Original Paper

Sonodynamic Therapy Inhibits Fibrogenesis in Rat Cardiac Fibroblasts Induced by TGF- β 1

Yuanyuan Guo^{a,e} Zengxiang Dong^a Yuanqi Shi^a Wei Wang^a Lu Wang^a Jing Sun^b
Xin Sun^a Zhen Tian^{b,c} Jianting Yao^a Zhitao Li^{b,c} Jiali Cheng^a Ye Tian^{a,b,c,d}

^aDepartment of Cardiology, the First Affiliated Hospital of Harbin Medical University, Harbin,

^bDepartment of Pathophysiology, the Key Laboratory of Cardiovascular Pathophysiology, Harbin Medical University, Harbin, ^cKey Laboratory of Cardiovascular Medicine Research (Harbin Medical University), Ministry of Education, Harbin, ^dHeilongjiang Academy of Medical Sciences, Harbin,

^eDepartment of Cardiology, the Second Affiliated Hospital of Harbin Medical University, Harbin, P. R. China

Key Words

SDT • Cardiac fibroblast • TGF- β 1 • GSK3 β • AKT

Abstract

Background/Aims: Sonodynamic therapy (SDT) is a localized ultrasound-activated therapy for atherosclerosis when combined with a sonosensitizer, 5-aminolevulinic acid (ALA), but whether it can prevent cardiac fibrosis has not been studied. In the present study, we evaluated the effects SDT on fibrogenesis in rat cardiac fibroblasts. **Methods:** The primary cardiac fibroblasts were isolated from rats, and induced to fibrogenesis with TGF- β 1. With this *in vitro* model, we tested the preventive effects of SDT on fibrogenesis and further the underlying mechanism. **Results:** TGF- β 1 stimulation up-regulated α -SMA and COL1/III protein levels in cardiac fibroblasts, and enhanced the progression of cells from the G0/G1 phase to the S phase. SDT inhibited the TGF- β 1 mediated cell proliferation and decreased the levels of α -SMA and COL1/III by activating AKT/GSK3 β pathway and blocking TGF- β 1/SMAD3 signaling. **Conclusion:** Our studies demonstrate an antifibrotic effect of SDT in rat cardiac fibroblasts, suggesting that SDT may intervene cardiac fibrogenesis by regulating myocardial fibrotic remodeling.

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Introduction

Myocardial fibrosis can arise from a wide range of pathological processes, and is correlated with adverse clinical outcomes. Cardiac fibroblasts (CFs), accounting for 60% to 70% of all cells in the adult heart, play an important role in the structural, mechanical,

Y. Guo and Z. Dong contributed equally to this study.

Ye Tian

Department of Cardiology, First Affiliated Hospital, Cardiovascular Institute, Harbin Medical University, 23 Youzheng Street, Harbin 150001, (China)
Tel. +86 451 85555943, Fax +86 451 87530341, E-Mail yetian@ems.hrbmu.edu.cn

biochemical and electrical characteristics of the heart [1-4]. Meantime, CFs is the major source of extracellular matrix (ECM, e.g. collagen I (COLI) and collagen III (COLIII)) in the heart [5]. However, under the certain condition, these cells can transform to myofibroblast and contribute to cardiac fibrosis [6]. Cardiac fibrosis is one of main reason for diastolic heart failure, which leads to about 50% of heart failure cases [7]. So far, no therapy is available for the treatment of cardiac fibrosis [8, 9]. Therefore, a novel antifibrotic therapy is urgently needed.

Sonodynamic therapy (SDT) is a novel approach for cancer and atherosclerosis treatment [10, 11]. SDT is to utilize ultrasound to locally activate sonosensitizer, which increases the reactive oxygen species (ROS) and induces the biological effect of cells [12-14]. In the present study, 5-aminolevulinic acid (ALA) is used in combination with SDT, because it is a natural precursor of a sonosensitizer, protoporphyrin IX (PpIX), and metabolized to PpIX in cells through the heme biosynthesis pathway. Previous studies indicated that mild oxidative stress displays neuro-protection in ischemia through activating nuclear factor erythroid 2-related factor (Nrf2) in astrocytes [15]. ROS and hypoxic preconditioning also show the cardio-protective effects [16, 17]. These results suggest that SDT may have a potential cardioprotection and inhibit cardiac fibrosis through modulating intracellular ROS levels. To test this hypothesis, we examined the effects of SDT on fibrogenesis in rat cardiac fibroblasts. Our results demonstrated that SDT reduced the transformation rate of fibroblast to myofibroblast by activating AKT/GSK3 β pathway and blocking TGF- β 1/SMAD3 signaling and might have a therapeutic potential for myocardial fibrosis in the future.

Materials and Methods

Reagents

5-aminolevulinic acid (ALA), propidium iodide (PI) and 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and it was purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO (Grand Island, NY, USA). Tyspin, penicillin-streptomycin and 4, 6-diamidino-2-phenylindole (DAPI) were from Beyotime Biotechnology (Beijing, China). TGF- β 1 was purchased from Pepro Tech (Rocky Hill, NJ, USA). LY294002 was obtained from Med Chem Express (Princeton, NJ, USA). All other drugs and chemicals used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

The Sprague-Dawley (SD) neonatal rats were obtained from Harbin Medical University. All animal protocols were in accordance with the regulations of the Ethics Committee of Harbin Medical University and approved by the ethical committee of Harbin Medical University. Cardiac fibroblasts were isolated from 1-to 3-day-old SD rats anesthetized with pentobarbital sodium. Briefly, neonatal rat hearts were finely minced and placed together in 0.25 % trypsin. Collected cell suspensions were centrifuged and re-suspended in DMEM supplemented with 10 % FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The re-suspension was plated onto culture flasks, and fibroblasts were obtained after differential adhesion for 90 min. Cells were passaged by trypsin after grown to confluence. Cell cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO₂ and 95 % air. All experiments were carried out with cardiac fibroblast in 2-4 generation.

Intracellular ALA-PpIX detection and ALA cytotoxicity

ALA incubation was conducted as described previously [13]. Briefly, the differentiated cells were washed twice with phosphate buffered saline (PBS) and incubated with 1.0 mM ALA in serum-free DMEM at 37 °C in the dark for 0-48 h. The cell medium was gently washed with PBS twice and observed using a fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY, USA). The fluorescence intensity of PpIX was measured with a fluorescence spectrometer at 405 nm excitation and 630 nm emission wavelengths (USB2000; Ocean Optics Inc., Dunedin, FL, USA). To investigate the cytotoxicity of ALA, the cells were seeded into a 96-well plate and incubated with various concentrations (0-10.0 mM) of ALA for 60 h. The survival rate of the cells was measured by MTT assay.

Ultrasonic exposure system

The cells were exposed to ultrasound after ALA incubation as described previously [13]. Briefly, the ultrasonic transducer, pulse generator, and power amplifier used in this study were designed and assembled in the Harbin Institute of Technology (Harbin, China). The homemade ultrasonic transducer (diameter: 35 mm, resonance frequency: 1.0 MHz, duty factor: 10 %, repetition frequency: 100 Hz) was placed in a water bath, 30 cm under the cells. The ultrasonic intensity was 0.1–1.0 W/cm² (Onda Corp., Sunnyvale, CA, USA), and the survival rate of the cells was measured by MTT assay.

Immunofluorescence assay

For microscopy experiments, cells were plated on chamber slides, fixed for 20 min with 4 % formaldehyde, permeabilized with 0.1 % Triton X-100, blocked with 3 % BSA, and stained with α -SMA (1:800, Sigma-Aldrich, USA) antibody overnight. After washing in PBS, cells were incubated with goat anti-mouse secondary antibody (Molecular Probes, Invitrogen) for 1 h at room temperature. After washing with PBS, DAPI was added and the slides were incubated for 10 min at room temperature. Following two more washes in PBS, the slides were covered with immune-mount and a coverslip. Carl Zeiss Axio VertA1 microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) was used to visualize cells and NIS Elements software was used to record images for further analysis.

Western blot

Total protein was extracted from the cultured CFs of rats for protein immunoblotting. Protein samples were separated in SDS-PAGE and blotted to nitrocellulose membrane. The blots were blocked with 5 % nonfat milk for 2 h at room temperature, then probed with primary antibody including GAPDH (1:800 dilution, Zsbg, China), α -SMA (1:2000, Sigma-Aldrich, USA), COL1 (1:1000 dilution, proteintech, China), COL3 (1:1000 dilution, proteintech, China), SMAD3 (1:1000 dilution, Elabscience, China), Phospho-SMAD3 (1:1000 dilution, Elabscience, China), AKT (Cell signal, 1:1000 dilution, USA), Phospho-AKT (Cell signal, 1:1000 dilution, USA), GSK3 β (1:1000 dilution, Elabscience, China), Phospho-GSK3 β (T216) (1:1000 dilution, Elabscience, China) in PBS and finally incubated at 4 °C overnight. The membranes were washed with PBS-T and then incubated with secondary antibody for 1 h at room temperature. Finally, the western blot bands were collected by Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified with odyssey v1.2 software by measuring intensity (area XOD) in each group with GAPDH as internal control. The results were expressed as fold changes by normalizing the data to the control values.

Flow cytometry and cell cycle analysis

Flow cytometry (BD FACSCantoII) was used to determine the cell cycle distribution of rat cardiac fibroblasts. The isolated cardiac fibroblasts were cultured at 37 °C in a humidified atmosphere of 5 % CO₂, and synchronized at the early G0/G1 phase by 0.5 % FBS for 24 h. Culture medium was then replaced with 10 % FBS normal culture medium, and the cells were administered with different treatment. The cells were removed from the plates with 0.25 % trypsin, washed with PBS and fixed with 4 °C 70 % ethanol. Ethanol was removed by centrifugation and cardiac fibroblasts were washed with PBS again. The cardiac fibroblasts were incubated with propidium iodide (PI) (PI: 30 mg/mL, RNase A 150 mg/mL and 0.1% Triton-X 100) at 37 °C for 45 min. The data of flow cytometry was acquired using Cell Quest software, and the percentage of cells in the G0/G1, S and G2/M phases were calculated with MODFIT software.

Statistical analysis

All quantitative data were expressed as the mean $\pm$ standard error of mean (SEM) and analyzed by SPSS 13.0 software. Two independent Student's *t*-tests and one-way ANOVA were used for difference comparisons. All statistical significance was two-tailed and significance level was set at 0.05. Differences were considered to be statistically significant when *P* < 0.05.

Result

Intracellular accumulation of ALA-PpIX in cardiac fibroblasts and cell viability after SDT treatment

Since PpIX is able to emit fluorescence, we determined the intracellular accumulation of ALA-PpIX by the mean fluorescence intensity with the inverted microscope. As shown

Fig. 1. Intracellular accumulation of ALA-PpIX in cardiac fibroblasts. (A) Fluorescent photomicrograph of cardiac fibroblasts showing intracellular PpIX after incubation with 1mM ALA. (B) The accumulation of PpIX in cardiac fibroblasts detected by fluorescence spectrometer at different time-point after incubation with 1 mM ALA. Data are expressed as mean $\pm$ SEM (n=6). *P<0.05 vs. control.

Fig. 2. Cell viability after ALA and SDT. (A) The survival rates of cardiac fibroblasts after incubation with ALA at different concentrations at 0.1-10 mM as determined by MTT assay. (B) The accumulation of PpIX in cardiac fibroblasts detected by fluorescence spectrometer at concentration (0.1- 6mM). (C) The survival rates of cardiac fibroblasts after 1 mM ALA incubation with ultrasound intensity for 0.1, 0.3, 0.5, 0.7, and 1 W/cm². Data are expressed as mean $\pm$ SEM (n=6). *P<0.05, **P<0.01 vs. control.

in Fig.1A and 1B, cellular uptake of ALA-PpIX elevated rapidly within 1 h and reached the plateau at 4 h. No significant increase of intracellular ALA-PpIX was observed over 5-48 h. Therefore, in the following experiments, 4 h were chosen as the ALA loading time.

ALA alone did not induce cytotoxicity at the concentration less than 6 mM, and cell viability was more than 97% (Fig 2A). Then, we determined the intracellular accumulation of ALA at different concentrations. As shown in Fig. 2B, cellular concentration of ALA-PpIX reached peak at ALA concentration of 1 mM. No significant increase of ALA uptake was observed when its concentration was over 1 mM. Therefore, in the following experiments, 1 mM was chosen as the drug-loading concentration. As shown in Fig. 2C, the ultrasound exposure alone did not show

Fig. 3. SDT blocks TGF- β 1-induced myofibroblast transformation and collagen synthesis. (A) Myofibroblast transformation measured in cells stained with an antibody to α -SMA (green), and DAPI (blue; labels nuclei) after treatment. (B) Quantification of α -SMA-positive staining. (C-E) Protein levels of α -SMA, COL I and COL III measured by western blot. Data are expressed as mean $\pm$ SEM (n=6). **P<0.01 vs. control; ## P<0.01; # P<0.05 vs. TGF- β 1 group. +SDT, +ALA and +US indicate TGF- β 1 plus SDT group, TGF- β 1 plus ALA group and TGF- β 1 plus ultrasound group separately.

influence on cell viability. When the ultrasound dose was set at 0.5 W/cm², the cell viability in SDT group was decreased to approximately 70% (P<0.05 compared with control group). Therefore, 0.1 W/cm² ultrasound intensity was selected in the following experiments.

SDT blocks TGF- β 1-induced myofibroblasts transformation and collagen synthesis

To determine how SDT influences the proliferation and transformation of cardiac fibroblast treated with TGF- β 1, we stained cells for interstitial expression of α -SMA, a marker of fibroblast transformation (Fig. 3A and 3B). Cells treated with TGF- β 1 displayed a significant increase of interstitial α -SMA-positive area; whereas SDT reduced fibroblast transformation. To further determine whether SDT inhibits TGF- β 1-induced myocardial fibrosis, the expression levels of α -SMA and collagen production were detected by western blot. SDT significantly inhibited the TGF- β 1-stimulated increases of α -SMA and COL I/III protein in CFs (Fig. 3C, 3D and 3E), suggesting that the myofibroblast differentiation driven by TGF- β 1 is inhibited by SDT.

SDT prevents cell cycle progression and proliferation induced by TGF- β 1

The effect of SDT on cell cycle progression was determined with flow cytometry in rat cardiac fibroblasts. TGF- β 1 treatment promoted fibroblast to enter S phase, suggesting the transformation of fibroblast to myofibroblast. However, SDT inhibited the cell cycle transition

Fig. 4. Effect of SDT on cell cycle progression. (A) Representative flow cytometry graphs in cells after treatment. (B) Mean values of cell cycle distribution in each group. (C) The survival rates of cardiac fibroblasts in each group by MTT assay. Data are expressed as mean $\pm$ SEM (n=4). *P<0.05 vs. control; # P<0.05 vs. TGF- β 1 group. +SDT, +ALA and +US indicate TGF- β 1 plus SDT group, TGF- β 1 plus ALA group and TGF- β 1 plus ultrasound group separately.

Fig. 5. Effect of SDT on the expression of p-SMAD3, p-AKT and p-GSK3 β . (A) Images of p-SMAD3, SMAD3 and GAPDH protein and mean values of protein level (relative to GAPDH) in cells. (B) Images of p-AKT, AKT and GAPDH protein and mean values of protein level (relative to GAPDH) in cells. (C) Images of p-GSK3 β , GSK3 β and GAPDH protein and mean values of protein level (relative to GAPDH) in cells. Data are expressed as mean $\pm$ SEM (n=6). **P<0.01 vs. control; ##P<0.01, # P<0.05 vs. TGF- β 1 group. +SDT, +ALA and +US indicate TGF- β 1 plus SDT group, TGF- β 1 plus ALA group and TGF- β 1 plus ultrasound group separately.

from G0/G1 to S phase. Moreover, ALA or ultrasound alone did not change the cell cycle in cardiac fibroblast (Fig. 4A and B). Above results indicated that SDT inhibits the proliferation of cardiac fibroblasts induced by TGF- β 1. SDT not only inhibited the fibroblast proliferation, but also promoted cell death (Fig 4C).

Fig. 6. SDT blocks TGF- β 1-induced myofibroblast transformation and collagen synthesis via AKT signaling. (A-B) Images and mean values of COLI, COLIII and GAPDH protein. (C-D) Images and mean values of α -SMA, p-SMAD3 and GAPDH protein. Data are expressed as mean $\pm$ SEM (n=6). **P<0.01 vs. control; ##P<0.01, # P<0.05 vs. TGF- β 1 group. +SDT, and +LY294002 indicate TGF- β 1 plus SDT group and TGF- β 1 plus LY294002 group separately.

SDT inhibits the TGF- β 1-stimulated fibrotic response through the AKT/GSK3 β pathway

The TGF- β 1/SMAD3 signaling pathway plays an important role in cardiac fibrosis [18]. SMAD3 phosphorylation and subsequent translocation to the nucleus are required for TGF- β 1 signaling. Because SDT displays an inhibitory effect on TGF- β 1-induced fibrosis, we explored whether SDT has any effect on the TGF- β 1-induced SMAD3 phosphorylation. In cultured cardiac fibroblasts, TGF- β 1 induced the robust SMAD3 phosphorylation after 48h treatment. However, SDT blocked the TGF- β 1-induced phosphorylation of SMAD3 (Fig. 5A). These results indicated that SDT inhibits the profibrotic TGF- β 1 signaling. It is well known that the activation AKT pathway counteract the effect of TGF- β 1 signaling by modulating the STAT3 phosphorylation. Therefore, we determined the protein levels of AKT and its phosphorylating status in cardiac fibroblasts after SDT. As shown in Fig. 5B, we found that SDT significantly augmented the levels of phosphorylated AKT in cardiac fibroblasts, GSK3 β with phosphorylated at Tyr-216 also elevated in cardiac fibroblasts after SDT (Fig. 5C). We further found that LY294002, a highly selective AKT inhibitor, completely blocked GSK3 β phosphorylation induced by SDT. Consistent with above observation, SDT significantly decreased the α -SMA and COLI/III protein in fibroblast treated with TGF- β 1. LY294002 dramatically reversed this effect of SDT, suggesting that SDT inhibits cardiac fibroblast transformation and collagen synthesis in cardiac fibroblasts through the activation of AKT signalling (Fig. 6).

Discussion

Cardiac fibroblasts play a vital role in pathological development and progression of myocardial remodeling. The biological effects of SDT on cells have been reported by several studies [11, 12]; however, few studies have been performed to evaluate the effect

of SDT on cardiac fibroblasts. The present study proved that SDT inhibited TGF- β 1-induced fibrogenesis through activating the AKT/GSK3 β /SMAD3 cascade in rat cardiac fibroblasts.

All SDT studies are focused primarily on cancer treatment [19-21]. Previous studies demonstrated the sonodynamic effect of ALA on macrophages that are responsible for the progression of atherosclerosis [13, 14]. SDT treats vascular disease through decreasing neointimal hyperplasia in the rabbit iliac artery [22]. In our previous study, we applied 0.5 W/cm² SDT (1mM of ALA and 5 min of ultrasound exposure) to induce the cell death of macrophages [23]. In this study, we confirmed that PpIX could be accumulated in rat cardiac fibroblasts through the administration of ALA. Intracellular ALA-PpIX reached the maximum when ALA incubated with fibroblast for 4 h (Fig. 1A and B). 0.5 W/cm² ultrasound irradiation with ALA at the concentration over 7 mM showed obvious cytotoxicity (Fig. 2A and C). Cellular uptake of ALA-PpIX reached peak levels when ALA is at the concentration of 1 mM. Considering SDT safety, we selected 1 mM ALA and 0.1 W/cm² ultrasound irradiation as a treatment conditions in our study.

TGF- β 1 is secreted by mesenchymal and inflammatory cells, and induces fibroblast to myofibroblast transformation [24]. Myofibroblasts are present in abnormal myocardium and characterized by the presence of microfilamentous contractile apparatus enriched with α -SMA. Persistent myofibroblast leads to excessive scarring, further losing tissue compliance [24]. In this study, cardiac fibroblast cells that were treated with TGF- β 1 displayed a significant increase of the interstitial α -SMA-positive area, suggesting TGF- β 1 induces the transformation of fibroblast to myofibroblast. SDT significantly inhibited TGF- β 1-stimulated increases in α -SMA and COL1/III protein expression and consequent the fibroblast transformation (Fig. 3C, 3D and 3E). Cell cycle analysis also indicated that SDT inhibited the proliferation of cardiac fibroblasts induced by TGF- β 1. Collectively, SDT inhibits the transformation and proliferation of cardiac fibroblasts.

How does SDT have antifibrosis effect? Using neonatal rat cardiac fibroblasts, we further investigated the antifibrotic signaling of SDT and its interaction with the fibrotic signaling of TGF- β 1. Many studies have indicated that GSK3 β regulates the activities of several metabolic, signaling and structural proteins [25-27], and play an important role in CF activation and fibrotic remodeling after myocardial infarction [24]. Forde et al. reported that GSK3 β deletion leads to hyperactivation of profibrotic TGF- β 1/SMAD3 signaling that is associated with excessive fibrosis and adverse ventricular remodeling [28]. GSK3 β is suppressed by the terminal phosphorylation of GSK3 β at Ser-9, whereas activated by the Tyr-216 phosphorylation [28]. In the present study, we found that SDT inhibited the TGF- β 1-induced phosphorylation of SMAD3 and upregulated the phosphorylation of GSK3 β at Tyr-216. These findings indicate that SDT prevents cardiac fibrosis through activate GSK3 β signaling and inhibits phosphorylation of SMAD3 in cardiac fibroblasts.

Various upstream kinases, such as PI3K, AKT, MAPK, p70 ribosomal S6 kinase, PKA and PKC, are responsible for phosphorylation of GSK3 β upon stimulation [29]. Therefore, phosphorylated GSK3 β (Tyr-216) levels was upregulated possibly because of the activation of these kinases. Although not all these kinases that are primarily responsible for phosphorylation of GSK3 β (Tyr-216) induced by SDT have been investigated in this study, we found that SDT-exposed cardiac fibroblasts had significantly higher levels of p-AKT than control group (Fig. 5) and LY294002 blocked the inhibitory effects of SDT on TGF- β 1-induced proliferation, transformation, and collagen synthesis in cardiac fibroblasts. These findings suggest that SDT exerts its antifibrotic effect in cardiac fibroblasts through activating the AKT/GSK3 β pathway.

In summary, this study demonstrated that SDT inhibits fibrogenesis in rat cardiac fibroblasts induced by TGF- β 1, and exerts its antifibrotic effect through activating the AKT/GSK3 β pathway. We extend the current understanding of the antifibrotic effects of GSK3 β , whose phosphorylation at Tyr-216 blocks TGF- β 1-induced nuclear translocation of p-SMAD3 in cardiac fibroblasts. Our studies suggest that SDT may intervene cardiac fibrogenesis by regulating myocardial fibrotic remodeling.

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Disclosure Statement

None declared.

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