ONLINE DATA SUPPLEMENT

Nox1, Nox4 and Nox5, promote vascular permeability and neovascularization in retinopathy

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Running title: Nox1, Nox4 and Nox5 promote retinal vasculopathy

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Supplemental Methods

Animals

All experiments were approved by the Alfred Research Alliance animal ethics committee (Applications E/1520/2015/M and E/1602/2016/M) and performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes. All animals were housed at 22°C in a 12-hour light/dark cycle and had free access to water and food. Investigators were blinded to the experimental groups.

Forty to 60 female Wistar Kyoto Rats (WKY) and spontaneously hypertensive rats (SHR) that were 6 to 8 weeks old were purchased from the Australian Animal Resources (Perth, Western Australia) and then studied for 8 weeks. Rats were randomly allocated to be non-diabetic and diabetic. Diabetes was induced with a single tail vein injection of streptozotocin (55mg/kg, Sigma-Aldrich, CA, USA). Diabetic rats were excluded from the study if their blood glucose levels were lower than 12 mmol/L and humanely euthanized. Rats were administered insulin (Humulin NPH, Eli Lilly, IN, USA) at a dose of 12.5 IU/kg body weight twice a week. Non-diabetic rats received a single tail vein injection of vehicle (0.1M citrate buffer, pH 7.4). Cohorts of 6 to 8-week old diabetic rats received the dual Nox1/4 inhibitor GKT136901 (500 µl, 60 mg/kg, Genkyotex, Switzerland)[1] or vehicle (1.2% [v/w] methyl cellulose and 0.1% [v/w] polysorbate-80 in sterile water) by oral gavage each day for 8 weeks. Tissues were collected at the end of the 8-week study period. The dose of GKT136901 was based on previous studies in diabetic animals.[1] Systolic blood pressure was measured by tail cuff plethysmography (Coda, Kent Scientific, CT, USA) at the end of the experiment.

The mouse model of oxygen-induced retinopathy (OIR) was performed as published previously.[2] Briefly, male and female Nox5 transgenic mice and littermate controls at postnatal day (P) 7 of age and their nursing mothers were placed in a hyperoxia chamber

(75% O_2) cycled with 2 hours room air (22% O_2) each day for 5 days. On P12, mice were placed in room air until P18 to induce retinal neovascularization. Three litters of mice per group were evaluated. Only mothers with 5 to 7 pups were studied to ensure optimal body weight gain for the experiment. OIR was induced in Nox5 transgenic mice which were generated in-house.[3] Briefly, the Nox5 mouse strain (FVB/N background) was produced by ligating the purified Nox5β gene-coding region into the tetracycline-responsive promoter Pbi-1 (Clontech, Mountain View CA, USA). The VE-Cad-tTA (FVB/N background) mouse strain was generated by introducing the endothelial-specific VE-cadherin (VE-Cad, also known as *Cdh5*) promoter to the tetracycline-controlled transcriptional activator (tTA-off) gene. The VE-CAD-tTA and Nox5 transgenic mice were backcrossed to $ApoE^{-/-}$ mice (C57BL/6J background) for 10 generations to generate VE-Cad-tTA and Nox5 transgenic mice on a C57BL/J background. For the subsequent experiment, $ApoE^{-/-}$VECAD-tTA and $ApoE^{-/-}$ Nox5 transgenic mice were crossed to generate endothelial cell-specific Nox5 transgenic mice ($VE\text{-}Cad^{+}Nox5^{+}$). Littermates generated from this cross were used as controls ($VE\text{-}Cad^{-}Nox5^{-}$, $VE\text{-}Cad^{-}Nox5^{+}$, $VE\text{-}Cad^{+}Nox5^{-}$). The Nox5 expression under the Tet-off system was silenced by the continuous administration of doxycycline (1 mg/ml in drinking water, Sigma Aldrich) in drinking water until birth.

Immunohistochemistry for 8-hydroxyguanine and glial fibrillary acidic protein

Three-µm retinal paraffin sections were dewaxed and hydrated and antigen retrieval was performed by heating the sections in sodium citrate buffer (pH 6.0) as previously published.[4,5] Sections were incubated with an anti-8-hydroxyguanine (8-OHdG, 1/1000, cat. No. 4345-MC-050, Trevigen, MD, USA) or a glial fibrillary acidic protein (GFAP) antibody (1/500, cat. No. Z0334, Dako, CA, USA) in phosphate-buffered saline (PBS) overnight at 4°C. Sections were washed and incubated with an anti-mouse conjugated with biotin (1/500,

Dako) for 8-OHdG and anti-rabbit conjugated with Alexa Flour® 488 for GFAP for 45 minutes at room temperature. The signal was detected using the ABC avidin-biotin complex kit (Vector Labs, CA, USA) and sections were counterstained with hematoxylin. Quantitation was performed using Image-Pro Plus (Media Cybernetics, MD, USA) with the dropper tool to specifically detect the brown immunolabeling for 8-OHdG and green immunolabeling for GFAP. Values are expressed as a percentage of the total retinal field.

ELISA for retinal VEGF, MCP-1 and vitreal albumin

Retinal and vitreal samples were harvested as previously described.[4, 6] Briefly, vitreal samples (30 to 40 µl) were collected using a pipette tip from the vitreous cavity after making an incision near the peripheral retina. Vitreal samples were collected in 200 µl PBS containing a protease cocktail (1/100, Thermo Scientific, CA, USA). Retinal tissues were then dissected, and the remaining vitreal gel was carefully removed. Retinal tissues were digested in phosphate buffer (pH 8) containing 1/100 protease cocktail (Thermo Fisher Scientific, CA, USA) as previously described with minor modifications.[7] Retinas were homogenized with a mix of 0.5 to 1 mm-sized beads using a Bullet Blender (Next Advanced, NY, USA) for 5 minutes at a speed of 8 at 4°C. Total retinal protein concentration was quantitated using a Bradford assay kit (Bio-rad, CA, USA). Retinal and vitreal VEGF and MCP-1 levels were measured using a rat VEGF (cat. No. DY564, R&D systems, MN, USA), or a mouse VEGF (cat. No. DY493 R&D systems), or a rat MCP-1 ELISA kit (cat. No. 555130, BD, NJ, USA) and expressed as retinal VEGF or MCP-1 per total protein concentration. Vitreal albumin was measured using a rat albumin ELISA kit (cat. No. E111-125, Bethyl Laboratories, TX, USA) or a mouse albumin ELISA kit (cat. No. E99-134, Bethyl Laboratories) according to the manufacturer's instructions and expressed as total protein concentration.

Retinal vascular leakage

According to our previous methods,[5] rats were perfused with 200 to 300 ml of PBS (pH 7.4) via the right atrium using a perfusion pump (Masterflex C/L, IL, USA) at the flow rate of 50 ml per minute to remove intravascular albumin. Retinas were removed and digested as above for ELISA assays and albumin levels were measured according to the manufacturer's instructions and expressed as total protein concentration.

Real time PCR

RNA from retinal tissues and cells was extracted using a RNeasy Mini kit (Qiagen, Hilden, Germany) as previously published.[4, 5] The total RNA concentration was quantitated using Qiaxpert (Qiagen) and 1 μg of RNA was subjected to DNase treatment (Turbo DNA-free kit, Life Technologies, CA, USA) and reverse transcription (Roche, CA, USA). Real-time PCR was performed using a SYBR green master mix kit (Thermo Fisher Scientific) according to the manufacturer's instructions and mixed with the following primers to VEGF, ICAM-1, Angiopoeitin-2, Nox1, Nox2, Nox4 or Nox5 (Table S2). Signals were analyzed using QuantStudio 5 (Life Technologies) and the $2^{-\Delta\Delta Ct}$ method.

Lucigenin superoxide assay

Protocols were performed as previously published.[8, 9] Freshly isolated retinas were incubated in 0.2 ml Krebs/HEPES buffer containing 5 μM of lucigenin (Sigma-Aldrich, IL, USA, 100 μl in PBS) in the dark for 10 minutes at room temperature. Luminescence was measured every 5 minutes for 30 minutes using a luminometer (CLARIOstar, BMG Labtech, Gütersloh, Germany). The repeated measurements were averaged and expressed as relative luminometer units (RLU) normalized to total protein concentration for each retina.

Western blot

Protocols were performed as previously published.[10] Briefly, protein extract from retinal tissues or cultured cells were separated using electrophoresis, transferred to PVDF membranes and blocked with 5% skim milk. The membranes were incubated with one of the following primary antibodies, anti-occludin-1 (1/500, Cat. No. 711500, Life Technologies), anti-ZO-1 (1/500, cat. No, 61-7300, Life Technologies) or anti-Nox5 (1/500, cat. No. sc-67007, Santa Cruz, TX, USA) overnight at 4°C on a rotator. Membranes were washed overnight with PBS and re-probed with an anti-β-actin antibody (1/1000, cat. No. 3700, Cell Signalling, CA, USA). The signals were detected using a SuperSignal West Pico ECL kit (Thermo Scientific) on a ChemiDoc (Biorad) and quantitated using Image Lab software (Bio-rad).

Primary cultures of bovine retinal endothelial cells

Bovine retinal endothelial cells (BREC) were cultured as previously published.[4] Briefly, one million BRECs from passages 5 to 6 were cultured in BREC culture media on 10-mm gelatin –coated petri dishes until they reached 70% confluence.[10] Cells were incubated in DMEM (Life Technologies) containing either 5.5 (normal glucose) or 25 mmol D-glucose (high glucose) with GKT136901 (5 µM) or dimethyl sulfoxide (DMSO, 0.01%, Sigma-Aldrich).[5] After 72 hours, BREC and cell culture supernatants were harvested. Cell viability was measured using a lactate dehydrogenase assay kit (Thermo Fisher) and only samples with more than 80% viability were used for analysis. For real-time PCR, RNA was extracted using the RNA mini kit (Qiagen,) according to the manufacturer's protocol for cultured cells. Protein extraction was performed using RIPA buffer containing 1/100 protease inhibitor cocktail (Thermo Fisher). VEGF release was measured in cell culture supernatants using a

commercial bovine VEGF ELISA kit (Cat. No. VS0286B, Kingfisher Biotech, MN, USA) according to the manufacturer's instructions.

Nox5 siRNA transfection

In a separate set of experiments, BREC from passages 5 to 6 were subjected to transfection assays using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. The Nox5 silencing RNA (siRNA, Cat. No. sc-45486) and negative control scrambled siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. Briefly, one million BREC were cultured in BREC media on 10-mm gelatin-coated Petri dishes until they reached 70% confluence. Before the transfection, cells were serum starved overnight. For each sample, cells were transfected with 1 µl of 20 µM Nox5 siRNA or the negative control siRNA in 1.5 ml of OPTI-MEM (Life Technologies) for 24 hours. Cells were then changed to a fresh serum-free culture medium without antibiotics. After 24 hours, cells were incubated in DMEM (Life Technologies) containing either 5.5 (normal glucose) or 25 mmol D-glucose (high glucose) for 72 hours.

Flow cytometry for dihydroethidium

To measure cellular ROS, we used a previously published method.[11] BREC were harvested and incubated with dihydroethidium (DHE, 5 µm in PBS, Sigma-Aldrich, MO, USA) for 10 minutes at room temperature and washed with FACS buffer. DHE is readily taken up by cells, and in the presence of superoxide, is oxidized to 2-hydroxyethidium, which emits red fluorescence when excited at 535 nm. The fluorescent signal was detected by a flow cytometer (BD LSRII, NJ, USA) using a 610/20-nm filter and the 561 nm laser and is expressed as mean fluorescence intensity (MFI).

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Table S1. Body weight, blood glucose and systolic blood pressure in WKY and SHR.

	Body weight (g)	Blood glucose (mmol/L)	Systolic blood pressure (mmHg)
WKY Non-diabetic	233.3 ± 14.3	10.7 ± 0.9	108.3 ± 3.7
WKY Diabetic	179.4 ± 8.6***	31.2 ± 0.6***	103.2 ± 2.8
WKY Diabetic + GKT	180.1 ± 16.3***	28.0 ± 1.9***	105.1 ± 4.6
SHR Non-diabetic	182.5 ± 9.1	9.7 ± 0.6	149.7 ±3.9‡‡‡
SHR Diabetic	144.0 ± 21.7†††	28.9 ± 1.9†††	143.7 ± 2.6‡‡‡
SHR Diabetic + GKT	146.3 ± 22.7†††	31.0 ± 1.6†††	144.1 ± 4.0‡‡‡

GKT, GKT136901. *n*=21 to 26 rats per group. ***P<0.001 to WKY non-diabetic. †††P<0.001 to SHR non-diabetic. ‡‡‡P<0.001 to WKY groups. Values are mean±SEM.

Table S2. Real-time PCR Primers

Gene	Species	Forward primer (5'-3')	Reverse primer (5'-3')
Nox1	Rat	GACCAATGTGGGACAATGAGTTT	GGAGGGCCGCATAAGAAAA
Nox2	Rat	AAGTGATTGGCCTGAGATTCATC	GAGCAACAAGCCAGTCACCA
Nox4	Rat	GAATGAAGGGCAGAATCTCAGA	CAACCTCGCCTTCCACAAAC
VEGF	Rat	GGGCTGCTGCAATGATGAA	TTGATCCGCATGATCTGCAT
Angiopoietin-2	Rat	GCCCAGGTCGCGATGAT	TTGCCTCACTCAGCATCTTGTACA
TNFα	Rat	TGATCGGTCCCAACAAGGA	TGGGCTACGGGCTTGTCA
ICAM-1	Rat	TAAATGGACGCCACGATCAC	CAGAAAGTATCCGCTGTGAGTGTT
Nox1	Bovine	GCAATGATGTGAATAGCTGTGTGTAG	GGACAGAGGGACTCTTCAAAGC
Nox2	Bovine	ATCCCTGCTCCCACTAACATCA	GAGCAACAAGCCAGTCACCA
Nox4	Bovine	GACACACTGGGACAATGTAGAATAATAATATACT	GAGCAACAAGCCAGTCACCA
Nox5	Bovine	CCCACCATTGCTCGCTATG	CCGAATGTGCAGCCAGATAGT
VEGF	Bovine	GCGGCTATGGGTAGTTCTGTGT	CGAACGTACTTGCAGATGTGACA
ICAM-1	Bovine	CAGTTCCCAGCGGCAAGA	GAGCCCGCCAAGGTCAT
Nox1	Mouse	CATTTTGCAACCGTACACTGAGA	CAGAAGCGAGAGATCCATCCA
Nox2	Mouse	AGGCAGAACCAACACTTAACCTTT	CATCCAAGCTACCATCTTATGGAA
Nox4	Mouse	GACTGGAAACCATACAAGTTAAGAAGACT	ACTGAGGTACAGCTGGATGTTCAC
VEGF	Mouse	AGCAGAAGTCCCATGAAGTGATC	TCAATCGGACGGCAGTA
ICAM-1	Mouse	GGAGGTGGCGGGAAAGTT	TCCAGCCGAGGA
Nox5	Human	CCCACCATTGCTCGCTATG	CCCGAATGTGCAGCCAGATAGT

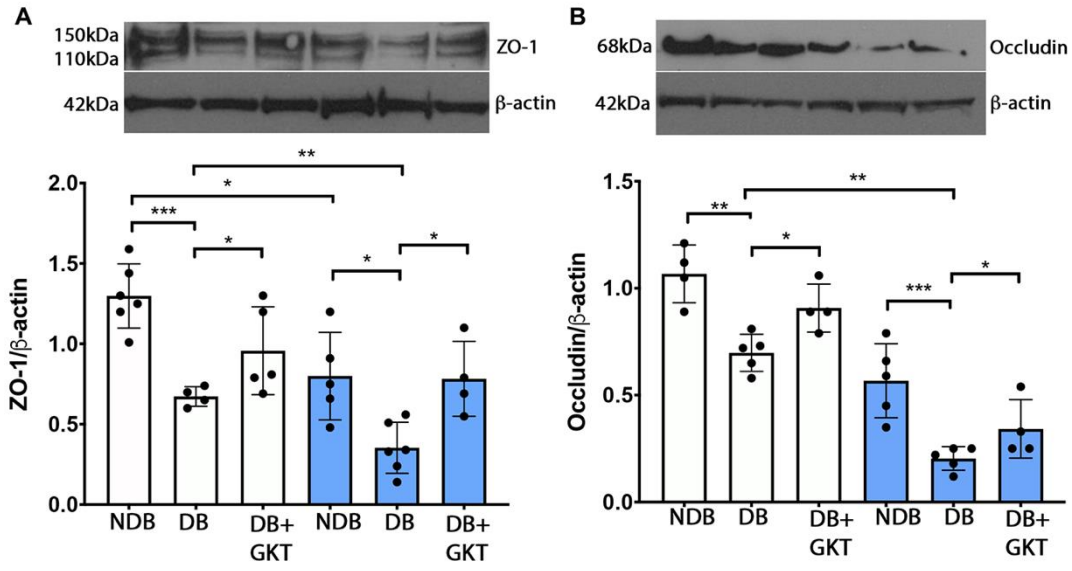

Figure S1. Diabetes-induced changes in ZO-1 and occludin levels in retina are exacerbated by hypertension and reduced by Nox1/4 inhibition.
NDB, non-diabetic. DB, diabetic. GKT, GKT136901. (**A**) ZO-1 protein levels and (**B**) occludin protein levels in retina. *n*=4 to 6 rats per group. **P*<0.05, ***P*<0.01 and ****P*<0.001. Values are mean±SD.

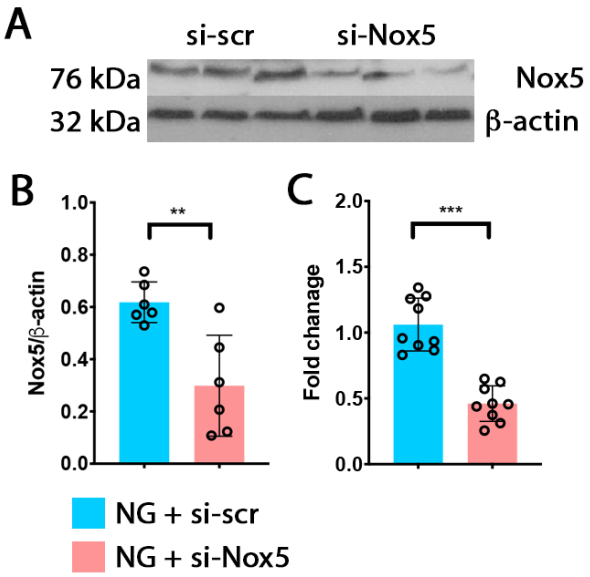

Figure S2. Nox5 protein and mRNA levels in primary cultures of retinal endothelial cells.

NG, normal glucose. (**A**) Western blot showing Nox5 protein at ~76 kDa and β-actin at ~43kDa. Nox5 silencing RNAs (si-Nox5) reduced Nox5 (**B**) protein and (**C**) mRNA levels compared to control scrambled RNAs (si-scr). ***P*<0.01 and ****P*<0.001. *n*=6 to 9 samples from at least 2 to 3 independent experiments. Values are mean±SD.

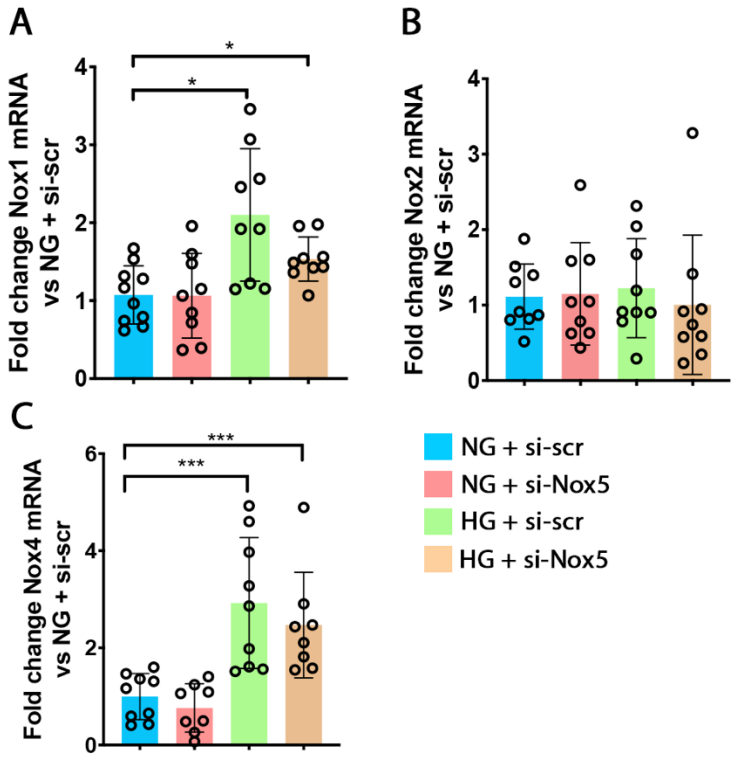

Figure S3. Nox1, Nox2 and Nox4 expression levels in bovine retinal endothelial cells were unaffected by Nox5 silencing RNAs.
NG, normal glucose. HG, high glucose. The mRNA levels of (**A**) Nox1, (**B**) Nox2 and (**C**) Nox4 following exposure to high glucose and Nox5 silencing RNAs (si-Nox5) or control scrambled RNAs (si-scr). **P*<0.05 and ***P*<0.01. *n*=8 to 10 samples from 3 independent experiments. Values are mean±SD.

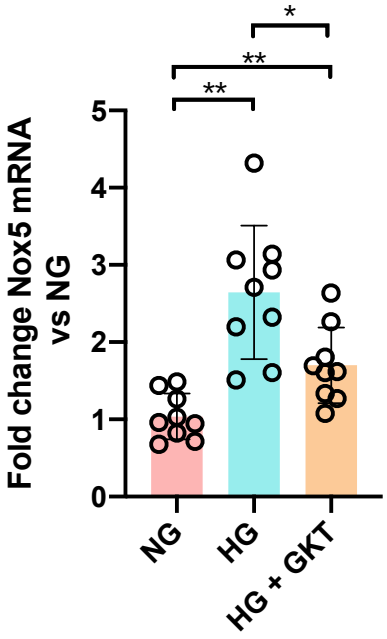

Figure S4. Nox5 mRNA levels in bovine retinal endothelial cells treated with GKT136901. NG, normal glucose. HG, high glucose. GKT136901 (GKT) reduced the mRNA levels of Nox5 following exposure to high glucose. *P<0.05 and **P<0.01. n=9 samples from 3 independent experiments. Values are mean±SD.

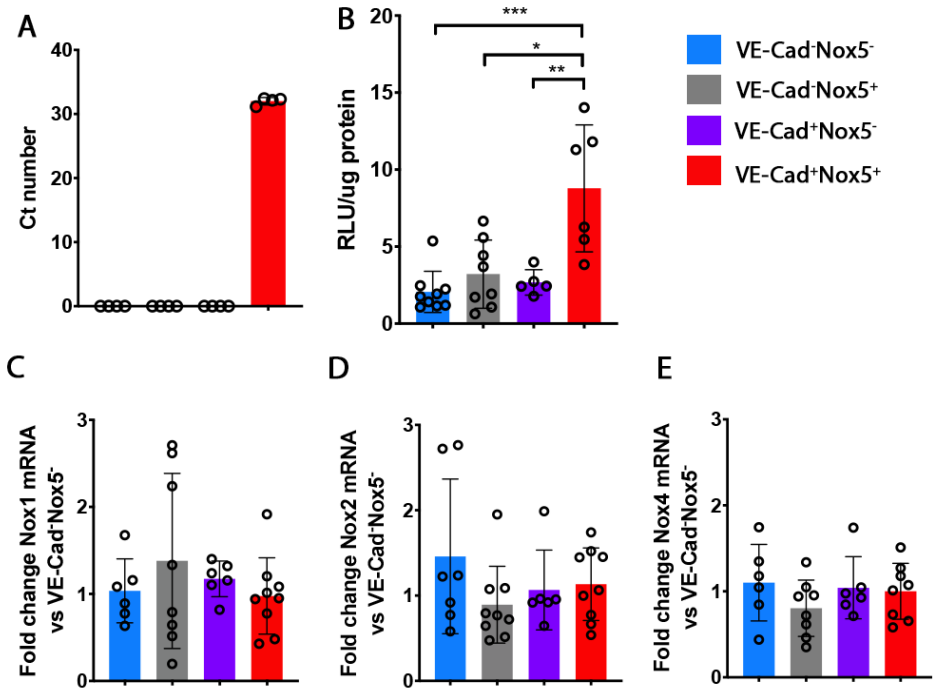

Figure S5. The expression of Nox1, Nox2, Nox4 and Nox5 in retina from endothelial cell-specific Nox5 transgenic (VE-Cad^{+}Nox5^{+}) mice with OIR.

(**A**) Ct numbers of human Nox5 mRNA by real-time PCR in VE-Cad^{+}Nox5^{+} mice compared to control littermates with OIR. (**B**) Superoxide production by lucigenin assay was increased in retinas from VE-Cad^{+}Nox5^{+} mice compared to control OIR littermates. The mRNA levels of (**C**) Nox1, (**D**) Nox2 and (**E**) Nox4 in the retinas of VE-Cad^{+}Nox5^{+} OIR mice were comparable to OIR controls. n=6 to 9 mice per group *P<0.05 and **P<0.01. Values are mean±SD.