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Renal Denervation Lowers Atheroprone Endothelial Phenotypes and Atherosclerosis by Targeting the Mitochondria-Inflammation Circle

Jihong Han*

Key Laboratory of Metabolism and Regulation for Major Diseases of Anhui Higher Education Institutes, Hefei University of Technology, Hefei, 230009, China

Abstract

Endothelial cells' (ECs') mitochondrial redox equilibrium may become disturbed, which can lead to persistent inflammation and atherosclerosis. Endothelial dysfunction can be brought on by oxidative damage amplified by chronic sympathetic hyperactivity. We tested whether renal denervation (RDN), a technique for lowering sympathetic tone, can protect ECs by reducing the inflammation caused by mitochondrial reactive oxygen species (ROS) to prevent atherosclerosis.

Before consuming a high-fat diet for 20 weeks, RDN or a sham procedure was performed on ApoE-deficient (ApoE/) mice. The EC phenotype, atherosclerosis, and mitochondrial morphology were identified. To ascertain the mechanisms behind RDN-repressed endothelial inflammation, human artery ECs were given norepinephrine treatment in vitro. RDN decreased inflammation, oxidative stress, and atherosclerosis in EC mitochondria. The persistent sympathetic hyperactivity raised the activity of the enzyme monoamine oxidase A (MAO-A) and the amount of norepinephrine in the blood. impaired MAO- The production of atherogenic and proinflammatory molecules was increased in ECs as a result of ROS buildup and NF-B activation caused by the activation of mitochondrial homeostasis. Additionally, it inhibited PGC-1, a regulator of mitochondrial function, with the help of NF-B and oxidative stress. The inhibition of EC atheroprone phenotypic changes and atherosclerosis was achieved by disrupting the positive-feedback regulation between mitochondrial dysfunction and inflammation caused by the inactivation of MAO-A by RDN.

Keywords: Renal denervation; Endothelial dysfunction; Mitochondrial dysfunction; Inflammation; Atherosclerosis

Introduction

The main pathophysiology of coronary artery disease (CAD), the leading cause of death worldwide, is atherosclerosis. Atherosclerosis risk factors include numerous comorbidities like hypertension, diabetes mellitus, obesity, and ageing. Atherosclerosis is thought to emerge from vascular endothelial dysfunction, which is defined by maladaptive changes in endothelial functional phenotypes .Reactive oxygen species (ROS) are primarily produced by mitochondria, which are also a main target for damage brought on by ROS.The involvement of mitochondria, particularly those found in endothelial cells (ECs), in the development of atherosclerosis has not been thoroughly studied, despite the fact that mitochondrial dysfunction is a common pathogenic mechanism for many diseases. Inflammation and innate immunological signalling cascades that lead to endothelial activation and dysfunction are both caused by the alteration of mitochondrial redox homeostasis. Strategies that lower or neutralize the amount of ROS produced by mitochondria may have a variety of positive benefits, including a reduction in inflammatory response, promotion of endothelium repair, and a suppression of atherosclerosis [1-3].

Recent research suggests that endothelial dysfunction frequently coexists with sympathetic nervous system (SNS) activation. According to a recent study, arteries are innervated, and neurotransmitters may have an impact on inflammatory leukocyte adherence to ECs. This suggests that SNS is also a factor in atherogenesis. However, there is an ambiguous connection between inflammation, oxidative stress, and sympathetic hyperactivity.

The sympathetic neurotransmitter norepinephrine is metabolized by the mitochondrial enzyme monoamine oxidase-A (MAO-A), which also produces hydrogen peroxide (H_2O_2) as a byproduct of this enzymatic reaction. It's interesting to note that heightened sympathetic drive and norepinephrine spillover are linked to MAO-A activation. As a result of the excessive H_2O_2 production in this phase, atherosclerosis' pathophysiology of vascular injury is caused by mitochondrial oxidative damage. One of the primary players in the MAO-A/H_2O_2 axis is p53, which functions as a suppressor of peroxisome-proliferator-activated receptor-coactivator-1 (PGC-1), a crucial link between inflammatory, redox, and metabolic regulatory pathways. These findings, along with the fact that endothelium is enriched in MAO-A, imply that persistent MAO-A/H_2O_2 axis activation in the setting of chronic sympathetic hyperactivity can trigger an inflammatory response in ECs [4-7].

Through the ablation of the sympathetic nerves that pass through the adventitia of the renal artery, renal denervation (RDN) can successfully diminish systemic sympathetic activity. In therapeutic settings, it has been applied as a novel therapy for resistant hypertension. Additionally, RDN has demonstrated potential therapeutic benefits on oxidative stress, inflammation, and insulin resistance, which are risk factors for endothelial dysfunction [8-11], and RDN enhances endothelial function in the context of diabetes, according to our prior findings. By enhancing endothelial function, namely through the pathways connected to the vicious cycle of MAO-A-induced mitochondrial ROS release and inflammation in ECs, our goal in this study is to ascertain if RDN can reduce atherosclerosis.

***Corresponding author:** Jihong Han, Key Laboratory of Metabolism and Regulation for Major Diseases of Anhui Higher Education Institutes, Hefei University of Technology, Hefei, 230009, China, E-mail: jihong08@nankai.edu.cn

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Subjective Heading

The First Affiliated Hospital of the University of Science and Technology of China's Institute Research Ethics Committee accepted the use of human serum samples in the experiments, which were carried out in compliance with the second principle of the Helsinki Declaration. After obtaining informed consent, blood samples were taken from CAD patients and non-CAD controls (14 men and 6 women, ages 50 to 68). The specific inclusion/exclusion standards and medical characteristics of non-CAD controls and CAD patients were described.

Eight-week-old male ApoE-deficient (ApoE-/-) mice were maintained at the Nankai University Animal Center in Tianjin, China, where they had free access to food and water. The RDN group (RDN) and the sham surgery group were randomly assigned to two groups of mice after one week of acclimation (Sham). While mice in the Sham group underwent abdominal surgery without RDN, mice in the RDN group got bilateral RDN surgically and chemically. Following surgery, all mice were given a pro-atherogenic high-fat diet (HFD) for 20 weeks, which comprises 21% fat and 0.5% cholesterol. After the mice were sedated and put to death in a CO2 chamber at the conclusion of the experiment, blood, kidney, renal artery, and aorta sample were collected. The Animal Welfare Board of Canada approved each study [11-13].

Endothelial Basal Medium 2 (EBM-2, Lonza, USA), which contains 2% foetal bovine serum (FBS), 50 g/mL penicillin/streptomycin, and growth factors, was used to cultivate human aortic ECs (HAECs, Lonza, USA). Using Lipofectamine RNAiMAX or Lipofectamine 2000, cells that were 70–80% confluent were transfected with either a PGC-1 CRISPR expression vector or an MAO-A siRNA from Santa Cruz, USA. Cells were switched to EBM-2 media containing FBS after a 24 hour transfection, and the appropriate therapy was administered. Purchased from ATCC (Manassas, USA), human monocytic cell line THP-1 cells were grown in RPMI 1640 media with 10% FBS and 50 g/mL penicillin/streptomycin.

Mice were positioned in a laying position on a platform after being given a general anaesthetic with isoflurane, and their abdomens were then opened. The surface of the renal arteries were painted with phenol solution (10% in ethanol) for 5 minutes using a tiny brush after the renal arteries and veins were identified and all visible nerves along vessels were cut. The other side received the same treatment. The same treatment was performed on the mice in the Sham group, with the exception that the nerves were not severed and the renal arteries were treated with 0.9% saline rather than phenol solution. The evaluation of renal artery morphology by hematoxylin and eosin (H&E) staining and norepinephrine content in kidney or serum at the conclusion of the experiment served to demonstrate the successful RDN [14,15].

Discussion

Following collection, mouse whole aorta and 6-mm frozen cross sections of the aortic root were made, and they were utilised to identify en face lesions and sinus lesions by Oil red O staining. Based on the standards for experimental atherosclerosis investigations outlined in the American Heart Association statement all the pictures taken under a microscope were used to quantitatively analyse the lesion area (intima) and Oil red O positive region. The area of necrotic cores, thickness of the fibrosis cap, and collagen content of the aortic root were also assessed using cross sections. Masson trichrome (Solarbio, China) with H&E staining. Through the use of dihydroethidium (DHE, Sigma, USA) staining, ROS levels in plaques were ascertained. TUNEL labelling, performed by Vazyme Biotech in China, was used to identify apoptotic cells. The Leica DM5000B microscope (Wetzlar, Germany) was used to take each picture, and Image J was used for quantitative analysis.

Renal arteries were embedded in paraffin, fixed in 4% paraformaldehyde, and cut into 4-m slices before immunohistochemical labelling. Briefly, slices were treated first with biotinylated goat anti-rabbit IgG and then with anti-tyrosine hydroxylase antibody (Proteintech, 25859-1-AP, 1:200). The stain strength (level of tyrosine hydroxylase staining) was graded using a blinded system as 0, negative, 1, weak, 2, mild, 3, moderate, or 4, strong.

Immunofluorescent staining was performed on frozen aortic root cross sections using primary antibodies against CD68 (Santa Cruz, sc-17832, 1:200), SM22 (Proteintech, 10493-1-AP, 1:200), CD31 (Santa Cruz, sc-376764, 1:200), MCP-1 (Proteintech, 66272-1-Ig, 1:200), endothelin-1 (AbcamHAECs were fixed in 4% paraformaldehyde, permeabilized by 0.5% Triton, blocked with 5% BSA and incubated with primary antibody against NF-κB (Santa Cruz, sc-8008, 1:200), 8-OHdG (Santa Cruz, sc-66036, 1:200), or PGC-1α (Proteintech, 66369-1-Ig, 1:200), followed by incubation with FITC or rhodamine-conjugated secondary antibody.HAECs or mouse tissues' total proteins were extracted using a protease inhibitor-containing protein lysis solution (PMSF plus cocktail). In order to evaluate protein expression by Western blot as reported an equal number of total proteins from each sample were employed. After scanning each Western blot image, the band density was semi-quantitatively examined.

A reverse transcription kit (Invitrogen, USA) was used to create cDNA from total RNA isolated from mouse tissues. Quantitative real-time PCR (qPCR) was then conducted using a SYBR green PCR master mix and primers with the sequences specified in Table S3 of the Supplementary Materials. GAPDH mRNA in the matched sample was used to normalise the expression of mRNA for each gene.

Using the Mindray Biochemical Analyzer BS-190, the levels of mouse serum triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein AI (Apo-AI), and apo-B were measured (Mindray, Shenzhen, China). An enzymatic technique was used to assess the quantities of free fatty acids (FFA) in mouse serum (Biolabo, France). With the aid of the appropriate test kits, the presence of the oxidative stress markers malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, glutathione (GSH) content, and total antioxidant capacity (T-AOC) in mouse serum was determined (Solarbio, China).

ELISA kits were used to test the levels of norepinephrine (Abnova, China), MAO-A (Abbexa, UK), mouse serum cytokines (R&D, USA), and MAO-A levels/activity (Mbbiology, China) in serum samples from humans. Following transfection and treatment, conditioned media from HAECs was gathered for cytokine measurement using ELISA kits from Thermo Scientific (USA). Following sacrifice, mouse thoracic aortic tissues were gathered, fixed in 2.5% glutaraldehyde, and then postfixed in 1% osmium teroxide for 2 hours. In accordance with standard protocol, samples were dehydrated in gradient ethanol solutions. An electron microscope was used to view the generated ultrathin slices (Hitachi, Japan).

The dichlorofluorescin diacetate (DCFDA, Solarbio, China) technique was used to measure the intracellular ROS generation. After treatment, cells were placed on 35 mm confocal culture dishes

and exposed to DCFDA for 30 min at 37 °C. Immediately after, the cells were immediately observed using a confocal microscope (ZEISS, LSM710, Germany) with excitation/emission wavelengths of 480/530 nm.

Using the MitoSox red fluorescent dye, mitochondrial superoxide production was assessed in intact cells (Invitrogen, USA). Following treatment, cells were stained for 15 minutes at 37 °C with 1.5 mmol/L MitoSox red and 10 ng/mL Hoechst blue dye (Solarbio, China), followed by two washes with PBS. A confocal microscope was used to measure the MitoSox fluorescence in random fields (ZEISS, LSM710, Germany). Using ImageJ, all acquired fluorescence pictures were examined.

Cell toxicity was measured as a percentage of cell viability using the widely used CCK-8 assay (Solarbio, China). Norepinephrine was administered to HEACs for 48 hours after they had been plated in 96-well dishes with 1 104 cells per well. Each well received a treatment, then 10 L of CCK-8 solution was added, and the wells were allowed to incubate for two hours. The absorbance at 450 nm was measured in each well using a microplate reader. Cell viability was normalised to the control group. The ROS levels in the RDN group were shown to be lower than those in the Sham group using DHE staining .DNA oxidative damage caused by the MAO-A/H2O2 axis can be detected using the 8OHdG staining method. RDN equally decreased aortic endothelial 8OHdG levels

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Norepinephrine plays a major role in mediating sympathetic activity. It's interesting to note that norepinephrine circulation and persistently elevated sympathetic tone can be caused by cardiovascular risk factors. The effect of norepinephrine on the viability of HAECs was initially assessed by the CCK8 assay in order to further determine the relationship between RDN and endothelial function. Norepinephrine had no impact on cell survival at low doses, but it drastically decreased it at 100 mol/L (Fig. S3A), demonstrating its cytotoxicity to ECs. In fact, norepinephrine raised intracellular ROS levels in HAECs in a concentration- and time-dependent manner, as demonstrated by DCFDA labelling (Figs. S3B and S3C). Norepinephrine significantly altered the shape of the mitochondria in conjunction with an increase in ROS generation. The examination of the images using the Mitochondrial Network Analysis (MiNA) toolset and the MitoTracker Red labelling show that the mitochondrial footprint and

Norepinephrine induces MAO-A expression in HAECs in a concentration-dependent manner, despite the fact that norepinephrine is the substrate for MAO-A activity. It's interesting to note that norepinephrine-induced ROS generation and DNA oxidative damage were significantly reduced when MAO-A expression was specifically decreased by siRNA . Additionally, norepinephrine-impaired mitochondrial functions were considerably enhanced by MAO-A siRNA. The effects of norepinephrine on mitochondrial membrane potential and ROS levels were counteracted by MAO-A siRNA. Through NF-B pathway activation, mitochondrial ROS generation can start an inflammatory response. Norepinephrine decreased the expression of I-B while increasing the expression of phosphorylated NF-B in HAECs. Consequently, norepinephrine increased NF-B nuclear translocation (Fig. 6B; S3D). However, norepinephrine mitigated the effects of diminished IB and elevated p-NF-B or enhanced NF-B nuclear translocation.

In a vicious cycle during inflammation, PGC-1 and NF-B moderate one another, and oxidative stress is a key factor. PGC-1 expression vector transfection effectively reduced norepinephrine-reduced IB and boosted NF-B phosphorylation and nuclear translocation in HAECs (Fig. 6L; S4E). Norepinephrine enhanced the levels of MCP-1 and IL-6 in the HAEC conditioned media, while PGC-1 overexpression prevented the increases from occurring because they were associated with NF-B expression .The stimulation of PGC-1 expression also prevented the adherence of THP-1 cells to HAECs that was caused by norepinephrine (Fig. S4D). All of the aforementioned information points to the possibility that inhibiting NF-B can reduce the oxidative damage that norepinephrine-induced mitochondrial dysfunction causes.

Conclusion

The fact that this study mainly concentrated on how RDN altered EC phenotypes as atherosclerosis progressed is a drawback. However, the proliferation and migration of smooth muscle as well as macrophage phenotypes can be modified by sympathetic signalling activation .Sympathetic hyperactivity may support numerous elements of atherogenesis in later stages, including atheroma development, progression, and rupture, given its functions in a variety of pathologic diseases. Further research is needed to understand the precise functions of RDN and MAO-A activity in different cell types during advanced atherosclerosis.

In conclusion, we identified the mechanisms underlying endothelial dysfunctions corrected by RDN during atherogenesis, laying the groundwork for the use of RDN in hypertensive patients whose atherosclerosis complicates their condition. Additionally, the discovery that elevated norepinephrine spillover controls EC phenotypes via a mitochondrial-inflammation circuit controlled by MAO-A may offer potentially lucrative therapeutic options for the therapy of atherosclerosis.

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Conflict of Interest

The authors declare that they are no conflict of interest.

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