Original Paper

Non-Lethal Sonodynamic Therapy Inhibits Atherosclerotic Plaque Progression in ApoE^{-/-} Mice and Attenuates ox-LDL-mediated Macrophage Impairment by Inducing Heme Oxygenase-1

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Key Words

SDT • Macrophage • HO-1 • Atherosclerotic plaque • AKT • ERK • Nrf2

Abstract

Background: Previous studies from our group showed that low-intensity sonodynamic therapy (SDT) has protective effects on atherosclerosis (AS). However, because the intensity of ultrasound passing through tissue is attenuated, the consequences of very low-intensity SDT, referred to as non-lethal SDT (NL-SDT), on atherosclerotic plaques are unclear. The aim of this study was to determine whether NL-SDT affects atherosclerotic plaques and to elucidate the possible underlying mechanisms. **Methods:** An AS model was established using ApoE^{-/-} mice fed a western diet. En face Oil Red O staining was used to measure atherosclerotic plaque size. Hematoxylin and eosin staining and immunohistochemical staining were used to observe plaque morphology and assess the location of macrophages and heme oxygenase 1 (HO-1). HO-1 mRNA and protein levels in AS plaques were evaluated by real-time PCR and western blotting. Human THP-1 cells and mouse peritoneal macrophages were used in this study. Western blotting was used to investigate the expression of cellular proteins after NL-SDT. Macrophage apoptosis was evaluated by TUNEL assays and flow cytometry with Annexin V/PI double staining. Intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were measured with 2'-7'-dichlorofluorescein diacetate (DCFH-DA) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) staining, respectively. **Results:** NL-SDT significantly inhibited AS progression and reduced the necrotic core area. NL-SDT induced HO-1 expression in lesional macrophages and in cultured macrophages. NL-SDT activated the protein kinase B (AKT) and extracellular signal-related protein kinase (ERK) pathways and the transcription factor NF-E2-related factor 2 (Nrf2).

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NL-SDT significantly reduced oxidized LDL (ox-LDL)-induced macrophage MMP collapse, ROS production and cell apoptosis. Zinc protoporphyrin (ZnPP), a HO-1-specific inhibitor, reversed the protective effects of NL-SDT. **Conclusion:** NL-SDT inhibits atherosclerotic plaque progression and increases plaque stability. *In vitro*, NL-SDT has a protective effect on ox-LDL-induced macrophage impairment via HO-1.

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Introduction

Atherosclerosis (AS) is the primary cause of heart disease and stroke. Unstable plaques are vulnerable and prone to rupture, which precipitates acute, occlusive luminal thrombosis and its consequences, including myocardial infarction, unstable angina, sudden cardiac death, and stroke [1]. Unstable plaques have a large necrotic core and thin fibrous cap with reduced extracellular matrix. Inhibiting the formation of unstable plaques decreases the occurrence of acute coronary heart disease. Macrophages are associated with atherosclerotic plaque instability, plaque progression and plaque rupture [2]. Meanwhile, macrophage uptake and storage of atherogenic lipoproteins in atherosclerotic plaques can increase endoplasmic reticulum (ER) stress, in turn inducing macrophage apoptosis and necrosis. These processes promote the progression and instability of atherosclerotic plaques [3].

Heme oxygenase 1 (HO-1), an inducible isoform of the heme oxygenase family, degrades heme into the metabolites carbon monoxide, biliverdin, and ferrous iron. Several reports have shown that HO-1 can significantly inhibit intraplaque hemorrhage and plaque instability [4, 5]. The metabolic products of HO-1 have anti-inflammatory and antioxidant properties, and HO-1 has also been shown to have protective effects against AS.

Sonodynamic therapy (SDT), a promising and noninvasive treatment in which application of ultrasound activates specific sonosensitizers, has been widely used to treat tumors [6]. Recently, our group found that different intensities of SDT have different biological effects [7]. *In vitro*, low-intensity SDT induces mitochondria-mediated macrophage apoptosis [8-10] and macrophage autophagy [11], inhibits RIPK1/RIPK3-dependent necroptosis [12] and regulates myocardial fibrotic remodeling [13], whereas high-intensity SDT induces necroptosis [7]. In a rabbit model of AS, low-intensity SDT stabilized early atherosclerotic plaques by inducing macrophage apoptosis and apoptotic cell clearance [14]. However, the underlying mechanism of SDT in treating AS is still unclear [8, 9, 15, 16]. Considering that ultrasound intensity attenuates as sound waves pass through human tissue towards an atherosclerotic plaque and that macrophage survival state in human atherosclerotic plaques varies [17], so the effects of very low-intensity SDT (non-lethal SDT, NL-SDT) in AS should be assessed.

Materials and Methods

Chemicals

Phorbol-12-myristate-13-acetate (PMA) and 5-aminolevulinic acid (ALA) were purchased from Sigma Aldrich (St. Louis, MO, USA). ox-LDL was purchased from Yiyuan Biotech (Guangzhou, China); 4, 6-diamidino-2-phenylindole (DAPI), 2'-7'-dichlorofluorescein diacetate (DCFH-DA) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) were purchased from Beyotime Biotechnology (Beijing, China). The inhibitors SP600125, LY294002, PD98059 and SB203580 were obtained from Santa Cruz Biotechnology (USA). Zinc protoporphyrin (ZnPP) was obtained from Sigma-Aldrich (St. Louis, MO, USA). X-tremeGENE siRNA transfection reagent and a TUNEL Assay Kit were purchased from Roche (Basel, Switzerland).

NL-SDT treatment

The NL-SDT equipment, including an ultrasonic generator, transducer and power amplifier, used in this study was assembled by the Harbin Institute of Technology (Harbin, China). Macrophages were treated with a homemade ultrasonic transducer (diameter: 3.5 cm, resonance frequency: 1.0 MHz; duty factor: 10%,

ultrasonic intensity: 0.1 and 0.4 W/cm²) placed in a water bath 30 cm underneath the cells, while mouse aorta was directly exposed to the ultrasonic transducer. The macrophages (1×10^6 cells/mL) were then seeded in 35 mm Petri dishes, consistent with the size of the therapeutic instrument probe before NL-SDT treatment, and incubated with 1 mM ALA (Sigma-Aldrich, St. Louis, MO, USA) in the dark for 3 hours prior to treatment with the NL-SDT equipment.

Animals

All animal protocols were performed in accordance with the regulations of the Ethics Committee of Harbin Medical University. ApoE^{-/-} C57BL/6 mice were obtained from Beijing HuaFuKang Biotechnology Co., LTD. (Beijing, China). All mice were male and 4-8 weeks old. The mice were fed a western diet (0.25% cholesterol, 21% fat, 19.5% casein, 34.16% sugar) for 4 weeks before NL-SDT. Three hours after intraperitoneal injection of ALA (100 mg/kg), the mice were anesthetized with pentobarbital. The ultrasonic transducer (diameter: 3.5 cm, resonance frequency: 1.0 MHz; duty factor: 10%, ultrasonic intensity: 0.4 W/cm²) was then positioned above the front heart region of the mouse (Fig. 1C). During the whole process and 1 day after the treatment, the mice were kept in the dark. To determine the long-term effects of NL-SDT, we repeated the above process every two weeks until 12th week (Fig. 1A). To measure HO-1 expression after NL-SDT, the mice were sacrificed in several days (Fig. 1B).

Cell culture

Male 4- to 8-week-old C57BL/6 mice weighing approximately 20-25 g were purchased from HuaFuKang Biotechnology Co. (Beijing, China). The mice were sacrificed by cervical amputation, and the peritoneal cavity was washed with 10 mL cold PBS containing 3% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Cells were harvested from the peritoneal cavity using a Pasteur pipette and inoculated onto a 35 mm plastic dish at 1.2×10^6 cells/mL for 1 hour in a humidified atmosphere at 37°C with 5% CO₂. The supernatant was then replaced with RPMI 1640 medium (HyClone, Logan, UT, USA) containing 10% FBS. Remaining adherent cells were peritoneal macrophages. The human acute monocytic leukemia THP-1 cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI 1640 medium containing 10% FBS, 20 µg/mL penicillin and 20 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. THP-1 monocytes were seeded onto coverslips in 35 mm Petri dishes at a density of 1×10^6 cells/mL and treated with 100 ng/mL PMA for 72 hours to induce differentiation into macrophages. The macrophages were incubated with 50 µg/mL ox-LDL for 18 hours in serum-free RPMI 1640. For NL-SDT, the cells were pretreated for 2 hours before exposure to ox-LDL. ZnPP (0.1 µM) and other inhibitors were added at the indicated concentrations 2 hours before NL-SDT.

Flow cytometry analysis

We stained cells using an Annexin V-FITC and PI Detection Kit (BD Pharmingen, USA) before flow cytometry. Annexin V-FITC binds to phosphatidylserine (PS) on the outer leaflet of the plasma membrane,

Fig. 1. (A) (B) Design used to study ApoE^{-/-} mice. (C) Schematic diagram of the ultrasonic exposure set-up. Ultrasonic exposure set-up for applying SDT to mice.

while PI is excluded by cells with intact membranes. PI positivity is therefore a sign of necrosis or late apoptosis, whereas cells positive for Annexin V-FITC but negative for PI are generally defined as early apoptotic cells. Trypsinized cells (6×10^5) were gently washed with serum-containing media and then PBS. Next, the cells were resuspended in 100 μ L binding buffer with 5 μ L of Annexin V-FITC and 5 μ L of PI for 10 min at room temperature in the dark, followed by the addition of 400 μ L binding buffer. Finally, we analyzed cells by flow cytometry within 1 hour. The results were assessed using BD FACSDiva Software v7.0 (Becton-Dickinson, USA). For statistical analysis, more than 10,000 total cells per group were counted, and each assessment was repeated three times.

TUNEL assay

DNA fragmentation of apoptotic cells was detected using a TUNEL assay kit according to the manufacturer's instructions. After drug and NL-SDT treatment, cells or frozen sections of mouse aortic root were fixed in 4% paraformaldehyde solution in PBS for 30 min at room temperature. The cells or frozen sections were then incubated with 3% H_2O_2 in methanol for 10 min at room temperature and treated with a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4°C, incubated in the TUNEL reaction mixture for 60 min at 37°C, incubated in DAPI fluorescent dye for 15 min at 37°C, and then visualized by fluorescence microscopy (Zeiss, Germany). The nuclei of apoptotic cells appeared green, and green fluorescence overlapping blue nuclear fluorescence indicated apoptosis. Apoptotic cells were counted from four randomly selected fields in each sample, and the counts are expressed as a percentage of the total number of cells.

Reactive oxygen species (ROS) detection

Intracellular ROS generation was determined by measuring the fluorescence of 2,7-dichlorofluorescein (DCF); DCFH-DA was added to the cells to a final concentration of 20 μ M and incubated at 37°C for 30 min. Immediately after NL-SDT treatment, the cells were observed using fluorescence microscopy (Zeiss, Germany) at 488 nm excitation and 525 nm emission wavelengths.

Mitochondrial membrane potential (MMP) detection

MMP was assessed using the fluorescent probe JC-1, which is a dual emission potential-sensitive probe that localizes to the mitochondrial matrix when the MMP is high. From there, the formation of polymers (J-aggregates) produces red fluorescence. JC-1 cannot aggregate in the mitochondrial matrix when the MMP is low, and the JC-1 monomer produces green fluorescence. The monomeric form of JC-1 appears in mitochondria after MMP loss. One hour after SDT, cells were incubated with 5 μ g/mL JC-1 for 20 min at 37°C in the dark and then observed using fluorescence microscopy (Zeiss, Germany).

Western blotting

Total protein from cells or mouse aorta was extracted with RIPA buffer. Nucleoprotein and cytosolic protein were extracted with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China). Protein concentration was measured with a BCA protein assay kit (Beyotime, China). Total protein (25–50 μ g protein) was separated by 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 5% nonfat milk 1 hour at room temperature. The membranes were incubated at 4°C overnight with antibodies against the following proteins: HO-1 (Proteintech Group, China), β -actin (Santa Cruz Biotechnology, USA) and Nrf2 (Abcam, Cambridge, UK) (all 1:1,000), p-ERK, ERK, p-p38, p38, p-AKT, AKT, p-JNK, JNK (Cell Signaling Technology, USA) and Lamin B (Boster, Wuhan, China) (all 1:500). The membranes were washed with Tris-buffered saline with Tween, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; Beijing Zhongshan Biotechnology, Beijing, China), rewashed and visualized using an enhanced chemiluminescence detection kit (Haigene, China) on a Bio-Rad ChemiDoc™ MP Imaging System (California, USA).

Real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from 2–3 mouse aortas per group using TRIzol reagent (Invitrogen, USA). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (ABI, USA). Quantitative PCR was performed on an ABI 7500 Fast Real-Time PCR System with FastStart Universal SYBR Green MasterMix

(Roche, Basle, Switzerland). GAPDH served as an endogenous control. Quantification of mRNA determined using the $2^{-\Delta\Delta CT}$ method. Mouse HO-1 primers were as follows: 5'-ACGCATATACCCGCTACCTG-3' (sense) and 5'-CCAGAGTGTTCATTCGAGCA-3' (antisense). Primers for mouse GAPDH were purchased from Sangon Biotech (Shanghai, China).

Oil Red O staining

Oil Red O staining was performed to assess lipids within atherosclerotic plaques. The entire aorta of the mouse was cut open and stained with Oil Red O for 30 min and then washed with ethanol 3 times. Images were taken using a Digital Single Lens Reflex (Canon, Japan).

HE and immunohistochemical staining

Vessel parameters of mice were analyzed by hematoxylin and eosin (HE) staining. All tissues were sliced to 5- μ m thickness, mounted on glass slides coated with poly L-lysine and subjected to HE staining according to routine histopathological methods.

Immunohistochemical staining was performed as follows. Paraffin sections were prepared using a standard protocol. They were blocked in endogenous peroxidase with 3% H_2O_2 in PBS for 15 min. Then, the slides were washed 2 $\times$ 5 min in PBS plus 0.025% Triton X-100 with gentle agitation. They were blocked in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature, drained for a few seconds and wiped around the sections with tissue paper. Primary antibody diluted in TBS was applied with 1% BSA, and the samples were incubated overnight at 4°C. Next, they were rinsed 2 $\times$ 5 min in PBS with 0.025% Triton with gentle agitation. Enzyme-conjugated secondary antibody, diluted to the concentration recommended by the manufacturer in PBS, was applied to the slides and incubated for 30 min at room temperature. The slides were developed with DAB for 10 min at room temperature and counterstained with hematoxylin. Finally, they were dehydrated, cleared, mounted and visualized by light microscopy (Olympus, Japan). A macrophage antibody (CD68) (Santa Cruz Biotechnology, USA; 1:50) and an anti-HO-1 antibody (Proteintech Group, China; 1:100) were used.

Confocal fluorescence microscopy

Following the NL-SDT treatment, cells were fixed with 4% formaldehyde for 10 min, permeabilized in 0.1% Triton X-100 for 5 min and then blocked in 10% normal goat serum for 1 hour at room temperature. The cells were then incubated with fluorescent Nrf2 antibody (Abcam, Cambridge, UK) at a working dilution of 1:100 in 1% BSA overnight at 4°C. Cell nuclei were stained with DAPI. Immunofluorescence was visualized using a Zeiss LSM 700 confocal system (Zeiss, Germany).

RNA interference of Nrf2

siRNA against Nrf2 was synthesized by GenePharma (Shanghai, China) with the following sequences: siNrf2-1#: 5'-GAAUGGUCCUAAAACACCAAtt-3'; siNrf2-2#: 5'-CAGUCUUCUUGCUACUAAtt-3' [18].

Negative control siRNA was provided by GenePharma. siNrf2 and negative control siRNA were transfected into THP-1-derived macrophages with X-tremeGENE siRNA transfection reagent using a routine process. Protein was collected 48 hours after the transfection.

Statistical analysis

Quantitative data are shown as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Statistical Analysis System (SAS) software version 9.2 (SAS institute, Cary, NC). Student's t-test and one-way ANOVA followed by Bonferroni's post hoc test were used to analyze all experimental data. Values of $P < 0.05$ were considered statistically significant.

Results

NL-SDT inhibited atherosclerosis progression and reduced necrotic core area in atherosclerotic plaques in the aortic root in ApoE^{-/-} mice

To analyze the effects of NL-SDT on atherosclerotic plaques, ApoE^{-/-} mice with atherosclerotic lesions in the aortic root were assessed; the mice developed the lesions after

Fig. 2. NL-SDT inhibited atherosclerotic plaque progression in ApoE^{-/-} mice. Mice received multiple rounds of NL-SDT, as described in the materials and methods (n=10–12 mice per group). (A) Oil Red O staining in the upper aorta above the thoracic aorta and the percentage of en face plaque area. *P < 0.05 compared with the control group. (B) HE staining was used to assess the plaque area in atherosclerotic plaques. Scale bar represents 500 μ m. *P < 0.05 compared with the control group. (C) HE staining was used to assess the necrotic core area in atherosclerotic plaques. Scale bar represents 500 μ m. *P < 0.05 compared with the control group. (D) TUNEL staining of aortic plaques in the two groups is shown. Quantification of lesional apoptotic cells. The results are represented as the percentage of TUNEL-positive cells to the total cell number within the atheroma. Scale bar represents 200 μ m. *P < 0.05 compared with the control group.

12 weeks on a western diet (Fig. 2A, B and C). As shown in Fig. 2A, compared to the control group, the NL-SDT group had a decreased plaque area. Because only the thoracic aorta can be covered by the ultrasonic transducer, we only analyzed Oil Red O staining in the upper part of the aorta. We found that the plaque area in the NL-SDT group was smaller than that in the control group ($6.02 \pm 1.20\%$ versus $11.38 \pm 0.69\%$, $P < 0.05$, Fig. 2A). We also analyzed plaques in consecutive sections of mouse aortic root by HE staining and obtained similar results as those shown in Figure 2A (Fig. 2B). As shown in Fig. 2C, the necrotic core area of the plaques significantly decreased after NL-SDT (0.047 ± 0.011 mm² versus 0.104 ± 0.020 mm², $P < 0.05$).

NL-SDT decreased cell apoptosis of atherosclerotic plaques in ApoE^{-/-} mice

To study the protective effects of NL-SDT on plaques, we used a TUNEL assay to detect apoptotic cells in consecutive sections of mouse aortic root. As shown in Fig. 2D, there were fewer apoptotic cells in the NL-SDT group than the control group (31.39 ± 8.69 cells/mm² versus 86.51 ± 23.03 cells/mm², $P < 0.05$).

NL-SDT up-regulated HO-1 expression in macrophages in early atherosclerotic plaques in mouse aortas

We hypothesized that NL-SDT decreased cell apoptosis in atherosclerotic plaques due to changes in HO-1 expression. To test this hypothesis, we detected HO-1 expression in early atherosclerotic plaques. After 4 weeks on a western diet, ApoE^{-/-} mice developed early atherosclerotic plaques in the aortic root [19]. At this time point, the atherosclerotic mice were randomly assigned to the following groups: control, ALA, ultrasound and NL-SDT. As

Fig. 3. NL-SDT up-regulated HO-1 expression of lesional macrophages in the early atherosclerotic plaques of mouse aorta. ApoE^{-/-} mice were given a western diet for 4 weeks before they were randomly assigned into different groups (n=6-8 mice per group). (A) HO-1 expression in aorta plaques was detected by western blotting one day after NL-SDT in each group. (B) HO-1 expression in aorta plaques after NL-SDT at different time points. (C) HO-1 mRNA levels in aorta plaques one day after NL-SDT. *P < 0.05. (D) HE and immunohistochemistry results of early lesions one day after NL-SDT. The upper and lower panels represent the control and NL-SDT groups. The figures show HE, macrophages and HO-1 staining. Scale bars represent 500 μ m in the left figure and 200 μ m for the other three figures in each group.

shown in Fig. 3A, HO-1 protein expression was higher in the NL-SDT group than in other groups. Changes in HO-1 expression after NL-SDT were also analyzed. On 1 day after NL-SDT, HO-1 expression significantly increased; 3 days after NL-SDT, the level decreased; and 7 days or 14 days later, expression significantly decreased (Fig. 3B). Based on the above results, we analyzed HO-1 mRNA expression in mouse atherosclerotic plaques at 1 day after NL-SDT, and we found that the relative level of HO-1 mRNA was increased compared to that of the control group (19.15 ± 7.22 versus 2.19 ± 0.41 , $P < 0.05$) (Fig. 3C).

NL-SDT-induced HO-1 expression predominantly localized to macrophages in atherosclerotic plaques

As shown in the control group in Fig. 3D, the formation of early plaques in mouse aorta corresponded to increased macrophage infiltration and the absence of cholesterol crystals. After NL-SDT, HO-1 expression increased, and HO-1 and macrophage staining overlapped in the atherosclerotic plaques. These results indicate that NL-SDT predominantly induced HO-1 expression in macrophages in atherosclerotic plaques.

Apoptosis induced by SDT at different ultrasonic intensities

As shown in Fig. 4, in the control group, percentages of apoptotic cells were $11.0 \pm 1.1\%$. In the SDT groups, the percentage of apoptotic cells increased significantly when the ultra-

Fig. 4. (A) The effect of ultrasonic intensity-mediated SDT on macrophage apoptosis as assessed by flow cytometry. (B) Statistical analysis of the results in panel A. ** $P < 0.01$ compared with the control group.

Fig. 5. NL-SDT induced HO-1 expression in macrophages via the AKT and ERK pathways. Macrophages were treated with or without ALA and ultrasound, as described in the materials and methods. (A) Western blotting of HO-1 expression in mouse peritoneal macrophages. ns $P > 0.05$ and *** $P < 0.001$ compared with the control. (B) Western blotting of HO-1 expression in THP-1-derived macrophages. ns $P > 0.05$ and *** $P < 0.001$ compared with the control. (C) Time-dependent HO-1 expression after NL-SDT in macrophages was detected by western blotting. (D) THP-1-derived macrophages were treated with or without ALA and ultrasound, and 6 hours later, total cellular protein was subjected to western blotting for detection of phospho-ERK, phospho-p38, phospho-AKT, phospho-JNK, ERK, p38, AKT and JNK. (E) THP-1-derived macrophages received NL-SDT for the indicated times. Total cellular protein was subjected to western blotting as indicated in panel D. (F) THP-1-derived macrophages were pretreated with inhibitors of ERK, p38, AKT and JNK and then received NL-SDT. HO-1 protein expression was detected by western blotting.

sound intensity increased to 0.8 W/cm² ($17.42 \pm 1.1\%$ versus $11.0 \pm 1.1\%$, $P < 0.01$). But the difference of percentage of apoptotic cells among control group, 0.1 W/cm² and 0.4 W/cm² were not statistically significant ($P > 0.05$).

Fig. 6. NL-SDT induced the expression and the translocation of Nrf2 from the cytosol to the nucleus. (A) At different time points after NL-SDT, Nrf2 immunofluorescence in THP-1-derived macrophages was visualized by confocal microscopy. Scale bars represent 20 μ m (B) Cytosolic and nuclear proteins were extracted from THP-1-derived macrophages after NL-SDT. Nrf-2 expression was detected by western blotting. Lamin B served as an internal control for nucleoprotein. * $P < 0.05$, ** $P < 0.005$ compared with the control. (C) Nrf2 protein expression was detected by western blotting 48 hours after THP-1-derived macrophages were transfected with siNrf2. * $P < 0.05$ compared with the control. (D) THP-1-derived macrophages were transfected with siNrf2 and given NL-SDT; 8 hours after NL-SDT, Nrf2 and HO-1 protein expression was detected by western blotting. * $P < 0.05$ compared with the control.

NL-SDT up-regulated HO-1 expression in macrophages in vitro

To investigate whether NL-SDT induces macrophage HO-1 expression *in vitro*, HO-1 expression was measured in mouse peritoneal macrophages and THP-1-derived macrophages. HO-1 protein expression was first detected 6 hours after NL-SDT. As shown in Fig. 5A, NL-SDT significantly enhanced this expression ($P < 0.001$). HO-1 expression was also slightly increased in the ALA and ultrasound groups, but these differences were not statistically significant ($P > 0.05$). The results of the THP-1-derived macrophage experiments were consistent with those of the experiments using mouse peritoneal macrophages (Fig. 5B). To assess changes in HO-1 expression over time, THP-1-derived macrophages were examined at different time points after NL-SDT. As shown in Fig. 5C, HO-1 expression increased 1 hour after NL-SDT and peaked 8 hours after NL-SDT. Twelve hours after NL-SDT, HO-1 expression decreased but was still higher than that of the control group ($P < 0.05$).

NL-SDT induced HO-1 expression in macrophages via the ERK and AKT pathways and activated Nrf2 in vitro

As shown in Fig. 5D, the ERK and AKT pathways were significantly activated 6 hours after NL-SDT: the phosphorylation levels of these two proteins increased compared to those in the control group. In the ultrasound and ALA groups, ERK and AKT were also activated, but their protein expression was less than that in the NL-SDT group. We also observed that NL-SDT did not activate the p38 and c-Jun N-terminal kinase (JNK) pathways (Fig. 5D). Many studies have reported that signaling pathway activation varies with time, especially at early stages after stimulation; therefore, we looked at pathway activation at different time points after NL-SDT, especially at the early stage. As shown in Fig. 5E, signal transduction pathway activation changed with time. The ERK pathway was activated 0.5 hours after NL-SDT, and

Fig. 7. NL-SDT prevented ox-LDL-induced THP-1-derived macrophage apoptosis by up-regulating HO-1 expression. (A) Cell apoptosis was detected using a TUNEL assay kit. Scale bar represents 200 μ m. (B) Statistical analysis of macrophage apoptosis in each group from panel A. *** $P < 0.001$ and ** $P < 0.01$ compared with the NL-SDT + ox-LDL group.

Fig. 8. NL-SDT reduced ox-LDL-induced ROS production and decreased MMP in THP-1-derived macrophages by up-regulating HO-1 expression. (A) Intracellular ROS generation was determined by measuring DCFH fluorescence as described in the materials and methods. Scale bar represents 100 μ m. (B) Statistical analysis of ROS production in panel A. ** $P < 0.01$ and *** $P < 0.001$ compared with the NL-SDT + ox-LDL group. (C) Fluorescence images of Jc-1 in macrophages from each group. Scale bar represents 100 μ m. (D) Statistical analysis of macrophage Jc-1 red/green ratio in panel C. *** $P < 0.001$ compared with the NL-SDT + ox-LDL group.

this activation was maintained for 6 hours. The AKT pathway had a similar pattern, but the p38 and JNK pathways were not activated. These results also confirmed the results in Fig. 5D. To further investigate the roles of ERK, AKT, p38 and JNK in NL-SDT-induced HO-1 expression, we individually pre-treated THP-1-derived macrophages with JNK inhibitor (SP600125), AKT inhibitor (LY294002), ERK inhibitor (PD98059) and p38 inhibitor (SB203580) before NL-SDT. The results showed that the AKT and ERK inhibitors significantly suppressed NL-SDT-induced up-regulation of HO-1 expression (Fig. 5F).

NF-E2-related factor 2 (Nrf2) is a transcription factor that interacts with the antioxidant response element (ARE) in the promoter of the gene encoding HO-1. Confocal microscopy and western blotting were used to examine the location and expression of Nrf2 following NL-SDT. As shown in Fig. 6A and B, under normal conditions, Nrf2 localized to the cytoplasm. In contrast, after NL-SDT, Nrf2 accumulated in nuclei. These results suggest that NL-SDT can activate Nrf2. We also found that NL-SDT up-regulates Nrf2 protein expression in a time-dependent manner (Fig. 6B). To determine whether NL-SDT-induced activation of Nrf2 upregulated HO-1 expression, we used Nrf2-specific siRNA to silence Nrf2. As shown in Fig. 6C, Nrf2 protein expression was reduced by the Nrf2-specific siRNA compared to the negative

Fig. 9. Model describing the protective effect of NL-SDT through HO-1 induction based on our observations. NL-SDT induced HO-1 expression through the ERK and AKT pathways. Nrf2 was up-regulated and translocated to the nucleus after NL-SDT was applied. Up-regulation of HO-1 expression had protective effects on ox-LDL-induced cellular impairment.

control siRNA (NC). Furthermore, siNrf2-1# had a better inhibitory effect than siNrf2-2# (relative protein level: siNrf2-1# 0.40 ± 0.11 versus NC 1, $P < 0.05$; siNrf2-2# 0.68 ± 0.11 versus NC 1, $P < 0.05$). After Nrf2 was inhibited, NL-SDT-induced HO-1 protein expression was reduced ($P < 0.05$) (Fig. 6D).

NL-SDT prevented ox-LDL-induced macrophage apoptosis by up-regulating HO-1 expression in vitro

To assess whether NL-SDT-induced up-regulation of HO-1 expression had a protective effect on macrophage survival, we used ox-LDL to stimulate macrophages *in vitro* to mimic the internal environment of plaque. Using a TUNEL assay, we found that apoptosis of THP-1-derived macrophages increased significantly after treatment with ox-LDL for 18 hours, and NL-SDT significantly inhibited this effect ($4.81 \pm 0.45\%$ versus $9.97 \pm 0.85\%$, $P < 0.001$) (Fig. 7A and B). To demonstrate that the protective effect of NL-SDT on macrophage apoptosis induced by ox-LDL is dependent on HO-1, ZnPP was used to block HO-1. ZnPP weakened the protective effect of NL-SDT, as shown in Fig. 7A and B ($P < 0.01$).

NL-SDT reduced ox-LDL-induced ROS production in macrophages by up-regulating HO-1 expression

As mentioned above, the ROS level in macrophages increased after ox-LDL stimulation. In Fig. 8A and B, the green fluorescence of DCFH represents ROS, which significantly increased in the ox-LDL group, and NL-SDT inhibited the ox-LDL-induced increase in ROS. However, when we pre-treated macrophages with ZnPP, the protective effect of NL-SDT was abolished. ROS production significantly decreased in the NL-SDT + ox-LDL group compared with the ox-LDL group (relative ROS level: $230.0 \pm 49.5\%$ versus $412.3 \pm 73.9\%$, $P < 0.001$), and ZnPP significantly reversed the protective effect of NL-SDT (relative ROS level: $385.0 \pm 57.5\%$ versus $230.0 \pm 49.5\%$, $P < 0.01$).

NL-SDT inhibited the ox-LDL-induced decrease in mitochondrial membrane potential in macrophages by up-regulating HO-1 expression

In Fig. 8C and D, the percentage of cells with normal MMP is equal to the jc-1 red/green ratio. MMP showed no difference between the control and NL-SDT groups, and ox-LDL decreased the MMP. NL-SDT significantly inhibited the ox-LDL-induced MMP decrease (NL-SDT+ox-LDL group, $66.8 \pm 4.8\%$ versus ox-LDL group, $29.8 \pm 4.6\%$; $P < 0.001$), but when macrophages were pre-treated with ZnPP, the protective effect of NL-SDT on mitochondria was reversed (Znpp+NL-SDT+ox-LDL group, $37.71 \pm 3.1\%$ versus NL-SDT+ox-LDL group, $66.8 \pm 4.8\%$; $P < 0.001$). Considering that ZnPP is a specific inhibitor of HO-1, these results indicate that the protective function of NL-SDT is based on HO-1.

Discussion

Our group previously reported that low-intensity SDT promotes early plaque stabilization by inducing macrophage apoptosis and does not induce inflammation or

necrosis [14]. It has also been reported that foam cell apoptosis is beneficial in early plaques, but in advanced plaques, macrophage death can result in increased inflammation and might promote plaque instability [20]. In the current study, we found that NL-SDT attenuate the ApoE^{-/-} atherosclerotic plaque progression and stabilize plaques (Fig. 2A, B and C). In contrast to low-intensity SDT, NL-SDT inhibits but does not induce lesional cell apoptosis (Fig. 2D). These results indicate that the mechanism underlying NL-SDT-mediated attenuation of atherosclerotic plaque progression is different from that of low-intensity SDT. Previous studies reported that HO-1 inhibits apoptosis [21] and can be induced by photodynamic therapy (PDT) [22]. In the current study, we found that NL-SDT also induces lesional macrophages to express HO-1 (Fig. 3A, B and C). To the best of our knowledge, no reports have examined whether NL-SDT can induce HO-1 expression *in vivo*, and our study is the first to use ApoE^{-/-} mice to verify this hypothesis. HO-1 expression was also observed in the control group (Fig. 3D), which might have been associated with atherosclerotic plaque progression. Previous reports have indicated that endogenous HO-1 expression can be detected in advanced human atherosclerotic lesions and localizes to endothelial cells, macrophages, and foam cells [23, 24]. Increased blood pressure [25, 26], smoking [27, 28], and oxidized lipids [29], which are regarded as risk factors for AS, can up-regulate endogenous HO-1 expression. However, this protective up-regulation of HO-1 is not sufficient to inhibit plaque progression [30]. In the current study, NL-SDT promoted HO-1 expression to inhibit plaque progression. HO-1 acts as the rate-limiting enzyme in the degradation of heme, in addition to having anti-inflammatory and antioxidant properties. HO-1 also has anti-apoptotic effects [21, 31, 32], which is consistent with the finding that NL-SDT inhibits cell apoptosis (Fig. 2D and Fig. 7). After mice were given NL-SDT treatment once, HO-1 expression in aorta atherosclerotic plaques increased at first and then decreased to the normal level, which was maintained through the seventh day (Fig. 3B). This duration might not be sufficient to inhibit the progression of AS; thus, we also treated mice with multiple rounds of NL-SDT.

In vitro, we used SDT at a 0.1 W/cm² intensity, which did not induce macrophage apoptosis (Fig. 4) but enhanced HO-1 expression in mouse peritoneal macrophages and THP-1-derived macrophages (Fig. 5A and B). This expression was time-dependent (Fig. 5C). Prior studies have shown that HO-1 expression is related to many signaling pathways, especially the mitogen-activated protein kinase (MAPK) and AKT pathways [33, 34]. Therefore, we also focused on these two pathways and found that NL-SDT can activate the AKT pathway. This result is consistent with Wu et al.'s [35] observation that high-density lipoprotein (HDL) induces HO-1 expression in endothelial cells by activating the PI3K/AKT pathway, inhibiting inflammation. Additionally, Carchman et al. [36] reported that lipopolysaccharide (LPS) induces HO-1 expression in liver cells through the P38-MAPK pathway, which has a cell-protective effect. We showed that NL-SDT activates the ERK pathway, which is an important member of the MAPK family, in THP-1-derived macrophages. Furthermore, inhibitors of ERK and AKT partially inhibited NL-SDT-induced HO-1 expression (Fig. 5F). Several reports have indicated that Nrf2 translocates to the nucleus in response to various stimuli, followed by the subsequent induction of HO-1 expression [37, 38]. In the present study, we also found that NL-SDT increases nuclear Nrf2 localization and up-regulates Nrf2 protein expression (Fig. 6A and B). When we silenced Nrf2, HO-1 expression was inhibited (Fig. 6D). However, we did not examine the relationship between AKT, ERK and Nrf2 in this study, although there is evidence that AKT and MAPKs can activate Nrf2 in the cytoplasm and increase Nrf2 nuclear translocation [39-42]. Our findings demonstrated that activation of AKT, ERK and Nrf2 is involved in the induction of HO-1 by NL-SDT in macrophages.

The protective function of HO-1 on AS has been demonstrated in many studies. In the current study, NL-SDT was shown to induce macrophage expression of HO-1 *in vivo* and *in vitro*. Our results also indicated that NL-SDT-induced HO-1 expression protected against ox-LDL-induced macrophage injury. Ox-LDL is an initiating factor of AS. When macrophages internalize excess lipids beyond the capacity of cell metabolism, ER stress will be triggered, which can lead to cellular dysfunction and apoptosis [43-46]. In THP-1 macrophages, we found that NL-SDT prevented oxLDL-induced activation of the proapoptotic mediators JNK

and CHOP, which was followed by reduced ER stress (unpublished data). *In vitro*, we also showed that NL-SDT can defend against ox-LDL-induced macrophage apoptosis, oxidative stress and MMP decrease and that this protective function can be suppressed by ZnPP, which is a specific inhibitor of HO-1 (Fig. 7 and Fig. 8).

Based on previous reports and our data, we proposed a hypothetical model to describe the protective function of NL-SDT-induced HO-1 expression, as shown in Fig. 9. In conclusion, by enhancing macrophage expression of HO-1 to inhibit the progression of atherosclerotic plaques and ox-LDL-induced macrophage injury, NL-SDT exerted a protective function. The results of this study provide insight into the clinical use of SDT. The mechanism of SDT treatment of atherosclerotic plaques is very complex, and more studies are required to explore it further.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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