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Lin Lu

Soohwa Jang
Thomas Jefferson University

Jiaqi Zhu
Thomas Jefferson University

Qing Qin
Thomas Jefferson University

Lijun Sun
Thomas Jefferson University

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Authors

Lin Lu, Soohwa Jang, Jiaqi Zhu, Qing Qin, Lijun Sun, and Jianxin Sun



Nur77 mitigates endothelial dysfunction through activation of both nitric oxide production and anti-oxidant pathways

Lin Lu^{a,1}, Soohwa Jang^{b,1}, Jiaqi Zhu^{b,1}, Qing Qin^b, Lijun Sun^b, Jianxin Sun^{b,*}

^a Department of Cardiovascular Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025, PR China

^b Center for Translational Medicine, Thomas Jefferson University, Philadelphia, PA, 19107, USA

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ABSTRACT

Background: Nur77 belongs to the member of orphan nuclear receptor 4A family that plays critical roles in maintaining vascular homeostasis. This study aims to determine whether Nur77 plays a role in attenuating vascular dysfunction, and if so, to determine the molecular mechanisms involved.

Methods: Both Nur77 knockout (Nur77 KO) and Nur77 endothelial specific transgenic mice (Nur77-Tg) were employed to examine the functional significance of Nur77 in vascular endothelium in vivo. Endothelium-dependent vasodilatation to acetylcholine (Ach) and reactive oxygen species (ROS) production was determined under inflammatory and high glucose conditions. Expression of genes was determined by real-time PCR and western blot analysis.

Results: In response to tumor necrosis factor alpha (TNF- α) treatment and diabetes, the endothelium-dependent vasodilatation to Ach was significantly impaired in aorta from Nur77 KO as compared with those from the wild-type (WT) mice. Endothelial specific overexpression of Nur77 markedly prevented both TNF- α - and high glucose-induced endothelial dysfunction. Compared with WT mice, after TNF- α and high glucose treatment, ROS production in aorta was significantly increased in Nur77 KO mice, but it was inhibited in Nur77-Tg mice, as determined by dihydroethidium (DHE) staining. Furthermore, we demonstrated that Nur77 overexpression substantially increased the expression of several key enzymes involved in nitric oxide (NO) production and ROS scavenging, including endothelial nitric oxide synthase (eNOS), guanosine triphosphate cyclohydrolase 1 (GCH-1), glutathione peroxidase-1 (GPx-1), and superoxide dismutases (SODs). Mechanistically, we found that Nur77 increased GCH1 mRNA stability by inhibiting the expression of microRNA-133a, while Nur77 upregulated SOD1 expression through directly binding to the human SOD1 promoter in vascular endothelial cells.

Conclusion: Our results suggest that Nur77 plays an essential role in attenuating endothelial dysfunction through activating NO production and anti-oxidant pathways in vascular endothelium. Targeted activation of Nur77 may provide a novel therapeutic approach for the treatment of cardiovascular diseases associated with endothelial dysfunction.

1. Introduction

The endothelium functions as functional barrier between the blood and out layer of vascular wall and critical for the maintaining of vascular tone [1]. Cardiovascular risk factors, such as hypercholesterolemia, obesity, and diabetes, initiate vascular inflammation and endothelial dysfunction, thus enhancing the development of cardiovascular disease, such as atherosclerosis and hypertension [1,23].

Nitric oxide (NO) is a major regulator to maintain the vascular homeostasis [45]. NO has been shown to possess many vascular protective

properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and vascular inflammation^{5 6 7}. In normal vascular endothelium, NO is synthesized in the blood vessel wall mainly by endothelial nitric oxide synthase (eNOS) through conversion of L-arginine to L-citrulline and may be scavenged by excess reactive oxygen species (ROS) [5]. Accumulating evidence suggests that under pathological conditions, due to the enhanced oxidative stress, the eNOS becomes dysfunctional resulting in production of superoxide instead of NO, which referred to as "eNOS uncoupling" [8,9]. Uncoupled eNOS generates superoxide radical (O₂^{·-}) and/or hydrogen peroxide (H₂O₂) from oxygenase domain, which eventually leads to decreased NO

* Corresponding author. Center for Translational Medicine, Thomas Jefferson University, 1020 Locust Street, Room 368G, Philadelphia, PA, 19107, USA.

E-mail address: Jianxin.sun@jefferson.edu (J. Sun).

¹ These authors contributed equally.

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Abbreviations

NR4As	Orphan nuclear receptor 4A family
TNF- α :	Tumor necrosis factor alpha
eNOS	Endothelial nitric oxide synthase
HO-1	Heme oxygenase-1
SODs	Superoxide dismutases
GCH1	GTP cyclohydrolase I
NO	Nitric Oxide
HG	High glucose
Ach	Acetylcholine
SNP	Sodium nitroprusside
ROS	Reactive oxygen species
STZ	Streptozotocin
DHE	Dihydroethidium
BH4	Tetrahydrobiopterin
GPx-1	Glutathione peroxidase-1
qPCR	Quantitative real-time polymerase chain reaction
ChIP	Chromatin immunoprecipitation
HUVECs	Human umbilical vein endothelial cells
NBRE	NGFI-B response element
STZ	Streptozotocin

bioavailability and conversion of NO into peroxynitrite (ONOO⁻) that has deleterious effects leading to endothelial dysfunction [7,10,11]. Superoxide dismutases (SODs) have been shown to be important anti-oxidant enzymes in scavenging O₂⁻ and maintaining normal endothelial function [12,13]. Emerging evidence indicates that increased production of ROS and decreased NO bioavailability are essentially implicated in endothelial dysfunction associated with human diseases, such as atherosclerosis, hypertension, diabetes, and aging [14, 15].

Several co-factors including 6R-5,6,7,8-tetrahydrobiopterin (BH4) are essentially involved in regulating eNOS activity [5,16]. BH4 is one of the major co-factors that have been shown to regulate NO production through maintaining and stabilizing the eNOS coupling dimer state, thus facilitating electron transfer from the reductase domain to its oxygenase domain [17,18]. BH4 levels are mainly regulated by Guanosine triphosphate cyclohydrolase I (GCH1)-mediated *de novo* biosynthetic pathway from guanosine 5'-triphosphate (GTP) [8,9,18], and dihydrofolate reductase (DHFR)-mediated conversion of BH4 from BH2, which is known as salvage pathway [10,19]. Indeed, loss of endothelial GCH1 has been shown to increase production of superoxide and decrease NO production, thus impairing acetylcholine-induced vasodilation [20–22]. These studies suggest that BH4 is the key determinant implicated in maintaining normal endothelial function and preventing development of cardiovascular disease.

Orphan nuclear receptor 4A members (NR4As) are first identified as immediate early-response genes and their expression is induced by various external stimuli, including growth factors, inflammatory cytokines, and hormones [23]. NR4As consist of three members, namely Nur77 (NR4A1), Nurr1 (NR4A2), and NOR-1 (NR4A3). Like other nuclear receptors, NR4As contain the DNA-binding domain, zinc-finger and C-terminal domain [24,25]. It has been increasingly recognized that Nur77 is essentially involved in the development of certain human diseases, including atherosclerosis, diabetes, and obesity [26]. We and others have shown that Nur77 is increased by inflammatory cytokines, high glucose, and high fat diet in various types of cells and tissues, hence, constituting a potent negative feed-back mechanism to suppress the progression of various diseases, including diabetes, obesity, and inflammation [27–30]. Recently, Nur77 knockout (Nur77 KO) mice have been shown to increase susceptibility to high fat-induced obesity, further indicating the pathophysiological relevance of Nur77 in

metabolic disease [31,32]. At this time, little is known about the role of Nur77 in endothelial dysfunction. In the present study, we found that Nur77 prevents endothelial dysfunction under inflammatory and high glucose conditions at least in part through upregulating the expression of GCH1 and SODs in vascular ECs.

2. Material and methods

2.1. Animal experiments

Nur77 KO mice were purchased from the Jackson Laboratory (stock no. 006187) to generate Nur77 KO mice and WT littermate controls. Nur77 KO mice have been backcrossed into the C57BL/6 strain for at least 10 generations in our laboratory. Nur77 KO mice are congenic and have the same genomic background as WT used in the present study. Animal protocols were approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University. Endothelial specific Nur77 transgenic (Nur77-Tg) mice were generated by Cyagen biosciences Inc. The transgenic animals were genotyped by PCR using the primers: F1: 5'-ATCGTCCCACTCCCATGACAG-3'; R1: 5'-AGCTGGTA-GAGGAAGGTGTACC-3'. The size of transgenic PCR product is 272 bp. Both male and female mice (2–3 months) were used in this study. All strains have no abnormal phenotype regarding reproducibility, growth and mortality rate. To generate diabetic mice, 50 mg/kg of streptozotocin (STZ; Sigma) in 0.1 M sodium citrate buffer (pH4.5) were intraperitoneally injected for 5 consecutive days after 6 h fasting. The control group were received same volume of vehicles. Mice were fed with regular chow diet ad libitum and sacrificed at 4 weeks after first STZ injection. Body weight and blood glucose were monitored on a weekly basis and blood glucose was measured using Contour®NEXT ONE glucose meter (ASCENSIA Diabetes Care). Diabetes was diagnosed if blood glucose was ≥ 250 mg/dL. For glucose tolerance test, mice were fasted for 12 h and injected with 2 g/kg of D-glucose, the blood glucose level was monitored at indicated time point.

2.2. Cell culture and adenovirus transduction

Human umbilical vein endothelial cells (HUVECs) were purchased from Gibco and cultured in endothelial cell medium (ECM) (ScienCell) supplemented with endothelial cell growth supplement (ECGS), 1 % penicillin/streptomycin, and 5 % fetal bovine serum (FBS) BulletKit (Lonza) under 5 % CO₂ and 95 % O₂ at 37 °C. Adenoviruses harboring Flag-tagged human Nur77 cDNA (Ad-Flag-Nur77) and LacZ were made by using AdMax (Microbix Biosystems) as we previously described [27].

2.3. Isolation of mouse aortic endothelial and smooth muscle cells

Mouse aortic endothelial cells (MAECs) were isolated and cultured essentially as described previously [33]. Briefly, mice were sacrificed and the abdominal aorta was cut at the middle to release the blood, and then perfused with 1 ml of PBS containing 1000 U/ml of heparin from the left ventricle. The aorta was dissected out from the aortic arch to the abdominal aorta and then flushed with PBS for twice. Then 100 μ L of collagenase type II (2 mg/ml in DMEM; Worthington Biochem) were instilled into the aorta and the whole aorta was incubated in serum free DMEM for 20 min at 37 °C. The aorta was then opened and flushed with 5 ml 20 % FBS/DMEM for twice. After spinning down at 1500 rpm for 3min, all the cells were collected, re-suspended with fresh ECM, and transferred to collagen I-coated 12-well plate (1 aorta/well) and incubated till 80 % confluence. Purity of endothelial cell population was confirmed by flow cytometry or immunostaining using VE-cadherin and CD31 antibodies. 0.25 % trypsin/EDTA was used for subculture and the cells at 3 to 5 passage were used for experiments. Smooth muscle cells were isolated as described previously [34]. Briefly, the thoracic aortas were isolated from 4-5-week old mice and then removed all the surrounding adipose and adventitia. The cleaned aortas were cut into small

rings, and then digested with collagenase II solution (10 mg/ml) at 37 °C with 5 % CO₂ for overnight. Cells were then pipetted and centrifuged at 180×g for 5 min. Cell pellets were suspended with DMEM medium containing 15 % FBS and plated on gelatin-coated dishes.

2.4. Isometric tension study

Mice were sacrificed under anesthesia with 2.5 % isoflurane and 2 % oxygen and thoracic aorta was excised quickly and placed in ice-cold physiological saline solution (PSS), gently removed adherent tissue as much as possible. Then aorta were segmented into 2–3 mm size and mounted in an wire myograph (620 M Wire Myograph, DMT, Ann Arbor, MI) with physiological salt solution (PSS) containing (in mM, pH7.4): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 11 D-glucose, 3 CaCl₂ at 37 °C, continuously oxygenized with 95 % O₂ and 5 % CO₂. Mounted aortic rings were equilibrated for 30 min and forced tension was gently adjust up to 4 mN. Vessel viability were tested using 60 mM KCl and washed fresh PSS solution. Sub-maximal contractile concentration was determined by incremental concentration of phenylephrine (PE, 1 nM–10 μM) and calculate the appropriate dose of phenylephrine which could induced 80–90 % of contraction compared to maximal contraction. After equilibrating the aortic rings with fresh PSS solution for 30 min, vessel rings were induced pre-contraction by PE and following treatment with increasing concentrations of acetylcholine (1 nM–10 μM) and sodium nitroprusside (1 nM–10 μM) to evaluate the endothelial-dependent and endothelial-independent vasodilation, respectively. For TNF-α incubation, aortic rings were isolated and trimmed with ice-cold PBS then incubated with 50 ng/mL of murine TNF-α with DMEM basal medium (low glucose, without FBS and antibiotics) for 24 h at 37 °C.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIzol following the manufacturer's instruction and quantified using Nanodrop instrument (ThermoFisher Scientific). One microgram of total RNA was used to synthesize the cDNA by high-capacity cDNA reverse transcription kit (Applied Biosystem, Foster City, CA) and performed real-time quantitative PCR with Power SYBR green PCR master mix (Applied Biosystem) and specific primers as indicated in [Supplemental Table 1](#). GAPDH was used to normalize mRNA expression and the 2^{-ΔΔCT} method was used to analyze the relative mRNA expression as described previously [35]. TaqMan MicroRNA assay kit (cat #: 4427975, ThermoFisher Scientific) was used to detect miR-133a expression. To perform mRNA stability assay, 10 μg/ml actinomycin D were added and the total RNA was collected at indicated time points as we described previously [36].

2.6. Western blot analysis

Cell lysates were extracted using radioimmunoprecipitation assay (RIPA) buffer containing (in mM, pH 7.5) 20 Tris-HCl, 150 NaCl, 1 EGTA, 1 % NP-40 with protease and phosphatase inhibitor and protein concentration was quantified using bicinchoninic acid (BCA) reagents (ThermoFisher Scientific). 10–12 % SDS-acrylamide gel were used for electrophoresis and transfer into nitrocellulose membrane and then blocked with 3 % BSA in TBST. Anti-Nur77 antibody was purchased from Novus Biologicals (USA, Cat# : NB100-56745), anti-GCH1 antibody was purchased from Abcam (Cat# ab307507), anti-SOD1 antibody was purchased from Sigma (Cat#:SAB5701040), and anti-GAPDH antibody was purchased from Santa Cruz (Cat#: sc-365062). Antibodies were diluted with 3 % BSA in TBST and incubated at 4 °C for overnight. Next day, blots were washed with TBST for 3 times and then incubated with IRDye® 800 CW goat anti-rabbit or IRDye 680RD goat anti-mouse secondary antibody (1:10,000, LI-COR) for 1 h at room temperature. Band intensity was quantified with Image J (version 1.32j, NIH) from three to five independent experiments.

2.7. Transient transfection and luciferase assay

The human SOD1 promoter was amplified by PCR using human genomic DNA (Clontech) with primers with primers 5'-GCCCCGGA-CAAAGTAGTTT-3' and 5'-GAGACTACGACGCAAACCAG-3' and cloned into the luciferase reporter plasmid pGL3-Basic (Promega) [27]. SOD1 mutant promoter was made using the QuikChange II Site-Directed Mutagenesis Kit (cat#:200523, Agilent). HUVECs were seeded in 24-well plates and incubated overnight. The cells were transfected with either 200 ng pGL3-SOD1 promoter luciferase reporter in the presence or absence of indicated expression vectors using Lipofectamine™ LTX (Invitrogen) transfection reagent. 24 h after transfection, cells were directly lysed in the lysis buffer (Promega) and 20 μl of the cell lysates were assayed for luciferase activity with a luciferase reporter assay system (Promega) and determined with a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek) according to the manufacturer's instructions. Firefly luciferase activity was normalized for transfection efficiency by the corresponding Renilla luciferase activity. All transfection experiments were performed at least 5 times in duplicate.

2.8. Aortic ring sprouting assay

Aortic ring assay was performed as we described previously [37]. Briefly, the thoracic aorta segment was carefully separated and divided into a uniform ring with approximately 0.5 mm wide. Aortic rings were then inoculated into a 96-well plate containing 40 μL Matrigel/well, and another 40 μL Matrigel was added to cover each ring. In total, 100 μL complete ECM was then added in the presence and absence of high glucose (25 mM). The medium was then changed every 2 days, and photographs were taken under a microscope during the 5–7 days.

2.9. Reactive oxygen species measurement

Cells were plated onto 96-well plate and maintained with ECM with supplements. After stimulation with either TNF-α or high glucose (25 mM), cells were washed with pre-warmed HBSS and incubated with 25 μM of DCFDA (2',7'-dichlorofluorescein diacetate) (Sigma) for 30 min at 37 °C. Mannitol as an osmotic control for HG conditions. Cells were briefly washed with pre-warmed HBSS and observed the fluorescence signal using EVOS FL Auto Imaging System (ThermoFisher). For tissue ROS measurement, unfixed thoracic aortic ring were cryopreserved with Tissue Plus® O.C.T. compound (FisherHealthCare, Huston, TX) at –80 °C until used. Blocks were sectioned into 6 μm using cryostat (Leica Biosystems, Wetzlar, Germany) and immediately stained with 10 μM DHE (dihydroethidium) (Sigma) for 30 min at room temperature and mounted with DAPI containing ProLong® Gold Antifade Mountant (ThermoFisher). Images were photographed using FlouView1000 confocal microscope (Olympus, Tokyo, Japan) and quantified using Image J (version 1.32j, NIH) software. ROS production was quantitated from the ratio of ethidium/DAPI fluorescence. All data were obtained by randomly selecting 10 areas and summarized as mean ± SD from each experiment.

2.10. Nitric oxide measurement

Cell supernatants were freshly collected and kept onto ice until the measurement. Nitrite (NO₂-) and nitrate (NO₃-) conversion rate was determined by chemiluminescence NO detector (Siever 280i NO Analyzer) using 6 mM potassium iodide and acetic acid. Acquired data were converted from standard calibration curve into NO_x concentration (μM) as we described previously [37].

2.11. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate) according to the instructions of the

manufacturer we described previously [35]. Soluble chromatin was prepared from HUVECs transduced with either Ad-LacZ or Ad-Flag-Nur77 (MOI = 50) for 48 h. DNA-bound Nur77 subunit was immunoprecipitated by incubating with anti-Flag M2 antibody, which was purchased from Sigma (cat #: F1804) for overnight at 4 °C with rotation. After reversal of cross-links and digestion of bound proteins, the recovered DNA was quantified by PCR using primer pairs that cover a Nur77 consensus sequence in human SOD1 promoter as follows: sense, 5'- ATCTGTAGGGTTGTGCCTT -3'; antisense, 5'- AGTCGCTACACCTCAATCCC -3'.

2.12. Transfection of miR-133a mimic

The miR-133a mimic (cat #: 4464066) and negative miRNA mimic control (cat #: 4464058) were obtained from Thermo Fisher Scientific. HUVECs were cultured in 6-well plates at 50 % confluence. The miR-133a mimic and negative miRNA mimic control were diluted into 125 μ l OptiMEM to a concentration of 100 nM and then mixed with 7.5 μ l Lipofectamine RNAiMAX (cat #: 13778075, Invitrogen) in 125 μ l OptiMEM at room temperature for 15 min before adding into plates. Transfection medium was replaced by regular cell culture medium after 6 h of transfection.

2.13. Statistical analysis

Data were presented as the mean \pm SD. All statistical analyses were performed using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA), with $p < 0.05$ considered as significant. Normality of distribution was assessed by using Shapiro-Wilk test. Differences between 2 groups with normally distributed data were analyzed using a two-sided unpaired t -test. When the difference among three or more groups was evaluated, one-way analysis of variance (ANOVA) or, when appropriate, two-way ANOVA followed by Tukey's or Bonferroni's post-hoc test was used. For non-normally distribution or sample number less than 6, two-tailed Mann-Whitney test (2 groups) or Kruskal-Wallis test (multiple groups) was performed, followed by Dunn post hoc analysis.

3. Results

3.1. Generation of Nur77 endothelial specific transgenic mice

To investigate the role of Nur77 in endothelial dysfunction, we attempted to perform both gain- and loss-of-function studies by using Nur77 knockout and Nur77 transgenic mice. Nur77 global knockout mice are viable, fertile, appear to develop a normal adult vasculature as reported previously [38]. These mice have been widely utilized to study the roles of Nur77 in cardiovascular and inflammatory diseases^{28 29 30}. To further investigate the role of Nur77 in regulating endothelial

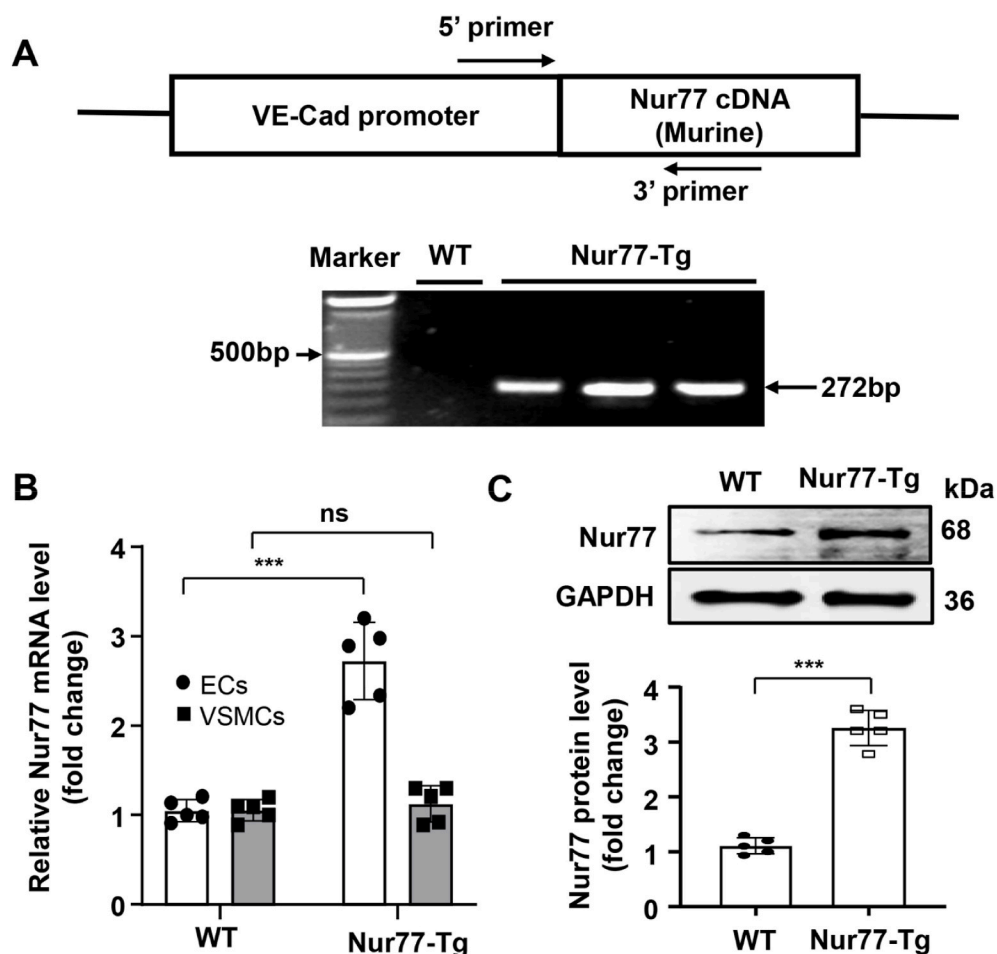


Fig. 1. Generation and characterization of endothelial-specific Nur77 transgenic mice. (A) Schematic representation of the construct used for generating Nur77 endothelial specific transgenic mice (Nur77-Tg) (top) and PCR product of genotyping transgenic mice (bottom). (B) Relative expression of Nur77 in mouse aortic endothelial cells and smooth muscle cells isolated from WT and Nur77-Tg mice as determined by RT-PCR. *** $P < 0.001$ compared with WT group, using 2-tailed Student's t -test. $n = 5$. ns: not statistically significant. (C) Expression of Nur77 in mouse aortic endothelial cells isolated from WT and Nur77-Tg mice as determined by western blot. *** $P < 0.001$ compared with WT group, using 2-tailed Student's t -test. $n = 5$.

dysfunction, we have generated Nur77 endothelial specific transgenic mice (Nur77-Tg) using a construct bearing a VE-cadherin promoter fused with murine Nur77 cDNA (Fig. 1A). As shown in Fig. 1B, the expression of Nur77 was increased by approximately 2.8-fold in aortic endothelial cells isolated from Nur77-Tg as compared with the wild-type (WT) mice, as determined by qPCR, while the expression of Nur77 in aortic smooth muscle cells was unaltered between Nur77-Tg and WT mice (Fig. 1B). The protein level of Nur77 in aortic endothelial cells was increased by about 3-fold, compared with the WT mice, as determined by western blot (Fig. 1C).

3.2. Nur77 prevents endothelial dysfunction

To determine whether Nur77 is involved in maintaining the normal vasomotor function, isometric tension study was performed using Nur77 knockout mice (Nur77 KO), Nur77-Tg mice, and their WT littermates under inflammatory conditions. Consistent with previously report, WT and Nur77 KO aortic rings exhibited similar response to PE [39]. Thoracic aortic rings were isolated from Nur77 KO and age- and sex-matched WT mice and then incubated with 50 ng/ml of TNF- α for 24 h. As shown in Fig. 2A, TNF- α treatment significantly impaired acetylcholine (ACh)-induced vessel relaxation and this impairment was further exacerbated in the aortic rings derived from Nur77 KO mice. Furthermore, we found that endothelial specific overexpression of Nur77 markedly prevented TNF- α -induced impairment of vessel relaxation in response to ACh (Fig. 2C). Endothelial-independent vasodilation, which was determined in the presence of sodium nitroprusside (SNP), was not significantly affected (Fig. 2B and D).

To examine whether Nur77 is involved in endothelial dysfunction under other pathological conditions, Nur77 KO, Nur77-Tg, and their WT littermates were subjected to STZ (50 mg/kg) injection for 5 consecutive days to create diabetic mice. The diabetic incidence and severity were confirmed by monitoring blood glucose levels (Supplemental Fig. 2). Blood glucose levels were gradually increased until 4 weeks after STZ

injection. Compared with vehicle-injected group, the levels of blood glucose between animal groups (Nur77-Tg, Nur77-KO and WT mice) were not significantly altered, suggesting Nur77 barely affects the development of STZ-induced diabetes in mice. However, endothelial dysfunction in STZ-treated mice was greatly exacerbated in Nur77 KO mice (Fig. 3A), but this dysfunction was largely prevented in Nur77-Tg mice (Fig. 3C). Endothelial-independent vasodilation, which was determined in the presence of sodium nitroprusside, was not significantly affected (Fig. 3B and D). Together, these data suggest that Nur77 is essentially involved in endothelial dysfunction under both inflammatory and diabetic conditions.

3.3. Nur77 inhibits ROS generation and increases NO production in ECs

It had been known that the functional endothelium is mainly maintained by robust NO production and associated ROS inhibition [6]. Since our data showed that overexpression of Nur77 markedly attenuated endothelial dysfunction, we attempted to investigate whether Nur77 regulates ROS generation and NO production in ECs. To this end, we measured the high glucose-induced ROS generation in the mouse aorta obtained from Nur77 KO, Nur77-Tg, and their WT littermates. As shown in Fig. 4A, high glucose treatment of vessels from wild-type mice significantly increased the production of ROS, which was further increased in Nur77 KO mice. Notably, endothelial specific overexpression of Nur77 substantially prevented high-glucose-induced production of ROS in the vascular wall (Fig. 4B). Likewise, high glucose-induced ROS production in cultured ECs was markedly inhibited in Nur77 overexpressing ECs (Fig. 4C). Furthermore, we found that NO production was significantly increased in Nur77 overexpressing ECs (Fig. 4D).

To further elucidate the functional role of Nur77 in regulating EC function, we examined the vessel sprouting using aortic rings from Nur77 KO, Nur77-Tg, and their WT littermates. As shown in Fig. 5A and B, high glucose treatment significantly impaired the aortic ring

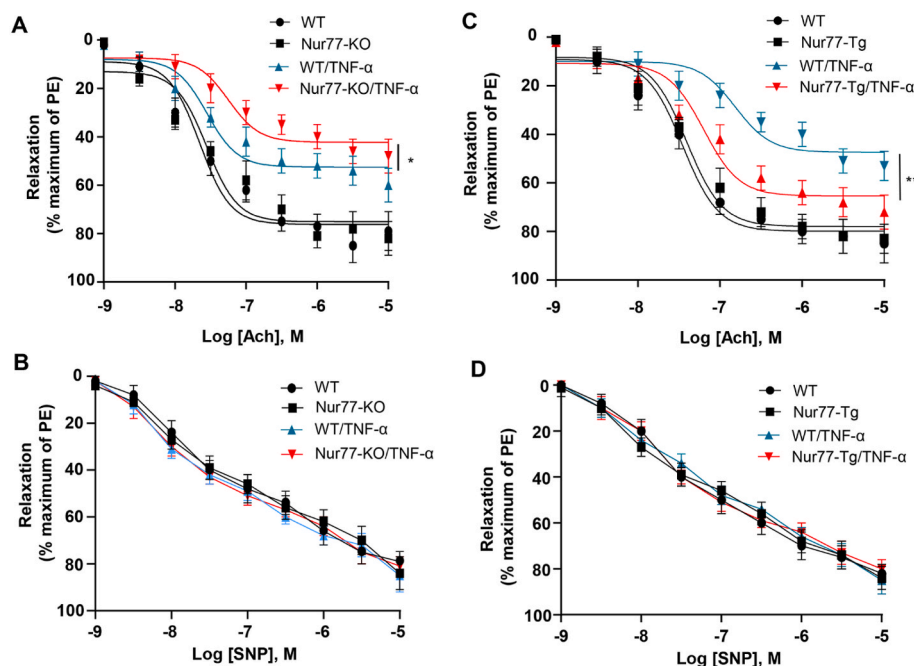


Fig. 2. Endothelial Nur77 prevents TNF- α induced endothelial dysfunction. Vasorelaxation assay was performed using mouse aorta isolated from WT, Nur77-KO, and Nur77-Tg mice after incubation with 50 ng/ml of murine TNF- α for 24 h. (A) Aortic ring pre-contraction was induced by the administration of phenylephrine (PE, 1 μ M) and endothelial-dependent vasorelaxation was induced by following treatment of cumulative concentration of acetylcholine (ACh). TNF- α treatment-induced impairment of vasorelaxation in WT mice was deteriorated in Nur77-KO mice. (B) Endothelial-independent vasorelaxation was not affected by Nur77 deficiency. (C) TNF- α treatment-induced impairment of vasorelaxation was prevented in Nur77-Tg. (D) Endothelial-independent vasorelaxation was not affected by endothelial specific overexpression of Nur77. * $P < 0.05$ and ** $P < 0.01$, unpaired two-sided t -test. $n = 9$.

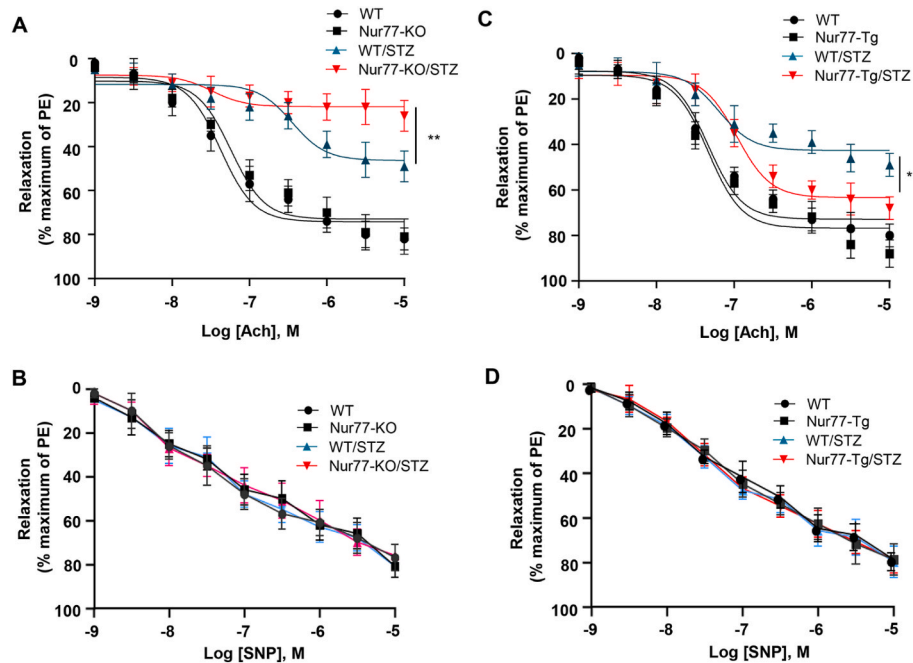


Fig. 3. Endothelial Nur77 prevents diabetes-induced endothelial dysfunction. WT, Nur77-Tg and Nur77-KO mice were subjected to STZ injection (IP, 50 mg/kg) for five consecutive days. Blood glucose levels and body weights were monitored until 4 weeks after first injection. Vasorelaxation assay was performed using mouse aorta isolated from WT, Nur77-KO, and Nur77-Tg mice after STZ injection-induced diabetes. (A) Aortic ring pre-contraction was induced by the administration of phenylephrine (PE, 1 μ M) and endothelial-dependent vasorelaxation was induced by following treatment of cumulative concentration of acetylcholine (ACh). Diabetes-induced impairment of vasorelaxation in WT mice was further deteriorated in Nur77-KO mice. $**P < 0.01$, unpaired two-sided *t*-test. *n* = 9. (B) Endothelial-independent vasorelaxation was not affected by Nur77 deficiency under both normal and diabetic conditions. (C) Diabetes-induced impairment of vasorelaxation was prevented in Nur77-Tg mice. $**P < 0.01$, unpaired two-sided *t*-test. *n* = 9. (D) Endothelial-independent vasorelaxation was not affected by endothelial specific overexpression of Nur77.

sprouting in wild-type mice and this sprouting was further deteriorated in aortic rings obtained from Nur77 KO mice, suggesting that maintaining the normal Nur77 expression is essential for preventing HG-induced vessel dysfunction. In our study, we found that EC overexpression of Nur77 did not significantly prevent HG-induced suppression of vessel sprouting as compared with WT mice. As aortic ring assay involves multiple steps including cell proliferation and migration, interaction of endothelial cells with pericytes and smooth muscle cells, and extracellular matrix signaling, it is likely that other pathways than NO and ROS production are potentially involved in HG-induced impairment of aortic ring sprouting and they are not sufficiently affected by Nur77 expression. Together, these results suggest that endothelial Nur77 is essential for maintaining vascular function under HG conditions.

3.4. Nur77 increases the expression of GTP cyclohydrolase I and superoxide dismutases in ECs

Our results suggest that Nur77 mitigates endothelial dysfunction mainly through acting on the redox-signaling pathway in endothelial cells. Thus, we performed an initial screening to identify genes that were directly or indirectly linked to NO and ROS production in aortic ECs isolated from WT and Nur77-Tg mice. By performing RT-PCR, we determined the genes involved in NO production including eNOS, GTP cyclohydrolase I (GCH1), and dihydrofolate reductase (DHFR), ROS generation including NADPH oxidases (Nox2 and Nox4), and several key antioxidant proteins including superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (Gpx), glutathione reductase (GR), thioredoxin (Trx1 and Trx2), peroxiredoxins (Prx1 and Prx2), and heme oxygenase-1 (HO-1). Consistent with previous reports [40,41], Nur77 overexpression increased the expression of eNOS and glutathione peroxidase (GPx-1) (Fig. 6A and B). Among other genes, we found that the expression of GTP cyclohydrolase I (GCH1) and superoxide

dismutases (SODs) was markedly increased in ECs isolated from Nur77-Tg mice as compared with wild-type mice (Fig. 6C–F). Moreover, the expression of GCH1 and SOD1 was further confirmed by western blot analysis (Fig. 6H and K). Interesting, the expression of heme oxygenase-1 (HO-1) was decreased in Nur77 overexpressing vessels (Fig. 6G). To corroborate our findings in mouse ECs, we determined the expression of both GCH1 and SOD1 in HUVECs overexpressing Nur77. As shown in Fig. 7A–C, adenovirus-mediated overexpressing of Nur77 increased the expression of GCH1 and SOD1 by approximately 2.5-fold and 6-fold, respectively, at a MOI of 50. Together, these results suggest that Nur77 protects endothelial dysfunction at least in part through increasing expression of several key enzymes involved in both NO production and ROS scavenging.

3.5. Molecular mechanisms underlying upregulation of GCH1 and SOD1 expression by Nur77

Nur77 is an orphan nuclear receptor that has been shown to regulate gene transcription through directly binding to the specific DNA-response elements as a monomer, homo- or hetero-dimers [42]. Previously, Nur77 has been shown to increase eNOS and GPx-1 expression through both post-transcriptional and transcriptional mechanisms [40,41]. To elucidate molecular mechanisms underlying upregulation of GCH1 by Nur77, we initially performed a sequence analysis of GCH1 promoter and did not reveal the presence of a putative Nur77 response elements (NBRE) in human GCH1 promoter region (-1 kb), suggesting that post-transcriptional mechanism may be involved in the regulation of GCH1 mRNA levels by Nur77. To this end, we determined the GCH1 mRNA stability and found that Nur77 overexpression markedly prolonged the GCH1 mRNA half-life (Fig. 8A). miR-133a has been shown to decrease GCH1 mRNA stability [21], thus we attempted to determine whether miR133a is involved in regulating GCH1 expression by Nur77. As shown in Fig. 8A, transfection of miR-133a mimic in ECs prevented

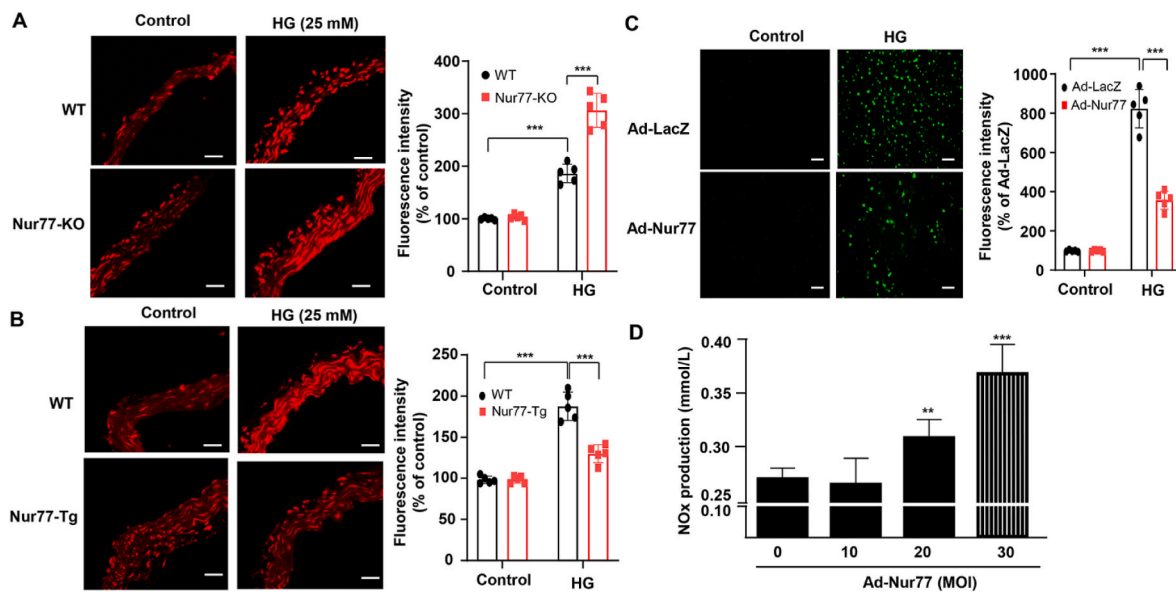


Fig. 4. Nur77 attenuates high glucose-induced ROS production in ECs. **A**, Thoracic aortic rings from Nur77-KO and their WT littermates were incubated control and high glucose medium for 48 h and then cryopreserved with Tissue Plus® O.C.T. compound. The rings were sectioned into 6 μm and immediately stained with 10 μM DHE (dihydroethidium) for 30 min at room temperature and images were photographed using FlouView1000 confocal microscope and quantified using Image J. ROS production was estimated from the ratio of ethidium/DAPI fluorescence. All data were obtained by randomly selecting 10 areas and summarized as mean \pm SD from each experiment. *** P < 0.001, using Two-way ANOVA coupled with Tukey's post hoc test. Scale bars: 50 μm . n = 5. **B**, Thoracic aortic rings from Nur77-Tg and WT littermates were incubated control and high glucose medium for 48 h and the ROS production was determined as described above. *** P < 0.001, using Two-way ANOVA coupled with Tukey's post hoc test. Scale bars: 50 μm . n = 5. **C**, HUVECs were transduced with Ad-LacZ and Ad-Nur77 (MOI = 50). 48 h after transduction, cells were stimulation with high glucose (25 mM) for 48 h, followed by washing with pre-warmed HBSS and then incubated with 25 μM of DCFDA (2',7'-dichlorofluorescein diacetate) for 30 min at 37 $^{\circ}\text{C}$. Cells were briefly washed with pre-warmed HBSS and the fluorescence signal was recorded using EVOS FL Auto Imaging System. *** P < 0.001, using Two-way ANOVA coupled with Tukey's post hoc test. Scale bars: 1000 μm . n = 5. **D**, HUVECs were transduced with either Ad-LacZ or Ad-Nur77 at indicated MOI. 48 h after transduction, supernatants were collected for the NO measurement by NO analyzer. ** P < 0.01, *** P < 0.001 versus MOI = 0 using 1-way ANOVA coupled with Tukey's multiple-comparison post hoc test. n = 5.

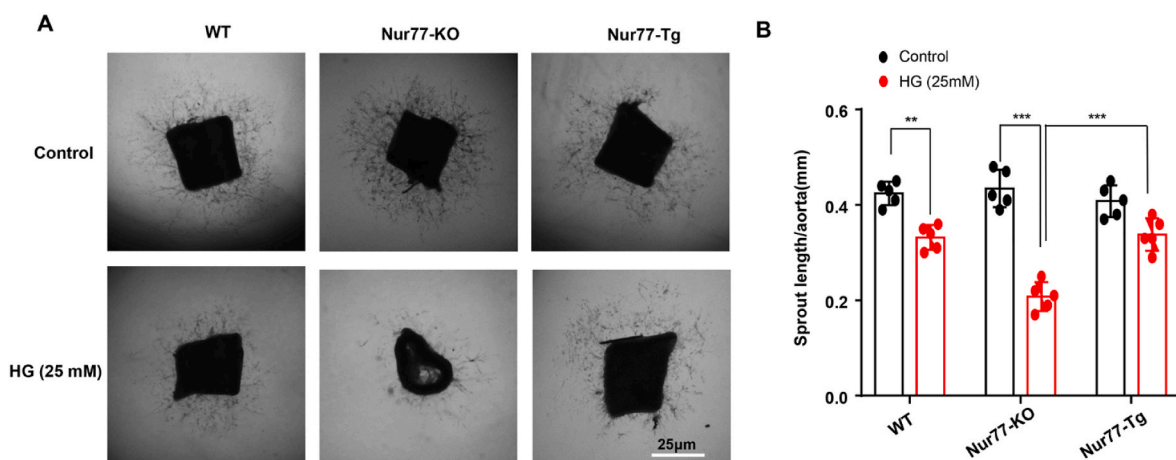


Fig. 5. Endothelial overexpression of Nur77 promotes vessel sprouting under high glucose conditions. The thoracic aortic rings isolated from WT, Nur77-Tg and Nur77-KO mice were cultured in Matrigel containing endothelial growth medium (EGM) in the presence and absence of high glucose (HG, 25 mM) for 5–7 days. The new vessel sprouting was captured and quantitated. Scale bar: 25 μm ** P < 0.01, *** P < 0.001, using Two-way ANOVA coupled with Tukey's post hoc test. n = 5.

Nur77-induced increase of GCH1 mRNA stability. Furthermore, we found that miR-133a expression was significantly decreased in Nur77 overexpressing HUVECs and aortic ECs isolated from Nur77 transgenic mice (Fig. 8B and C). Consistent with a previous report [21], HG and TNF- α treatment inhibited the expression of GCH1, which was prevented in Nur77-overexpressing ECs (Fig. 8D). Together, these results suggest that Nur77 increases GCH1 expression at least in part through suppressing miR-133a in ECs.

To dissect the molecular mechanism(s) underlying Nur77-induced

SOD1 expression in ECs, we analyzed human SOD1 promoter region and found the presence of a putative Nur77 response element (NBRE) (AAAGGTAA) located between -445 and -452 base pairs in human SOD1 promoter. Furthermore, we found that overexpression of Nur77 significantly increased human SOD1 promoter activity (-770 + 87 bp) in ECs (Fig. 9A). Mutation of this putative NBRE site abolished the responsiveness of the human SOD1 promoter to Nur77, indicating that this site mediates the transcriptional activation of human SOD1 by Nur77. Moreover, Nur77 binding to the identified NBRE site in human

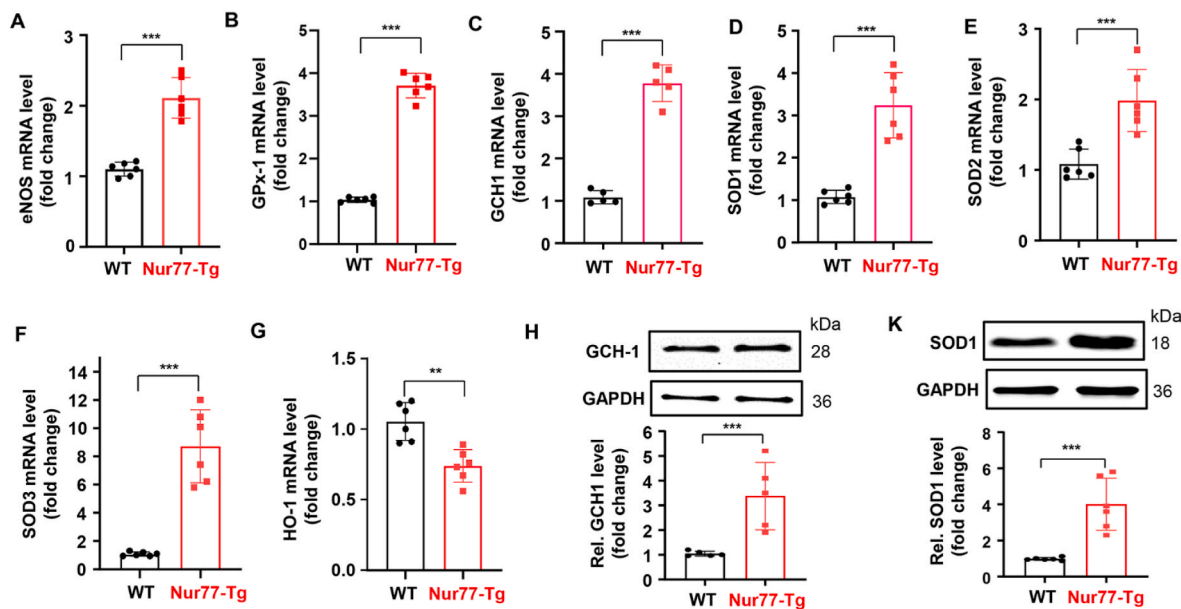


Fig. 6. Effects of endothelial Nur77 expression on the expression of the enzymes involved in NO and ROS production. A-G, Relative expression of the enzymes involved in NO and ROS production in aortic ECs isolated from Nur77-Tg and their WT littermates was determined by qPCR. H and K, the expression of GCH1 and SOD1 in aortic ECs isolated from Nur77-Tg and WT littermates was determined by western blot. ** $P < 0.01$, *** $P < 0.001$, using unpaired two-sided t-test. $n = 5-6$.

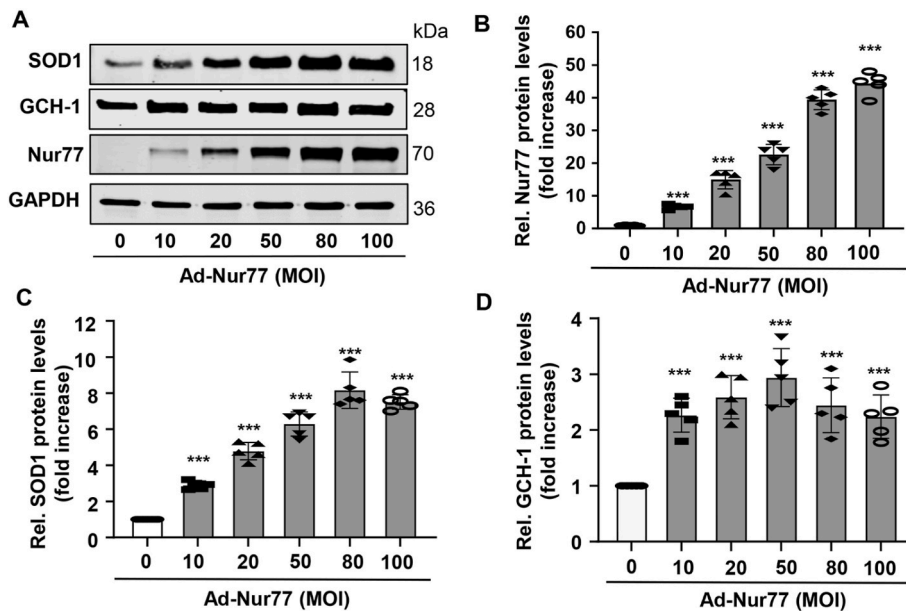


Fig. 7. Nur77 promotes GCH1 and SOD1 expression in HUVECs. A, HUVECs were transduced with adenovirus Nur77 (Ad-Nur77) or adenovirus LacZ (Ad-LacZ) at indicated multiplicity of infection (MOI) for 48 h and the expression of Nur77, SOD1, and GCH1 was determined by western blot analysis and then quantitated by densitometric analysis (B-D). Representative blot images (top) and quantified data (bottom) were obtained from 5 individual experiments. *** $P < 0.001$ versus MOI = 0 using 1-way ANOVA coupled with Tukey's multiple-comparison post hoc test. $n = 5$.

SOD promoter was verified by ChIP assay in HUVECs overexpressing Flag-Nur77 (Fig. 9B), further demonstrating that Nur77 increases SOD1 expression through directly binding to the human SOD1 promoter. To define the role of Nur77 in regulating SOD1 expression under HG conditions, we isolated aortic ECs from WT and Nur77 KO mice. We found that HG had minimal effects on the expression of SOD1 in aortic ECs isolated from WT mice, but significantly decreased the SOD1 expression in Nur77-deficient ECs (Fig. 9C). These findings strongly suggest that Nur77 is required for maintaining SOD1 levels under HG conditions.

4. Discussion

Endothelial dysfunction has been linked to a variety of cardiovascular diseases, including atherosclerosis, hypertension, and heart failure [1]. Identification of novel regulators controlling endothelial dysfunction is essential for developing novel therapies against these diseases. In the present study, we identified Nur77 as a novel regulator for controlling endothelial function under both inflammatory and diabetic conditions. We showed that Nur77 potently suppresses endothelial dysfunction by simultaneously suppressing ROS production and

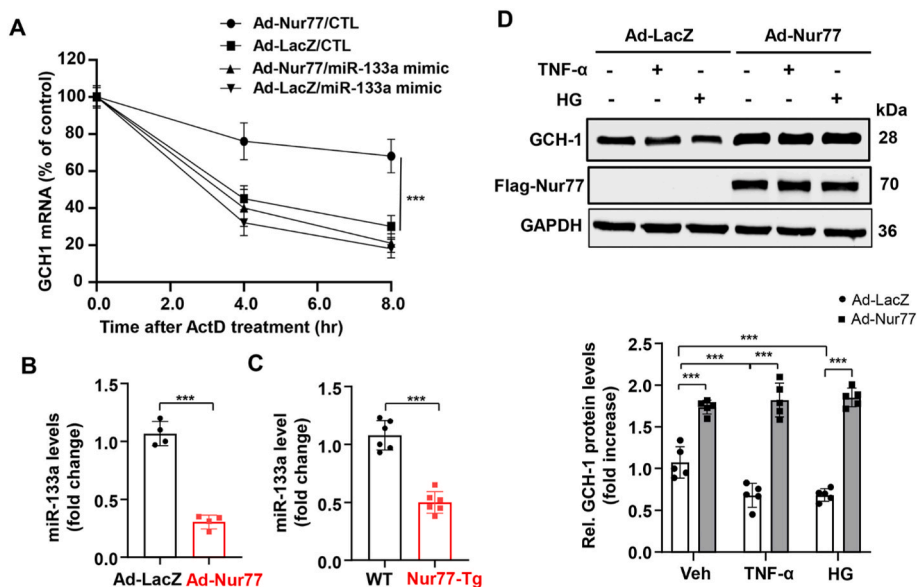


Fig. 8. Nur77 regulates GCH1 expression through targeting miR-133a in ECs. A, HUVECs were transduced with either Ad-LacZ or Ad-Nur77 (MOI = 50) for 24 h and then transfected with miR-133a mimic (Mimic) and normal negative control (CTL) for 48 h. Cells were then treated with 10 μg/ml actinomycin D. Total RNAs were extracted at indicated time points and the levels of GCH1 mRNA were determined by quantitative real-time PCR. ****P* < 0.001, unpaired two-sided *t*-test. *n* = 5. (B) HUVECs were transduced with Ad-LacZ and Ad-Nur77 for 48 h, miR-133a levels was determined by quantitative real-time PCR. ****P* < 0.001, unpaired two-sided *t*-test. *n* = 4. (C) The levels of miR-133a were determined in mouse ECs isolated from WT and Nur77-Tg mice. ****P* < 0.001, unpaired two-sided *t*-test. *n* = 6. D, HUVECs were transduced with adenovirus Nur77 (Ad-Nur77) or adenovirus LacZ (Ad-LacZ) at MOI = 20. 48 hrs after transduction, cells were stimulated with either vehicle, HG (25 mM), or TNF-α for 48 h. The expression of indicated proteins was then determined by western blot and then quantitated by densitometric analysis. ****P* < 0.001 using Two-way ANOVA coupled with Tukey's post hoc test. *n* = 5.

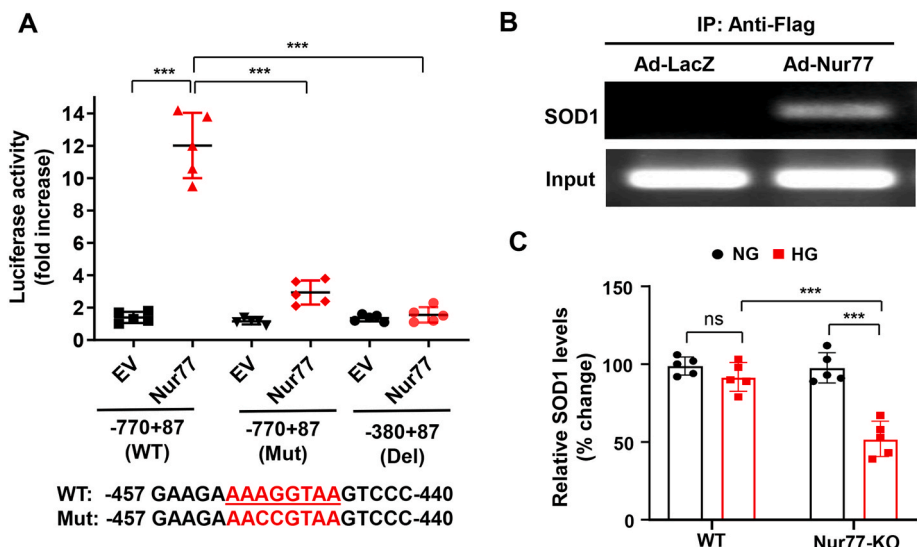


Fig. 9. Nur77 transcriptionally upregulates SOD1 expression. A, HUVECs were transfected with various human SOD1 promoter fragments (100 ng) and pRL-RSV (10 ng) in the presence of pFlag-Nur77 (Nur77) or empty vector (EV, 300 ng). Thirty-six hours after transfection, luciferase assays were performed and normalized by constitutive renilla luciferase. ****P* < 0.001, using Two-way ANOVA coupled with Tukey's post hoc test. *n* = 5. B, Nur77 binds to human SOD1 promoter. HUVECs were transduced with the indicated adenoviruses at an MOI of 50. 48 h after transduction, the recruitment of Nur77 to the SOD1 promoter was determined by ChIP assays. C, Aortic ECs were isolated from wild-type (WT) and Nur77 knockout (KO) mice and subjected to the treatment of either high glucose or mannitol for 48 h. The expression of SOD1 was then determined by qPCR. ****P* < 0.001 using Two-way ANOVA coupled with Tukey's post hoc test. ns: not statistically significant. *n* = 5.

enhancing nitric oxide generation in vascular ECs. Furthermore, we found that Nur77 overexpression markedly increased the expression of GCH1 and SOD1, both of which are essential for preserving endothelial function under oxidative stress. Mechanistically, we found that Nur77 increases GCH1 and SOD1 expression through post-transcriptional and transcriptional mechanisms, respectively in ECs. Multiple mechanisms including elevated reactive oxygen species (ROS), proinflammatory

factors, and deficiency of nitric oxide (NO) bioavailability contribute to endothelial dysfunction. Thus, therapeutic strategies targeting multiple pathways simultaneously could be more efficient than strategies targeting one single pathway to mitigate endothelial dysfunction. Our results, and that of others, suggest that targeting Nur77 can effectively prevent endothelial dysfunction by targeting more than one mechanism at a time (nitric oxide production, ROS scavenging, and vascular

inflammation).

The endothelium is formed by a single layer of EC located in the intima layer of the arteries and plays an important role in regulating endothelial cell activation, smooth muscle cell proliferation, coagulation, and angiogenesis [43,44]. Endothelial dysfunction, as characterized by increased ROS production and decreased bioavailability of endothelium-derived NO, is essentially involved in the development of several cardiovascular diseases such as atherosclerosis, restenosis, and heart failure [1,3]. Recently, the cardiovascular function of NR4A receptors has received a significant attention [42]. In response to atherogenic and chronic inflammatory stimuli, such as oxidized LDL, lipopolysaccharide and TNF- α , the expression of Nur77 has been shown to be markedly induced in endothelial cells, thus functioning as a negative regulator to prevent EC activation and atherosclerosis development through multiple mechanisms [27,28,30,42]. We have recently shown that Nur77 potently inhibits endothelial activation and inflammation through inhibiting NF- κ B and inflammasome activation [27,30,45]. Furthermore, Nur77 overexpression has been shown to attenuate thrombotic formation, vascular hyper-permeability, and increases cell survival and angiogenesis in ECs [36,46,47]. Interestingly, a recent study suggests that Nur77 is essentially involved in preventing hyperglycemia-induced oxidative stress and vascular dysfunction by metformin [39]. However, the direct evidence showing the biological significance of Nur77 in regulating endothelial dysfunction remains obscured. Herein, we demonstrate that Nur77 exerts additional beneficial activities for the vessel wall through simultaneously increasing NO production and decreasing ROS generation, thus potently preventing endothelial dysfunction under both inflammatory and diabetic conditions. In the present study, we found endothelial specific overexpression of Nur77 significantly attenuated the overall ROS production in the mouse aorta after stimulation of TNF- α and HG. Thus, it is attempted to speculate that the protective effects of Nur77 on endothelial cells may create an environment that decreases ROS production in various components of the vascular system through both autocrine and paracrine pathways.

Hyperglycemia is one of the major risk factors for CVDs and it has been shown to elevate ROS production and inflammation leading to impaired vasodilation and endothelial dysfunction [14]. Overproduction of ROS can be neutralized by directly inhibiting ROS generating enzymes and/or indirectly upregulating anti-oxidant enzymes. Glutathione peroxidase-1 (GPx-1), as an antioxidant enzyme counteracting oxidative stress, plays an important role in modulating intracellular ROS. Nur77 has been previously shown to upregulate GPx-1 expression in pancreatic β cells at transcriptional levels [41]. Consistent with this report, we found that endothelial specific overexpression of Nur77 increased GPx-1 expression by approximately 3.7-fold in vascular ECs. Superoxide dismutases (SODs) have been shown to be the major anti-oxidant enzymes in the vessel wall to inhibit oxidative inactivation of nitric oxide and peroxynitrite formation [48]. SODs have three isoforms, including the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3) [48]. Recent studies have shown that overexpression of SODs, particularly SOD1 and SOD3, restores acetylcholine-dependent vasodilation response in multiple disease states [12,49,50]. In the present study, our results showed that overexpression of Nur77 significantly increases the expression of three SODs in endothelium. To study the mechanisms involved, we focused on the Nur77-mediated upregulation of SOD1 because SOD1 is the major intracellular SOD in scavenging superoxide and it accounts for 50%–80% of total SOD activity in aorta [48]. We found that in human ECs, Nur77 markedly upregulated SOD1 expression through directly binding to the SOD1 promoter. SOD1 has been shown to be either downregulated or upregulated in aorta under high glucose conditions [51,52]. In this study, we show that HG barely affected the expression of SOD1 in aortic ECs isolated from wild-type mice, but markedly inhibits SOD1 expression in Nur77-deficient ECs, suggesting that Nur77 is required for maintaining SOD1 expression

under HG conditions. SOD3 is the major Cu/Zn-containing SOD in the vascular extracellular space (ecSOD) and it is predominantly expressed in vascular smooth muscle cells rather than endothelial cells [53]. At this time, the mechanism by which endothelial Nur77 overexpression regulates SOD3 expression remains unknown, but warrants further investigation.

Heme oxygenase-1 (HO-1) metabolizes heme to generate carbon monoxide (CO), biliverdin, and iron [54]. The role of HO-1 in regulating endothelial dysfunction has been extensively studied, but it remains controversial [54]. Pharmacological induction or gene transfer of HO-1 has been shown to ameliorate vascular dysfunction in animal models of atherosclerosis, post-angioplasty restenosis, myocardial infarction, and hypertension [55–57]. However, several studies have demonstrated that HO-1 promotes endothelial dysfunction in hypertensive and obese animals [58,59]. Intriguingly, in this study, we found that Nur77 inhibits HO-1 expression in vascular ECs. Whether this inhibition contributes to Nur77-induced prevention of endothelial dysfunction in diabetic mice warrants further investigation.

In healthy blood vessels, the expression of eNOS is tightly regulated through complex mechanisms, involving transcriptional, post-transcriptional, and post-translational controls [4]. Previous studies have shown that Nur77 regulates eNOS expression at post-transcriptional levels, through prolonging eNOS mRNA stability in ECs [40]. Consistent with this finding, we found that eNOS expression is increased about 2-fold in ECs isolated from Nur77 transgenic mice. At posttranslational levels, BH4 is a critical determinant of endothelial NOS (eNOS) activity and coupling [2]. BH4 deficiency correlates with diminished NO production and endothelial dysfunction in cardiovascular diseases such as hypertension and diabetes [2,18]. GCH1 is the rate-limiting enzyme in BH4 biosynthesis and its expression is reduced in inflammatory vascular disease and diabetes, thus contributing to eNOS uncoupling and endothelial dysfunction [21,60]. Furthermore, ectopic expression of miR-133a has been shown to reduce GCH1 protein and BH4 levels in vascular endothelium in diabetes [21]. In the present study, we found that overexpression of Nur77 significantly increased GCH1 protein levels through prolonging GCH mRNA stability in ECs. Furthermore, we found that the expression of miR-133a was inhibited in Nur77 overexpressing ECs. Further studies using unbiased experimental approaches such as transcriptomics and proteomics will comprehensively define the molecular mechanism(s) underlying the protective effects of Nur77 in vascular ECs.

The protective effects of nuclear receptor Nur77 on endothelial dysfunction and vascular inflammation have been reported previously [61], however, the detailed molecular mechanisms by which Nur77 mitigates endothelial dysfunction remain less explored. In this regard, our study defined a novel anti-oxidant role of Nur77 and characterized the associated molecular mechanisms in vascular ECs. Furthermore, our results provide a proof-of-concept that targeted activation of Nur77 in ECs may represent a novel therapeutic strategy for prevention and treatment of endothelial dysfunction associated cardiovascular diseases.

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CRediT authorship contribution statement

Lin Lu: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sooхва Jang:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jiaqi Zhu:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Qing Qin:** Visualization, Validation, Investigation, Formal analysis, Data curation. **Lijun Sun:** Visualization,

Validation, Investigation, Formal analysis, Data curation. **Jianxin Sun:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2024.103056>.

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