

Protoporphyrin IX-mediated sonodynamic therapy promotes autophagy in vascular smooth muscle cells

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Abstract. Sonodynamic therapy (SDT) is effective in treating intimal hyperplasia and promoting plaque stability in animal models. The present study aimed to evaluate the effects of SDT with the sonosensitizer protoporphyrin IX (PpIX) on vascular smooth muscle cell (VSMC) viability and autophagy. Cultured VSMCs cells were divided into the following groups: i) Control, ii) ultrasound, iii) PpIX and iv) SDT. Flow cytometry and laser confocal detection were used to measure Annexin V stained VSMCs following different treatments. Alterations in mitochondrial membrane potential (MMP) were evaluated via JC-1 staining. Autophagosome formation was observed using electron and fluorescence microscopy. Western blotting was used to analyze the expression levels of the autophagy markers light chain 3 (LC3-I) and LC3-II. The results demonstrated that SDT did not trigger apoptosis nor induce a significant decline in MMP of VSMCs. However, SDT significantly increased autophagosome formation and increased the LC3-II/LC3-I ratio. The findings demonstrated that PpIX-SDT increased autophagy without inducing mitochondrial-dependent apoptosis in VSMCs.

Introduction

Atherosclerosis is a major cardiovascular disease. Sonodynamic therapy (SDT), which is a noninvasive technique and uses ultrasonic energy to activate certain drugs (i.e. sonosensitizer)

to produce chemical reactions and reactive oxygen species, has been shown to have potential to attenuate progression of atherosclerotic plaque formation (1-5).

Currently, porphyrins and their analogs are the predominant sonosensitizers used in the clinic (6). Among these porphyrin-related sonosensitizers, protoporphyrin IX (PpIX) was found to be accumulated specifically in atherosclerotic plaques at a much higher concentration, i.e. 10 times higher, than in normal vessel walls (7), indicating that PpIX is a potential atherosclerosis-selective sonosensitizer.

Cell autophagy, the degradation process of cellular proteins or organelles in the cell (8), is an important defense and protection mechanism. Previous studies have shown that autophagy plays an important role in atherosclerosis (9,10). For instance, autophagy can selectively remove macrophages in the artery atheromatous plaque and participate in degradation of low-density lipoprotein (LDL) cholesterol (11). In addition, induced autophagy in vascular smooth muscle cells (VSMCs) can prevent oxidized LDL (oxLDL)-triggered VSMC foam cell formation (12). In contrast, deficient VSMC autophagy affects blood vessel contractability by changing the Ca²⁺ steady-state response (13). Therefore, VSMC differentiation and autophagy play important roles in the initiation and development of atherosclerosis, and have been therapeutic targets for treatment of atherosclerosis.

SDT has been shown to promote macrophage apoptosis and maintain stability of atherosclerotic plaques (4); however, the role of VSMCs in the SDT-treated plaques remains unclear. In the present study, we aimed to investigate the PpIX-mediated sonodynamic effects on VSMCs in order to provide molecular mechanisms by which SDT was clinically employed to treat atherosclerosis.

Materials and methods

Reagents. PpIX was purchased from Sigma-Aldrich, reconstituted in 100% DMSO (Sigma-Aldrich, St. Louis, MO, USA) to 0.1 g/ml and stored at room temperature. The VSMC line A7r5 (ATCC) was derived from murine thoracic vascular aorta. JC-1 probe was provided by Beyotime Institute of Biotechnology (Haimen, China). DAPI and propidium iodide (PI) were obtained from Sigma-Aldrich. Light chain 3B (LC3B) antibody and anti-rabbit secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies

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against microtubule-associated protein 1 LC3 were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Hyclone Laboratories, Inc. (HyClone, Logan, UT, USA). All other reagents were obtained from Sigma Chemical Co. Ltd.

Cell preparation and SDT procedure. A7r5 cell line was maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Cells were grown at 37°C in a humidified 5% CO₂/95% air atmosphere. During the SDT treatment procedure, VSMCs were exposed to ultrasound generated by the ultrasonic generator and power amplifier (Harbin Institute of Technology, Harbin, China) at 1.0 MHz with an intensity of 1.0 W/cm² in the presence or absence of PpIX, which was maintained at a concentration of 2 µg/ml. During the sonication procedure, the temperature of the solution inside the Petri dishes increased less than 0.5°C, as monitored with a thermometer.

Flow cytometry analysis. Cells were seeded into 35 mm Petri dishes and divided into 4 groups: Control group, ultrasound group, PpIX group, and SDT group. Each group consisted of 8 dishes. Five min after ultrasound treatment, cells were placed in a 37°C and 5% CO₂ incubator for 6 h, followed by sequential incubation with 5 µl Annexin V-FITC for 15 min and 10 µl PI for 5 min in the dark room. Apoptosis rates were analyzed by flow cytometry (Epics Altra II; Beckman Coulter, Brea, CA, USA). Experiments were repeated three times for each group under the same conditions.

Laser scanning confocal microscopy. Six h after SDT, cells were incubated with 5 µl Annexin V in the dark for 15 min. Laser scanning confocal microscopy was used to observe apoptosis with excitation at 488 nm and emission at 525 nm wavelengths (SP8; Leica, Mannheim, Germany). Experiments were repeated three times for each group under the same conditions.

Mitochondrial membrane potential (MMP) assessment. MMP was assessed using the probe JC-1, a sensitive fluorescent dye used to detect changes in MMP (1). Briefly, 6 h after SDT, VSMCs were incubated with 10 mg/ml JC-1 for 10 min at 37°C in the dark and monitored with a fluorescence microscope. Red-orange fluorescence was attributable to a potential-dependent aggregation in the mitochondria. Green fluorescence, reflecting the monomeric form of JC-1, appeared in the cytosol after mitochondrial membrane depolarization.

Transition electron microscopy (TEM). Four groups of cells were collected by centrifugation, washed with 1X phosphate buffered saline (PBS) (0.01 M pH 7.2-7.4), embedded with agar, fixed with 2.5% glutaraldehyde, post-fixed with osmium tetroxide, dehydrated in gradient alcohol, and embedded in Epon812 (Yiwei Info Technology Co. Ltd., Shanghai, China). The blocks were then cut into ultra-thin sections, stained with uranium acetate, and observed and photographed under a TEM (Hitachi, Tokyo, Japan).

Immunofluorescence staining and fluorescence microscopy imaging. Six hours after different treatments, VSMCs were

washed with 1X PBS and fixed with 4% paraformaldehyde for 30 min. VSMCs were then incubated with the primary antibody of interest at 4°C overnight. VSMCs were then washed with 1x PBS three times and stained with fluorescent second antibody at 37°C for 30 min in the dark. DAPI was used for nuclear staining. After fluorescence quenching, images were acquired with the same exposure settings using a fluorescence microscopy with standard excitation filters (Olympus Corporation, Tokyo, Japan).

Western blot analysis. SDS-PAGE and immunoblotting were performed according to standard procedures to detect the expression levels of autophagy marker proteins, such as LC3. Briefly, cells were lysed with RIPA buffer on ice, and supernatant samples were resolved in 10-15% SDS-PAGE depending on the size of the target protein, followed by transfer onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Thereafter, membranes were incubated in blocking buffer at room temperature for 1 h, followed by incubation with anti-LC3 antibody at 4°C overnight. The protein-antibody complexes were then tagged with IR Dye 680-labeled secondary antibodies at room temperature for 1 h. The infrared fluorescence was detected with the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA). β-actin was used as a loading control. The ratios of LC3-II/LC3-I to β-actin in each experiment were calculated by Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All values are expressed as means ± SD of at least three independent experiments, and one-way ANOVA was used to analyze the statistical difference among the multiple groups. A P-value less than 0.05 was considered statistically significant.

Results

PpIX-SDT did not increase VSMC apoptosis. First, we investigated whether PpIX-SDT had any effect on VSMC apoptosis. VSMCs in SDT and control groups were stained with Annexin V-FITC, followed by flow cytometric analysis. As shown in Fig. 1A, no significant difference in apoptosis between SDT and control group was observed, which was further confirmed by confocal microscopy (Fig. 1B).

PpIX-SDT did not induce loss of MMP in VSMCs. As shown in Fig. 2, normal VSMCs with JC-1 staining exhibited orange fluorescence, and neither light nor PpIX alone induced any changes in MMP. However, SDT treatment induced a diffuse orange staining pattern in VSMCs, which was representative of no significant decline in MMP.

PpIX-SDT induces VSMC autophagy. Next, we explored VSMC autophagic activity in the SDT and other groups with electron microscopy. The number of autophagy bodies, including nuclear pyknosis, karyorrhexis, cell shrinkage, without the formation of apoptotic body, in the SDT group were significantly higher compared to the other groups (Fig. 3). To further confirm that SDT induced autophagy in VSMCs, we measured the levels of LC3-II, a widely used indicator

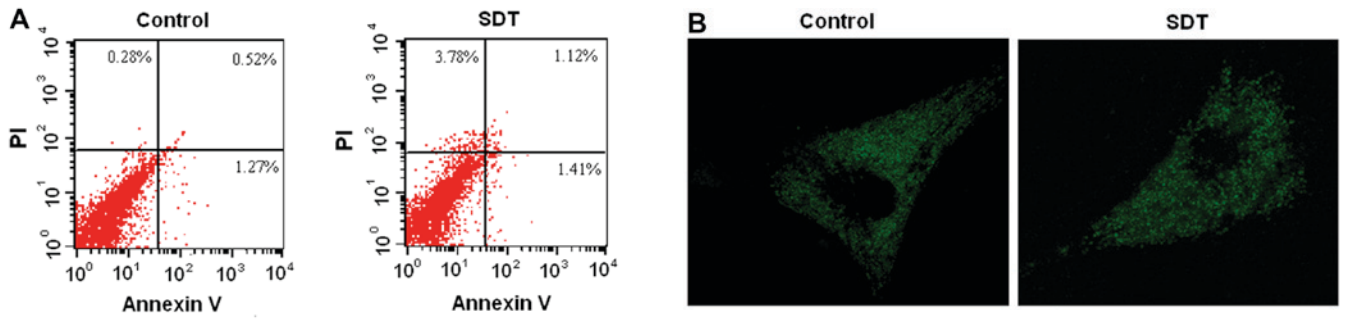


Figure 1. PpIX-SDT does not significantly induce VSMC apoptosis. (A) Flow cytometry with double staining of Annexin V and PI. (B) Confocal microscopy observation with Annexin V low dye in the unimpaird cell membranes.

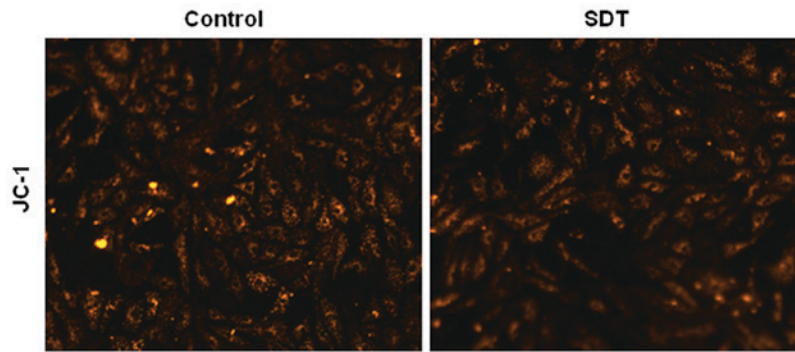


Figure 2. PpIX-SDT does not significantly alter VSMC mitochondrial membrane potential (MMP). Fluorescent probe JC-1 was used to detect changes in MMP. Normal cultured VSMCs contained orange fluorescent mitochondria in the cytoplasm.

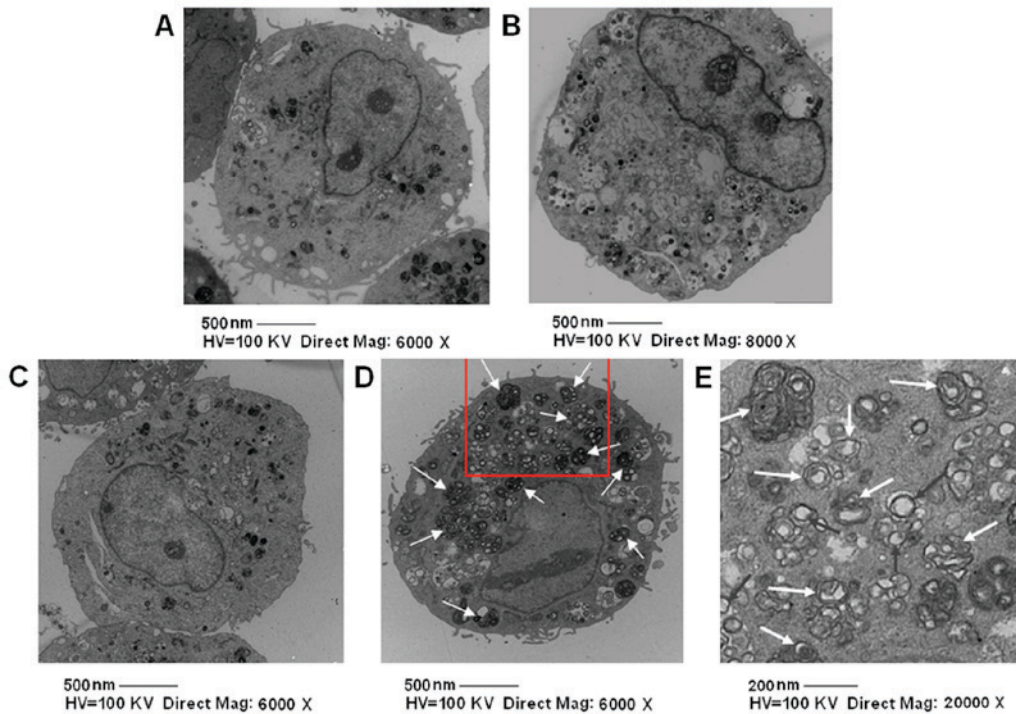


Figure 3. SDT increases autophagosome formation and promotes extensive vacuolization. TEM reveals ultrastructural changes in VSMCs. (A) Control group; (B) PpIX group; (C) ultrasound group; (D) SDT group; (E) autophagosomes (white arrows) were found in the SDT group (magnification, x20,000) and were swollen and vacuolated (black arrows).

of autophagy (13), in VSMCs of different groups. As shown in Fig. 4, a significant increase in fluorescence was observed in the SDT treatment group compared to the control group. We

also measured the expression levels of LC3-I and LC3-II in VSMCs in different groups by western blot analysis. As shown in Fig. 5, the LC3-II/LC3-I ratio was significantly increased

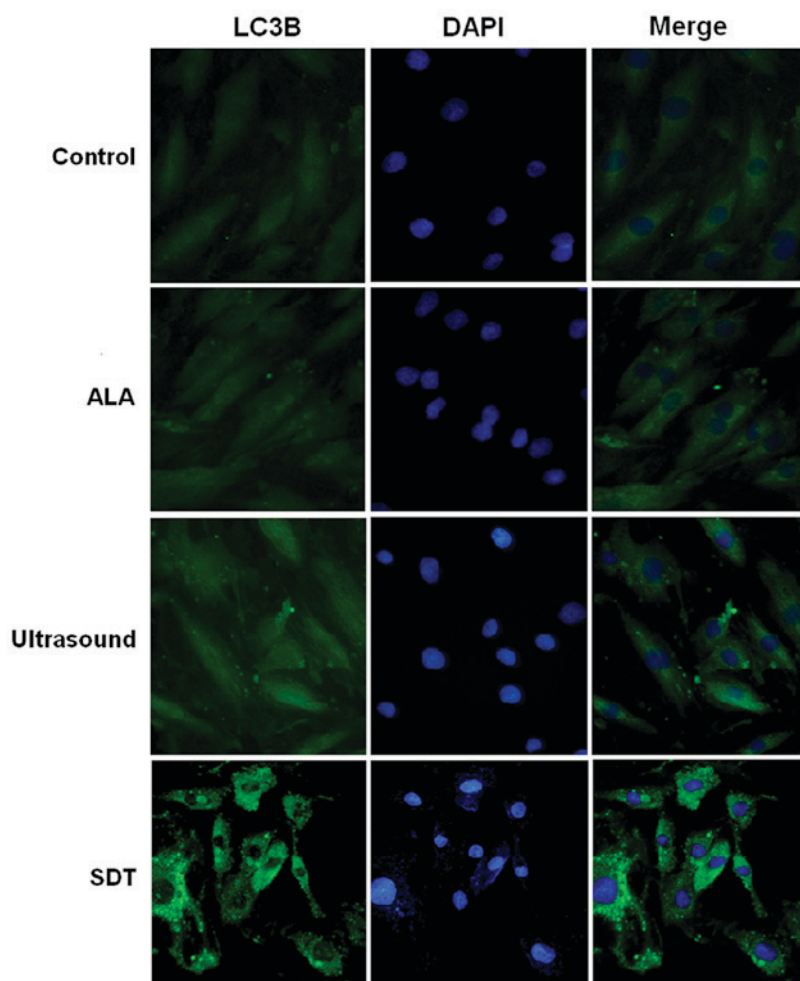


Figure 4. PpXT-SDT increases VSMC autophagic activity. Fluorescence microscopy showed a punctate pattern of fluorescence that was indicative of increased autophagy in VSMCs.

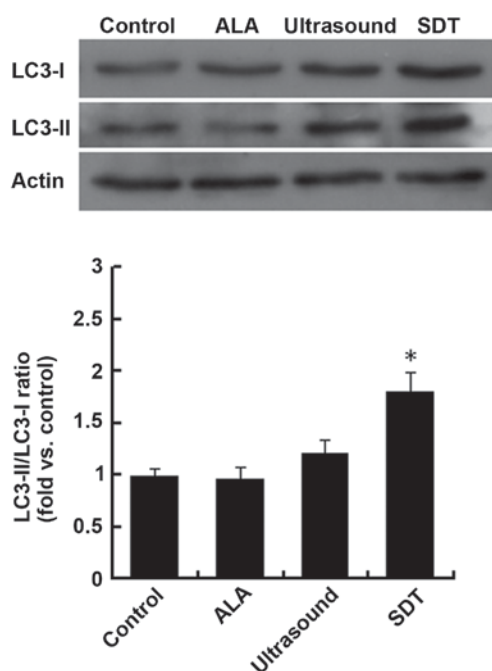


Figure 5. PpXT-SDT increases the ratio of LC3-II/LC3-I in VSMCs. The ratios of LC3-II/LC3-I were quantified by Quantity One software. Data are presented as mean \pm SD of three independent assessments. * $P < 0.05$ vs. control.

in the SDT group compared to the control group. Thus, we conclude that SDT promotes VSMC autophagic activity.

Discussion

Recently, Michiels *et al*, reported that PpIX-SDT promoted VSMC phenotype transformation from a dedifferentiated to a differentiated status (14), which partially explained why SDT inhibited in-stent restenosis in animal models (4). PpIX has low toxicity and a short dark period in the cells, as compared with other sonosensitizers (15,16). In addition, Michiels *et al* (14) showed that PpIX-SDT had no effect on VSMC viability. Consistent with the above findings, we did not observe that PpIX-SDT altered the viability of VSMCs, despite having increased the ultrasound intensity to 1.0 W/cm². Together, these findings suggest that PpIX-SDT is a relatively safe therapeutic approach for treating atherosclerotic plaques.

Previously, Cheng *et al* (17,18) reported that PpIX-SDT induced cell death in THP-1 macrophages via a mitochondria-dependent pathway. However, our results showed that PpIX-SDT did not significantly induce cell apoptosis compared to the control group in VSMCs, which coincided with the finding that MMP was not altered by PpIX-SDT treatment. The maintenance of MMP observed in our study is probably

attributed to the limited ultrasonic energy and acoustic sensitivity agent concentration.

Cell autophagy is generally regarded as a cell survival mechanism because cells degrade non-essential, aging or non-functional proteins, and/or cytoplasmic organelles, and recycle those degraded components to maintain normal cellular homeostasis (19). Increasing evidence suggests that autophagy is involved in a wide range of diseases, including atherosclerosis (20,21). During the development of atherosclerotic plaques, harmful material, such as a large number of reactive oxygen species, cause oxidative stress, leading to the maintenance of basal activity of cell autophagy, which can protect cells from the oxidative stress and promote plaque stability (22-24). Therefore, these cells were protected from cell death, i.e. apoptosis, and atherosclerotic plaques can form and steadily grow. Platelet derived growth factor (PDGF) was shown to mediate autophagy and adjust the response of VSMCs to phenotypic transformation induced by oxidative damage (25). However, excessive activation of cell autophagy eventually resulted in plaque cell death, and tended to be harmful. Because of the elastic strength of the plaque's fibrous cap, which mostly depends on smooth muscle cells and their secreted collagen (26), VSMC death usually leads to unstable plaque (27), and even plaque rupture. Hence, VSMC survival plays an important role in plaque stability (26). During the formation of atherosclerotic plaques, cell autophagy induced by mild oxidative stress contributes to the removal of damaged organelles. However, if induced autophagic activity is not sufficient to eliminate all damaged cell components, excessive oxidative stress and mitochondrial cytochrome C release can induce cell apoptosis. Therefore, it is generally believed that low levels of VSMC autophagy promote plaque stability, but high levels of cell autophagy are not conducive to stable plaques (9,28).

In our study, we observed increased autophagic activity following SDT treatment, which was supported by the following evidence: i) increased autophagosome formation revealed by electron microscopy, ii) increased protein levels of LC3B, an autophagy molecular marker, revealed by immunofluorescence staining, and iii) an increased LC3-II/LC3-I ratio, as revealed by western blot analysis. LC3, originally named microtubule associated protein 1A and 1B, referred to as MAP1LC3, plays an important role in autophagy. The mammalian LC3 family has three members: LC3A, LC3B and LC3C. Once synthesized, the C-terminus of LC3 is immediately sheared by autophagy related protein 4 (Atg4) and subsequently produces LC3-I, which is localized in the cytoplasm. In autophagy, LC3-I will be modified by Atg7 and Atg3 and subsequently generate LC3-II, which is located in the autophagosome. Thus, LC3 is widely recognized as an autophagy molecular marker (29), and the LC3-II/LC3-I ratio may be used to evaluate the activity of autophagic flux in cells/organs. Taken together, the findings suggest that VSMC autophagy increases following PpIX-SDT treatment.

Autophagy is crucial for normal VSMC function, phenotype, and survival. Inhibition of autophagy as a therapeutic strategy in the treatment of neointimal stenosis and atherosclerosis would be unfavorable. Conversely, stimulation of autophagy could be a valuable new strategy in the treatment of arterial disease (30-33). Our study demonstrates that PpIX-SDT induces autophagy in cultured VSMCs. Future

studies are required to elucidate the molecular mechanisms by which PpIX-SDT activates autophagy, and to explore whether SDT promotes plaque stability in *in vivo* atherosclerotic models.

In the present study, we demonstrate that PpIX-SDT induces VSMC autophagy, characterized by increased autophagosome formation. In addition, we show that PpIX-SDT does not trigger any significant increase in apoptosis or decrease in MMP. These results point to the possibility that induction of VSMC autophagy may be one of the mechanisms whereby SDT promotes plaque stability *in vivo*.

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