Supporting Information

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SI Methods

Animals. Male Sprague–Dawley rats weighing between 200 and 225 g were used for the biochemical fractionation of kidney cortex. Rats were killed, and both kidneys were removed (1). A slice of kidney cortex at the pole was used for biochemical fractionation, Western blot analyses, and NADPH oxidase activity. All rats had unrestricted access to food and water, and were maintained in accordance with Institutional Animal Care and Use Committee procedures. Type 1 diabetes was induced with streptozotocin as described (1), and at day 14, all rats were killed.

Cell Culture. Rat glomerular mesangial cells (MCs) were isolated and characterized as described (1). These cells were used between 15th and 30th passages and maintained in DMEM supplemented with antibiotic/antifungal solution and 17% FBS. The rat aortic vascular smooth muscle cells (a gift of A. Hahn) and endothelial cells were also kept in DMEM supplemented with antibiotic/antifungal solution and 17% FBS. Mouse glomerular epithelial cells (podocytes) (gift from P. Mundel, Mount Sinai School of Medicine, New York) were maintained in RPMI MEDIUM 1640 supplemented with 10% FBS. Mouse glomerular endothelial cells (gift from M. P. Madaio, University of Pennsylvania, Philadelphia) were grown in DMEM/F12 supplemented with 10% FBS.

RNA Interference. A SMARTpool consisting of four short or siRNA duplexes specific for rat Nox4 was obtained from Dharmacon. The SMARTpool of siRNA for Nox4 was transfected at 400 nM in a double transfection using X-tremeGENE (Roche Applied Science) as described (2). Briefly, MCs were plated in antibiotic free media to obtain a ≈40% confluencey on the day of transfection; 100–400 nM scrambled control (nontargeting siRNA obtained from Dharmacon) or specific Nox4 siRNA were added to the cells. 24 h later, the media was aspirated, and fresh media minus antibiotics were added to the cells. The transfection was repeated and 24 h later the cells were harvested (for a total of 48 h posttransfection) for Western blot analysis or purification of mitochondria followed by NADPH oxidase assay.

Crude Subcellular Fractionation. Crude subcellular fractionation of MCs and kidney cortex was adapted from ref. 3. MCs were washed three times and scraped in ice-cold PBS followed by centrifugation at 800 × *g* to pellet the cells. The cell pellet was resuspended in 5 times the volume of Buffer A (20 mM Hepes/10 mM KCl/1.5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/1 mM DTT/0.1 mM PMSF, and Complete Protease Inhibitors from Roche Applied Science) supplemented with 250 mM sucrose. The resuspended cell pellet was incubated on ice for 15 min before the cells were broken with a Dounce homogenizer (100 strokes). A portion was removed as the "total" fraction. The remaining broken cell mixture was centrifuged in three sequential steps: 1,000, 10,000, and 100,000 × *g*. The 10,000 × *g* pellet was considered the "mitochondrial" fraction, and the 100,000 × *g* supernatant the "cytosol." The 100,000 × *g* pellet was considered the "membrane" fraction. Equal volumes, 1/5th, of each fraction were resolved on SDS/PAGE, and Western blot analysis was performed.

However, kidney cortex was processed the same manner protein concentrations were normalized by using the Bio-Rad Bradford reagent, and equal protein concentrations were analyzed by SDS/PAGE.

Subcellular Membrane (Mb) Fractionation. Isolation of subcellular Mb fractions was performed as previously described (4). Kidney cortex was homogenized with a Dounce homogenizer (100 strokes) in an isolation medium consisting of 220 mM mannitol/70 mM sucrose/20 mM Tris·HCl/1 mM EDTA/5 mM EGTA, pH 7.4. The homogenate was centrifuged at 500 × *g* for 10 min at 4 °C, and the pellet consisting of nuclei and unbroken cells was discarded; the resulting supernatant was centrifuged at 1,000 × *g* for 10 min at 4 °C and the related pellet (1,000 × *g* Mb fraction) was washed twice before being resuspended in isolation medium to which a mixture of protease inhibitors had been added (1 mM PMSF/1 mM leupeptin/1 mM pepstatin A). The 1,000 × *g* supernatant was collected, centrifuged at 3,000 × *g* for 10 min at 4 °C, and the resulting pellet washed twice leading to the 3,000 × *g* Mb fraction. A similar procedure was used to prepare the 6,000 and 17,000 × *g* fractions of subcellular Mbs (4). Protein concentrations were normalized by using the Bio-Rad Bradford reagent and equal protein concentrations (40 μg) was analyzed by SDS/PAGE.

Purification of Mitochondria. Mitochondria were purified from rat kidney cortex or MCs by using a combination of differential and Percoll gradient centrifugation adapted from ref. 5. All procedures were carried out at 4 °C. Kidney cortex or MCs were homogenized in 1× MSHE buffer (0.21 M mannitol/0.07 M sucrose/10 mM Hepes, pH 7.4/1 mM EDTA/1 mM EGTA). The following protease inhibitors were added just before use: 1 mM DTT, 2 μg/mL leupeptin, 21 mM phenylmethylsulfonyl fluoride. Unbroken cells and nuclei were pelleted at 500 × *g*. The supernatant, containing the mitochondria, was centrifuged at 9,500 × *g* to pellet the mitochondria. The mitochondria were washed twice and resuspended in 1× MSHE buffer. Mitochondria (400 μL) were layered onto a Percoll solution (1.5 mL; 50% Percoll and 50% 2× MSHE) and centrifuged at 50,000 × *g* in a Beckman Coulter Optima Max Ultra centrifuge using a TLA 120.2 rotor for 1 h. The brown mitochondrial band was collected by fractionating the gradient. To examine the purity of isolated mitochondrial preparations, the mitochondria were suspended in cold 4% formaldehyde, 1% glutaraldehyde (4F1G) in PBS and fixed overnight. For easier handling, the mitochondria were centrifuged at 5,000 × *g*, suspended in warm 3% gelatin, then the allowed to solidify. The semisolid blocks were fixed again in 4F1G then processed for ultramicrotomy and examination by electron microscopy.

This pure mitochondria fraction was used for NADPH oxidase activity and Western blot analysis. For protein detection in pure mitochondria isolated from MCs, 200 μL of fractions were collected from the top of the Percoll/MSHE gradient and 1/10th of each fraction was resolved by SDS-Page, and Western blot analysis was performed.

Mitochondria Isolation Kit. MCs were harvested from the cultures and fractionated into cytosol (C) and mitochondria by using a Pierce mitochondria isolation kit (Pierce) according to the instruction of the manufacturer.

NADPH Oxidase Assay. Pure intact mitochondria fraction from Percoll/MSHE gradient isolated from MCs was directly assayed for NADPH-dependent superoxide production using lucigenin-enhanced chemiluminescence (1, 2, 6). To start the assay, mitochondria were then added to 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin,

and 100 μM NADPH. Photon emission in terms of relative chemiluminescence (light) units (RLU) was measured every 20 or 30 s for 10 min in a luminometer. There was no measurable activity in the absence of NADPH. A buffer blank (<5% of the cell signal) was subtracted from each reading. Superoxide production was expressed as RLU/mg protein. Protein content was measured by using the Bio-Rad protein assay reagent. NADPH-driven hydrogen peroxide production was also measured in the pure intact mitochondrial (Mit) fractions by using Amplex Red Assay Kit (Invitrogen/Molecular Probes) as described (7). The amount of hydrogen peroxide generated was determined by measuring the absorbance of the Amplex Red reagent at 565 nm.

Western Blot Analysis. For immunoblotting, proteins were separated by using SDS/PAGE and transferred to poly(vinylidene difluoride) membranes. The membranes were blocked with 5% low fat milk in Tris-buffered saline, and then incubated with the indicated primary antibodies. Rabbit polyclonal Nox4 antibody directed against recombinant GST-mouse Nox4- (299–515) was designed in our laboratory (Fig. S1). A commercially available Nox4 antibody was used (Novus) when indicated. Prohibitin, LAMP-1, ADP ribosylation factor (ARF) antibodies were obtained from Abcam. Na^+/K^+-ATPase was from Upstate Biotechnology. Calnexin antibodies were purchased from Stressgen. Cytochrome (Cyt) c oxidase subunit II was from Santa Cruz Biotechnology, and the subunit VI was from MitoSciences. The appropriate HRP-conjugated secondary antibodies were added and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using NIH Image/Image J software.

Immunoprecipitation of NADPH Oxidase Activity with Nox4 Antibodies. The assays were conducted using Percoll gradient-purified mitochondria permeabilized with 0.6% Digitonin. For immunoprecipitations, the amount of mitochondria used (150 μg) was normalized to an equal amount of total protein as determined by Bradford analysis. Permeabilized mitochondria were immunoprecipitated overnight using 7.8 μg of our purified Nox4 antibody, commercial Nox4 antibody [Novus (49)] or rabbit IgG (Sigma) and 30 μL of protein A-Sepharose (Amersham Pharmacia Biotech). The immunoprecipitates were washed with 1XMSHE buffer and analyzed for NADPH-dependent superoxide generation.

Messenger RNA Analysis. Messenger RNA was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method as previously described (8). Total RNA was isolated from rat MCs by using an RNeasy Mini kit from Qiagen. Messenger RNA expression was quantified by using a Realplex mastercycler (Eppendorf) with SYBR Green dye and rat RT^2 qPCR Primers (SABiosciences) for Nox4 (sequence accession no. NM_053524.1) and normalized to GAPDH.

Immunofluorescence Confocal Microscopy. Cells grown on 4-well chamber slides were stained with 500 nM MitoTracker Green (MTG) for 15 min or 1 μM MitoTracker Deep Red (MTR) 633 for 30 min. Cells were subsequently fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. The cells were blocked with 5% normal goat serum or 5% normal donkey serum in PBS for 30 min, and incubated with appropriate primary antibodies for 30 min. Cyanin-3-, FITC-, or Alexa Fluor 488-conjugated secondary antibodies were then applied to the appropriate cells for 30 min. The cells were washed 3 times with PBS, mounted with antifade reagent with DAPI, and visualized on Olympus FV-500 confocal laser scanning microscope. MTG fluorescent intensity was determined at 488-nm excitation and 520-nm emission. MTR 633 fluorescent intensity was determined at 650-nm excitation and 668-nm emission.

Mitochondrial superoxide generation was assessed in live cells with MitoSOX Red (Invitrogen/Molecular Probes), which is a fluorogenic dye that is taken up by mitochondria, where it is readily oxidized by superoxide, but not by other reactive oxygen species (ROS) or reactive nitrogen species. Cells were loaded with 1 μM MitoSOX Red in phenol-free DMEM for 10 min at 37 °C. Cells were washed with warm buffer. MitoSOX Red fluorescent intensity was determined at 515-nm excitation and 580-nm emission. The brightness intensity of MitoSOX signal was semiquantified by using either the Image-Pro Plus 4.5 software (Media Cybernetics) or NIH Image/ImageJ software as described (1, 9). The data shown represent three separate experiments, and are expressed as relative fluorescence intensity.

Statistical Analysis. Results are expressed as mean ± SE. Statistical significance was assessed by Student's unpaired t test. Significance was determined as probability (p) <0.05.

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2. Block K, et al. (2007) NAD(P)H oxidases regulate HIF-2alpha protein expression. *J Biol Chem* 282:8019–8026.
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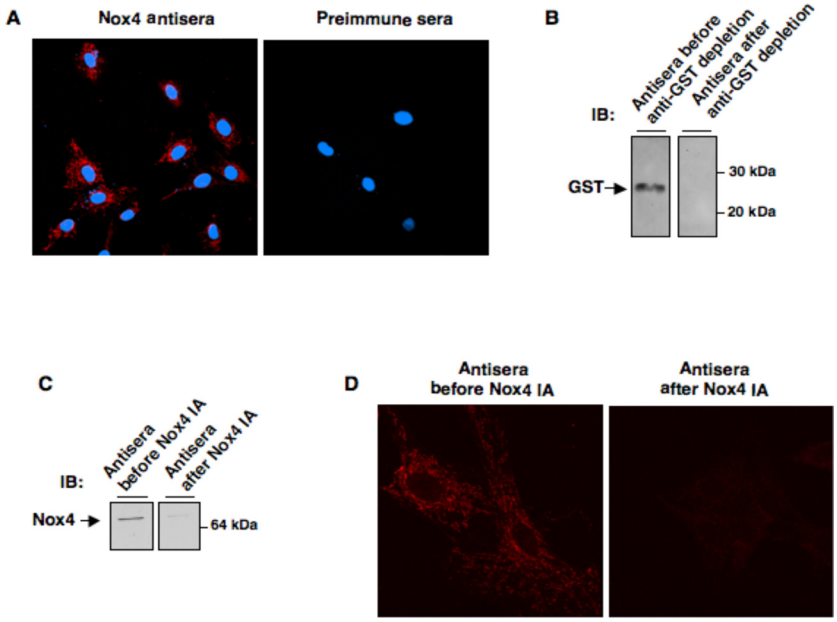

Fig. S1. Characterization of Nox4 antibody. A fragment (amino acids 299–515) of the mouse Nox4 C terminus was cloned into pGEX-2T vector and expressed as a GST-mouse Nox4 fusion protein by using *Escherichia coli* BL21. New Zealand White rabbits were immunized s.c. using the recombinant fusion protein attached to glutathione-Sepharose 4B beads emulsified in Freund's complete adjuvant. (*A*) Preimmune serum or Nox4 antisera were used to localize Nox4 by immunofluorescence in MCs. (*B*) The antiserum was passed repeatedly over fresh batches of glutathione-Sepharose 4B beads saturated with GST to deplete the antisera of GST antibodies. Note that the resulting antisera fail to recognize GST as assessed by Western blot analysis. (*C*) Nox4 antibodies were immunoadsorbed (IA) from the GST-purified antisera by repeated passage over Nox4 fusion protein bound to glutathione-Sepharose 4B beads. Immunoblotting of MC lysate with the IA serum confirmed the loss of the 70-kDa immunoreactive band. (*D*) Immunofluorescence using antisera before or after immunoadsorpbtion of Nox4 antibodies. Note loss of immunofluorescence staining after the adsorption of the antisera with the Nox4 fusion protein.

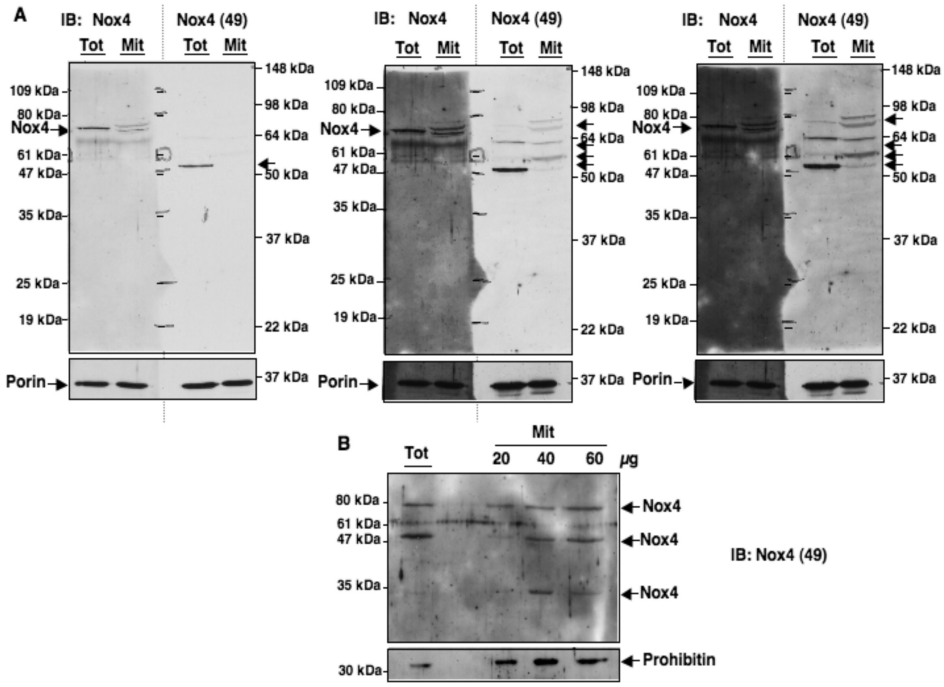

Fig. S2. Detection of different Nox4 migratory bands in total and crude Mit fractions. (*A*) Nox4 protein was detected by using our Nox4 antibody (Nox4) or a commercial antibody [Nox4 (49)] by Western blot analysis in crude subcellular renal cortex total (Tot) or crude Mit fractions (Mit) isolated from rat renal cortex as in Fig. 1 and outlined in *Materials and Methods*. Porin was used as a loading marker. To visualize all Nox4 immunoreactive bands, different exposures of the same immunoblot are shown left to right. (*B*) Detection of Nox4 by Western blot analysis using commercial Nox4 antibody [Nox4 (49)] in Tot and increasing amounts of Percoll-purified Mit isolated from rat renal cortex. Prohibitin was used as a marker for mitochondria.

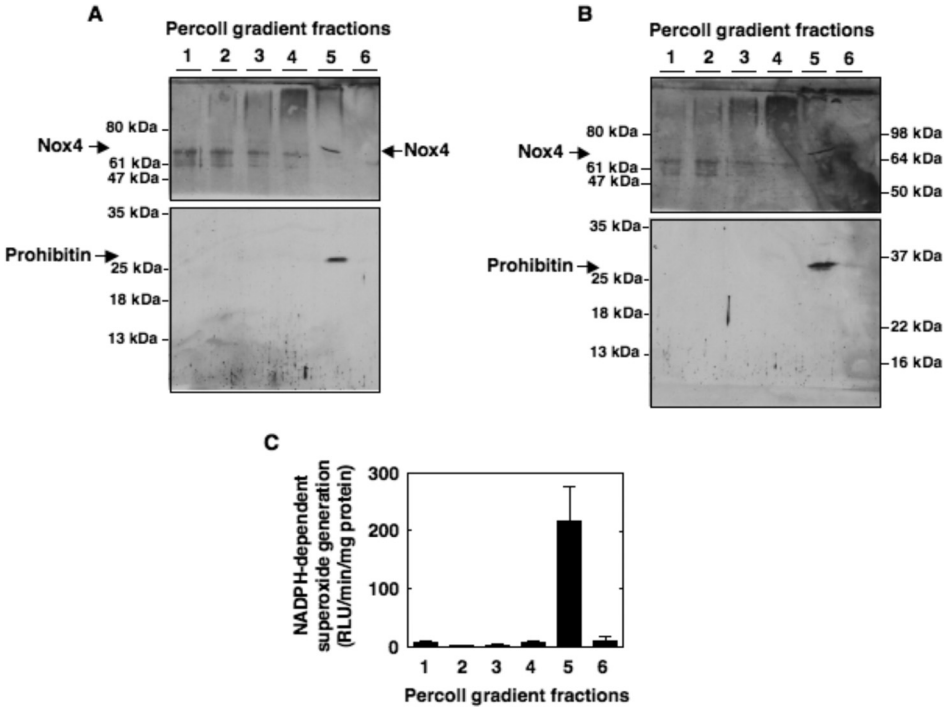

Fig. S3. (*A* and *B*) Pure mitochondria were isolated by using differential centrifugation and Percoll gradient; 200 μL fractions were collected from the top of the gradient and immunoblotting of each fraction (1/10th volume) was performed with Nox4 or prohibitin antibodies. (*C*) Pure mitochondria were isolated by using differential centrifugation and Percoll gradient. Fractions were collected from the top of the gradient, and NADPH-dependent superoxide generation was measured in each fraction as described in *Material and Methods*.

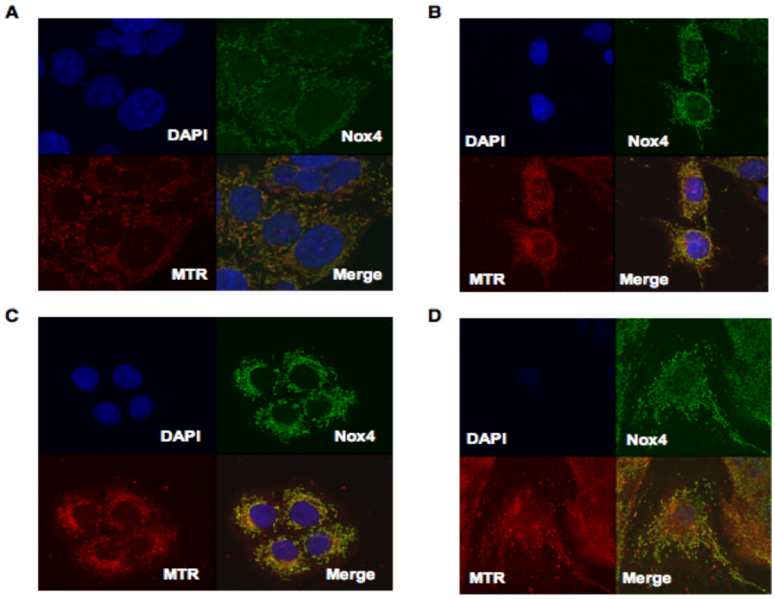

Fig. S4. Subcellular localization of Nox4 in glomerular endothelial cells (*A*), glomerular epithelial cells (*B*), aortic endothelial cells (*C*), and aortic vascular smooth muscle cells (*D*). Mitochondria were visualized with MTR 633 in live cells. Nox4 was then stained with rabbit anti-Nox4 using a FITC-linked donkey anti-rabbit secondary antibody, and nuclei were counterstained with DAPI.

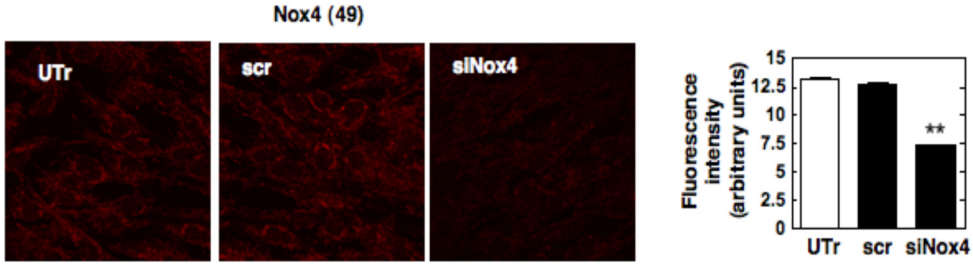

Fig. S5. Nox4 was visualized by immunofluorescence in untransfected (UTr) and siNox4- or scr-transfected cells with the commercial antibody [Nox4 (49)]. Fluorescence intensity was semiquantified, and values are the means ± SE from three independent experiments. **, $P < 0.01$ versus UTr cells.

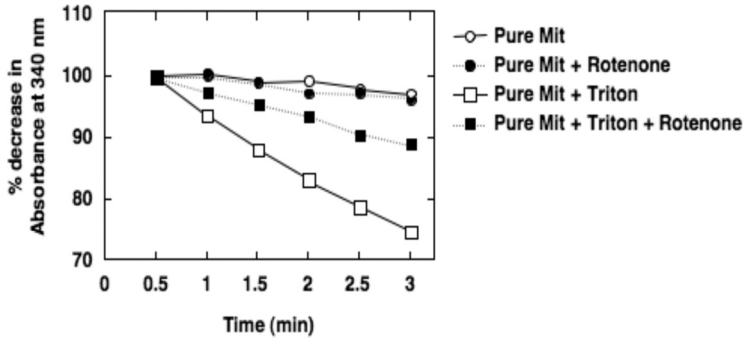

Fig. S6. Absence of rotenone-sensitive NADH oxidase activity in the Percoll gradient-purified mitochondrial preparation. NADH oxidation was monitored by the decrease in absorbance at 340 nm by spectrophotometry using 5 μg of Percoll-purified mitochondria with ubiquinone-2 as an electron acceptor as previously described (10, 11). Absorbance was measured for 3 min with or without addition of rotenone (1 μM) to the reaction mixture. The data are expressed as percentage decrease in absorbance at 340 nm. The data show that no (or minor) changes in absorbance at 340 nm are detected in the pure Mit fraction, and that NADH oxidation was not altered after treatment with rotenone, demonstrating the intactness of the mitochondria. In contrast, in conditions where the pure Mit fraction is permeabilized with Triton X-100, thereby allowing the accession of NADH to its binding site, NADH oxidation is markedly augmented and sensitive to rotenone, indicating that the NADH oxidase activity measured is due to complex I.

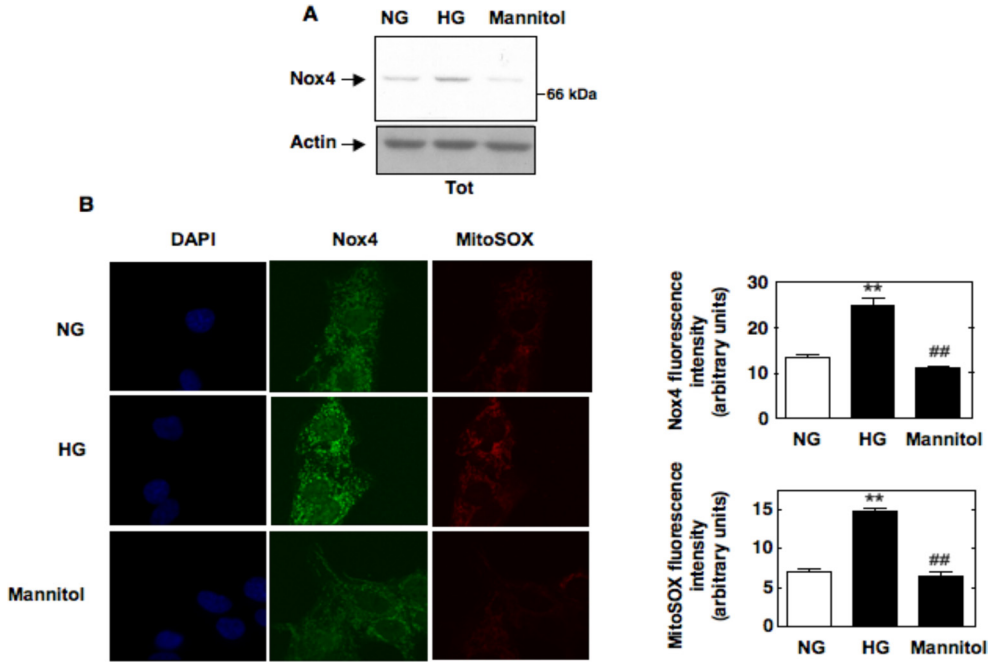

Fig. S7. (*A*) MCs were treated with normal glucose (NG, 5 mM D-glucose), high glucose (HG, 25 mM D-glucose), or mannitol (20 mM mannitol + 5 mM D-glucose) for 1 h. Equivalent amounts of cell lysates were analyzed by Western blot analysis for Nox4 expression by using our antibody. Actin was used as a loading control. (*B*) Representative images obtained by confocal fluorescence microscopy of MitoSOX Red fluorescence in MCs after exposure to NG (5 mM D-glucose), HG (25 mM D-glucose), or mannitol (20 mM mannitol + 5 mM D-glucose) for 1 h. After fixation and permeabilization, it was cell stained with our Nox4 antibody and appropriate FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI. In histograms on the left, fluorescence intensity was semiquantified, and values are the means ± SE from three independent experiments. **, $P < 0.01$ versus NG. ##, $P < 0.01$ versus HG.

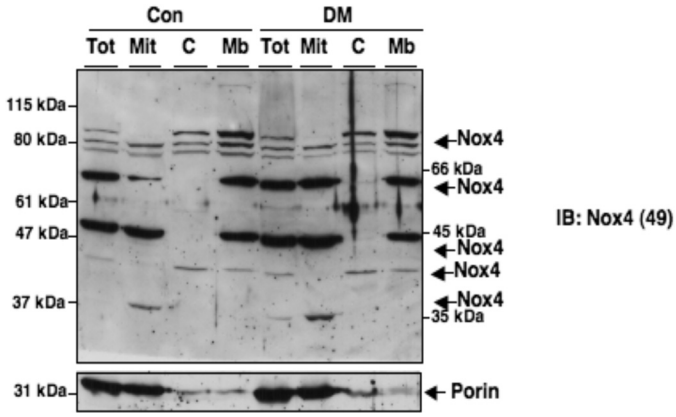

Fig. S8. Nox4 protein expression was detected by Western blot analysis using a commercially available antibody [Nox4 (49)] from subcellular fractions, Tot, pure Mit, C, or Mb isolated from control (Con) or diabetic (DM) rat kidney cortex. Porin was used as a mitochondrial marker.

Table S1. Probability of Nox oxidase localization to the mitochondria

Protein	MitoProt score, %
Rat Nox4	97
Rat Nox1	10.3
Rat gp91^{phox}/Nox2	0.81
Rat Nox3	11
Human COX IV	97
Human tuberin	1.6