High glucose promotes mesangial cell apoptosis by oxidant-dependent mechanism

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Kang, Barinder P. S., Stanley Frencher, Venkatesh Reddy, Alex Kessler, Ashwani Malhotra, and Leonard G. Meggs. High glucose promotes mesangial cell apoptosis by oxidant-dependent mechanism. Am J Physiol Renal Physiol 284: F455–F466, 2003. First published November 5, 2002; 10.1152/ajprenal.00137.2002.—Reactive oxygen species (ROS) are recognized as important mediators of biological responses. Hyperglycemia promotes the intracellular generation of superoxide anion and hydrogen peroxide. In several cell lines, oxidant stress has been linked to the activation of death programs. Here, we report for the first time that high ambient glucose concentration induces apoptosis in murine and human mesangial cells by an oxidant-dependent mechanism. The signaling cascade activated by glucose-induced oxidant stress included the heterodimeric redox-sensitive transcription factor NF-κB, which exhibited an upregulation in p65/c-Rel binding activity and suppressed binding activity of the p50 dimer. Recruitment of NF-κB and mesangial cell apoptosis were both inhibited by antioxidants, implicating oxidant-induced activation of NF-κB in the transmission of the death signal. The genetic program for glucose-induced mesangial cell apoptosis was characterized by an upregulation of the Bax/Bcl-2 ratio. In addition, phosphorylation of the proapoptotic protein Bad was attenuated in mesangial cells maintained at high-glucose concentration, favoring progression of the apoptotic process. These perturbations in the expression and phosphorylation of the Bcl-2 family were coupled with the release of cytochrome c from mitochondria and caspase activation. Our findings indicate that in mesangial cells exposed to high ambient glucose concentration, oxidant stress is a proximate event in the activation of the death program, which culminates in mitochondrial dysfunction and caspase-3 activation, as the terminal event.

mesangial cell; reactive oxygen species; superoxide anion; nuclear factor-κB; mitochondria

CELL DEATH BY APOPTOSIS is a tightly orchestrated event under the control of genetic programs, which have been highly conserved during the evolutionary process (16). The identification of extracellular stimuli that promote cell death by apoptosis has become an area of intense investigation. Importantly, recent evidence that high-glucose concentration triggers the generation of reactive oxygen species (ROS) in mesangial cells (6, 18) raises questions concerning the effect of oxidant stress on mesangial cell survival. The enhanced production of free radicals has been linked to increased mesangial matrix deposition, increased glomerular volume, and urinary transforming growth factor-β excretion (6). These alterations in the growth phenotype and biochemical properties of mesangial cells are suppressed in genetically engineered mice (6) with constitutively activated SOD. ROS have also been implicated in the activation of death programs (42) and ischemic preconditioning (10, 48). The late phase of diabetic glomerulopathy is characterized by the loss of resident glomerular cells, sclerosis of glomeruli, and occlusion, events that correlate strongly with the decline in glomerular filtration rate (29). Cell death may occur by necrosis or apoptosis (4, 16). Necrosis is a process by which irreversible injury to the cell membrane results in the loss of structural integrity and the release of intracellular contents. Apoptosis, which is frequently not detected morphologically, is characterized by nuclear condensation, membrane blebbing, and the formation of apoptotic bodies (4, 16, 45). Mesangial cells possess the genetic program for apoptosis (33, 35–37), and this mechanism of cell death has been reported during the resolution phase of inflammatory glomerular lesions (2, 26). An important question concerns the effect of glucose-induced oxidant stress on mesangial cell survival and whether ROS generation by this mechanism activates the genetic program for apoptosis. Recruitment of the redox-sensitive transcription factor NF-κB, plays a pivotal role in the regulation of cell survival (19, 20, 22, 32, 39, 44, 46). Hyperglycemia has been reported to promote nuclear translocation and activation of NF-κB in several cell lines (27, 46). Presently, there are no data available concerning the effect of glucose-induced NF-κB activation on mesangial cell survival. Alternatively, the generation of ROS has been implicated in the activation of cell death programs (48), providing a rationale to examine the effect...
of oxidant stress on the fate of mesangial cells exposed to a high-glucose environment.

In the present study, we have employed an SV40-transformed murine glomerular mesangial cell (MCC) line and normal human mesangial cells (NHMCs) to investigate the effect of high glucose on mesangial cell survival. SV40 murine mesangial cells retain the phenotype and biochemical characteristics observed in wild-type mesangial cells (24, 43). NHMCs were harvested from normal human kidney tissue (BioWhittaker). To document the presence of oxidative stress, we first measured the activity of the antioxidant enzymes, SOD, and catalase in MCCs maintained at 5 and 25 mM glucose. To detect the presence of intracellular $O_2^-$ and $H_2O_2$, MCCs and NHMCs were loaded with the oxidant-sensitive dye 2,3,4,5,6 pentfluorodihydroxotetramethylrosamine (Redox Sensor red CC-1) (15). The cytotoxic effect of glucose-induced ROS generation was evaluated in the presence and absence of the free radical scavengers N-acetyl L-cysteine (NAC) and diphenyleleniodonium (DPI). To assess the effect of the prooxidant environment on the integrity of mitochondria, immunoblots were performed to evaluate the subcellular distribution of cytochrome c. Finally, we demonstrate that inhibition of ROS-dependent NF-κB activation protects against glucose-induced mesangial cell apoptosis.

**METHODS**

**Reagents.** NAC, DPI chloride, ascorbic acid, HEPES, penicillin, streptomycin, chelerythrine, and D-glucose were purchased from Sigma. Calyculin A, leupeptin, PMSF, and protease inhibitor cocktail were purchased from Calbiochem-Behring. O2 was obtained from the American Type Culture Collection and Lifesciences. All culture media were procured from GIBCO-BRL and BioWhittaker.

**MCC culture.** SV40-transformed MCCs (MES-13) were obtained from the American Type Culture Collection and maintained in a 3:1 mixture of DMEM and Ham's F-12 medium containing 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (14 mM), and glucose (100 mg/dl) at 37°C in an atmosphere containing 5% CO$_2$-95% air. Cultures were passaged twice a week with the above-mentioned medium. Under these conditions MES-13 exhibit the phenotypic characteristics of mesangial cells, including staining for desmin, vimentin, Thy 1, and types I and IV collagen by immunofluorescence (31, 43). After reaching 80% confluence before passage, the cell line was centrifuged onto the manufacturer's instructions. In brief, MCCs and NHMCs were plated in 24-well cell culture plates (Corning), and, after experimental treatments, cells ($2 \times 10^4$) were washed with PBS (pH 7.4). Attached MCCs and NHMCs were incubated with a cell lysis buffer (Roche Diagnostic) and centrifuged, and the resultant supernatant (20 μl; 2 mg/ml protein), which contained cytoplasmic histone-associated DNA fragments characteristic for apoptosis, was applied onto a streptavidin-coated microtiter plate. The peroxidase-conjugated anti-DNA antibody reacts with the DNA component of nucleosomes. After removal of unbound antibodies by washing, the amount of nucleosomes was quantitated by the peroxidase-dependent ELISA kit (Roche Diagnostic). The activity of peroxidase was determined photometrically with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] as a substrate. The values from quadruplicate absorbance (at 405 nm) measurements were averaged. The values were normalized and data are presented as ratios of experimental/control.

**In situ terminal deoxynucleotidyl transferase (TUNEL) assay.** A TUNEL assay was performed to study the double-stranded cleavage of DNA in MCCs. Briefly, cells grown on chambered culture slides at 5 and 25 mM glucose for 16 h were washed in PBS, fixed with 10% neutral buffered formalin for 1 h at room temperature, and incubated with protease K ($20 \mu g/ml$) for 30 min in a moist chamber at room temperature. Mesangial cells were washed again with PBS and covered with 50 μl of terminal deoxynucleotidyl transferase reaction mixture containing 5 units of terminal deoxynucleotidyl transferase, 1.5 mM CoCl$_2$, and 0.5 mM 2'-deoxyuridine-5-triphoshate coupled to biotin (biotin-16-DUTP). All reagents were purchased from Boehringer Mannheim Biochemicals. Cultures were incubated in this solution for 60 min at 37°C in a humidified chamber, washed in PBS, and then incubated in staining buffer [4× concentrated SSC buffer and 5% (wt/vol) nonfat dry milk] for 30 min at room temperature in a moist chamber. After incubation, the cells were exposed to the staining solution containing 5 μg/ml of FITC-labeled Extravidin (Sigma), $4 \times$ concentrated SSC buffer, and 5% nonfat dry milk for 30 min in a moist chamber,
washed with PBS, and finally mounted with Vectashield (Vector Labs) containing 4 ',6 '-diamidino-2-phenylindole dye to visualize nuclei. The staining was performed in quadruplets for each group, and 30 random fields (average 600 nuclei) were studied in each replicate. Double-stranded cleavage of DNA was determined by green (FITC) fluorescence in the nuclei.

**Catalase and SOD activity.** Cells (2 × 10⁵) were lysed in 0.5 ml of buffer (M-PER Mammalian Extraction reagent; Pierce) containing 0.1 mM Na₃VO₄, 10 mM NaF, 0.5 mM PMSF, 1% Nonidet P-40, and protease inhibitor cocktail set I (Calbiochem-Novabiochem). The lysate samples were kept on ice for 1 h and centrifuged at 10,000 rpm for 20 min, and the supernatants from different groups were used for catalase and SOD activities. A sample volume was normalized for an equal amount of proteins in cells cultured in 5 and 25 mM glucose for 16 h. Catalase activity was determined at 520 nm by using a catalase assay kit (Calbiochem-Novabiochem) in triplicate; enzymatic activity was calculated by using a catalase standard curve and expressed as catalase units per milligram protein. SOD activity was determined by employing a WST-1 assay kit (Calbiochem-Novabiochem). The SOD activity was calculated at 525 nm from the ratio of the absorption of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in the presence and absence of lysates.

**Immunofluorescent detection of glycoxidative stress.** Glucose-mediated oxidative stress in MMCs and NHMCs was studied by staining of 2,3,4,5,6-pentafluorodihydrotetramethylrosamine (PF-H₂TMRos or Redox Sensor red CC-1, Molecular Probes) using fluorescence microscopy. MMCs and NHMCs were maintained for 16 h under one of the following conditions: SFM+5 mM glucose, SFM+25 mM glucose, or SFM+25 mM glucose+NAC (50 µM). At the end of the incubation, cells were loaded at 37°C for 20 min with Redox Sensor red CC-1 (1 µM) and a mitochondria-specific fluorescent dye, MitoTracker green FM (50 nM; Molecular Probes). Redox Sensor red CC-1 is oxidized in the presence of O₂⁻ and H₂O₂. Culture slides were washed and mounted with PBS and visualized at ×40 magnification by using a Nikon fluorescence microscope (Nikon Eclipse E800) equipped with a triple filter cube and charge-coupled device camera (Nikon DXM1200). The staining was performed in quadruplets for each group, and 30 random fields (average 600 cells) were studied in each replicate. Images were captured by using Nikon ACT-1 (version 1.12) software and combined for publishing format with Adobe Photoshop 6.0 software.

**EMSA for NF-κB activity.** Nuclear extracts of mesangial cells were prepared with an NE-PER kit (Pierce). Approximately 5 × 10⁶ cells were used for each determination. Nuclear proteins were assayed for NF-κB p50 and p65/c-Rel DNA binding activity by using NF-κB/c-Rel gelshift plus assay kit (Geneika Biotechnology). The NF-κB and Rel ready-to-label wild-type double-stranded oligo probes were also supplied by Geneika Biotechnology. The oligonucleotides were labeled with ³²P[γ³²P]ATP and further purified by using a NUCTRAP probe purification column (Stratagene). For the oligonucleotide-protein complex (bandshift), nuclear extracts and purified hot probes were premixed and incubated at 10°C for 20 min. Unlabeled wild-type and unlabeled mutant oligonucleotides were included to determine the specificity of the competition bandshifts. The oligonucleotide-protein complex was immediately loaded on 5% nondenaturing acrylamide gel (38:2) precooled at 4°C in 1× Tris-borate-EDTA (pH 8.0) buffer, and the gels were electrophoresed at 100 V for 2 h, dried, and exposed for autoradiographic visualization. The bands were quantified by using the computerized image analysis software Un-Scan-IT (Automated Digitizing System).

**Immunoelectrophoresis analysis.** MMCs were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1 mM Na₃VO₄, 1 mM NaF, 0.5 mM PMSF, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 0.5 µg/ml leupeptin, and 0.5 µg/ml aprotinin. Samples were separated by 8% (wt/vol) SDS-PAGE for p53 and phospho-p53 Ser112 or 12% (wt/vol) acrylamide gels for Bax, Bcl-2, Bad, phospho-Bad Ser112, and phospho-Bad Ser136. Proteins were transferred onto nitrocellulose membranes by using a semidyed transfer cell apparatus (Bio-Rad). Primary rabbit polyclonal antibodies for Bax and Bcl-2 (1:800; Santa Cruz Biotechnology); Bad, phospho-Bad Ser112, phospho-Bad Ser136 (1:800; Cell Signaling Technology), and mouse monoclonal antibody for p53 (1:800; Santa Cruz Biotechnology) and phospho-p53 Ser392 (1:800; Calbiochem-Novabiochem) were used. For Bax, Bcl-2, p53, and phospho-p53 Ser392, secondary antibody was used at a dilution of 1:5,000. For Bad, phospho-Bad Ser112, and phospho-Bad Ser136, a dilution of 1,000 of secondary antibody was used. The blots were developed by using the ECL kit (Amersham-Pharmacia), and the bands were scanned and quantified as described above.

**Preparation of subcellular fractions.** MMCs were harvested from the cultures and fractionated into cytosol and mitochondria by using an ApoAlert cell fractionation kit (Clontech Laboratories). Briefly, cells were incubated with fractionation buffer (Clontech Laboratories) on ice for 10 min, homogenized in an ice-cold Dounce tissue grinder, and centrifuged at 700 g for 10 min at 4°C. The pellet was discarded and the supernatant was further centrifuged at 10,000 g for 25 min at 4°C. The supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were collected separately, and protein concentration in these fractions was determined by Bio-Rad assay. The fractions were subjected to SDS-PAGE (12% gels) to ascertain the separation of cytosolic and mitochondrial fractions. Anti-cytochrome c oxidase subunit IV (COX IV) antibody (1:1,000; Molecular Probes) was used to probe the protein COX IV as a marker in mitochondria and its absence in cytosol by Western blot analysis. For detection, horseradish peroxidase-linked secondary antibody was used at a dilution of 1:3,000. Fractions obtained from cytosolic and mitochondrial compartments were also probed with cytochrome c antibody by immunoelectrophoresis. Mouse monoclonal anti-cytochrome c antibody (BD Biosciences) was used at a dilution of 1:250, and the protein was detected by horseradish peroxidase-linked secondary antibody (1:3,000). Cytochrome c was detected and quantified in the cytosolic fraction from different groups as described above.

**Cleaved caspase-3 expression.** Lysates of MMC were subjected to 4–20% gradient gel electrophoresis, transferred onto nitrocellulose, and probed for the presence of cleaved caspase-3. For this, blots were incubated with rabbit polyclonal antibody (1:800; Cell Signaling Technology), washed, and treated with secondary antibody (1:5,000). The cleaved caspase-3 expression was quantified as described above.

**Statistical analysis.** Data are expressed as means ± SE. Comparison between two values was performed by unpaired Student’s t-test. For multiple comparisons among different groups of data, the significant differences were determined by the Bonferroni method. Significance was defined at P ≤ 0.05.

**RESULTS**

**High glucose promotes MMC apoptosis.** To determine the effect of high ambient glucose concentration on MMC survival, cells were plated and cultured in SFM containing 5 or 25 mM glucose for 16 h. Apoptotic cell death was quantified by immunoelectrophoresis analysis. MMCs were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1 mM Na₃VO₄, 1 mM NaF, 0.5 mM PMSF, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 0.5 µg/ml leupeptin, and 0.5 µg/ml aprotinin. Samples were separated by 8% (wt/vol) SDS-PAGE for p53 and phospho-p53 Ser112 or 12% (wt/vol) acrylamide gels for Bax, Bcl-2, Bad, phospho-Bad Ser112, and phospho-Bad Ser136. Proteins were transferred onto nitrocellulose membranes by using a semidyed transfer cell apparatus (Bio-Rad). Primary rabbit polyclonal antibodies for Bax and Bcl-2 (1:800; Santa Cruz Biotechnology); Bad, phospho-Bad Ser112, phospho-Bad Ser136 (1:800; Cell Signaling Technology), and mouse monoclonal antibody for p53 (1:800; Santa Cruz Biotechnology) and phospho-p53 Ser392 (1:800; Calbiochem-Novabiochem) were used. For Bax, Bcl-2, p53, and phospho-p53 Ser392, secondary antibody was used at a dilution of 1:5,000. For Bad, phospho-Bad Ser112, and phospho-Bad Ser136, a dilution of 1,000 of secondary antibody was used. The blots were developed by using the ECL kit (Amersham-Pharmacia), and the bands were scanned and quantified as described above.
death was detected by ELISA cell death assay, which detects histone-associated DNA fragments within the cytoplasmic fraction of cells with high specificity. As shown in Fig. 1, MMCs maintained in SFM + 5 mM glucose for 16 h exhibited a detectable baseline level of apoptosis. A 50% increase in apoptotic cell death was detected when the glucose concentration in the media was increased to 25 mM (P ≤ 0.01). To control for the potential effect of osmolarity on MMC apoptosis, in separate studies, cell death was assessed under osmolar equivalent conditions of 5 mM glucose and 20 mM mannitol for 16 h. The percentage of apoptotic MMCs was similar to baseline values detected in cells maintained in SFM + 5 mM glucose. As shown in Fig. 2, TUNEL staining confirmed the increased number of apoptotic nuclei in cells maintained in SFM + 25 mM glucose. Taken together, these results indicate that high ambient glucose concentration promotes the death program in MMCs.

**High glucose promotes intracellular ROS generation.** High ambient glucose concentration has been reported to alter the redox status of cells through the overproduction of ROS (27, 31). H2O2 is a toxic product of both aerobic metabolism and pathogenic ROS production. The heme-containing enzyme catalase metabolizes H2O2 by dismutation, resulting in the formation of O2− and H2O. O2− is the first product of the univalent reduction of oxygen. SOD catalyzes the dismutation of O2− by conversion to H2O2 + O2. To determine whether high ambient glucose concentration induces a prooxidant environment in MMCs, catalase and SOD activity were measured 16 h after plating in SFM at 5 or 25 mM glucose. As shown in Fig. 3, A and B, 25 mM glucose increased the activities of both catalase and SOD. The increment in catalase activity was 25% (P ≤ 0.01), whereas SOD activity increased twofold (P = 0.01). To provide a more direct assessment of oxidative stress, we performed additional experiments with the redox-sensitive dye Redox Sensor red CC-1. These studies were also performed in NHMCs to determine whether glucose promotes oxidative stress in this primary mesangial cell line. MMCs (Fig. 4, A–C) and NHMCs (Fig. 4, D–F) were loaded with Redox Sensor red CC-1 and the mitochondria-specific dye MitoTracker green FM. Redox Sensor red CC-1 is oxidized in the presence of O2− and H2O2. As shown in Fig. 4, B and E, bright yellow-orange fluorescence was seen in mitochondria due to the colocalization of oxidized red CC-1 dye (red fluorescence) and MitoTracker green FM dye (green fluorescence). This effect is inhibited by NAC (Fig. 4, C and F). The findings indicate that exposure of MMCs and NHMCs to 25 mM glucose alters the intracellular redox status by increasing the production of ROS.

**Role of ROS in glucose-induced apoptosis.** H2O2 has been reported to promote oxidant-induced apoptosis in rat mesangial cells (17). The prooxidant environment of MMCs and NHMCs maintained at 25 mM glucose implicates oxidant stress as a potential trigger for apoptosis. To determine whether glucose-induced intracellular ROS generation activates the death program, ascorbic acid and the cell permeable thiol antioxidants NAC and DPI were added separately to the culture media. As shown in Fig. 5A, NAC, DPI, and ascorbic acid reduced MMC apoptosis to baseline values, whereas the PKC inhibitor chelerythrine had no detectable effect on glucose-induced cell death. Figure 5B shows identical experiments at 5 mM glucose, indicating the absence of cytotoxic effects of the antioxidants and chelerythrine at the concentrations used. To determine whether NHMCs exhibit a similar pattern of glycoxidative stress, an identical experimental protocol was performed. As shown in Fig. 6, 25 mM glucose increased NHMC apoptosis by ~50% (P ≤ 0.001). The glucose-induced component of apoptosis is markedly attenuated by the antioxidants NAC, DPI, and ascorbic acid (P ≤ 0.001). Similar to MMCs, chelerythrine has no detectable effect on glucose-induced NHMC apoptosis. These findings strongly suggest that 25 mM glucose induces MMC and NHMC apoptosis by an oxidant-dependent mechanism.

**ROS-dependent activation of NF-κB.** In several cell lines, NF-κB has been identified as a target for ROS-dependent signals (27, 46). In the inactive state, NF-κB is sequestered in the cytoplasm, which is associated with an endogenous inhibitor protein of the IκB family (19). Diverse stimuli activate NF-κB through the phosphorylation of IKK. The NF-κB-IκB complex is phosphorylated by IKK, resulting in ubiquination and pro-
Fig. 2. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining of MMC apoptotic nuclei. MMCs were maintained in C or H for 16 h. A and D: phase-contrast images of MMCs maintained under C or H conditions, respectively. B and E: TUNEL-positive MMC nuclei. C and F: MMC nuclei labeled with 4',6''-diamidino-2-phenylindole dye, a nuclear-specific counterstain. Arrows, apoptotic nuclei. Magnification, ×40.

Fig. 3. Effect of 25 mM glucose on antioxidant enzymatic activity. MMCs were maintained in C or H for 16 h. Catalase (A) and SOD (B) activities were measured in C and H as an index of oxidant stress. Values are means ± SE and represent 3–4 independent experiments. *P ≤ 0.01, C vs. H.
teosomal degradation of IkB, promoting nuclear translocation of NF-κB. To determine whether NF-κB is activated by ROS-dependent signals in MMCs maintained in SFM +25 mM glucose, gel shift assays were performed with nuclear proteins and an NF-κB binding site-specific probe. As shown in Fig. 7, A and B, NF-κB binding complexes were detected in MMCs maintained at 5 and 25 mM glucose. The identity of the bands was determined by competitive bandshift analysis using unlabeled consensus or mutant oligonucleotide (Fig. 7, C and D). As shown in the densitometric analysis (Fig. 7F), nuclear proteins from mesangial cells maintained at 25 mM glucose exhibited an upregulation of p65/c-Rel binding (P < 0.001). This upregulation in p65/c-Rel binding activity was suppressed by the antioxidants NAC and DPI (P < 0.001). Conversely, as shown in Fig. 7E, p50 DNA binding activity was inhibited by 25 mM glucose (P < 0.001). Inclusion of antioxidants NAC and DPI restored p50 binding activity to baseline. NAC had no detectable effect on binding activities of p50 or p65/c-Rel dimers at 5 mM glucose. Taken together, the aggregate data indicate that in serum-starved MMCs, 25 mM glucose selectively activates the p65/c-Rel dimer of NF-κB by an oxidant-dependent mechanism. ROS-dependent signals appear to exert a deleterious effect on p50 binding, which can be reversed in the presence of antioxidants.

Expression of apoptosis-related factors: Bcl-2 family of proteins. To determine whether MMC apoptosis in response to 25 mM glucose is characterized by alteration in the Bax/Bcl-2 ratio and phosphorylation status of Bad and p53, immunoblot analyses were performed. As shown in Fig. 8A, the ratio of Bax/Bcl-2 was increased in lysates of mesangial cells maintained at 25 mM glucose. The upregulation of Bax/Bcl-2 ratio was completely prevented by NAC (P < 0.001). Interestingly, NAC has an inhibitory effect on Bax/Bcl-2 ratio at 5 mM glucose as well. The phosphorylation status of Bad (Fig. 8B and C) was also altered in MMCs maintained at 25 mM glucose. Phosphorylation at serine residues is recognized as a mechanism of inactivating the proapoptotic function of Bad (16, 30). As shown in Fig. 8B and C, phosphorylation at Ser112 and Ser136 was markedly attenuated in MMCs ex-
posed to 25 mM glucose. The inclusion of NAC in the culture media of MMCs at 25 mM glucose enhanced the phosphorylation at Ser112 and Ser136 of the Bad protein. Because NF-κB is known to regulate p53 expression (22, 44) and Bax is a target gene for p53 (23, 47), we examined the phosphorylation status of the p53 protein. Ser392 is located at the COOH terminus of p53 and linked to transcriptional activation (7). Phosphorylation of Ser392 was upregulated in MMCs exposed to 25 mM glucose (Fig. 8D), implicating p53 in the alterations of Bax and Bcl-2 expression (7). This upregulation of Ser392 phosphorylation was blocked by addition of antioxidants and PKC inhibitor on NHMC apoptosis. NHMCs were maintained under one of the following conditions for 16 h: C, H, H+inhibitor (HD, HN, HCL, and HA), and C+inhibitor (CN). The inhibitors added to the culture media were D (10 μM), N (50 μM), A (100 μM), and CL (2 μM). Apoptosis was detected by the ELISA cell death assay. For each assay, 20 μl of lysate (2.0 mg/ml) were used. Values are means ± SE and represent 6–8 independent experiments. *C vs. HN (P ≤ 0.05). ***C vs. H or HCL (P ≤ 0.001). ###C vs. HN, HD, HA, or CN (P ≤ 0.001).

Fig. 6. Effect of antioxidants and PKC inhibitor on NHMC apoptosis. NHMCs were maintained under one of the following conditions for 16 h: C, H, H+inhibitor (HD, HN, HCL, and HA), and C+inhibitor (CN). The inhibitors added to the culture media were D (10 μM), N (50 μM), A (100 μM), and CL (2 μM). Apoptosis was detected by the ELISA cell death assay. For each assay, 20 μl of lysate (2.0 mg/ml) were used. Values are means ± SE and represent 6–8 independent experiments. *C vs. HN (P ≤ 0.05). ***C vs. H or HCL (P ≤ 0.001). ###C vs. HN, HD, HA, or CN (P ≤ 0.001).

Fig. 8. Quantitative immunoblot analyses of Bax, Bcl-2, Bad, and p53 expression. MMCs were maintained in C, CN, H, and HN for 16 h. A: ratio of Bax and Bcl-2 protein expression in the above groups. B and C: ratios of phospho-Bad (Ser112)/Bad and phospho-Bad (Ser136)/Bad, respectively. D: ratio of phospho-p53/p53 protein expression in the same groups. The data shown are representative immunoblots of 3–5 independent analyses. *P < 0.001, C vs. H, HN, or CN. #P ≤ 0.01, H vs. HN or CN.

Fig. 7. Effect of glucose-induced ROS generation on NF-κB binding activity. MMCs were maintained under one of the following conditions for 16 h: C, H, HN, HD, or CN. Nuclear extracts were prepared from MMCs and analyzed for p50− (A) and p65/c-Rel−binding activity (B) by EMSA. C and D: competitive bandshift analysis confirming specificity of binding complexes in A and B. C, labeled oligonucleotide; Wt, cold oligonucleotide; and Mu, mutant oligonucleotide. Jurkat cells were used as positive control (+) for p50 and p65 dimers. E and F: densitometric analyses of p50 and p65 DNA binding activity. Values are means ± SE and represent 3–4 independent experiments. *P ≤ 0.05, ***P ≤ 0.001, C vs. H, HN, HD or CN. ###P ≤ 0.001, H vs. HN, HD, or CN.
of NAC to the culture medium. Taken together, the data indicate that in MMCs maintained at 25 mM glucose, oxidative stress promotes directional shifts in the expression and phosphorylation status of the Bcl-2 family of proteins that favors progression of the apoptotic process.

**Cytochrome c release and caspase activation.** To determine whether perturbations in the Bcl-2 family of proteins are coupled with the release of cytochrome c from the mitochondrial compartment and caspase activation, immunoblots were performed on cytosolic- and mitochondria-enriched fractions of MMCs. As shown in Fig. 9A, top, 25 mM glucose (H) increased the release of cytochrome c from mitochondria compared with MMCs maintained at 5 mM glucose (C). To control for possible contamination of cytosol by mitochondrial proteins, immunoblots were probed with an antibody against COX IV. To control for variations in loading conditions, Coomassie blue-stained gels (Fig. 9B) are shown that document equal loading conditions in cytosolic and mitochondrial fractions. As shown in Fig. 9A, bottom, COX IV was not detected in the cytosol of MMCs maintained at 5 or 25 mM glucose. The inclusion of NAC in MMC cultures maintained at 25 mM glucose markedly attenuated cytochrome c release (Figs. 9, A and C). Immunoblot analysis of the cytochrome c enriched mitochondrial fraction did not detect changes in cytochrome c content among the different groups.

**DISCUSSION**

In the present study, we demonstrate that in vitro exposure of MMCs and NHMCs to 25 mM glucose activates the genetic program for apoptosis. Glucose-induced apoptosis was independent of mechanical strain and osmolar forces and triggered by oxidant stress. Direct evidence is also provided for perturbations in the pro- and antiapoptotic members of the Bcl-2 family, culminating in the release of cytochrome c from mitochondria and caspase activation. Finally, we demonstrated that inhibition of the redox-sensitive transcription factor NF-κB prevents glucose-induced MMC apoptosis.

**High glucose promotes mesangial cell apoptosis.** Hyperglycemia dominates the pathophysiology and clinical course of type 1 and type 2 diabetes. Compelling evidence from the Diabetes Control and Complications Trial indicates that rigorous control of blood glucose reduces the risk of long-term microvascular complications (6a). In the present study, we demonstrate for the first time that high ambient glucose concentration activates the genetic program for MMC and NHMC apoptosis. The cytotoxic property of high glucose was independent of osmolar forces, because the percentage of apoptotic MMCs with an osmolar equivalent glucose-mannitol media did not differ from control values. The
latter property may serve to protect mesangial cells from transient elevations in osmolarity or reflect the unique ability of high glucose to trigger activation of intracellular signaling molecules involved in the expression of the death program. Hyperglycemia has recently been reported to induce apoptosis in cardiac myocytes (7, 8), whereas in vascular smooth muscle cells, hyperglycemia was found to protect against apoptotic cell death (14). These observations suggest that the cytotoxic properties of glucose vary across cell lines. Several factors may operate to limit detection of this form of cell death under in vivo conditions. First, cell loss is not a prominent characteristic during the early phases of diabetic glomerulopathy (38), in which thickening of the basement membrane and glomerular hypertrophy are the most characteristic deviations from normal (25). Second, during the late phases of this disorder, expansion of the mesangial matrix dominates the histological picture (28) along with sclerosis and occlusion of glomeruli (4, 29). Third, cell death by apoptosis does not result in sclerosis or residual scarring (16) and, in the absence of an immunocytochemical analysis, cannot be detected morphologically (7). Taken together, our finding that high glucose, independent of hemodynamic or physical forces, promotes mesangial cell apoptosis raises additional questions concerning the fate of mesangial cells in diabetic nephropathy.

**High glucose, ROS, and mesangial cell apoptosis.** Multiple lines of evidence have established a role for ROS as important mediators of cell biology (9, 21, 34). O$_2^-$ is the first product of the univalent reduction of oxygen. O$_2^-$ is converted to H$_2$O$_2$ and oxygen by SOD (6). Both O$_2^-$ and H$_2$O$_2$ have been reported as capable of activating death programs (42). Recent work has emphasized the importance of mitochondrial generation of ROS, in response to high-ambient-glucose concentration, as the trigger for hyperglycemia-related metabolic events, including the covalent modification of proteins by advanced glycation end products (27). In the present study, we demonstrate that high glucose also promotes the generation of ROS in MMCs and NHMCs, implicating ROS as potential mediators of glucose-induced mesangial cell apoptosis. Two main sites for the generation of ROS have been identified at the inner mitochondrial membrane, the NADH dehydrogenase at complex I, and the interface between ubiquinone and complex III (27). Although ROS are not classically thought of as signaling molecules, alterations in the redox status of cells has been shown to modulate the activation of transcription factors (9) and ionic channels (13). Previous work has documented that H$_2$O$_2$ activates the death program in mesangial cells (17); however, this is the first report demonstrating that high ambient glucose concentration promotes mesangial cell apoptosis by an oxidant-dependent mechanism. Although hyperglycemia is known to be a potent stimulus for the activation of PKC isozymes, which modulate a myriad of biological functions, the PKC inhibitor chelerythrine did not attenuate or increase MMC or NHMC apoptosis. Conversely, the antioxidant NAC, DPI, and ascorbic acid suppressed transmission of the death signal in both cell lines. Our findings are consistent with the growing body of work implicating ROS in the pathogenesis of diabetic complications (3, 12, 41). In this regard, the balance of evidence points toward the O$_2^-$ as the mediator of cell injury (6, 27); however, evidence also supports a role for the cytotoxic effects of H$_2$O$_2$ (17, 42). Taken together, the present study provides evidence that glycooxidative stress is an activating signal for the apoptosis gene program in mesangial cells.

**Oxidant-dependent NF-κB activation and mesangial cell apoptosis.** NF-κB comprises an inducible family of transcriptional factors that are important regulators of host immune and inflammatory responses. In addition, NF-κB-dependent signaling pathways have been implicated in cell survival (19, 20, 22, 32, 39, 44, 46). Stimulation of the NF-κB signaling pathway occurs by phosphorylation and degradation of the NF-κB inhibitory protein IκB, with subsequent translocation of NF-κB to the nucleus (5, 19). Our results indicate a differential effect of 25 mM glucose on NF-κB binding activity, which is ROS dependent. The upregulation in p65/c-Rel activity was markedly suppressed in the presence of NAC or DPI, implying that ROS preferentially target this dimer. Interestingly, p50 binding activity, which was downregulated by high glucose, was restored by NAC and DPI, suggesting an inhibitory effect of ROS on this dimer. Previous studies in other cell lines (27, 46) have documented an association between glucose-induced ROS generation and NF-κB activity, suggesting this transcription factor may modulate cellular responses to a high-glucose environment. The observation that NAC and DPI not only blocks the recruitment of NF-κB but also prevents MMC apoptosis is consistent with such a hypothesis. The cumulative results strongly suggest that hyperglycemia recruits NF-κB via ROS-dependent signals and implicates oxidant-dependent activation of NF-κB in the signaling cascade of glucose-induced MMC apoptosis.

**Oxidant stress and proapoptosis gene program.** A growing body of evidence supports the cytotoxic potential of ROS (12, 13, 42) and their direct participation in the activation of the death program (9, 17). The present study is the first report documenting that glucose-induced oxidant stress activates the genetic program for mesangial cell apoptosis. Until recently, the redox-sensitive transcription factor NF-κB was viewed as prosurvival and antiapoptotic (22, 44). It is now recognized that NF-κB may also assume a proapoptotic function through the regulation of apoptosis genes. The nuclear transcription factor p53 regulates proapoptotic gene programs (20, 32), and NF-κB has been reported to be a prerequisite for the induction of p53-mediated apoptosis (32). Although our data do not establish a cause and effect relationship between NF-κB and the genetic program for apoptosis, the marked attenuation of mesangial cell death in association with p65/c-Rel inhibition is consistent with such a hypothesis. Alternatively, we demonstrate phosphorylation of p53 at Ser392, a modification of the p53 protein linked with...
transcriptional activation (7). Our results also indicate that MMC apoptosis was accompanied by an increase in the Bax/Bcl-2 ratio, an alteration that favors progression of apoptosis (16). High-glucose concentration attenuated phosphorylation of Bad at Ser112 and Ser136. The latter finding is consistent with the proapoptosis function of Bad (30). Of note, the antioxidant NAC restored phosphorylation of Bad to levels detected at 5 mM glucose. The increase in p53 phosphorylation detected at 25 mM glucose was prevented by the addition of NAC, as was the increase in the Bax/Bcl-2 ratio. It seems reasonable to infer that either oxidant stress directly activates p53 (33) or NF-κB and p53 may cooperate to regulate the expression of Bax (20, 32, 44). Additional studies will be required to test this hypothesis.

Oxidant stress and mitochondrial dysfunction. The mitochondria are key determinants of cell death and cell survival (1, 11). Cytochrome c release by mitochondria and caspase activation are critical events in triggering oxidant-induced apoptosis (11). Anti- and pro-apoptotic proteins of the Bcl-2 family possess a COOH-terminal domain, which serves to target proteins to specific cell compartments (16). Several mechanisms are utilized by the antiapoptotic protein Bcl-2 to interrupt transmission of death signals, direct antioxidant effect, protein-protein interaction, and inhibition of cytochrome c release from mitochondria (16). This latter mechanism enables Bcl-2 to directly interfere with cytochrome c-dependent activation of caspases, a key event in the execution of the death signal. The antiapoptotic function of Bcl-2 may be neutralized by translocation of Bad and Bax from the cytosol to the mitochondria, with the subsequent formation of heterodimers (16, 42). Cytochrome c release is tightly regulated by protein-protein interactions among Bcl-2, Bax, and Bad (42). A growing body of evidence suggests that release of cytochrome c from mitochondria commits a cell to die by either apoptosis or necrosis (11). Cytochrome c was detected in the cytosolic fractions of MMC maintained at 25 mM glucose, strongly suggestive of an oxidant-induced mitochondrial dysfunction (1, 11, 42). Of note, we were unable to detect alterations in the cytochrome c content of mitochondrial fractions in experimental and control groups. This may be due to the enriched cytochrome c content of mitochondria compared with the relatively small fraction released to the cytosol. Moreover, the expression of cleaved caspase-3 was upregulated by 25 mM glucose. The antioxidants NAC and DPI markedly attenuated the glucose-induced upregulation of cleaved caspase-3 expression. Taken together, our data indicate that oxidant-induced perturbations in the Bcl-2 family of proteins facilitate the release of cytochrome c from mitochondria, initiating the terminal cascade of the death signal.

The present study has certain limitations. First, the duration of exposure to 25 mM glucose was brief compared with an in vivo model of hyperglycemia. Second, although beyond the scope of this investigation, infection of mesangial cells with constitutively active mutants of IκBα to establish whether NF-κB is necessary and sufficient for mesangial cell apoptosis was not performed. Moreover, we did not demonstrate increased p53 DNA binding activity to the p53-dependent gene Bax. Finally, the application of an in vitro system to study the fate of resident cells in the diabetic glomerulus may not mimic the in vivo condition. Future investigations directed at the issues not addressed in the present study will be important in further elucidating the molecular events that direct expression of the death program.

In summary, the results of the present study have established apoptosis as a biological response to high ambient glucose concentration in MMCs and NHMCs. As depicted in APPENDIX A, activation of the death program is ROS dependent, and recruitment of NF-κB is an integral component of the death signaling pathway. Although the precise mechanism by which NF-κB orchestrates the transmission of the death signal remains to be defined, inhibition of NF-κB binding activity by NAC was found to prevent mesangial cell apoptosis. Perturbations in the Bcl-2 family of proteins, cytochrome c release, and caspase activation are consistent with evolving concepts in which mitochondria are viewed as key determinants of cell survival and death. Future investigations must consider the in vivo consequences of hyperglycemia-induced oxidant stress on mesangial cell survival and the progression of diabetic nephropathy.

APPENDIX A

HIGH GLUCOSE

ROS

NF-κB

p53

Bax

Bad

Bcl-2

cytochrome c

Caspase↑


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