 h under control or HG in the presence and absence of 24 nM GFX 109203X, PKC depletion, 100 μM PDTC, 5 mM NAC, and 500 μM trolox for 48 h. GFX 109203X, PDTC NAC, and trolox were added 1 h before the stimulation of HG. *P < 0.05 compared with control glucose, †P < 0.05 compared with HG without PKC inhibition or antioxidants.

We visualized intracellular ROS in MC by confocal microscopy. CM-H$_2$DCFDA enters into cells and makes DCFH, which fluoresces when it reacts with hydroperoxides or H$_2$O$_2$ (41,42). HG generates DCF-sensitive ROS in MC within 15 min (data not shown), and it depends on glucose uptake and metabolism. In this study, 3-O-methyl-D-glucose failed to induce intracellular ROS generation in MC. In contrast, 3-O-methyl-D-glucose significantly induced ROS in endothelial cells (17), suggesting that different mechanisms may operate in HG-induced ROS generation in different cell types under different conditions. Our observation that NF-κB is activated through ROS in MC cultured under HG provides evidence that ROS can act as signaling molecules of HG as in other membrane receptor signaling (43,44).

Our observation that H$_2$O$_2$ activates NF-κB in a dose-dependent manner is different from previous studies showing lack of effect in murine and human MC (45,46). This difference is possibly due to difference in time frame of observation given the instability of H$_2$O$_2$ and transient nature of H$_2$O$_2$-induced NF-κB activation.

Increased PKC activity has been implicated in HG-induced vascular complications, including diabetic nephropathy (47). This study demonstrated that a PKC inhibitor and PKC depletion can significantly suppress HG-induced ROS generation as well as NF-κB activation, suggesting that PKC plays a role in HG-induced ROS generation leading to NF-κB activation. Our data agree with a recent report demonstrating PKC-dependent ROS generation in vascular cells cultured under HG (48). On the other hand, ROS can regulate the activation of PKC through redox changes in sulfhydryl groups of cysteine-rich regions of PKC or through activation of phospholipase D leading to production of diacylglycerol. Studer et al. (49) have demonstrated that antioxidants effectively inhibit PKC activation in MC cultured under HG. A recent study by Nishikawa et al. (8) also suggests the role of ROS in PKC activation in endothelial cells cultured under HG.

In agreement with previous reports indicating the requirement of NF-κB for full induction of MCP-1 (20–23), this study demonstrates that NF-κB is involved in the induction of MCP-1 in MC cultured under HG. Lack of MCP-1 mRNA and protein upregulation in IxBαM-tranfected MC cultured under HG and effective inhibition of HG-induced MCP-1 protein secretion by PKC inhibition or different antioxidants at doses inhibiting NF-κB activation are the supportive evidence. MCP-1, a specific chemoattractant for monocytes implicated in recruiting and activating monocyte/macrophage to the glomerulus in proliferative glomerular disease, has recently been recognized as a possible mediator in the development and progression of diabetic renal injury (24,25). Both HG (24) and glycated albumin (25) upregulated MCP-1 expression in human MC and urinary levels of MCP-1 increased with increasing albuminuria in diabetic patients (25). Although a recent report (50) demonstrated a possible role of NF-κB activation in intercellular adhesion molecule-1 expression in rat MC cultured under HG, the biologic significance of NF-κB activation in MC under HG remains to be elucidated.

In conclusion, HG rapidly activates NF-κB in MC through activation of PKC and generation of intracellular ROS and HG-induced NF-κB activation in MC may be one of the early mechanisms involved in the development and progression of diabetic nephropathy through upregulation of MCP-1.

**Acknowledgment**

This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF 981–0714–106–2).
References


