myo-Inositol Oxygenase Overexpression Accentuates Generation of Reactive Oxygen Species and Exacerbates Cellular Injury following High Glucose Ambience

A NEW MECHANISM RELEVANT TO THE PATHOGENESIS OF DIABETIC NEPHROPATHY*

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Diabetic nephropathy (DN) is characterized by perturbations in metabolic/cellular signaling pathways with generation of reactive oxygen species (ROS). The ROS are regarded as a common denominator of various pathways, and they inflict injury on renal glomerular cells. Recent studies indicate that tubular pathobiology also plays a role in the progression of DN. However, the mechanism(s) for how high (25 mM) glucose (HG) ambience induces tubular damage remains enigmatic. myo-Inositol oxygenase (MIOX) is a tubular enzyme that catabolizes myo-inositol to N-glucuronate via the glucuronate-xylulose (G-X) pathway. In this study, we demonstrated that G-X pathway enzymes are expressed in the kidney, and MIOX expression/bioactivity was up-regulated under HG ambience in LLC-PK1 cells, a tubular cell line. We further investigated whether MIOX overexpression leads to accentuation of tubulo-interstitial injury, as gauged by some of the parameters relevant to the progression of DN. Under HG ambience, MIOX overexpression accentuated redox imbalance, perturbed NAD+/NADH ratios, increased ROS generation, depleted glutathione, reduced GSH/GSSG ratio, and enhanced adverse changes in the profile of the antioxidant defense system. These changes were also accompanied by mitochondrial dysfunctions, DNA damage and induction of apoptosis. Accentuated activity of profibrogenic cytokine, and expression of fibronectin, the latter two being the major hallmarks of DN. These perturbations were largely blocked by various ROS inhibitors (Mito Q, diphenyleneiodonium chloride, and N-acetylcysteine) and MIOX/NOX4 siRNA. In conclusion, this study highlights a novel mechanism where MIOX under HG ambience exacerbates renal injury during the progression of diabetic nephropathy following the generation of excessive ROS via an unexplored G-X pathway.

Diabetic nephropathy (DN) is characterized by metabolic changes with perturbed cellular signaling events in renal cells followed by an increased synthesis of extracellular matrices (ECMs) and mitochondrial dysfunctions (1, 2). These changes are related to accentuation of cellular flux of glucose intermediates (3), polyols (4), protein kinase C activity (5, 6), expression of TGF-β (7), NF-κB binding and cell cycle proteins (6, 8), and generation of advanced glycation end products and reactive oxygen species (8–10). The latter are regarded as central to the pathogenesis of DN with glomerulosclerosis and tubulointerstitial fibrosis in the kidney (11, 12). With respect to glomerulosclerosis, Hascall and co-workers (13, 14) have proposed a novel paradigm suggesting hyperglycemia-induced hyaluronan synthesis that leads to autophagy and influx of monocytes causing inflammatory damage to the renal glomerulus. Such complex interrelated cellular signaling events, involving also various forms of MAP/ERK kinases and Smad proteins, have been investigated largely in glomerular cells (1–3, 15, 16), and the information concerning tubulo-interstitial cells is limited (17, 18). Of note is that all of the cellular elements of the kidney respond to hyperglycemia by activation of more or less similar intracellular signaling events with some variations depending on the expression of a given molecule (e.g. aldose reductase (ALR) and myo-inositol oxygenase (MIOX) in medullary and cortical tubular cells, respectively, and PKC-α and -β isoforms in glomeruli (19–21)). ALR is known to be involved in the pathogenesis of diabetic nephropathy, where an accentuated activity of the "polyol pathway" is seen with perturbed NADPH/NAD+ and NAD+ /NADH ratios, and as a result, the medullary tubular cells undergo a "redox imbalance" and notable oxidative stress. Along these lines, MIOX has been described to be up-regulated in high glucose ambience and hyperglycemic states (20, 22). Interestingly, in our exploratory studies on renal biopsies of patients with DN, we noted that up-regulation of MIOX is concomitantly associated with increased NADPH

*The abbreviations used are: DN, diabetic nephropathy; ECM, extracellular matrix; ΔΨm, mitochondrial membrane potential; ROS, reactive oxygen species; ALR, aldose reductase; MIOX, myo-inositol oxygenase; NOX, NADPH oxidase; G-X, glucuronate-xylulose; SOD, superoxide dismutase; MBB, monobromobimane; DPI, diphenyleneiodonium chloride; NAC, N-acetylcysteine; TMRE, tetramethylrhodamine ethyl ester; DCF, dichlorofluorescein; DCF-DA, dichlorofluorescein diacetate; COX, cyclooxygenase; mtDNA, mitochondrial DNA; HG, high (25 mM) glucose; LG, low (5 mM) glucose; EV, empty vector.

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Expression of MIOX and NOX4 in Tubules

FIGURE 1. Expression of MIOX and NOX4 in human DN, as assessed by immunohistochemistry (IHC). An increased expression of MIOX is seen in the "hypertrophic renal tubules" of a patient with DN (top left), as compared with a control non-diabetic patient (bottom left). Concomitantly, an increased expression of NOX4 is seen in the identical region of tubules in the patient with DN (top right). The control shows a very low level of NOX4 expression in the renal tubules (bottom right). The glomeruli of the DN patient show typical lesions of diabetic nodular glomerulosclerosis, but no increase in the expression of either MIOX or NOX4 (green arrowheads) is seen.

The assumption that G-X pathway enzymes are also expressed in tubules, one can envision the following sequence of events in the accentuation of tubular injury. First, the G-X pathway initiated by MIOX/ALR1 under high glucose ambi-
tude would be accompanied by mitochondrial dysfunctions and initiation of apoptotic events, whereas there would also be simultaneous activation of fibrogenic cytokine TGF-β with consequential fibrosis; in concert, both would probably exacerbate tubulo-interstitial injury in DN. We used these two major readouts (i.e. cellular apoptosis and excessive ECM synthesis) to assess the effects of MIOX overexpression because they are regarded as the known final outcomes in the progression of diabetic nephropathy.

Experimental Procedures

Reagents—The reagents were purchased from the following vendors. From Sigma-Aldrich, we purchased polyclonal anti-fibronectin (catalogue no. F3648), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalogue no. G9583), anti-NADH dehydrogenase 1 (NADH) (cataloque no. HPA001401), anti-SOD2 (cataloque no. HPA001814), anti-actin (cataloque no. H4600) and monoclonal anti-β-actin (cataloque no. A5448), antibodies; NADP+; NADH; culture media; monobenzoxylamine (MBB; reduced glutathione); diphenyleneiodonium chloride (DPI); N-acetylcycteine (NAC); and the caspase-3 assay kit. From Focus Biomolecules, we purchased anti-GAPDH (catalogue no. J-005). From Cell Signaling Technologies, we purchased anti-MIOX IV antibody (catalogue no. 3E11) and cleaved caspase-3 antibody (catalogue no. D175). From Santa Cruz Biotechnology, Inc., we purchased mouse monoclonal anti-p53 (catalogue no. sc422), anti-catalase (cataloque no. sc50508), anti-Bcl2 (catalogue no. sc492), and anti-Bax (catalogue no. sc526) antibodies and NOX4 siRNA (cataloque no. sc41586). From Abgent, we purchased ALR1 antibody (catalogue no. AF1050a). From Abcam, we purchased anti-glutathione peroxidase antibody (catalogue no. ab27325) and the NAD/NADH assay kit. From Cayman Chemicals, we purchased the glutathione assay kit. From Molecular Probes, we purchased DAPI, tetramethylrhodamine ethyl ester (TMRE), dichlorofluorescein diacetate (DCF-DA), and Mitotracker Red. From Life Technologies, Inc., we purchased TO-PRO-3. From Assaypro, we purchased DAPI, tetramethylrhodamine ethyl ester (TMRE), dichlorofluorescein diacetate (DCF-DA), and Mitotracker Red. From Life Technologies, Inc., we purchased TO-PRO-3. From Cell Signaling Technologies, we purchased DAPI, tetramethylrhodamine ethyl ester (TMRE), dichlorofluorescein diacetate (DCF-DA), and Mitotracker Red.
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**Immunohistochemical Studies on Kidney Tissues**—For these studies, the ABC (avidin–biotin complex) method (Vector Laboratories) was employed. Four-μm-thick kidney sections were deparaffinized by xylene treatment and then hydrated in graded series of decreasing concentrations of ethanol. They were then successively incubated with rabbit anti-MIOX, anti-NADPH oxidase 4 (NOX4), or anti-ALR1 (Abgent), biotinylated anti-rabbit IgG, and streptavidin conjugated with horseradish peroxidase (HRP) with interspersed brief washes with PBS. In some experiments, a heat-induced epitope retrieval (antigen retrieval) method, using R-buffers (EM sciences), was employed to enhance the detection of antigens expressed in the kidney. The sections were then treated with SIGMAFAST™ 3′,3′-diaminobenzidine solution for 3–5 min at 22 °C to develop the HRP reaction product. The sections were counterstained with hematoxylin, coverslip-mounted, and examined.

**Cell Culture Studies**—The LLC-PK1 cell line (a renal tubular cell line) was purchased from ATCC. The pcDNA3.1 vector, Lipofectamine 2000, G418, Zeocin, and psiRNA-h7SKGFpzeo G1 vector were obtained from Invitrogen and InvivoGen Corp. The cells were grown in M199 medium with basal low concentration of D-glucose (5 mM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. They were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For high glucose ambience experiments, the cells were exposed to 5–25 mM D-glucose for 12–15 h, whereas L-glucose served as a control. In some experiments, the time period was extended to 24–30 h.

**RT-PCR Analyses of Kidney Tissues and LLC-PK1 Cells**—Total RNA was isolated from tubular enriched fractions of 10 murine kidney cortices free of medulla by the acid guanidinium thiocyanate-phenol-chloroform extraction method (32). Similarly, total RNA was also isolated from LLC-PK1 cells maintained in culture Petri dishes. The isolated RNA was precipitated in the presence of RNase-free glycogen (20 mg/ml) and then pelleted by centrifugation at 10,000 × g. The pellet was resuspended in 0.5 ml of DNase buffer (0.1 M sodium acetate, 5 mM MgSO₄, pH 5.5) and digested with 100 units of RNase-free DNase (Invitrogen), in the presence of RNasin (1 unit/ml; Promega). Following a phenol-chloroform extraction and ethanol precipitation, the RNA was reprecipitated with ethanol. About 2 μg of total RNA was subjected to the first strand cDNA synthesis using Moloney murine leukemia virus-RT and oligo(dT)₁₅ primer. The cDNA was suspended in 250 μl of 10 mM Tris-EDTA buffer, pH 7.4, and kept at −70 °C until further use. PCR was performed using 2 μl of cDNA and Taq DNA polymerase (5 units/μl) by employing standard protocol conditions provided by the vendor (Invitrogen). Relative sense and antisense primers for various enzymes of the G-X pathway were as follows: 5′-AGA CCC TTC CCT GGT CTA TCG A-3′ and 5′-TAA AGA CAC GATCCA GCA GCG-3′ (MIOX, PCR product ~93 bp); 5′-AGT AGC CAG TCC TGG TTAAG A-3′ and 5′-TTT CGG CTG AAC CTG CCA T-3′ (ALR1; PCR product ~100 bp); 5′-GGC TCA GTG GAA GCA GTG CAT-3′ and 5′-TTT GTCG CCTT TCGT GCG GAG-3′ (l-glutamate dehydrogenase; PCR product ~109 bp); 5′-TCA ATG TCG TTC CGG TGC TT-3′ and 5′-CGG CGG TGT TGA CGT AGT ATT T-3′ (d-xylulose reductase; PCR product ~100 bp); 5′-G GTG TCA GAT GTC TGC GAC CAA G-3′ and 5′-TAT GGG TGG TCA GTG CAC GTT G-3′ (l-xylulose reductase; PCR product ~100 bp); 5′-TCA GCA GCG AGG TTA AAG-3′ and 5′-CTT GAG TTC CAA ATT CAT GAA GAA-3′ (xylulose kinase; PCR product ~104 bp).

**Generation of Prokaryotic Constructs and Stable Transfectants**—A porcine MIOX cDNA was generated by 5′-PCR using sense (5′-GGCGGGGAAATTCAGAGAACCCAGACCCCTTC-3′) and antisense (5′-GGCGGGGAAATTCCTACCCAGACGACGACACCGGGGC-3′) primers and cloned into pcDNA3.1 digested with EcoRI. The expression orientation of pcDNA3.1-RSOR/MIOX vector was identified by nucleotide sequencing. For MIOX in vitro gene disruption experiments, primer sequences of complements cDNA strands were designed using the siRNA Wizard program. The respective primer sequences were 5′-ACC TTC TCG AAA CCG GTG GTA GAA AGC GAT TGA GTA CGG AAA GAG TCT ACC ACG CGC GGC TGC TTT-3′ and 5′-CAA AAA GAA ACC GCG TGT AGT GGT AGA TCT TTC ATC CAC TAC TAC AGC CGG TTT CG-3′. Oligonucleotides were synthesized by IDT Technologies. Following annealing of complementary strands, dsDNA was cloned into psiRNA-h7SKGFpzeo G1 plasmid digested with BbsI/BbsI (InvivoGen). RSOR/MIOX siRNA was designed as psiRNA-h7SKGFpzeo G1-RSOR/MIOX. The empty plasmid served as a control. The empty plasmid and the one containing siRNA construct were then transfected into LLC-PK1 cells, and stable transfectants were selected by growing cells in the presence of 800 μg/ml G418 for 4–5 min, followed by centrifugation at 12,000 g/ml) by employing standard protocol conditions provided by the vendor (Invitrogen). Relative sense and antisense primers for various enzymes of the G-X pathway were as follows: 5′-AGA CCC TTC CCT GGT CTA TCG A-3′ and 5′-TAA AGA CAC GATCCA GCA GCG-3′ (MIOX, PCR product ~93 bp); 5′-AGT AGC CAG TCC TGG TTAAG A-3′ and 5′-TTT CGG CTG AAC CTG CCA T-3′ (ALR1; PCR product ~100 bp); 5′-GGC TCA GTG GAA GCA GTG CAT-3′ and 5′-TTT GTCG CCTT TCGT GCG GAG-3′ (l-glutamate dehydrogenase; PCR product ~109 bp); 5′-TCA ATG TCG TTC CGG TGC TT-3′ and 5′-CGG CGG TGT TGA CGT AGT ATT T-3′ (d-xylulose reductase; PCR product ~100 bp); 5′-G GTG TCA GAT GTC TGC GAC CAA G-3′ and 5′-TAT GGG TGG TCA GTG CAC GTT G-3′ (l-xylulose reductase; PCR product ~100 bp); 5′-TCA GCA GCG AGG TTA AAG-3′ and 5′-CTT GAG TTC CAA ATT CAT GAA GAA-3′ (xylulose kinase; PCR product ~104 bp).

**Western Blotting Studies**—Cells subjected to various treatments were lysed with ice-cold radioimmunoprecipitation assay lysis buffer, containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 2 mM Na₃VO₄, 5 mM Na₂P₂O₇ containing protease inhibitor mixture (Sigma-Aldrich), for 30 min at 4 °C. Lysates were homogenized in a Dounce homogenizer for 4–5 min, followed by centrifugation at 12,000 × g at 4 °C for 10 min. Supernatants were collected for protein expression studies, and the protein concentration of each sample was measured using the Bradford method. After adjusting protein concentration (100 μg/100 μl) in each of the samples, equal amounts of protein (20 μg) from each of the experiments were dissolved in SDS sample buffer, boiled, and subjected to SDS-
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Determination of the Source of Intracellular ROS Generation in MIOX-overexpressing Cell Line and the Effect of NOX siRNA on ROS under High Glucose Ambience—ROS generation was measured using fluoroprobe CM-H$_2$DCF-DA as indicated above. The dye following its diffusion into the cell becomes deacetylated by cellular esterase and then oxidized by intracellular ROS into a fluorescent compound, DCF, which was detected by flow cytometry. About 2 × 10$^5$ LLC-PK1 cells or MIOX-overexpressing LLC-PK1 cells were seeded into 6-well plates and allowed to attach overnight. MIOX-overexpressing LLC-PK1 cells were then treated with ROS inhibitors Mito Q (1 μM) or DPI (10 μM) for 6 h. These inhibitors have been described to inhibit the mitochondrial and NADPH oxidase ROS generation, respectively (38–40). In another set of experiments, cells were transfected with either NOX4 siRNA, scramble oligonucleotide, or NAC (10 mM). They were then maintained in culture for 24 h under high glucose ambience. Cells were gently scraped off from the plate with a rubber policeman and transferred into FACS tubes for staining. A 5 mM stock of CM-H$_2$DCF-DA in DMSO was diluted in PBS to make a final concentration of 5 μM. Cells were then stained with 5 μM CM-H$_2$DCF-DA for 15 min at 37 °C in dark. They were washed twice with PBS and resuspended into 300 μl of PBS and processed for acquisition of fluorescence by a flow cytometer. Flow cytometric analyses were performed using standard operational procedures, and the fluorescence of a total of 10,000 cells was acquired for each sample. Mean fluorescence intensity of the CM-H$_2$DCF-DA was determined by employing FACSDiva software (BD Biosciences).

Assessment of Cellular Distribution of Cytochrome c—Confocal microscopy was employed to delineate the distribution of cytochrome c, as described previously (41). Cells were incubated with 25 nM Mitotracker dye (MitoTracker Red Molecular Probes, Inc.) at 37 °C for 10 min. Slides were washed with PBS and fixed in 4% formaldehyde. They were then incubated with primary anti-cytochrome c antibody followed by incubation with FITC-labeled secondary antibody. Cells were also subjected to DAPI staining to visualize the nuclei. Images were taken using different excitation filters and merged.

In addition, confocal microscopy was performed for co-localization of cytochrome c and COX IV. The COX IV is considered a marker of mitochondrial activity that does not dissociate from this organelle following oxidant stress or H$_2$O$_2$ treatment (42, 43). Briefly, 2 × 10$^5$ cells were seeded on coverslips in a 6-well plate and allowed to attach overnight. Cells that had undergone various treatments were fixed with 4% paraformaldehyde for 5 min in ice and washed three times with PBS. They were then permeabilized with ice-cold methanol for 10 min at −20 °C.

Cells were washed twice in PBS and incubated in a blocking solution (1× PBS, 1% BSA, 0.3% Triton X-100) for 1 h. After blocking, fixed cells were immersed in a solution containing rabbit anti-COX IV or mouse anti-cytochrome c antibody diluted in blocking solution for 2 h at 22 °C. Cells were then washed three times with PBS and incubated in FITC or rhodamine-conjugated secondary antibodies for 1 h in the dark. FITC-conjugated anti-rabbit IgG was used for labeling COX IV, and rhodamine-conjugated anti-mouse IgG was used for labeling cytochrome c. Cells were then washed three times with PBS. Cellular nuclei were stained with 0.5 μg/ml DAPI for 5 min. Cells were re washed twice with PBS. Coverslips were then mounted with a drop of DAKO fluorescent mounting medium, and images were obtained using a Nikon A1R spectral confocal microscope.

Assessment of Mitochondrial Membrane Potential ($\Delta \Psi_m$)—To measure the loss of $\Delta \Psi_m$, confocal microscopy of cells stained with TMRE was employed (44). For these analyses, LLC-PK1 cells subjected to various treatments, high glucose versus low glucose, and those overexpressing MIOX and subjected to siRNA treatments were plated onto 35-mm glass bottom culture dishes (MatTak Corp.) and maintained at 37 °C in a defined medium. After high glucose treatment, the cells were placed in a phenol red-free DMEM (Corning Technologies, Inc.) containing 50 nM TMRE for 30 min and then examined by confocal microscopy. To visualize the nuclei, cells were stained with DAPI.

Assessment of Mitochondrial Ultrastructural Morphology—Electron microscopy was employed to delineate the ultrastructural integrity of the mitochondria of LLC-PK1 cells subjected to various treatments. Treated cells were immediately immersion-fixed in Noyes’s paraformaldehyde-glutaraldehyde fixative for 4 h at 4 °C. They were then gently scraped from the culture Petri dishes, following which, a pellet was prepared by centrifugation at 10,000 × g for 10 min. The pellet was treated with 0.5% uranyl acetate, dehydrated in a graded series of ethanol, and then processed for embedding in Epon (44). About 0.5-μm-thick sections were prepared and stained with 1% toluidine blue. The area representing a maximum number of cells with en face view were selected for thin sectioning with an average thickness of 60 nm and examined with an electron microscope at 80 kV. Ten cells/variable were photographed at a magnification of ×5,000, and 25 mitochondria were measured along the long axis. We designated intactness of mitochondria based on the morphology of a majority of mitochondria (>70%) that were more than 1 μm in length along the long axis in control cells, as described previously (45).

Detection of Mitochondrial DNA (mtDNA) Damage—Damage to high and low molecular weight mtDNA was evaluated by PCR amplification procedures, as described previously (46). Long range PCR was used to co-amplify high and low molecular weight mtDNA fragments in LLC-PK1 cell culture samples. For high molecular weight DNA, PCR primers used were as follows: 5′-ATT GCG AGA CGC TGG AGC CG-3′ (sense) and 5′-TAC CCC CGG TCG TGT AGC GG-3′ (antisense). Primers for low molecular weight DNA were as follows: 5′-GGG GTT ATG CAC GCG ATA GCA-3′ (sense) and 5′-GGG TGAGGCC GGT GTA TTG-3′ (antisense). PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide to visualize the ~8- and ~0.45-kb DNA products. Band intensity was measured with a PhosphorImager (Molecular Dynamics).

Assessment of Apoptosis—Samples from LLC-PK1 cells having undergone various treatments were evaluated for the extent of apoptosis. An in situ cell death detection kit (Roche Applied Sciences) was employed, in which nicked or blunt ends of the DNA strands or the 3′-OH ends of the genomic DNA are labeled with fluorescein-dUTP, and the reaction is catalyzed by...
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terminal deoxynucleotidyltransferase. This procedure is also
known as the TUNEL method. For these studies, the cells were
immersion-fixed in 4% phosphate-buffered (pH 7.4) parafor-
maldehyde solution for 20 min at 22 °C. They were then per-
meabilized with 0.1% Triton X-100 at 4 °C for 1 min and pro-
cessed for the TUNEL reaction as per instructions provided by
the manufacturer and examined with a UV microscope
equipped with epi-illumination.

Caspase-3 Activity Assay—The assay was performed using a
colorimetric caspase-3 assay kit (Sigma). The assay is based on
hydrolysis of peptide substrate acetyl-Asp-Glu-Val-Asp p-ni-
troanilide by caspase-3 into p-nitroaniline, which has maxi-
mum absorbance at 405 nm. Briefly, 5 × 10⁶ empty vector-
transfected LLC-PK1 and MIOX-transfected LLC-PK1 cells
were seeded overnight. Cells were treated with HG (25 mM) and
NAC (10 mM) for 24 h. Cells were scraped off from plates and
pelleted by centrifugation. The pellet was washed with PBS
and suspended in 100 μl of 1× supplied lysis buffer. Cells were
then incubated on ice for 20 min with intermittent gentle vor-
texing. Lyzed cells were then centrifuged at 16,000 × g for 10
min at 4 °C. Supernatants were transferred into a new tube and
used for the caspase-3 activity assay. Each sample was assayed
in duplicate in a 96-well plate in 200-μl reaction volume. 10 μl
of lysate containing 100 μg of protein/sample, 170 μl of 1×
assay buffer, and 20 μl of caspase-3 substrate were added and
mixed by gentle shaking. Wells were then covered with adhe-
sive film and incubated at 37 °C for 6 h. Absorbance was read at
405 nm after 6 h.

Measurement of Fibronectin and TGF-β in Culture Medium
and Cellular Expression of Fibronectin—Quantitative measure-
ments of both of the parameters was performed using a cul-
ture medium. Fibronectin in the culture supernatant of condi-
tioned medium was measured by using a quantitative competitive
ELISA kit according to the vendor's instructions (Assaypro), and it was expressed as ng/ml of total cellular protein.

TGF-β1 in culture supernatants of conditioned medium
(100 μl) was first activated by the addition of 20 μl of 1 N HCl
and then incubated at 25 °C for 10 min followed by neutraliza-
tion with 20 μl of 1 N NaOH. Total TGF-β1 was measured using
a sandwich ELISA kit according to the manufacturer's instruc-
tions (Assaypro), and its amount in the samples was expressed
as pg/ml of total cellular protein. In addition to the amount of
TGF-β1 in the culture medium, its bioactivity was determined
using a mink lung epithelial cell line stably transfected with
plasmogen activator-inhibitor-1 promoter linked to the lucif-
erase reporter gene (30). Mink cells were seeded onto a 96-well
tissue culture plate (1.6 × 10⁴/well) and allowed to adhere for
3 h. A serial dilution of TGF-β1 standard or conditioned media
from LLC-PK1 cells (1:1 dilution) was added to the
medium of the mink cells and incubated for 15 h. Luciferase
activity was determined using Promega Luciferase assay re-
agent, as described previously (31). Values obtained in various
samples were compared with TGF-β1 standards. For cellular
fibronectin expression, cells were treated with cold methanol at
−20 °C for 4 min and rinsed twice with PBS solution. After
blocking in 3% of bovine serum albumin, the cells were incu-
bated with mouse monoclonal anti-fibronectin at 4 °C for 12 h,
washed with PBS twice, and then re-incubated with anti-mouse
IgG conjugated with FITC at 22 °C for 4 h. Then sections were
rewashed with PBS and counterstained with TO-PRO-3-iodo-
dide to demarcate the nuclei.

Statistical Analysis—All results are depicted as mean or
mean ± S.D. Data were analyzed by one-way analysis of vari-
ance, and a p value of <0.05 was considered statistically
significant.

Results

The findings described here include events that indicate that
following increased expression of MIOX in tubular cells under
high glucose ambience, there is an accentuated perturbation in
regional redox and mitochondrial homeostasis leading to cellu-
lar apoptosis. In addition, there is an increased synthesis of
ECM proteins, reflective of tubulo-interstitial injury in diabetic
nephropathy.

Expression of G-X Pathway Enzymes—By immunohisto-
chemical and RT-PCR analyses, the existence of a G-X pathway
in kidney cortical tubular cells was explored. Antibodies to the
first two enzymes (i.e., MIOX and ALR1) are available, and by
immunohistochemical analyses, their expression was seen pri-
marily confined to the renal cortex, especially in the renal prox-
timal tubules (Fig. 2A, B and C). By RT-PCR analysis, expression of
every enzyme (i.e., MIOX, ALR1, gulonate dehydrogenase, xylu-
lose reductase, and xylulose kinase) could be demonstrated in
kidney tissues (Fig. 2D), which confirmed the existence of a G-X
pathway in the kidney cortical tubules, as it has been described
in the eye lens previously and is akin to the polyol pathway
(25–28).

Expression and Activity of MIOX following Transfection in
High Glucose Ambience—The expression of MIOX increased in
response to various D-glucose concentrations (5–25 mM) in a
dose-dependent manner, as assessed by Western blotting anal-
yses (Fig. 3, A (a) and B). Exposure of cells to various concen-
trations of n-glucose in the presence of pcDNA (empty vector;
EV) exhibited a negligible increase in the expression of MIOX.
Treatment with MIOX-specific siRNA caused a notable reduc-
tion in the expression of MIOX (Fig. 3, A (a) and B). Transfec-
tion of LLC-PK1 cells with MIOX-pcDNA increased the
expression of MIOX at basal as well as high concentration
(5–25 mM glucose) (Fig. 3, A (b) and C). This increased expres-
sion was normalized to almost basal levels following the treat-
ment of transfected cells with MIOX siRNA in high glucose
ambience (Fig. 3, A (b) and C). The exposure with L-glucose,
serving as a control, induced no significant change in the
expression of MIOX with or without pcDNA transfection or
siRNA treatment (Fig. 3, A (c) and D). The expression of β-ac-
tin, another control, exhibited no notable change in its expres-
sion following transfection, exposure to HG ambience, or
siRNA treatment, compared with LLC-PK1 cells maintained
under low glucose (LG; 5 mM) basal conditions. Band density
normalized to respective β-actin band density of four blots of
each variable is shown in bar graphs in Fig. 3, B–D.

Basal MIOX enzyme activity in the cellular extract from cells
maintained at LG was designated as 100%, and a relative per-
centage increase in various samples was calculated. A dose-de-
pendent increase in the enzyme activity was observed at various
concentrations of n-glucose (5, 15, and 25 mM) (Fig. 3E, col-

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At 25 mM (HG), a 50% increase in enzyme activity was observed. No increase in activity was observed in the presence of L-glucose (Fig. 3E, column 4). With the transfection of EV, no further increase was observed compared with that seen under high D-glucose (25 mM) ambience (Fig. 3E, column 5). However, MIOX-pcDNA transfection led to an almost doubling of the enzyme activity even at low D-glucose concentration (Fig. 3E, column 6). Interestingly, transfection of MIOX-pcDNA led to another 25% increase in MIOX activity in the presence of high D-glucose (25 mM) ambience (Fig. 3E, column 7). This increase in MIOX activity in the transfectants was largely unaffected with the treatment of nonspecific scramble oligonucleotide under HG ambience (Fig. 3E, column 8). Treatment of such transfected cells with MIOX-specific siRNA led to a substantial decrease in MIOX activity under high-glucose ambience (Fig. 3E, column 9). Although the changes in MIOX activity under various conditions were not as impressive as its expression, it was nevertheless considerable.

Effect of Glucose on \( \text{H}_2\text{O}_2 \) Generation and Relative Depletion of Cellular Reduced Glutathione following MIOX Transfection—For determination of \( \text{H}_2\text{O}_2 \) production, LLC-PK1 cells were grown under various conditions and then made quiescent by transferring to culture medium containing 0.1% FBS. The medium was replaced with Hanks’ balanced salt solution containing \( \text{H}_2\text{O}_2 \)-sensitive fluorophore DCF-DA (10 \( \mu \text{M} \)) and examined by a microscope equipped with UV illumination. Under basal LG ambience, a mild DCF-associated green fluorescence was observed, and no significant change in the fluorescence was observed in cells transfected with EV (Fig. 4A(a)). Treatment with high glucose induced a robust increase in DCF-associated fluorescence (Fig. 4A(b)). DCF fluorescence was markedly enhanced in cells transfected with MIOX-pcDNA under high glucose ambience, HG (Fig. 4A(c)), suggesting that MIOX transfection boosts the generation of \( \text{H}_2\text{O}_2 \). Treatment of such transfected cells with MIOX siRNA markedly reduced the fluorescence (Fig. 4A(d)), indicating a substantial contribution of MIOX in the generation of \( \text{H}_2\text{O}_2 \).

For determination of cellular GSH, cultured medium of treated cells was replaced with Hanks’ balanced salt solution containing MBB (10 \( \mu \text{M} \)) and examined by a microscope equipped with UV illumination. Under basal LG ambience, a bright MBB-associated bluish fluorescence was observed, whereas no differences in the intensity of fluorescence were noted with the transfection of empty vector (Fig. 4B(a)). Treatment with high glucose induced a notable decrease in MBB-associated fluorescence (Fig. 4B(b)). DCF fluorescence was markedly enhanced in cells transfected with MIOX-pcDNA under high glucose ambience, HG (Fig. 4A(c)), suggesting that MIOX transfection boosts the generation of \( \text{H}_2\text{O}_2 \). Treatment of such transfected cells with MIOX siRNA markedly reduced the fluorescence (Fig. 4A(d)), indicating a substantial contribution of MIOX in the generation of \( \text{H}_2\text{O}_2 \).
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Adaptive Changes in the Profile of Antioxidant Defense System and Oxidant Stress-responsive Enzymes in Response to Perturbation in the Intracellular Redox—In view of the generation of oxygen radicals and depletion of cellular GSH with MIOX transfection under HG ambience, expression of various molecules relevant to the antioxidant defense system was investigated by Western blotting analyses. Expression of six key proteins intimately related to the antioxidant systems and oxidant stress responsiveness was investigated. They were as follows: SOD1, SOD2, NOX4, heme oxygenase-1, catalase, and glutathione peroxidase. By Western blotting analyses, a variable low level expression of each of these proteins was observed under LG ambience or in cells transfected with EV (Fig. 4E, lane 1). In general, a similar trend for the change in the expression of all six proteins in response to different treatments was observed although the degree of increase/decrease was somewhat...
able. With HG treatment, a notable increase in their expression was observed, whereas concomitant transfection with EV led to no further increase in the expression (Fig. 4E, lanes 2 and 3). Transfection with MIOX-pcDNA also yielded a notable increase in the expression of all the proteins, and it was higher compared with that seen with HG treatment (Fig. 4E, lane 4 versus lane 2). A tremendous increase in the expression of proteins was observed with the transfection of MIOX-pcDNA in cells maintained under HG ambience up to 15 h (Fig. 4E, lane 5). Treatment of these transfectants with MIOX siRNA in the presence of HG significantly reduced their expression, although the restoration was still much above the basal levels (Fig. 4E, lane 6), suggesting that MIOX transfection also contributed to HG-induced adaptive up-regulation of the antioxidant defense system. Interestingly, at the 30 h time point, the expression of various enzymes was reduced (Fig. 4E, lane 7), suggesting exhaustion of redox defense mechanisms. Band densities normalized to respective β-actin band densities of each set of the above experiments in Fig. 4 depicted in bar graphs in Fig. 4, F–K, corresponding to the lanes depicting expression of SOD1, SOD2, NOX4, heme oxygenase-1, catalase, and glutathione peroxidase, respectively.

Delineation of the Source of Intracellular ROS Generation in MIOX-overexpressing Cell Line and Effect of NOX siRNA on ROS under High Glucose Ambience—To delineate the source of ROS in MIOX-overexpressing cells, they were treated with Mito Q and DPl, compounds that are known inhibitors of ROS generated from mitochondria and NADPH oxidase, respectively (38–40). Both Mito and DPl inhibited DCF-DA-associated fluorescence, as gauged by flow cytometric analyses (Fig. A–E). Although mean fluorescence intensity decreased with individual treatments of these inhibitors, neither of them could completely inhibit the fluorescence, suggesting that the ROS in MIOX-overexpressing cells are derived from both intracellular sources. Because we observed in our previous experiments (Fig. 4E) that there were perturbations in expression of tubular renal NOX4 and in view of the fact that its stimulation is influenced by the diabetic state (30), the following question we addressed. Can NOX4-specific inhibitors reduce the generation of ROS in MIOX-overexpressing cells under high glucose ambience? NOX4 siRNA did inhibit the generation of ROS, both under basal conditions and under high glucose ambience (Fig. 5, F–L).

However, siRNA treatment did not reduce the DCF-DA-associated fluorescence to basal levels, indicating that there are additional sources of ROS generation (e.g. mitochondria) in MIOX-overexpressing cells under high glucose ambience.

Perturbation in Mitochondrial Homeostasis following MIOX Transfection under High Glucose Ambience—Because ROS were found to be derived from both NADPH and mitochondrial sources following MIOX overexpression under HG ambience, we proceeded to delineate changes that would be reflective of mitochondrial dysfunctions relevant to cellular redox imbalance. LLC-PK1 cells were stained with Mitotracker (red fluorescence) to localize the mitochondria and were then stained with anti-cytochrome c antibody (green fluorescence) and counterstained with DAPI (blue) to outline the nuclei by confocal microscopy. Under basal conditions of LG ambience, cytochrome c and Mitotracker localized to mitochondria and yielded a deep orange-colored fluorescence (Fig. 6A (a)). Treatment with HG led to a moderate degree of leakage of cytochrome c into the cytosolic compartment (Fig. 6A (b), bluish arrowheads). However, MIOX transfection under high glucose ambience led to marked diffusion of cytochrome c into the cytosol (Fig. 6A (c), red arrowheads). On the other hand, siRNA treatment of MIOX-pcDNA-transfected cells maintained under HG ambience largely restored the co-localization of cytochrome c and Mitotracker, yielding an orange-colored fluorescence (Fig. 6A (d)), suggesting preservation of mitochondrial integrity. Cytochrome c release experiments were also repeated with the use of another mitochondrial marker, COX IV, which does not dissociate from mitochondria under oxidant stress (42, 43). Following various treatments, cells were stained with anti-COX IV (green fluorescence) and anti-cytochrome c (red fluorescence). Under low glucose conditions, cytochrome c and COX IV co-localized in the mitochondria and yielded a yellow-colored fluorescence (Fig. 6B (a), and HG treatment led to a moderate degree of leakage of cytochrome c (Fig. 6B (b), bluish arrowheads). Whereas MIOX transfection under HG ambience led to a marked diffusion of cytochrome c into the cytosol (Fig. 6B (c), bluish arrowheads), siRNA treatment largely restored the co-localization of cytochrome c and COX IV, yielding a yellow-colored Fluorescence (Fig. 6B (d), as observed under control low glucose conditions.

FIGURE 4. A, effect of HG on ROS generation following MIOX transfection. A low level DCF-associated green fluorescence was observed under LG in the presence or absence of empty vector (A (a)). HG induced a robust increase in fluorescence, which was further accentuated with transfection of MIOX-pcDNA (A (b) and c)). Fluorescence was markedly reduced with MIOX siRNA treatment (A (d)). B, effect of HG on the relative depletion of cellular GSH following MIOX transfection. A slight MBB-associated blue fluorescence was observed under LG ambience in the presence or absence of EV (B (a)). HG significantly reduced fluorescence, which was further decreased with the transfection of MIOX-pcDNA (B (b) and c)), and it was restored with treatment with MIOX siRNA (B (d)). In addition, the GSH/GSSG ratio was also determined (C). Under HG, the ratio was decreased, and a further decrease was seen in cells overexpressing MIOX. MIOX siRNA treatment prevented such a decrease to a large extent. D, perturbation in NAD+/NADH ratio following MIOX transfection in HG ambience. About a 50% decrease in the NAD+/NADH ratio was observed under HG ambience compared with LG concentration, and no further decrease was seen with transfection of EV (Columns 1–4). A comparable decrease in the ratio was observed following MIOX-pcDNA transfection alone under LG ambience, and it further decreased in the presence of HG ambience (columns 5 and 6). Treatment with siRNA improved the NAD+/NADH ratio in MIOX-transfected cells maintained under HG ambience, whereas scramble oligonucleotide had no significant effect (columns 7 and 8). E, profile of antioxidant defense system and oxidant-responsive enzymes in response to perturbation in the intracellular redox. Western blotting analyses of antioxidant enzymes (i.e. SOD1, SOD2, NOX4, heme oxygenase-1 (HO-1), catalase, and glutathione peroxidase (GPX) revealed a variable increase in their expression up to 15 h of the culture period under HG ambience. Concomitant transfection with EV did not cause any further increase in their expression, as compared with that observed at LG concentration (lanes 1–3). Transfection with MIOX-pcDNA yielded a comparable increase, and it was notably increased under HG ambience, whereas it was reduced to basal levels by MIOX siRNA treatment (lanes 4–6). Interestingly, the expression of various enzymes of the scavenging system was reduced in the 30 h time frame of the culture. Band densities normalized to respective β-actin band densities of various blots of each variable (i.e. SOD1, SOD2, NOX4, heme oxygenase-1, catalase, and glutathione peroxidase) compared with their respective controls at the 15 h time point are shown in bar graphs in corresponding panels F–K (n = 4; *p < 0.01; #, p < 0.05). Error bars, S.D.
In view of cytochrome c leakage from mitochondria under HG ambience and MIOX overexpression, mitochondrial integrity was investigated by measuring their membrane potential, as assessed by confocal microscopy after staining with TMRE followed by counterstaining nuclei with DAPI. Normally, under LG ambience, TMRE is seen uniformly localized to mitochondria (Fig. 6C (a)). A significant loss of TMRE staining was observed with treatment with HG (Fig. 6C (b)). Transfection of cells with MIOX-pcDNA under HG ambience caused a marked loss of $\Delta \Psi_m$, as indicated by loss of TMRE fluorescence in LLC-PK1 cells (Fig. 6C (c)), whereas siRNA treatment of cells transfected with MIOX-pcDNA largely restored the cellular TMRE fluorescence (Fig. 6C (d)), indicating a recovery of $\Delta \Psi_m$ from HG-MIOX-pcDNA injury.

Perturbations observed in the integrity of mitochondria led us to directly examine their ultrastructure by electron microscopy. Cells maintained in medium containing 5 mM glucose (LG) had tubular morphology of mitochondria with intact inner and outer membranes and cristae (Fig. 6D (a)). Cells grown in medium containing HG had constrictions in the main body of mitochondria with a certain degree of deformation of their cristae (Fig. 6D (b), arrowhead). Overexpression of MIOX induced further deformations of mitochondria under HG ambience. Mitochondria were highly deformed with an increased number of constrictions and a relative loss of cristae (Fig. 6D (c), arrowheads), suggesting that mitochondria are undergoing a tremendous degree of fission. Constrictions were significantly lessened following siRNA treatment of these transfected cells, and they regained their cristae structures and tubular morphology (Fig. 6D (d)), indicating that an adverse effect on morphology of mitochondria to a large extent was contributed by MIOX overexpression. Quantitative measurements indicated that under basal conditions, about 70% of intact mitochondria were in length along the long axis, which was decreased to 47 and 35% under HG ambience and HG + MIOX overexpression, respectively. The population of intact mitochondria was restored to 63% following siRNA treatment, whereas mild constrictions still persisted in some of the mitochondria (Fig. 6D (d)).

Impairment of Mitochondrial DNA and Induction of Apoptosis following MIOX Transfection under High Glucose Ambience—In view of the deformations of mitochondria observed by electron microscopy, we proceeded to investigate whether MIOX overexpression and high glucose can induce mitochondrial DNA damage. Under basal conditions of low glucose ambience, bands corresponding to high molecular DNA (~7.8 kb) and low molecular DNA (~0.4 kb) were visualized by PCR analyses (Fig. 7A, lane 1). However, DCF-DA-associated fluorescence, as delineated by flow cytometric analyses (A–E), suggesting that the ROS are derived from mitochondria as well as the NADPH oxidase system. Next, we addressed the question of whether NOX4-specific inhibition could reduce the generation of ROS in MIOX-overexpressing cells under high glucose ambience. NOX4 siRNA inhibited generation of ROS, both under basal conditions and under high glucose ambience (F–I). However, siRNA treatment could not reduce the mean fluorescence intensity to basal levels, suggesting an additional source of ROS generation (e.g. mitochondria) in MIOX-overexpressing cells under high glucose ambience ($n = 4$; *, $p < 0.01$; #, $p < 0.05$). Error bars, S.D.
FIGURE 6. A and B, effect of MIOX overexpression on mitochondrial dysfunction with release of cytochrome c under HG ambience. Under LG ambience, the cytochrome c (green) and Mitotracker (red) co-localized to mitochondria and yielded a deep orange/yellow-colored fluorescence (A (a)). Treatment with HG led to a moderate leakage of cytochrome c into the cytosolic compartment (A (b), bluish arrowheads). The MIOX-pcDNA transfection under HG ambience led to leakage of cytochrome c into the cytosol (A (c), red arrowheads). The siRNA treatment of MIOX-pcDNA-transfected cells maintained under HG ambience restored the co-localization of cytochrome c and Mitotracker, yielding a yellow-colored fluorescence (A (d)). Cytochrome c release experiments were also performed using another mitochondrial marker, COX IV, which does not dissociate from mitochondria under oxidant stress. Under low glucose conditions, cytochrome c (red) and COX IV (green) co-localized in the mitochondria and yielded a yellow-colored fluorescence (B (a)), and HG treatment led to a moderate degree of leakage of cytochrome c (B (b), bluish arrowheads), whereas MIOX transfection under HG ambience led to a notable dissociation of cytochrome c into the cytosol (B (c), bluish arrowheads), and siRNA treatment largely restored the co-localization of cytochrome c and COX IV, yielding a yellow-colored fluorescence (B (d)). C, effect of MIOX overexpression on mitochondrial membrane potential under HG ambience. TMRE staining is seen uniformly localized to the mitochondria (C (a)). A moderate loss of TMRE staining was observed with the treatment with HG (C (b)). Transfection of cells with MIOX-pcDNA under HG ambience caused a loss of staining (i.e. loss of ΔΨm) (C (c)), whereas siRNA treatment restored the cellular TMRE fluorescence to basal levels (C (d)). D, effect of MIOX overexpression on mitochondrial ultrastructure under HG ambience. By electron microscopy, cells maintained in medium containing LG showed normal morphology in the majority (~70%) of mitochondria (>1 μm in length along the long axis) with intact inner and outer membranes and cristae (D (a)). Under HG ambience, the population of tubular mitochondria decreased to 47%, and there were constrictions in the body of the mitochondria accompanied by a relative loss of their cristae (D (b), arrowhead). Overexpression of MIOX further decreased the population of intact mitochondria to 35% and induced marked constrictions and at times U-shaped angulation or other deformations of mitochondria in the presence of HG ambience (D (c), arrowheads), suggesting that mitochondria are undergoing a significant degree of fission. MIOX siRNA treatment significantly lessened the constrictions, and a majority (~63%) of mitochondria regained their tubular morphology (D (d)).
in a mild increase in the expression of a high molecular band (Fig. 7A, lane 2). Cells subjected to HG ambience in the presence or absence of empty vector showed a notable decrease in the intensity of the band of high molecular weight DNA (Fig. 7A, lanes 3 and 4 versus lanes 1 and 2). Transfection of cells with MIOX-pcDNA also caused a significant decrease in the intensity of the high molecular weight (Fig. 7A, lane 5 versus lanes 1 and 2), whereas treatment with MIOX siRNA restored the intensity of the HM band (lane 7 versus lane 6), suggesting that MIOX overexpression in the presence of HG readily damages high molecular weight DNA. Induction of apoptosis in LLC-PK1 cells following MIOX overexpression under HG ambience. Under basal conditions of LG, no significant apoptosis was observed (Fig. 7B, a). An HG ambience induced apoptosis in about 5% of cells (Fig. 7B, b, arrows). Upon MIOX-pcDNA transfection, apoptosis increased 2–3-fold (Fig. 7B, c, multiple arrowheads). Treatment with MIOX siRNA remarkably reduced apoptosis, and a few tiny nuclear fragments were seen (Fig. 7B, d, arrowheads).

**Effect of NAC on MIOX- and HG-induced Apoptosis and Expression of Bax, Bcl2, and Cleaved Caspase-3 and Activity of Caspase-3—**Cells under HG ambience or transfected with MIOX-pcDNA had increased expression of apoptotic pro-

**FIGURE 7.** A, impairment of mitochondrial DNA following MIOX overexpression under HG ambience. Under HG ambience, two bands corresponding to high molecular weight DNA (~7.8 kb) (HM) and low molecular weight DNA (~0.4 kb) (LM) were seen by PCR analyses (lane 1). A mild increase in the intensity of the high molecular weight band is observed following transfection of EV (lane 2). Treatment with HG in the presence or absence of EV showed a notable decrease in the intensity of the HM weight DNA (lanes 3 and 4 versus lanes 1 and 2). MIOX-pcDNA transfection caused a significant decrease in the intensity of the HM band (lane 5 versus lanes 1 and 2). Treatment of such transfected cells with HG ambience resulted in a marked decrease in the intensity of the HM band (lane 6 (asterisk) versus lanes 1 and 2), whereas treatment with MIOX siRNA restored the intensity of the HM band (lane 7 versus lane 6), suggesting that MIOX overexpression in the presence of HG readily damages high molecular weight DNA. B, induction of apoptosis in LLC-PK1 cells following MIOX overexpression under HG ambience. Under basal conditions of LG, no significant apoptosis was observed (Fig. 7B, a). An HG ambience induced apoptosis in about 5% of cells (Fig. 7B, b, arrows). Upon MIOX-pcDNA transfection, apoptosis increased 2–3-fold (Fig. 7B, c, multiple arrowheads). Treatment with MIOX siRNA remarkably reduced apoptosis, and a few tiny nuclear fragments were seen (Fig. 7B, d, arrowheads).
FIGURE 8. Effect of NAC on MIOX- and HG-induced apoptosis and expression of Bax, Bcl2, and cleaved caspase-3 and activity of caspase-3. A–C, cells under HG ambience or transfected with MIOX-pcDNA had increased expression of the apoptotic protein Bax (A (a), lanes 2 and 4 versus lane 1). The increase was augmented following concomitant treatment with HG and MIOX transfection (A (a), lane 5), and NAC treatment reduced the expression of Bax (A (a), lanes 3 and 6). Expression of anti-apoptotic protein Bcl2 decreased with HG treatment as well as by MIOX transfection (A (b)). The decrease was augmented with the concomitant treatment with HG and MIOX transfection (A (b), lane 5), and it was restored by NAC treatment (A (b), lane 6). Band densities normalized to respective β-actin band densities of Western blots are shown in bar graphs in B and C. D–F, both HG treatment and MIOX transfection induced an increased expression and activity of cleaved caspase-3 (D (a), lanes 2 and 4 versus lane 1; F, columns 2 and 4 versus column 1). They were further increased with concomitant treatment with HG and transfection of MIOX (D (lane 5) and F (column 5)). NAC treatment reduced the expression and activity of cleaved caspase-3 (D (lanes 3 and 6) and E (columns 3 and 6)). Band density normalized to respective β-actin band density is included in E. G, no significant apoptosis was seen under LG ambience in cells transfected with EV (G (a)). MIOX transfection or HG ambience induced a moderate degree of apoptosis (G (b and c)). Apoptosis was accentuated following MIOX-pcDNA transfection under HG ambience (G (d)). Apoptosis was reduced with NAC treatment (G (e and f)), suggesting that ROS modulate the events related to apoptosis. Band density normalized to respective β-actin band density of various blots and caspase-3 activity, compared with their respective controls, is shown in bar graphs in B, C, E, and F (n = 4; *, p < 0.01; #, p < 0.05).
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Notable degree of apoptosis (Fig. 8G (c)), which was markedly accentuated upon transfection with MIOX-pcDNA (Fig. 8G (d)). With the treatment with NAC, apoptosis was remarkably reduced both in cells maintained under HG ambience and those transfected with MIOX-pcDNA (Fig. 8G (e and f)). These findings suggested that MIOX- or HG-induced modulation of apoptogenic and anti-apoptogenic proteins is modulated via ROS.

Increased Expression and Bioactivity of TGF-β1 following Overexpression of MIOX under HG Ambience in LLC-PK1 Cells—Various signaling kinases have been shown to modulate the activity and expression of TGF-β1; however, information in regard to the modulation of this profibrogenic cytokine by ROS in the context of MIOX biology has not been reported. In view of the perturbations in the "cellular redox," the effect of MIOX and HG on the expression and activity of TGF-β1 was investigated. The amount of TGF-β1 was expressed as pg/mg protein, whereas its bioactivity was expressed as a percentage increase, considering 100% as basal level (i.e. LG ambience). No significant increase in expression or bioactivity was observed with transfection of empty vector (pcDNA) under LG ambience (Fig. 9, A and B (columns 1 and 2)). Exposure of cells to HG ambience induced an almost 2-fold increase in bioactivity and expression of TGF-β1 (Fig. 9, A and B, column 3). There was no significant further increase with transfection of empty vector (Figs. 9, A and B (column 4)). Interestingly, transfection of MIOX-pcDNA under LG ambience increased the bioactivity or expression of profibrogenic cytokine to levels comparable with those seen following exposure of LLC-PK1 cells to HG (Fig. 9, A and B (column 5)). However, overexpression of MIOX in the presence of HG had an additive effect, and an almost 3-fold increase in the bioactivity and expression of TGF-β1 was seen as compared with basal levels (Fig. 9, A and B (column 6)). Treatment of MIOX siRNA under these experimental conditions resulted in a marked reduction of this profibrogenic cytokine (Fig. 9, A and B, column 7), authenticating the specificity of MIOX in modulating the expression or bioactivity of TGF-β1.

Modulation of Fibronectin Expression by MIOX under High Glucose Ambience—Because TGF-β1, modulates the transscription of a number of ECM proteins, we investigated the expression of fibronectin. By immunofluorescence microscopy, a very low level of fibronectin expression was observed under LG ambience, and no significant change was seen in cells transfected with EV (Fig. 9D (a)). An increased expression of fibronectin was observed in LLC-PK1 cells under HG ambience (Fig. 9D (b)). A remarkable increase in the expression of fibronectin was observed following overexpression of MIOX under HG ambience (Fig. 9D (c)). To assess the specificity of MIOX effects, cells were treated with RSOR siRNA. Under HG ambience, the cells treated with MIOX siRNA had a notable reduction of immunofluorescence (Fig. 9D (d)), which suggested that the induction of fibronectin expression is also mediated by MIOX.

Fibronectin expression was also measured in culture medium by ELISA and expressed as ng/mg protein. The culture medium of cells maintained in LG conditions or transfected with EV pcDNA had ~10 ng/mg fibronectin (Fig. 9C, columns 1 and 2). The cells subjected to HG ambience had a ~3-fold increase in the de novo synthesis/expression of fibronectin, which was unaffected by the transfection of EV (Fig. 9C, columns 3 and 4). The cells transfected with MIOX-pcDNA and maintained in LG ambience also had a significantly increased expression of fibronectin (Fig. 9C, column 5). However, those maintained in HG ambience had a marked increase in the de novo synthesis/expression of fibronectin (Fig. 9C, column 6). The specificity of this MIOX effect on the expression of fibronectin was authenticated by treating the cells with MIOX siRNA, where a remarkable decrease in the expression was observed (Fig. 9C, column 7). These results indicated that the culture medium findings accurately reflect those observed in intact cells by immunofluorescence microscopy (Fig. 9C (a–d)). In order to ensure that MIOX-mediated fibronectin expression was modulated via ROS, experiments utilizing the ROS inhibitor NAC were carried out. An increased expression of fibronectin was seen in cells co-transfected with MIOX-pcDNA, and it was markedly increased with the concomitant exposure of cells to HG ambience (Fig. 9D (a–c)). With the treatment with NAC, the expression of fibronectin was reduced in cells transfected with MIOX and those concomitantly exposed to HG ambience, confirming that MIOX transfection alone increased the fibronectin expression via ROS (Fig. 9D (b–d)).

Discussion

The observations made in this investigation suggest that a G-X pathway enzyme system exists in the kidney (Figs. 1 and 2), and overexpression of MIOX in LLC-PK1 cells, a proximal tubular cell line, induces a number of perturbations that are mainly related to oxidant stress under HG ambience. Besides the oxidant stress, mitochondrial homeostasis and activity of profibrogenic cytokines were also perturbed, and that led to an increased expression of ECM proteins like fibronectin, as has been described in the various kidney compartments in diabetic nephropathy (1).

Diabetic nephropathy has been the subject matter of intense investigation for half a century, especially the work carried out on the glomerular compartment because the latter highlights the prototypic morphologic changes seen in human renal biopsies (Fig. 1). These morphologic changes are basically reflective of preceding disturbances in various metabolic/cellular signaling pathways with generation of ROS leading to increased synthesis and amassing of ECM proteins in the glomerulus. The ROS are believed to be central to the pathogenesis of DN, as has been thoroughly documented in the glomerular compartment in various animal models and isolated glomerular cells in in vitro culture systems (1–3). The oxidant stress in hyperglycemic states may not be solely confined to the glomerular cells but also to the tubules, an idea marshaled by Bonventre (47) a few years ago. In support of this contention are our preliminary immunohistochemical studies on human biopsy material showing a highly accentuated expression of NOX4 with identical co-distribution and marked up-regulation of MIOX in diabetic nephropathy (Fig. 1). Interestingly, NOX4 expression was marginally increased in the glomerular compartment in diabetic nephropathy, suggesting that the oxidant stress may be a dominant feature of the tubular compartment where the MIOX is co-expressed.
In order to study tubular pathobiology under high glucose ambience, we utilized LLC-PK1 cells and an MIOX-overexpressing cell line. Both exhibited an increased dose-dependent expression and activity of MIOX under high glucose ambience, and the response was much more accentuated in the overexpressing cell lines. Both expression and activity were notably reduced following MIOX siRNA treatment of cells (Fig. 3), indicating that the culture system is well suited for the delineation of the mechanism(s) related to generation of ROS via the G-X pathway in the tubular compartment. The generation of ROS with altered redox state of pyridine nucleotides (NADPH/NADP⁺ and NADH/NAD⁺ ratios) has been well described in glomerular cells in diabetic nephropathy with a comparable sequence of events in \textit{in vitro} systems under high glucose ambience (1, 2, 16). However, whether ROS generation could occur by MIOX via a G-X pathway has not been described, but it is

**FIGURE 9.** A and B, effect of MIOX overexpression on TGF-β1 expression and bioactivity under HG ambience in LLC-PK1 cells. No significant increase in the expression or bioactivity was observed with transfection of EV (pcDNA) under basal LG conditions (columns 1 and 2). HG ambience induced a moderate increase in the bioactivity and expression of TGF-β1, and no significant further increase was seen with EV transfection (columns 3 and 4). Interestingly, transfection of MIOX-pcDNA under LG ambience increased the bioactivity and expression of TGF-β1 comparable with those seen under HG ambience (column 5). Overexpression of MIOX in the presence of HG markedly increased the bioactivity and expression of TGF-β1, and it was significantly reduced with MIOX siRNA treatment (columns 6 and 7). C, modulation of fibronectin expression by MIOX under high glucose ambience. Under LG ambience, fibronectin expression was quite low in the “cell culture medium” with no significant change following EV transfection (C, columns 1 and 2). Under HG ambience, a ~3-fold increase in fibronectin expression was seen, which was unaffected by EV transfection (C, columns 3 and 4). MIOX-pcDNA transfection in LG ambience also caused a comparable increase in fibronectin expression (C, column 5), whereas MIOX-pcDNA transfection under HG ambience caused a marked increase in fibronectin expression, which was significantly reduced by MIOX siRNA treatment (C, columns 6 and 7). At the cellular level, a low level of fibronectin expression was observed under LG ambience, and no significant change was seen in cells transfected with EV (D (a)). A moderate increase in expression of fibronectin was observed in cells under HG ambience (D (b)). A marked increase in fibronectin expression was observed with MIOX overexpression under HG ambience (D (c)), which was reduced to basal levels following MIOX siRNA treatment (D (d)). In order to affirm that MIOX-mediated fibronectin expression was modulated via ROS, the cells were treated with NAC. With the treatment with NAC, expression of fibronectin was reduced in cells transfected with MIOX as well as in those concomitantly exposed to HG ambience, confirming that MIOX transfection alone increased the fibronectin expression via ROS (D, a and c versus b and d) (n = 4; *, p < 0.01; #, p < 0.05).

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conceivable. MIOX is a non-heme di-iron enzyme that oxidizes myo-inositol to glucuronic acid via a G-X pathway (48), which also oxidizes the less abundant chiro isomer of inositol, and its metabolites enter the pentose pathway (24). MIOX is predominantly expressed in kidneys of pigs and humans (26, 49); however, it remains to be investigated whether this enzyme is responsible for causing oxidative stress, particularly in the renal proximal tubules. After review of the G-X pathway (Fig. 2A) and confirmation of its existence in the renal tubules (Fig. 2, B–D), it seems that there would be a redox imbalance once the events are initiated by high glucose ambience. In support of this contention are our studies, which suggest an altered NAD+/NADH ratio following transfection of MIOX-pcDNA under high glucose ambience (Fig. 4D). The altered ratio could be seen in MIOX-transfected cells alone, and the fact the ratio was partially normalized by siRNA treatment would suggest that these redox perturbations may be specific to MIOX-initiated G-X pathway. Certainly, one needs to keep in perspective other pathways that could alter the pyridine nucleotides (NADPH/NADP+/NAD+/NADH) ratios. MIOX-induced redox imbalance was further substantiated by staining the cells with DCF-DA. A markedly increased intensity of DCF staining was observed in cells overexpressing MIOX under high glucose ambience, suggesting intracellular generation of ROS (Fig. 4A). Most likely, the generation of H2O2 is mediated through transcriptional mechanisms because the MIOX promoter includes carbohydrate response binding as well as oxidant response elements (29). This increase in the generation of ROS was attributable to being mediated through MIOX because its siRNA could notably decrease DCF fluorescence. Concomitantly with the increase in ROS, there was a decrease in intracellular GSH, which could be readily appreciable in MIOX-overexpressing cells subjected to high glucose ambience (Fig. 4B). Like the reduced antioxidant GSH content, the GSH/GSSG ratio was reduced (Fig. 4C), which seems to be attributable to MIOX-mediated cellular events, because, following its siRNA treatment, the intensity of fluorescent MIOX and the ratio were largely restored. Such changes in the GSH/GSSG ratio have been described in diabetic patients as well (50).

In view of the antioxidant GSH content, we proceeded to examine the profile of some of the ROS-scavenging oxidant stress-responsive enzyme systems at various time intervals of the cell culture. The enzyme profile could vary, depending upon the interval during which cells undergo oxidant stress or the extremes of reduction/oxidation ROS scavenging systems (phosphorylated NADPH/NADP+ and reduced/oxidized GSH/GSSG) (51, 52). Overall, the basal levels of various scavenging enzymes, SOD1, SOD2, catalase, and glutathione peroxidase, and their induction profiles were more or less similar (Fig. 4E). During the early time frame, they were notably increased under high glucose ambience with accentuated response in cells transfected with MIOX, and similar increased expression of NOX4 and heme oxygenase was observed. The responsiveness of these scavenging and responsive enzymes was related to the MIOX-mediated pathway because siRNA treatment led to a decrease in their protein expression. Interestingly, during the early time frame, the adaptive response to the oxidant stress was quite striking. However, following 30 h of culture, their expression began to decline remarkably, suggesting exhaustion of the defensive systems, as has been known in diabetic states in other culture systems (52). The prototype of adaptive increase followed by decline in the expression of heme oxygenase-1 following high glucose ambience has also been highlighted in recent studies on retinal endothelial cells (53). Of note, the ROS generated in MIOX-overexpressing cells are derived from both mitochondria and the NADPH oxidase system, as assessed by flow cytometric analysis (Fig. 5). The fact that NOX1 siRNA treatment partially blocked the generation of ROS and also suggest that ROS are derived from dual sources in MIOX-overexpressing cells under high glucose ambience has been described in various investigations related to diabetic nephropathy in various animal models (1, 3, 9, 10, 54).

To further support the notion that ROS are mitochondrially derived in cells overexpressing MIOX under high glucose ambience, we proceeded to examine the pathologic perturbations in mitochondria. Previously, we have described that high glucose induces perturbations in the mitochondrial homeostasis (41). Here we document that some of these mitochondrial abnormalities are also related to events distinct to the G-X pathway. First, we observed that there is a tremendous degree of translocation of cytochrome c from the mitochondrial to the cytosolic compartment, which was largely reduced following MIOX siRNA treatment (Fig. 6, A and B). The cytosolic translocation would be expected to lead to activation of caspases and eventually cellular apoptosis. The translocation may be related to the compromise in the ΔΨm or cristolysis of the membrane cristae (Fig. 6C). Likewise, these alterations were dampened by siRNA treatment, suggesting a potential role of MIOX in the evolution of such mitochondrial events. More importantly, mitochondrial morphologic changes, as highlighted by electron microscopy, were quite remarkable, where a marked degree of constrictions along their longitudinal axis were observed in cells transfected with MIOX and subjected to high glucose treatment (Fig. 6D). Interestingly, these mitochondrial constrictions were reduced by siRNA treatment. Such deformations are probably related to the imbalance in the expression or activity of various mitofusion (Mfn, Opal) and mitochondrial fission (Drp1) proteins (54). For the mitochondrial changes described above, one also needs to take into account ROS generated in the mitochondria during various forms of cellular stresses. In fact, some of the ROS are believed to be generated in this critical metabolic organelle when there are perturbations in the electron flow, membrane potential, and NADPH pool (55). Also, the fact that we observed changes in the mitochondrial Mn-SOD, SOD2 (Fig. 4E), would suggest that ROS generation in the present scenario is derived from more than one source, which eventually would be expected to damage some of the other downstream critical molecules (e.g. DNA, including mitochondrial DNA), leading to apoptosis. Such damage to the mitochondrial DNA is underscored in our PCR studies, where a notable reduction in the intensity of the high molecular weight DNA band was observed (Fig. 7A). Similarly, marked damage to the nuclear DNA was observed, as reflected by accentuated apoptosis in MIOX-overexpressing cells (Fig. 7B). The fact that apoptosis was lessened and there was a partial recovery of mitochondrial high molecular weight DNA would indicate a
substantial contribution of the G-X pathway in the generation of ROS and oxidant stress under high glucose ambience.

Apoptosis is regarded as one of the final morphologic readouts in diabetic nephropathy, as highlighted by various in vitro and in vivo studies (56, 57). Apoptosis was associated with increased activity of caspase-3, and it was mainly confined to proximal tubular cells. Caspase-3 activity and apoptosis were reduced with the diminution of oxidant stress, indicating that the proximal tubular injury was caused by ROS. To establish a causal relationship between MIOX-induced injury to LLC-PK1 cells (a tubular cell line) and apoptosis under high glucose ambience, experiments with the ROS inhibitor NAC were carried out. Our data suggested that there is an increased activity of caspase-3 and apoptosis in LLC-PK1 cells under high glucose ambience, and the cellular injury was accentuated with the concomitant transfection of MIOX-pcDNA (Fig. 8). The fact that both apoptosis and caspases-3 activity/expression were reduced with NAC treatment would suggest that MIOX-induced ROS generation is causally linked to such injurious cellular perturbations. Likewise, a reduced expression of anti-apoptotic Bcl2 by MIOX under HG ambience and normalization of Bcl2 following NAC treatment would lend further support to the idea that apoptosis is directly related to excessive generation of ROS (Fig. 8). Also, increased expression of apoptogenic Bax (Fig. 8) along with the release of cytochrome c (Fig. 6) observed in this investigation would strengthen the notion that MIOX-mediated generation of ROS is directly related to accentuated apoptosis. Besides ROS-induced apoptosis and mtDNA damage, an association between ROS and TGF-β1-mediated fibrosis has been described in the literature in other organ systems, such as in the lung (58). Indeed, an intricate interplay between TGF-β1 and ROS has been reported in various studies (reviewed in Ref. 59). Even in the kidney, increased caspase-3 activity, apoptosis, and ROS are seen in the setting of experimental chronic renal scarring or fibrosis (60).

With respect to renal fibrosis, another important final readout of diabetic nephropathy is nodular glomerulosclerosis and tubulo-interstitial fibrosis, as highlighted in the Fig. 1. In regard to glomerulosclerosis, the contribution of ROS, PKC, and advanced glycation end products involving complex signaling pathways, is very well known, and this process of fibrosis is mediated via a critical profibrogenic cytokine, TGF-β1 (1–3). ROS may directly modulate the expression and activity of TGF-β1 (59). Alternatively, redox imbalance can also lead to activation of PKC, which then would modulate expression of TGF-β1. For instance, PKC activation is reversed by antioxidants and administration of PKC inhibitors, and that reduces TGF-β1-mediated expression of ECM proteins and thereby ameliorates microvascular complications of diabetes (61, 62).

However, at this point, it is unknown whether the pathogenetic role of the G-X pathway in tubulo-interstitial fibrosis is mediated by the profibrogenic cytokine, TGF-β1. Without going into a detailed exploration of known complex signaling pathways that are involved in the process of fibrosis, we mainly investigated the status of TGF-β1 following MIOX transfection and siRNA treatment in the high glucose milieu. Interestingly, expression as well as activity of TGF-β1 were markedly boosted in MIOX-overexpressing cells in the presence of high glucose, and they were significantly reduced following siRNA treatment (Fig. 9, A and B). This suggested that, most likely, ROS generated during the G-X pathway, directly or indirectly, modulated TGF-β1 levels. Conceivably, this profibrogenic cytokine via Smad and MAPK pathways modulates the transcription of various ECM proteins (e.g. collagen and fibronectin) and eventually their cellular or tissue levels (63, 64). In line with the TGF-β1 activity/expression, there was a parallel increase in the expression of fibronectin in the cellular and culture medium compartments of MIOX-overexpressing cells subjected to high glucose treatment (Fig. 9, C and D). The fact that the increased de novo synthesis of fibronectin could be reduced by siRNA treatment would suggest that this enhanced ECM protein expression is reasonably correlative with MIOX-initiated G-X pathway events. This excessive cellular expression of fibronectin and its secretion are reminiscent of the amassing of ECM in the extracellular space, as observed in the tubulo-interstitial compartment in diabetic nephropathy. To authenticate that ECM amassing or excessive synthesis of fibronectin is ROS-mediated by MIOX under HG ambience, ROS inhibitor (NAC) experiments were carried out. NAC could substantially decrease the de novo synthesis of fibronectin in cells overexpressing MIOX or in cells concomitantly exposed to HG ambience
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(Fig. 9E). These data strongly support the notion that ROS generated in the MIOX initiated pathway are responsible for excessive synthesis of ECM proteins, an injury reminiscent of tubulointerstitial fibrosis. It is anticipated that future studies using animal model systems would delineate the pathogenetic role of this G-X pathway in various renal disease processes with the ultimate goal to devise various therapeutic strategies for the amelioration of diabetic tubulopathy.

In summary, observations made in this investigation underscore the relevance of the G-X pathway in renal tubulo-interstitial injury. Importantly, this study demonstrates the existence of enzymes pertinent to the G-X pathway in murine kidney, and MIOX initiation of this pathway leads to altered expression of enzymes related to the G-X pathway in various renal disease processes with the accentuated generation of ROS. All of these perturbations eventually lead to increased apoptosis, bioactivity of TGF-β1, and accentuated synthesis of ECM proteins (Fig. 10), the events reflective of tubulo-interstitial injury, as described in in vivo pathologic states, such as in diabetic nephropathy.

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