Materials and Methods

Animals

Experiments were performed on 8–12 week-old 1) C57Bl/6 mice, 2) endothelium–specific miR34a knockout mice, 3) p66ShcRNAi transgenic mice, and 4) db/db (BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J) homozygous and heterozygous mice. The endothelium–specific miR-34a knockout mice (E-miR-34a$^{-/-}$) were generated by crossing *miR34a*$^{fl/fl}$ *mice with cadherin-5-Cre* mice, in which the Cre recombinase is driven by an endothelium–specific cadherin-5 promoter. *MiR-34a*$^{fl/fl}$ mice were used as controls. P66shcRNAi transgenic mice (p66-T) were created in a B6SJL background. The transgene consists of a short hairpin sequence 5involvementCT GTC ATC GCT G TTC AAG AGA C AGC GAT GAC-3′ directed to nucleotides 239 to 257 of p66Shc mRNA, transcribed by the U6 RNA polymerase III promoter. Nucleotides 239 to 257 in p66Shc mRNA are in the coding region for the CH2 domain of p66Shc. This domain is conserved in human and mouse, and not found in p46/52shc RNA. Mice were genotyped for the presence of the transgene in tail genomic DNA by PCR. Non-transgenic littermates (p66-N) were used as controls. Mice were fed standard chow (Research Diets Inc., New Brunswick, NJ) containing (kilocalories): 10% fat, 70% carbohydrate, and 20% protein (D12450B). Terminal experiments were performed after mice were anesthetized (2–5% isoflurane). The entire aorta was isolated and used for immunoblotting, real-time qPCR, and vascular function. All protocols were approved by the institutional animal care and use committee of University of Iowa.

Immunohistochemistry

Aortic sections were de-paraffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM). Sections were probed with appropriate primary antibodies. Sirt1 antibody (Santa Cruz Biotech, Dallas, TX), p-S36-p66Shc antibody (Abcam, Cambridge, MA), von Willebrand Factor antibody (Abcam), and 8-hydroxydeoxyguanosine (8-OHdG) antibody (Abcam) were used at a 1:50 to 1:200 dilution followed by a biotinylated secondary antibody, streptavidin peroxidase solution, DAB peroxidase substrate, and hematoxylin counterstain or by an AlexaFluor®647-conjugated anti-mouse IgG (1/500) as the secondary antibody. Sections were digitally imaged with Olympus BX-61. Endothelial layer was quantified using ImageJ.

Mouse Vascular Reactivity

Male mice, 8–12 weeks old, were anesthetized and euthanized by rapid cardiac excision. The whole aorta was carefully excised and placed in ice-cold Krebs buffer (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11 mM glucose, 0.0026 mM $CaNa_2EDTA$). The aortas were cleaned of excess fat, cut transversely into 5–10 rings (1.8-2.0mm wide). The arterial rings were placed in oxygenated chambers (95% O_2/5% CO_2) super fused with Krebs buffer solution and maintained at 37°C and pH 7.4. Each ring was suspended between two wire stirrups in a 6 ml organ chamber of a four–chamber myograph system (DMT). One stirrup was connected to a three–dimensional micromanipulator and the other to a force transducer. The contractile force was recorded electronically. All rings were stretched to 2, 000 mg in 500 mg increments over a 1 h period to optimize the contractile response to KCl. One dose of KCl (60 mM) was added to verify vascular smooth muscle viability. Cumulative dose–response curve for phenylephrine (PE) (10^{-9} to 10^{-5} M) was obtained by administering the drug in one-half log doses. Endothelium–dependent vasodilatation was determined by generating dose–response curves to acetylcholine (Ach) (10^{-9} to 10^{-5} M). Vasorelaxation evoked by acetylcholine was expressed as the percent of maximal contraction. Endothelium–independent vasodilatation was measured by the vasorelaxation evoked by cumulative sodium nitroprusside (SNP) in rings pre-constricted with phenylephrine (10^{-6} M) and was also expressed as percent maximal contraction.

Isolation and culture of mouse aortic endothelial cells

Mouse aortic endothelial cells were isolated as described previously[1] .Male mice, 8–12 weeks old, were anesthetized and euthanized by rapid cardiac excision. The whole aorta was carefully excised and placed in DMEM supplied with 20% FBS. The aortas were cleaned of excess fat and connecting tissues. A 24 gauge cannula was inserted into one end of aorta and tied using surgical sutures. After washing with serum-free DMEM, the aorta was filled with collagenase II (Sigma, 2mg/ml) through the cannula, and incubated for 45min at 37 ºC. The aorta was then flushed several times with DMEM. The endothelial cells in the flushed medium were spun down at 1,200 rpm for 5 min, and gently resuspended in endothelial growth medium (EGM-2, Lonza, Walkersville, MD, USA) supplied with 10% FBS. Penicillin at 100ug/ml was added. The cells were seeded in a 12-well plate (1 mouse aorta/well). The medium was changed after 4 h. In pilot experiments the purity of endothelial cell preparations was tested using vWF and eNOS (endothelial markers) and α-smooth muscle actin (Supplemental Figure IXA, B & C)

Immunocytochemistry

Aortic endothelial cells were fixed in 4% formaldehyde for 15 min at room temperature, followed by blocking and permeabilization (PBS with 5% goat serum and 0.2% Triton X-100) for 30-60 min at room temperature. Cells were incubated with an antibody against von Willebrand Factor (vWF) (Abcam, 1:200 dilution) overnight at 4 ºC. After washing with PBS 3 times, the cells were incubated with an Alexa 647 anti-sheep secondary antibody (Abcam, 1:400 dilution) at room temperature for 1 h. The cells were washed 3 times with PBS, and the slides were mounted with an anti-fade reagent with DAPI and photographed with confocal microscopy.

Cell culture, plasmid/siRNA transfections, and adenoviral infections

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA USA) and cultured in endothelial growth medium (EGM-2, Lonza, Walkersville, MD USA). Cells were transfected with plasmids, validated siRNA-p66Shc (Invitrogen, Carlsbad, CA), siRNA-p53 (Santa Cruz Biotechnology, USA), or negative control siRNA purchased from Invitrogen with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were infected with 6×10^{11} viral particles per ml of the control AdLacZ or the AdSIRT1 adenoviral stocks, and incubated at 37°C for 24 h. For high glucose experiments, cells were incubated in EGM-2 supplemented with 25 mM glucose. Cells incubated in 25 mM mannitol were used as controls.

Quantitative Real time PCR

Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method. Total RNA from cultured cells, serum, or tissues of mice, was isolated by the TRIZOL (Invitrogen) method. Real-time PCR was performed using the Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). The following primers (EXIQON) were used. Mouse Sirt1 forward 5′- AAT GCT GGC CTA ATA GAC TTG CA -3′, reverse 5′- CCG TGG AAT ATG TAA CGA TTT GG -3′; human Sirt1 forward 5′-TCGCAACTATACCCAGAACATAGACA-3′, reverse 5′-CTGTTGCAAAGGAACCATGACA-3′; human p53 forward 5′-GGTGCGTGTTTGTGCCTG-3′, reverse 5′-TCTTGCGGAGATTCTCTTCCT -3′; miR-34a 5′-UGGCAGUGUCUUAGCUGGUUGU-3′, human GAPDH: forward 5′-ATG ACA TCA AGA AGG TGG TG -3′; reverse 5′-CAT ACC AGG AAA TGA GCT TG -3′; Mouse GAPDH 5′-GGC AAA TTC AAC GGC ACA -3′; 5′-CGC TCC TGG AAG ATG GTG AT -3′. A universal reverse primer (cDNA synthesis kit, Quanta Biosciences) was used for quantification of miRs. Mouse GAPDH and RNU6 (Quanta Biosciences) were used as internal controls or an external spike-in synthetic oligonucleotide cel-miR-39 (Norgen Biotek, Canada) was used as a control for mRNA and miR quantification, respectively.

***Ex vivo* infections with adenoviruses and transfections with a miR-34a mimic**

MiR-34a mimic (5′-UGGCAGUGUCUUAGCUGGUUGU-3′, Ambion Life Technologies) and scrambled control (Ambion) or AdLacZ and AdSIRT1 were transfected into freshly isolated aortas from mice. MiR-34a mimic was mixed with Lipofectamine 2000 followed by addition to the medium. After 4 h, aortic rings were moved to fresh medium and further incubated for 24 h. After 24 h of miR-34a mimic transfection, AdLacZ or AdSIRT1adenovirus was directly added to the medium and incubated for another 24 h.

***In vivo* inhibition of miR-34a**

Locked nucleic acid miR-34a inhibitor (5′-AGCTAAGACACTGCC-3′,) or a scrambled nucleotide (5′-ACG TCT ATA CGC CCA-3′) was administered to db/db and db/+ mice using an ALZET osmotic pumps that were implanted subcutaneously in mice. Each mouse received a total dose of 400 µg of miR-34a inhibitor or scrambled nucleotide over 28 days.

STZ-induced mouse model of diabetes

Streptozocin (STZ) in sterile 0.1M citrate buffer was injected at 100 mg/kg intraperitoneally into mice starved for 6 hrs. Vehicle buffer was used in control mice. STZ-injected mice were monitored for body weight and blood glucose.

Luciferase reporter assays

The miR-34a promoter luciferase reporter plasmid was obtained from Addgene (Cambridge, MA). P66shc-WT and S36A mutant plasmids have been previously described[2]. The reporter was co-transfected with a renilla luciferase plasmid driven by a constitutive promoter reporter into cells. Firefly and renilla luciferase luminescence were measured using a Dual Luciferase reporter kit (Promega, Madison WI) as per manufacturer's recommendations. Firefly/renilla ratio was calculated to normalize for variations in transfection efficiencies.

Western blotting

Immunoblotting was performed as previously described[3]. Chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce, Grand Island, NY), blots imaged with a Gel Doc 2000 Chemi Doc system (BioRad, Hercules, CA), and bands quantified using Quantity One software (BioRad).

ROS measurements

Diffusible H_2O_2 produced by cells was measured in conditioned medium using an Amplex Red probe, as previously described[4].

In situ hybridization (ISH) and quantification of miR-34a

Aortic sections were de-paraffinized with xylene, followed by Proteinase K treatment (10 µg/ml for 5 min). ISH buffer (EXIQON, production # 90000) was added with miR-34a probe (EXIQON, 5′ --3′ /5DigN/AACAACCAGCTAAGACACTGCCA/3Dig-N/) or with a scramble-miR probe (EXIQON, 5′ --3′ /5DigN/GTGTAACACGTCTATACGCCCA/3Dig-N/) at 20 nM or 40 nM, and incubated for 72 h at 56°C. After washing, the aortic sections were incubated in blocking solution for 15 min (5 ml PBS + 50 mg BSA + 100 ul Sheep serum + 2.5 ul Tween 20), followed by incubation with anti-DIG-FAB overnight (1:800 in Antibody dilution solution). Slides were dipped in a solution containing BCIP/NBT and incubated at 30°C for 48 h. The aortic sections were stained with nuclear fast red followed by dehydration. The slides were mounted with DPX and observe under the microscope. ISH was quantified using ImageJ.

Free fatty acid preparation

Palmitate solution was prepared as reported previously[5]. Palmitate (Sigma) was dissolved in 0.1 M NaOH at 72 °C for 30 min. The solution was then diluted in 20% fatty acid-free BSA (Sigma) pre-heated to 50 °C and incubated for additional 10 min at 50 °C. Fatty acid-free BSA was used as vehicle. Palmitate was used at 500 uM. Pilot experiments showed no significant decrease in cell viability at this concentration.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 6.0) statistical software. Significance of difference between two groups was evaluated using t-test. For multiple comparisons, one-way ANOVA was used and post-hoc analysis was performed with Tukey's test. Date are expressed as Mean ± SEM and considered significant if P values ≤ 0.05. All shown data is representative of at least three independent experiments.

References

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