ONLINE APPENDIX

Supplemental Methods

Detection of extracellular hydrogen peroxide. Extracellular hydrogen peroxide (H_2O_2) was determined using Amplex red assay. When combined with horseradish peroxidase, N-acetyl-3, 7-dihydroxyphenoxazine (Amplex red) reacts with H_2O_2 to produce the fluorescent molecule resorufin, which is restricted to the extracellular space. Cells grown in 24-well plates were treated with Ad-LacZ or Ad-Nox4$^{\Delta FAD}$ for 48 h followed by hypoxia for 16 h. The media were removed and cells were incubated with the Amplex Red reaction mixture (50 μM Amplex Red and 0.1 U/mL horseradish peroxidase) in phenol red-free DMEM at 37°C for 30 minutes. The reactions were protected from light. Fluorescence in the media was detected at 585 nm using an excitation 530 nm. Background fluorescence measured by a reaction without cells was subtracted from each value.

Assay of NADPH oxidase activity in cultured retinal endothelial cells. NADPH-dependent ROS generation was measured in cultured RCEC as described previously with minor modification (1). Briefly, cells were washed three times with ice-cold PBS, scraped from the plate, and centrifuged 800 rpm, 4°C, for 10 min. The cell pellets were resuspended and homogenized in lysis buffer (20 mM KH2PO4, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Protein concentration was measured by BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL). 20 μg of homogenates were added to 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 10 μM CM-H_2DCFDA, and 100 μM NADPH. Fluorescence intensity was quantified using a fluorescence micro-plate reader (Perkin Elmer, Waltham, MA) with excitation at 485 nm and emission at 535 nm.

Measurement of retinal vascular permeability. Retinal vascular permeability was quantified by measurement albumin leakage from blood vessels into the retina using the Evans blue-albumin method. Briefly, Evans blue (30 mg/ml, Sigma-Aldrich, St .Louis, MO) dissolved in PBS was injected into anesthetized mice through the femoral vein using 31-guage insulin syringes at a dose of 30 mg/kg. The mice were kept on a heating plate and temperature was maintained by ATC1000 DC (World Precision Instruments, Sarasota, FL). After 2 h, the mice were perfused via left ventricle with 0.1 M citrate buffer (PH 4.2) containing 1% paraformaldehyde. Immediately after perfuse, the retinas were dissected and incubated in 150 μl formamide at 70 °C for 18 h. Supernatant containing extracted extra-vascular Evans blue were obtained by centrifuging at 70,000 rpm for 30 min at 4 °C. Absorbance was measured at OD620nm and concentration of Evans blue was calculated according to standard curve of Evans blue in formamide. Protein concentration was quantified by BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL). Results were expressed as micrograms of Evans blue per milligram of total protein.

Measurement of NADPH oxidase activity in mouse retina. Activity of NADPH oxidase in mouse retinas was assayed using dichlorofluorescein (DCF) following a documented protocol (2). Briefly, unfixed frozen eye sections (10 μm) were freshly prepared and incubated with 100 μM NADPH and 10μM CM-H_2DCFDA at 37°C for 60 minutes. The specificity of the reaction was determined by incubating retinal sections in buffer containing CM-H_2DCFDA with or without PEG-SOD, catalase, or DPI. NADPH-dependent ROS generation in the retina was

assayed by fluorescence microscopy (Olympus, Hamburg, Germany). Fluorescence intensity was quantified using NIH Image J software (NIH, Bethesda, MD).

References:

1. Eid AA, Gorin Y, Fagg BM, Maalouf R, Barnes JL, Block K, Abboud HE: Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes* 58:1201-1211, 2009
2. Al-Shabrawey M, Rojas M, Sanders T, Behzadian A, El-Remessy A, Bartoli M, Parpia AK, Liou G, Caldwell RB: Role of NADPH oxidase in retinal vascular inflammation. *Invest Ophthalmol Vis Sci* 49 3239-3244, 2008