Mesangial cell NADPH oxidase upregulation in high glucose is protein kinase C dependent and required for collagen IV expression

L. Xia,1,2 H. Wang,1,2 H. J. Goldberg,2,3 S. Munk,1,3 I. G. Fantus,2,3 and C. I. Whiteside1,3

1University Health Network, 2Mount Sinai Hospital, and 3Department of Medicine, University of Toronto, Toronto, Ontario, Canada

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Xia, L., H. Wang, H. J. Goldberg, S. Munk, I. G. Fantus, and C. I. Whiteside. Mesangial cell NADPH oxidase upregulation in high glucose is protein kinase C dependent and required for collagen IV expression. Am J Physiol Renal Physiol 290: F345–F356, 2006. First published August 30, 2005; doi:10.1152/ajprenal.00119.2005.—Excess collagen IV expression by mesangial cells contributes to diabetic glomerulosclerosis. We hypothesized that in high glucose reactive oxygen species (ROS) generation by NADPH oxidase is PKC dependent and required for collagen IV expression by mesangial cells. In rat mesangial cells cultured in 5 mM (NG) or 25 mM d-glucose (HG), RT-PCR and Western immunoblotting detected p22phox and p47phox mRNA and protein, respectively. Quantitative real-time RT-PCR analyzed collagen IV mRNA. With the use of confocal microscopy, ROS were detected with dichlorofluorescein and intracellular collagen IV by immunofluorescence. In HG, ROS were generated within 1 h, sustained up to 48 h, and prevented by a NADPH oxidase inhibitor, diphenylenechloride iodonium (DPI), or a conventional PKC isozyme inhibitor, Go6976. In NG, phorbol myristate acetate stimulated ROS generation that was inhibited with DPI. In HG, expression of p22phox and p47phox was increased within 3 to 6 h and inhibited by Go6976. In HG, Go6976 or transfection with antisense against p22phox reversed the 1.8-fold increase in collagen IV mRNA. In HG, the antioxidants Tempol or Tiron, or transfection with antisense against p22phox or the 1.8-fold increase in collagen IV mRNA. In HG, the antioxidants Tempol or Tiron, or transfection with antisense against p22phox or p47phox, prevented ROS generation and the 2.3-fold increase in collagen IV protein. Increased mitochondrial redox potential in HG was unaffected by transfection with antisense against p22phox. We conclude that in HG, mesangial cell ROS generation by upregulated NADPH oxidase is dependent on conventional PKC isoforms and also required for collagen IV expression.

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The present study investigated the mechanisms whereby high glucose causes sustained activation of mesangial cell NADPH oxidase and the relationship between ROS generation and collagen IV production. We postulated that increased expression of the key regulatory NADPH oxidase subunits, p22phox, p47phox, and/or Nox4, occurs early during exposure to high glucose and that this response is dependent on diacylglycerol (DAG)-sensitive PKC isoforms. As we reported previously, mesangial cells express PKC-α, -β, -δ, -ε, and -ζ (23, 35, 57). In this study, we focused particularly on the role of the conventional PKC isoforms, PKC-α and -β. The interaction between mesangial cell ROS generation and PKC signaling and the consequent expression of collagen IV in high glucose were explored.

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MATERIALS AND METHODS

Materials. DMEM, trypsin, streptomycin, penicillin, Oligofectamine, and Lipofectamine-2000 were purchased from Invitrogen (Burlington, ON, Canada). FCS, d-glucose, t-glucose, Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), Tween 20, phorbol 12-myristate 13-acetate (PMA), bensamidine, and PMSF and mouse monoclonal antibody against β-actin were purchased from Sigma (St. Louis, MO). Caphostin C was purchased from Kymiya Biomedical (Seattle, WA). Go6976 was purchased from EMD Biosciences (San Diego, CA). Rabbit polyclonal antibodies against p47phox, p22phox, and Nox4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against collagen α (type IV) was obtained from Rockland Immunodiagnostics (Gilbertsville, PA). Enhanced chemiluminescence (ECL) was purchased from Amersham Biosciences (Buckinghamshire, UK). Horseradish peroxidase-labeled goat-anti-rabbit secondary antibodies and FITC-conjugated donkey anti-rabbit IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). Diphenylenechloride iodonium (DPI), Mito Tracker Green FM (MTG), Redox Sensor red CC-1 (RSR), and carboxymethyl-H2-dichlorofluorescin diacetate were from Molecular Probes (Eugene, OR). All the primers for RT-PCR were synthesized by The Center for Applied Genomics at The Hospital for Sick Children (Toronto, ON, Canada).

Cell culture. Primary rat mesangial cells were isolated from Sprague-Dawley rat kidney glomeruli and passages 11 to 17 were used in this study. Mesangial cells were grown in DMEM, pH 7.4, supplemented with 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES. After confluence reached 60–80%, mesangial cells were growth-arrested in 0.5% FCS for 48 h and treated with either 5.6 mM d-glucose, 25 mM d-glucose, or 24.4 mM l-glucose + 5.6 mM d-glucose for up to 48 h. To

Fig. 1. Reactive oxygen species (ROS) generation by mesangial cells detected by dichlorofluorescein (DCF). A: mesangial cells were cultured for 48 h in 5.6 mM d-glucose (NG), 5.6 mM d-glucose + 24.4 mM l-glucose (LG), or 25 mM d-glucose (HG). B: mesangial cells were cultured in NG or HG for 1, 3, or 48 h and treated with 1 µM DPI for 1 h or 300 nM Go6976 for 24 h. C: mesangial cells cultured in NG or HG for 48 h were exposed to 100 nM phorbol myristate acetate (PMA) for 24 h, with or without DPI. The graphs show mean DCF fluorescence intensity as a percentage of NG control (n = 90–290 cells from 3 independent experiments). *P < 0.001 vs. NG control (C). **P < 0.001 vs. HG 1 h or NG plus PMA. #P < 0.001 vs. HG 3 h. ##P < 0.001 vs. HG 48 h or HG plus PMA. Magnification bar = 25 µm.
examine the effect of antioxidants, mesangial cells were cocultured with 10 mM Tiron, that is specific for superoxide anion, or 100 nM Tempol, a superoxide dismutase mimetic (7, 8, 10, 15, 55).

To obtain total cell lysate, mesangial cells grown on 10-cm plates were lysed by boiling 2× sample buffer (0.130 M Tris-base, 20% glycerol, 4% SDS). The cells were then disrupted by passage through a 26-gauge needle and centrifuged 15,000 g for 5 min and the supernatant was collected as total cell lysate.

Western immunoblotting. Protein concentration in total cell lysate was determined using a modified Lowry microassay (Bio-Rad, Hercules, CA). Protein (30 μg) was loaded on to 10–15% SDS-PAGE gels, and blotting was performed with specific primary and secondary antibodies as previously reported (24). Equal loading of each lane was monitored by Ponceau-S staining of the membrane and by comparison to immunoblotting for β-actin in total cell lysate of the same samples. The blots were visualized by the ECL detection system. Data illustrated are representative of at least three independent experiments.

RNA isolation and semiquantitative RT-PCR analysis of p22phox and p47phox. After the mesangial cells were growth-arrested in 0.5% FCS for 48 h, total cellular RNA was isolated using TRIZol reagent (Invitrogen) or TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. For quantifying p22phox and p47phox transcripts, 2 μg of RNA were reverse transcribed into cDNA with a First-Strand cDNA Synthesis Kit (Fermentas, Hanover, MD) using 40 U of Moloney murine leukemia virus reverse transcriptase and 0.2 mM dNTP, 10% dimethylsulfoxide, 0.008 μM of each primer, and 2.5 U PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA). For p22phox and p47phox, parallel control PCRs were carried out with primers for 18S rRNA. PCR was preformed with an initial denaturation at 94°C for 3 min and cycles consisting of denaturation at 94°C for 1 min, annealing at the temperature indicated below for 50 s, and primer extension at 72°C for 40 to 50 s. This was followed by a final extension at 72°C for 10 min. All cycle numbers were in the linear range. p22phox (457 bp) was amplified using 29 cycles with an annealing temperature of 52°C and the primers were selected based on published sequences (58): sense primer 5′-CTC TAT TGC AGG AGT GC-3′ and antisense primer 5′-TCA CAC GAC CTC ATC TGT CAC-3′. p47phox (212 bp) was amplified using 36 cycles with an annealing temperature of 60°C and the primers were selected based on GenBank sequence, AY029167: sense primer 5′-ACG CTC ACC GAG TAC TTC AAC-3′ and antisense primer 5′-GTC GAT GGC CCG ATA GGT-3′. The 18S rRNA (187 bp) was amplified using 18 cycles with an annealing temperature of 60°C and the primers were selected based on sequences provided by Invitrogen and GenBank, M11188: sense primers 5′-CGG CTA CCA CAT CCA AGG AA-3′ and antisense primer 5′-GCT GGA ATT ACC GCG GCT-3′. The GeneAmp PCR System 2400 (Perkin-Elmer, Boston, MA) was used. PCR products were electrophoresed on 1.2 or 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet light and a charge-coupled device (CCD) camera using a Gel Doc 2000 instrument (Bio-Rad). The data illustrated are representative of at least three independent experiments.

Quantitative real-time RT-PCR of collagen I(IV) mRNA levels. Total RNA was isolated from mesangial cells using the RNeasy kit (Qiagen Mississauga, ON, Canada) and 2 μg were reverse transcribed with an Omniscript RT kit (Qiagen) using random primers in a total volume of 20 μl according to the manufacturer’s protocol. Real-time PCR was performed to measure the mRNA levels of α1(V) collagen and β-actin, an internal control, for each sample in separate wells in
duplicate on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using 2 μl of cDNA, 300 nM primers, and SYBR Green PCR Master Mix (Applied Biosystems). The parameters included a single cycle of 94°C for 10 min followed by 40 cycles of 95°C for 15 s, annealing, and 60°C for 1 min. The primers were: α1 (IV) collagen forward, 5'-ATCCCTTTGTGATG-CACACACAG-3'; α1 (IV) collagen reverse, 5'-AAGCTGTAAGCAT-TCCGTAGTA-3' (Genbank: XM_214400); β-actin forward, 5'-AGGCCTCTGAACTCTAG-3'; and β-actin reverse, 5'-CAACACAGGCGTGGCTAC-3' (Genbank: NM_031144). The primers crossed intron and exon junctions. Specificity of the PCR products was established by melting curve analysis and by running the products on a 1.5% agarose gel to verify the size. α1 (IV) collagen levels were expressed relative to the levels in 5.6 mM glucose-treated cells. ΔCt was determined by subtracting the mean Ct (threshold cycle) value derived from β-actin, which was not affected by the treatments, from the α1 (IV) collagen Ct. ΔΔCt was obtained by subtracting the mean ΔCt of the 5.6 mM glucose-treated cells from the ΔCt of each sample. Relative expression was then calculated using the equation 2^ΔΔCt (42).

Transfection of antisense oligonucleotides against p22phox and p47phox. Mesangial cells were grown to 70–80% confluence and transiently transfected with phosphorothioate ODNs against p22phox and p47phox using Lipofectamine-2000, according to the manufacturer's instructions. The sequence for the p22phox antisense oligonucleotide was 5'-GAT CCC CAT GGT GAG GAC C-3' (18), and the scrambled sequence was 5'-TAG GCA TAG GAC C-3' (18). The sequence for p47phox antisense oligonucleotide was 5'-GGT GTC CCC CAT GGC TGG GCA G-3' (GenBank AY029167, nt 5–26), and the scrambled sequence was 5'-TGA GGC TCC GTC CGC TGG AGC G-3'. ODNs were added to the cells at the concentration of 1 μM for 24–48 h in 5.6 or 25 mM glucose. The expression of p47phox and p22phox was quantified by Western immunoblotting.

Analysis of intracellular ROS production. The intracellular formation of ROS was detected by loading cultured cells with the fluoroprobe carboxymethyl-H2-dichlorofluorescein diacetate (CM-H2-DCF-DA), a nonpolar compound that is converted into a nonfluorescent polar derivative by cellular esterase after incorporation into cells. This compound is rapidly oxidized to fluorescent H2O2 (5). Briefly, mesangial cells were incubated for 30 min at 37°C with 1 mM CM-H2-DCF-DA, a nonpolar compound that is converted into a nonfluorescent polar derivative by cellular esterase after incorporation into cells. This compound is rapidly oxidized to fluorescent H2O2 (5). Briefly, mesangial cells were incubated for 30 min at 37°C with 1 mM CM-H2-DCF-DA in the presence of intracellular H2O2. A single image of 0.5 μm thickness was observed by dual-channel confocal fluorescence microscopy (Zeiss; excitation λ = 488 nm, emission λ = 515 nm). The expression of p22phox and p47phox was quantified by Western immunoblotting.

Confocal imaging of intracellular collagen α (type IV). Mesangial cells were grown-arrested in either 5.6 or 25 mM d-glucose. Cells were fixed in 3.7% formaldehyde and permeabilized by methanol for 10 min at −20°C. Cells were blocked with 1% goat serum plus 0.1% BSA and then the cells were incubated with rabbit polyclonal antibody against collagen α (IV) (1:500). FITC-conjugated (goat anti-rabbit) secondary antibody (1:100) were applied and detected by confocal fluorescence image analysis. To determine the specificity of binding, the primary antibodies were boiled before incubation with the permeabilized cells. No immunofluorescence labeling of the mesangial cells was observed using the boiled primary antibody (data not shown).

Localization of mitochondria and detection of redox potential. After growth-arrest in 0.5% FCS for 48 h, mesangial cells were incubated without FCS (DMEM) containing 250 mM RSR for 10 min and coincubated with 100 nM MTG for 30 min at 37°C to detect changes in redox potential and to localize mitochondria, respectively. The cells were analyzed using dual-channel confocal fluorescence microscopy (Zeiss; excitation λ = 488 nm, emission λ = 568 nm, and emission λ = 590 nm for RSR). The average pixel intensity per cell for each fluorescent probe was analyzed, and the ratio of those measurements for each cell was calculated using Scion image analysis software. To determine the overlap of RSR with MTG as a measure of change in redox potential localized in the mitochondria, the merged emission (yellow) was observed by dual-channel confocal fluorescence microscopy (32).

Statistical analysis. All values are expressed as means ± SE. Statistical analysis was performed using InStat 2.01 statistics software (Graph Pad, Sacramento, CA). A comparison of two groups was performed using the two-tailed unpaired Student’s t-test. The significance of results among groups was determined with one-way ANOVA with Bonferroni multiple comparisons test when multiple mean comparisons were required. P < 0.05 was considered significant.

RESULTS

Mesangial cell ROS generation in high glucose. Figure 1A illustrates the production of ROS in mesangial cells exposed to 5.6 mM d-glucose, 5.6 mmol/l d-glucose + 24.4 mM L-glucose, or 25 mM d-glucose for 24 h. Enhanced ROS generation was observed in high d-glucose but not in L-glucose. These data confirm our previous reports (13, 24, 35). Figure 1B demonstrates increased generation of ROS...
Fig. 4. Effects of PMA stimulation, calphostin C (Cal C), and Go6976 on NADPH oxidase subunit expression. A and B: representative images of RT-PCR or immunoblots demonstrating mRNA or protein in total cell lysate following 5.6 (NG) or 25 mM d-glucose (HG) for 48 h with or without 100 nM PMA for the final 24 h (n = 3–5 per group). *P < 0.05 vs. NG. **P < 0.01 vs. NG. #P < 0.001 vs. NG. C: representative images of RT-PCR for mRNA following NG or HG for 48 h or HG for 6 h with or without Cal C (1 μM for 24 h). *P < 0.05 vs. NG. ***P < 0.01 vs HG 6 or 48 h. D: representative immunoblots demonstrating in total cell lysates following NG or HG (24 h) with or without 1 μM Cal C or 300 nM Go6976 for 24 h. 18S rRNA was used as the internal control for mRNA, and β-actin was used as a loading control for protein. The graphs show NADPH oxidase subunits mRNA (ratio of 18S rRNA) or protein levels relative to NG (n = 3–5 per group). *P < 0.05 vs. HG. ***P < 0.01 vs. NG. ****P < 0.001 vs. NG. #P < 0.001 vs. HG.
within 1 h of exposure to 25 mM d-glucose, which was sustained up to 48 h. Incubation with the NADPH oxidase inhibitor (DPI) for 1 h (24) or preincubation of the cells for 24 h with a conventional PKC isozyme inhibitor, G6976 (34), completely inhibited the generation of ROS in high glucose. Figure 1C shows that in 5.6 mM d-glucose, or 25 mM d-glucose, 100 nM PMA for 24 h stimulated ROS production by three- or fivefold, respectively. These initial results with pharmacological inhibition suggested that high glucose-induced ROS production in mesangial cells may be due to PKC-stimulated NADPH oxidase activation.

Mesangial cell expression of p22phox, p47phox, and Nox4. In Fig. 2A, p22phox, p47phox, and Nox4 in mesangial cells exposed to 25 mM d-glucose for 1 to 48 h were analyzed by immunoblotting. A sustained increase in p22phox and p47phox protein content was observed between 3 and 6 h, but no changes in Nox4 or β-actin were detected. Figure 2B shows a representative response of p22phox and p47phox protein level to increasing concentration of d-glucose for 48 h in total cell lysates. Mesangial cell p22phox and p47phox content increased between 15 and 25 mM d-glucose with no change in Nox4.

High glucose-induced expression of p22phox and p47phox mRNA in the cells exposed to high glucose conditions, mesangial cells were exposed to 25 mM d-glucose for 1 to 48 h. As displayed in Fig. 3A, RT-PCR revealed an increase in the transcription of p22phox between 1 and 3 h and in p47phox between 3 and 6 h in high glucose, using 18S rRNA as a control in each sample. Exposure to 5.6 mM d-glucose + 24.4 mM l-glucose for 48 h had no effect on the levels of p22phox and p47phox mRNA (Fig. 3B).

To determine whether increased expression of NADPH oxidase subunits was dependent on DAG-sensitive PKC isozymes, mesangial cells in 5.6 or 25 mM d-glucose were stimulated with 100 nM PMA for 24 h. p22phox and p47phox mRNA levels increased during this treatment in normal glucose to a level similar to that observed in high glucose. PMA stimulated a small further increase in p22phox and p47phox mRNA in high glucose (Fig. 4A). The protein expression of p22phox and p47phox, but not that of Nox4, was also enhanced during stimulation with PMA in both normal and high glucose (Fig. 4B). Treatment with the general PKC inhibitor calphostin C (1 μM for 24 h) prevented the increase in both p22phox and p47phox mRNA and protein as early as 3 to 6 h following exposure to 25 mM d-glucose (see Fig. 4, C and D). G6976 prevented the increase in p22phox and p47phox protein levels (Fig. 4D).

High glucose-induced ROS production is dependent on NADPH oxidase. To determine whether the expression of p47phox or p22phox protein accounted for the increased ROS production by mesangial cells in high glucose, mesangial cells were transfected with antisense or scrambled ODNs against the p22phox and p47phox NADPH subunits in 5.6 or 25 mM glucose. Mesangial cells transfected with antisense ODNs demonstrated reduced expression of p22phox or p47phox protein in either normal glucose or high glucose by immunoblotting (Fig. 5).

Mesangial cells were cultured on coverslips, transfected with antisense ODNs against p22phox or p47phox in 5.6 or 25 mM d-glucose, and at 48 h were loaded with DCF. In high glucose, the intensity of DCF increased by 3.5-fold above that observed in normal glucose. Transfection with antisense ODNs against p22phox caused a decrease in intensity of DCF by 50% in normal glucose and by 90% in high glucose (P < 0.001 antisense vs. control or scrambled; Fig. 6A). Similarly, the intensity of DCF was reduced by 10% in normal glucose and 80% in high glucose following transfection with antisense ODNs against p47phox (P < 0.001 antisense vs. control or scrambled; Fig. 6A). Treatment of the mesangial cells with antioxidants, Tempol or Tempol, also prevented the increase in ROS during exposure to 25 mM d-glucose over 48 h (Fig. 6B).

Collagen IV expression in high glucose is dependent on NADPH oxidase. Intracellular collagen IV protein was increased in high glucose by 2.3-fold compared with normal glucose, as shown in Fig. 7. However, intracellular collagen IV protein was not changed following exposure to 5.6 mM d-glucose + 24.4 mM l-glucose compared with 5.6 mM d-glucose (Fig. 7A). Transfection of mesangial cells with ODNs specific for p22phox or p47phox prevented accumulation of collagen IV in high glucose (P < 0.001 high glucose antisense vs. high glucose control or scrambled; Fig. 7B). Treatment of the mesangial cells with the antioxidants,
Fig. 6. Reactive oxygen species production inhibition with antisense phosphorothioate ODNs against p22\textsuperscript{phox} and p47\textsuperscript{phox}. A: representative confocal microscopic images detecting DCF in control cells and following transfection with 1 μM scr or antisense ODNs against p22\textsuperscript{phox} or p47\textsuperscript{phox} in 5.6 (NG) or 25 mM d-glucose (HG). B: pretreatment with 10 mM Tiron or 100 nM Tempol for 48 h. The graphs show DCF fluorescence intensity as a percentage of NG control (n = 31–79 cells from 3 independent experiments). *P < 0.001 vs. NG control (C) or scr. **P < 0.001 vs. HG control (C) or scr. Magnification bar = 25 μm.
Tiron or Tempol, also effectively prevented the increase in collagen IV during exposure to 25 mM D-glucose over 48 h, as illustrated in Fig. 7C.

To determine whether the generation of ROS causes increased transcription of collagen IV mRNA, real-time RT-PCR was performed. The mRNA level was elevated in high glucose by 1.8-fold compared with normal glucose as shown in Fig. 8A. Transfection with ODNs specific for p22phox or p47phox in 5.6 (NG) or 25 mM D-glucose (HG). C: pretreatment with 10 mM Tiron or 100 nM Tempol for 48 h. The graphs show collagen IV immunofluorescence intensity as a percentage of NG control (n = 35–83 cells from 3 independent experiments). *P < 0.001 vs. NG control (C) or scr. **P < 0.001 vs. HG control (C) or scr. Magnification bar = 25 μm.

Mesangial cell mitochondrial redox potential. Mesangial cells were transfected with antisense ODNs against p22phox and then loaded with the two fluorescent probes, MTG and RSR. The fluorescence of MTG identified the location of the mitochondria. The RSR probe emits fluorescence following oxidation and becomes localized either in mitochondria or lysosomes. With the use of dual-channel confocal imaging, the ratio of the RSR to MTG fluorescence intensity in the same cell was calculated. As shown in Fig. 9A, no changes in RSR fluorescence or the MTG fluorescence were observed in the cells incubated in 25 mM D-glucose compared with 5.6 mM D-glucose. Mesangial cells transfected with antisense ODNs against p22phox showed no changes in either the RSR or MTG fluorescence. As documented in Table 1, no significant difference in the ratio of RSR/MTG fluorescence was detected between normal-glucose and high-glucose controls. There was no significant difference between the RSR/MTG fluorescence ratios for the cells transfected with antisense ODNs against p22phox and control or scrambled ODNs.

Figure 9B illustrates the merged images of the MTG- and RSR-labeled cells. The almost complete overlap of the two fluorescent probes detected by the yellow emission indicates that the RSR was located predominantly in the mitochondria. No changes in the merged patterns were apparent in the cells...
transfected with scrambled or antisense ODNs against p22phox in either normal or high glucose. A significant increase in the merged fluorescence emission was detected in high glucose as seen in Table 1, indicating increase mitochondrial ROS production in high glucose.

**DISCUSSION**

In this study, we examined the relationship between high glucose-induced mesangial cell production of collagen IV and PKC-dependent ROS generation. Our findings identify NADPH oxidase as the major source of ROS produced by mesangial cells in high glucose (38, 49). We also demonstrated that within 3 to 6 h of exposure to high glucose, mesangial cell expression of both p22phox and p47phox, but not Nox4, is upregulated as demonstrated by enhanced mRNA and protein levels. This increased expression was also found to be glucose concentration dependent. Although Kitada et al. (33) showed increased expression of p47phox and p67phox in glomeruli of diabetic rats, they identified the role of NADPH oxidase with DPI (48). Our study definitively identifies, using specific antisense reagents to p22phox and p47phox, that mesangial cells exposed to high glucose sustain ROS production by NADPH oxidase. Upregulation of NADPH oxidase subunits likely contributes to this response.

To determine the potential role of DAG-sensitive PKC in regulating the expression of NADPH oxidase subunits, we identified that acute stimulation of mesangial cells with PMA in normal glucose caused increased expression of p22phox and p47phox. Gö6976, which selectively inhibits PKC-α and -β, prevented the enhanced expression of p22phox and p47phox protein. Mesangial cell collagen IV protein expression in high glucose was prevented by inhibiting ROS production by either transfection with antisense ODNs against p22phox or p47phox or by treatment of the cells with the antioxidants Tempol or Tiron. The increase in mRNA levels of collagen IV in high glucose was prevented by both transfection with antisense ODNs against p22phox and treatment with Gö6976. We previously demonstrated that mesangial cell expression of collagen IV in high glucose during stimulation with endothelin-1 is dependent on PKC (23). In the present study, we now identify that production of ROS by NADPH oxidase is also PKC dependent. These data indicate that collagen IV expression also requires the generation of ROS by NADPH oxidase. Lee et al. (39)
reported that high glucose-induced expression of fibronectin by peritoneal mesothelial cells is due to the interaction of ROS generation and PKC signaling. Taken together with our results, these findings suggest that in high glucose the interaction of PKC signaling and ROS generation is necessary for enhanced expression of extracellular matrix proteins (see Fig. 10).

Decreased degradation via inhibition of mesangial cell matrix metalloproteinase also contributes to accumulation of collagen IV contributing to diabetic glomerulosclerosis. Accumulation of collagen IV protein in mesangial cells exposed to high glucose is, in part, due to decreased degradation which was not addressed in this study. ROS generation by mesangial cell NADPH oxidase is reported to increase the expression of the tissue inhibitor of matrix metalloproteinase (TIMP-1; see Fig. 10) that consequently inhibits collagen degradation (44, 61).

By contrast to mesangial cells, endothelial cells exposed to high glucose generate ROS through mechanisms involving mitochondria (6, 9, 45, 59, 60). In an attempt to determine whether mesangial cell mitochondrial function may be altered in high glucose, we colabeled mesangial cell mitochondria with MTG and RSR to identify their location and change in redox status, respectively. The semiquantitative analysis of these fluorescent markers using dual-channel confocal imaging revealed no change in the ratio of the two markers during high-glucose exposure or following transfection with antisense ODNs against p22phox (Fig. 9A; Table 1). The merged fluorescence images indicate that the MTG and RSR fluorescent probes overlapped almost completely demonstrating that the oxidized RSR was located predominantly in the mitochondria. The significant increase in the merged emission signal in high glucose compared with that observed in normal glucose (Fig. 9B; Table 1) suggests that mitochondrial production of ROS was likely increased. No change in the merged fluorescence pattern was observed following transfection of the cells with ODNs against p22phox. These data support the observation in high glucose the increased production of ROS in mesangial cells is predominantly, although not exclusively, from NADPH oxidase (41).

High glucose-induced ROS generation by mesangial cells is blocked by calphostin C (22) (see Fig. 10). Similar findings are reported for cultured aortic smooth muscle and endothelial cells (26). PKC-dependent activation of NADPH oxidase is, in part, dependent on phosphorylation of p47phox by PKC-α, -βII, -δ, and -ζ (12). Sustained activation of mesangial cell NADPH oxidase, generating ROS in high glucose, is associated with enhanced expression of the NADPH oxidase subunits as demonstrated in other nonphagocytic cells such as vascular smooth muscle cells (11, 37). Etoh et al. (11) reported increased expression of the NADPH oxidase subunits, NOX4 and p22phox, in the glomeruli and distal tubular cells of diabetic rats and that these findings are reversed by normalizing glucose levels with insulin treatment. Craven et al. (8) demonstrated that overexpression of Cu2+/Zn2+ superoxide dismutase protects against early diabetic glomerular injury in transgenic mice. Together, these findings strongly support the potential role of sustained ROS generation by NAPDH oxidase subunits, in the pathogenesis of early diabetic nephropathy.

ROS-directed extracellular matrix protein synthesis in high glucose has been described in mesangial cells and in diabetic glomeruli (19–21). In diabetes, TGF-β1 stimulates mesangial cell expression of extracellular matrix proteins, including collagen IV (19, 29), plasminogen activator inhibitor-1 (PAI-1), and downregulates genes encoding proteases (4). Hydrogen peroxide upregulates the expression of TGF-β1 (21, 25), PAI-1

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**Table 1. Mesangial cell mitochondria redox potential detected by double labeling with RSR and MTG**

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Control</th>
<th>Scrambled p22phox</th>
<th>Antisense p22phox</th>
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<tbody>
<tr>
<td>5.6 mM</td>
<td>0.70±0.02 (n=130)</td>
<td>0.75±0.02 (n=94)</td>
<td>0.75±0.02 (n=116)</td>
</tr>
<tr>
<td>25 mM</td>
<td>0.78±0.02 (n=157)</td>
<td>0.78±0.02 (n=134)</td>
<td>0.76±0.03 (n=102)</td>
</tr>
<tr>
<td>Merged fluorescence</td>
<td>80±4 (n=130)</td>
<td>83±4 (n=94)</td>
<td>90±3 (n=116)</td>
</tr>
<tr>
<td>5.6 mM</td>
<td>127±3*(n=157)</td>
<td>138±4*(n=134)</td>
<td>137±4*(n=102)</td>
</tr>
</tbody>
</table>

Values are means ± SE. RSR, Redox Sensor Red CC-1; MTG, Mito Tracker Green FM. *P < 0.001 vs. NG control.

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Fig. 10. Summary of signaling pathways involved in high glucose-induced collagen IV expression in rat mesangial cells. Generation of ROS from upregulated NADPH oxidase subunits p22phox or p47phox is dependent on conventional PKC isozyme activation and required for collagen IV expression.
(21, 29), and fibronectin (19). Antioxidants effectively prevent the upregulation of TGF-β1, PAI-1, and fibronectin in mesangial cells cultured in high glucose (25, 32) and in diabetic kidneys (24). Our findings indicate that ROS are required for mesangial cell accumulation of collagen IV in high glucose. The mechanism(s) may involve TGF-β1, or a more direct effect on the collagen IV promoter-specific transcription factors, e.g., activator protein-1 (AP-1), stimulated by ROS (54).

In summary, mesangial cells exposed to high glucose generate ROS predominantly through the activation of NADPH oxidase. Here, we identify that conventional PKC isozyme-dependent upregulation of the NAPDH oxidase subunits, p22phox and p47phox, is associated with a sustained increase in ROS production during high-glucose exposure (Fig. 10). ROS are necessary for high glucose-induced collagen IV expression in cultured mesangial cells. These findings support the potential role for conventional PKC isozyme inhibition and antioxidants in the prevention and treatment of early diabetic nephropathy.

REFERENCES


2. Baricos WH, Cortez SL, Deboisblanc M, and Xin S. Phosphorylation of p47phox directs phox homology domain 


