

Inhibitory Effects of Low Decibel Infrasound on the Cardiac Fibroblasts and the Involved Mechanism

Wei Jin¹, Qin-Qin Deng¹, Bao-Ying Chen², Zhen-Xing Lu¹, Qing Li¹, Hai-Kang Zhao³, Pan Chang⁴, Jun Yu⁴, Zhao-Hui Pei¹

¹Department of Cardiology, Third Hospital of Nanchang, Nanchang, ²Department of Radiology, Second Affiliated Hospital, Fourth Military Medical University,

³Department of Neurosurgery, Second Affiliated Hospital, Xi'an Medical University, ⁴Central Laboratory, Second Affiliated Hospital, Xi'an Medical University Xi'an, China

The authors Wei Jin, Qin-Qin Deng, Bao-Ying Chen, and Zhen-Xing Lu contributed equally for this work.

Abstract

Introduction: Infrasound is a mechanical vibration wave with frequency between 0.0001 and 20 Hz. It has been established that infrasound of 120 dB or stronger is dangerous to humans. However, the biological effects of low decibel infrasound are largely unknown. The purpose of this study was to investigate the effects of low decibel infrasound on the cardiac fibroblasts. **Materials and Methods:** The cardiac fibroblasts were isolated and cultured from Sprague–Dawley rats. The cultured cells were assigned into the following four groups: control group, angiotensin II (Ang II) group, infrasound group, and Ang II+infrasound group. The cell proliferation and collagen synthesis rates were evaluated by means of [³H]-thymidine and [³H]-proline incorporation, respectively. The levels of TGF- β were determined by enzyme-linked immunosorbent assay. Moreover, RNAi approaches were used for the analysis of the biological functions of miR-29a, and the phosphorylation status of Smad3 was detected using western blotting analysis. **Results:** The results showed that low decibel infrasound significantly alleviated Ang II-induced enhancement of cell proliferation and collagen synthesis. **Discussion:** Compared with the control, Ang II markedly decreased the expression of miR-29a levels and increased the secretion of TGF- β and phosphorylation of Smad3, which was partly reversed by the treatment with low decibel infrasound. Importantly, knockdown of miR-29a diminished the effects of infrasound on the cardiac fibroblasts. In conclusion, low decibel infrasound inhibits Ang II-stimulated cardiac fibroblasts via miR-29a targeting TGF- β /Smad3 signaling.

Keywords: Fibrosis, heart, microRNA, infrasound

INTRODUCTION

Infrasound that is generated by mechanical vibration is a sound wave with a harmful frequency ranging below 20 Hz, which exists widely in the environment. Various works mainly focused on the damaging effects of infrasound with high intensity and how to protect us ourselves from them.^[1-4] Previously, we reported that infrasound of 5 Hz at 130 dB induced apoptosis of the rat cardiac myocytes by regulating the expression of apoptosis-related protein ultrastructure,^[1,2] with pathological changes of hemodynamics.^[3,4] However, the biological effects of low decibel infrasound are largely unknown. In this study, we exposed the cultured rat cardiac fibroblasts to low decibel (4–20 Hz, 79.75–86.11 dB), and the biological outcome and the mechanisms involved were evaluated to give clues to novel strategies against cardiac fibrosis.

MATERIALS AND METHODS

Cell culture

Animal experiments were approved by the University Ethics Committee (the Ethical Committee of Xi'an Medical University on research with approval ID 2014-SAH-1). As described previously,^[5] the hearts from 1- or 2-day Sprague–Dawley rats from the Center of Laboratory Animal of Fourth Military Medical University were

Address for correspondence: Prof. Zhao-Hui Pei, Department of Cardiology, Third Hospital of Nanchang, Nanchang, China.
E-mail: leak123@126.com;
Prof. Jun Yu, Central Laboratory, Second Affiliated Hospital, Xi'an Medical University, Xi'an, China.
E-mail: pclamper@163.com

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DOI:
10.4103/nah.NAH_14_16

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How to cite this article: Jin W, Deng Q-Qin, Chen B-Ying, Lu Z-Xing, Li Q, Zhao H-Kang, *et al.* Inhibitory effects of low decibel infrasound on the cardiac fibroblasts and the involved mechanism. *Noise Health* 2017;19:149-53.

minced and dissociated with 0.125% trypsin and 0.075% collagen type I. To purify the non-myocytes from myocytes, dissociated cells were preplated for 90 min at 37°C, with high humidity and 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), during which the cardiac fibroblasts attached readily to the bottom of the culture dish. To obtain cardiac fibroblasts-enriched fraction, the culture dishes containing attached non-muscle cells were plated into an incubator and passaged 48 h later with 0.25% trypsin. These cells were used at passages 3–5. The purity of the cardiac fibroblasts used in the experiments was over 95%, as identified by immunohistochemistry staining with anti- α -SMA (Biotechnology, Santa Cruz, CA). Cells were stimulated with angiotensin II (Ang II) (1 μ mol/L, Sigma) or in the control conditions for 1, 2, and 3 days with or without exposure to infrasound.

Acoustical treatment

The infrasound in this study was generated by the infrasound 8TM device from CH Instruments, Inc. (Austin, TX, USA). The infrasound 8TM device has three gears on behalf of the three kinds of combinations of the frequency and intensity of infrasound, which automatically generate infrasound <90 dB at 4–20 Hz. The apparatus has three buttons that represent 10 min, 20 min and continuous output, respectively. The treatment protocol of infrasound was according to the manual from CH Instruments, Inc. Briefly, gear 3 of infrasound 8TM device was selected. The acoustic spectrum automatically focused on 4–20 Hz with random change of the main frequency, and sound intensity level was at 79.75–86. The acoustical treatment duration was 2 h (9:00–12:00), once every 24 h for 1, 2, and 3 days, respectively.

Evaluation of DNA synthesis

Deoxyribonucleic acid (DNA) synthesis was determined by measuring the incorporation of [³H]-thymidine into the DNA of cells cultured in 96-well plates at a density of 5×10^3 cells/well. After induction of quiescence, the cells were exposed to Ang II or control condition for 1, 2, and 3 days with or without exposure to infrasound for 2 h a day. [³H]-thymidine (18.5 kBq/ml) was added to the growth medium of each well at the last 4 h. The radioactivity for each sample was counted by a liquid scintillation counter (LS-6500, Beckman Co., CA, USA).

Evaluation of collagen synthesis

Collagen synthesis in the cardiac fibroblasts was evaluated by the incorporation of [³H]-proline into the cells cultured in 96-well plates at a density of 5×10^3 cells/well. As described above, after 48 h serum deprivation, the cells were cultured for a total period of 1, 2, and 3 days with or without exposure to infrasound for 2 h a day. [³H]-proline (18.5 kBq/ml) was added to the growth medium of each well at the last 4 h. The radioactivity for each sample was counted by a liquid scintillation counter (LS-6500, Beckman Co., CA, USA).

Knockdown of miR-29a

To knockdown miR-29a (NR_031836), a pRNAT-H1.1/neo-miR-29a or pRNAT-H1.1/neo-scramble RNA interference construction (GenScript, Piscataway, NJ) was transiently transfected into the cardiac fibroblasts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM1 reduced serum medium (Invitrogen) as described previously.^[6] Expression levels were verified by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

RNA extraction and quantitative real-time PCR

Total ribonucleic acid (RNA) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A total amount of 1 μ g of RNA from each sample was reverse-transcribed using random primers and moloney murine leukemia virus (MMLV) reverse transcriptase according to the protocol of the manufacturer (Promega, Madison, Wisconsin, USA). Real-time PCR was performed using Bio-Rad iQ SYBR Green supermix in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Expression of miR-29a was determined using the primers as follows: forward, 5'-ACCCCTTAGAGGATGACTGAT-3' and reverse, 5'-AACCGATTTTCAGATGGTGCT-3'. Relative quantification was performed using the $\Delta\Delta$ Ct method. The data were normalized with U6 as endogenous control.

Enzyme-linked immunosorbent assay

TGF- β in the supernatant was detected by using enzyme-linked immunosorbent assay (ELISA) test according to the manual. Optical density (OD) values were used to determine the concentration based on the standard curve.

Western blot analysis

For preparation of whole-cell lysates, the cells were washed with ice-cold phosphate buffered saline (PBS) and lysed for 30 min on ice in radio-Immunoprecipitation assay (RIPA) buffer with 150 mM NaCl as described.^[7] Cell lysates were cleared at 20,000g for 10 min. After the adjustment of protein concentration, the lysates were boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 5 min and separated by SDS-polyacrylamide gel electrophoresis. The gels were blotted on a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, USA) and stained with the indicated first antibody [anti-phospho-Smad3 or anti-total-Smad3 (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA)]. Antibody binding was detected with horseradish peroxidase-coupled secondary antibody followed by chemoluminescence detection (ECL Plus; Amersham Pharmacia, Uppsala, Sweden).

Statistics

Data were expressed as the mean \pm standard error of mean (SEM) and analyzed using one-way analysis of variance, followed by Tukey's post hoc tests using the Statistical

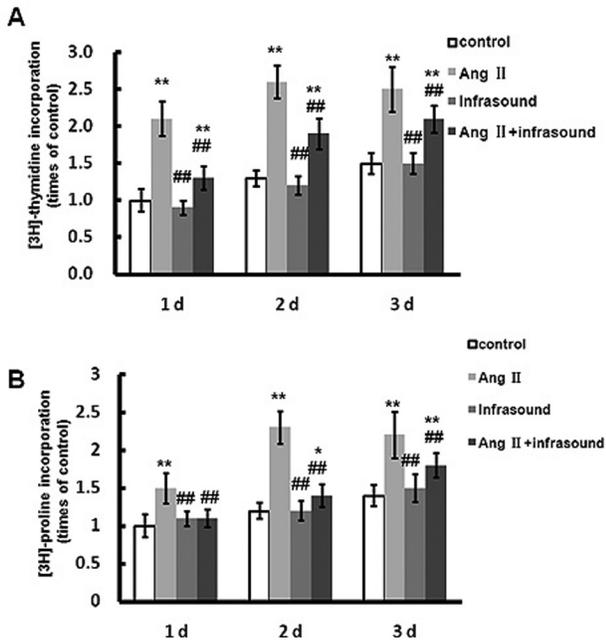


Figure 1: Effects of infrasound on the proliferation and collagen synthesis of cultured cardiac fibroblasts stimulated by angiotensin II. Quiescent cells were stimulated with Ang II (1 μmol/L) or in the control conditions for 1, 2, and 3 days with or without exposure to infrasound (4–20 Hz, 79.75–86.11 dB) for 2 h a day followed by the assessment of [³H] thymidine incorporation (a) or [³H] proline incorporation (b). The results were presented as times of control. The data were from four independent experiments with each assay in triplicate. **P* < 0.05, ***P* < 0.01 versus control group; ##*P* < 0.01 versus Ang II group

Program for Social Sciences version 15.0 software (SPSS Science, Chicago, IL, USA). *P* values < 0.05 were considered significant.

RESULTS

Low decibel infrasound inhibited the cardiac fibroblasts stimulated by Ang II

As shown in Figure 1, both [³H]-thymidine and [³H]-proline incorporation were significantly enhanced by Ang II (*P* < 0.01). At the time points of 1, 2, and 3 days, infrasound significantly alleviated the Ang II-induced increase of [³H]-thymidine and [³H]-proline incorporation in the cardiac fibroblasts (*P* < 0.01 versus control). However, the levels of DNA and collagen synthesis in Ang II+infrasound group were still significantly higher compared with those in the control group (*P* < 0.05). Importantly, infrasound did not significantly alter the activity of [³H]-thymidine and [³H]-proline incorporation in the cardiac fibroblasts not stimulated with Ang II (*P* < 0.05 versus control).

Low decibel targeted TGF-β/Smad3 signaling via miR-29a

It has been reported miR-29a was significantly reduced in a mouse model of pathological cardiac hypertrophy,^[8] and overexpression of the miR-29 family blocked TGF-β-Smad signaling.^[9,10] We, thus, speculated that the effects of infrasound might be related to the miR-29a as well as TGF-β-Smad signaling. According to the changes of [³H]-thymidine and [³H]-proline incorporation, the levels of

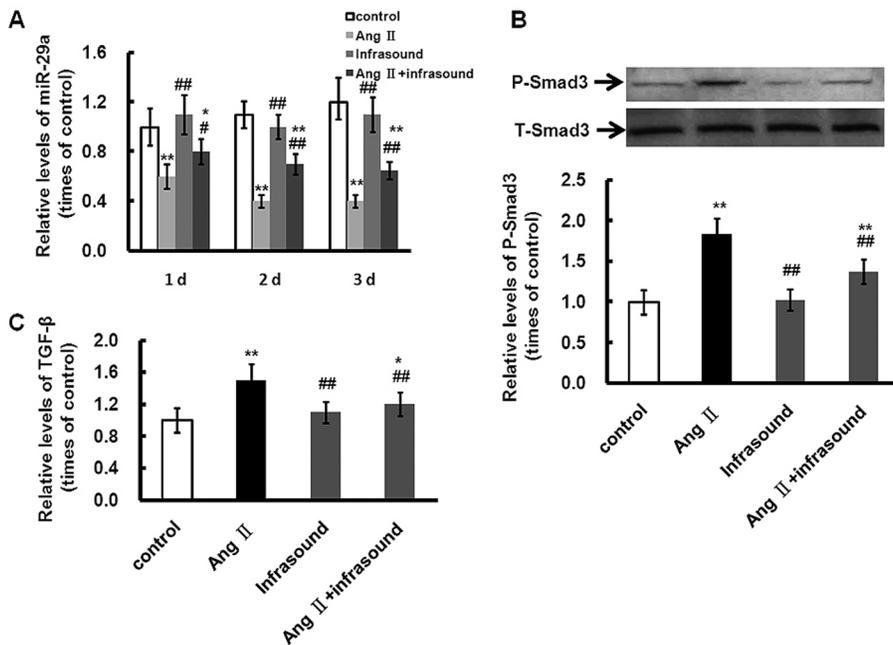


Figure 2: Effects of infrasound on the levels of miR-29a, phosphorylated Smad3, and TGF-β in the cultured cardiac fibroblasts stimulated by angiotensin II. (a) Levels of miR-29a detected by real-time PCR. (b) Phosphorylation status of Smad3 was determined by western blotting with total Smad3 as internal control. (c) TGF-β in the supernatant was detected by ELISA. The results were presented as times of control, and the data were from three independent experiments. **P* < 0.05, ***P* < 0.01 versus control group; #*P* < 0.05, ##*P* < 0.01 versus Ang II group

miR-29a were significantly decreased by Ang II, whereas such levels were increased by the treatment with infrasound [Figure 2a]. At the time point of 1 day, the supernatant levels of TGF- β [Figure 2b] and the phosphorylation status of Smad3 [Figure 2c] were both enhanced by Ang II, whereas such things were alleviated by the treatment with infrasound [Figure 2a].

To verify the role of miR-29a, we then knocked it down by shRNA [Figure 3a]. As a result, the reversal effects of infrasound on the Ang II-induced cell proliferation [Figure 3b] and collagen synthesis [Figure 3c] were diminished, along with elevated levels of P-Smad3 [Figure 4a] and TGF- β secretion [Figure 4b]. It was intriguing that infrasound still partly inhibited the

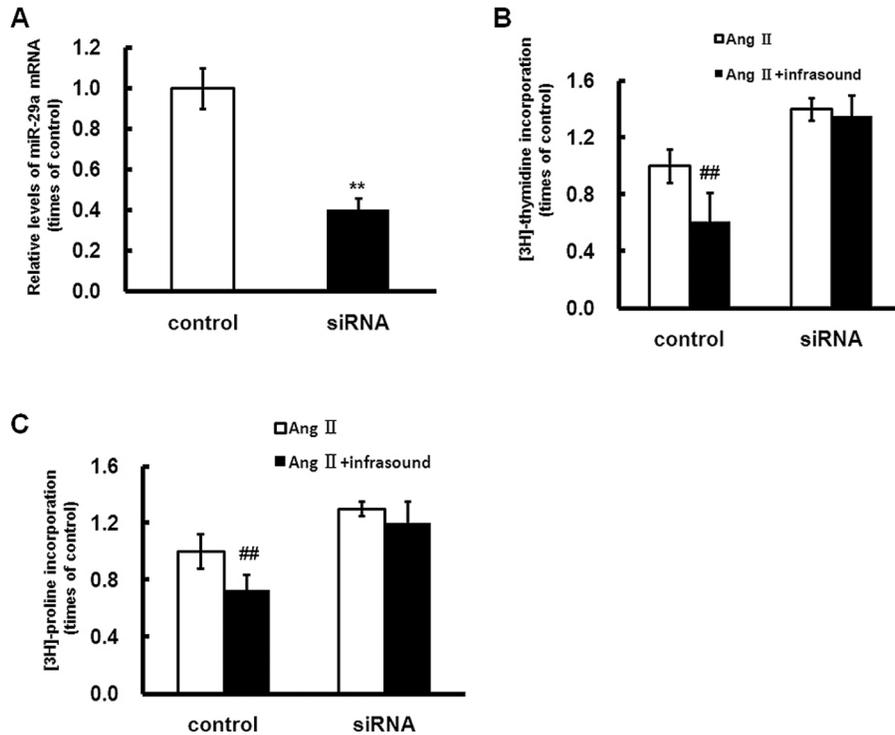


Figure 3: Silence of miR-29a and its effects on the infrasound-modulated proliferation and collagen synthesis of cultured cardiac fibroblasts stimulated by angiotensin II. (a) miR-29a in the cardiac fibroblasts was knocked down by siRNA interference with scrambled siRNA as control. (b) [3 H] thymidine incorporation. (c) [3 H] proline incorporation was determined in the siRNA or control cells treated with Ang II with or without exposure to infrasound. The results were presented as times of control. The data were from three independent experiments. ** $P < 0.01$ versus control group; ## $P < 0.01$ versus Ang II group

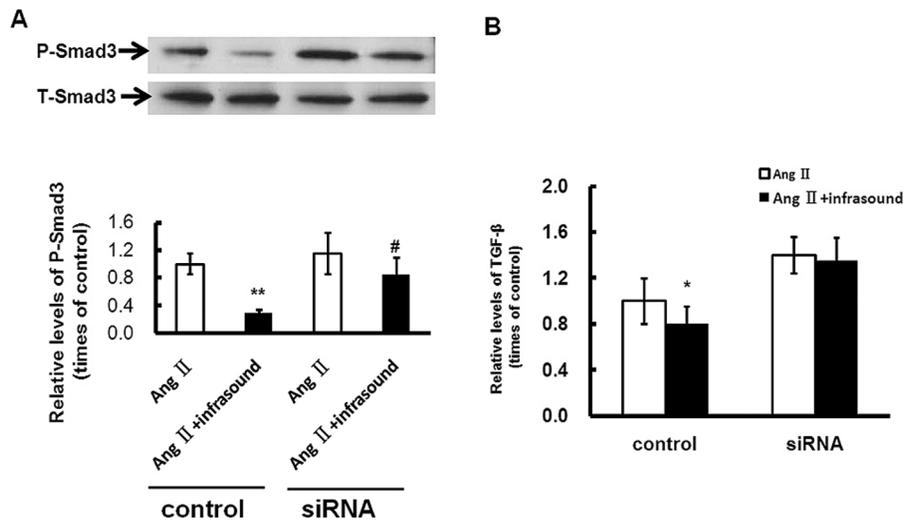


Figure 4: Levels of phosphorylated Smad3, and TGF- β in the siRNA or control cells treated with Ang II with or without exposure to infrasound. (a) Phosphorylation status of Smad3 was determined by western blotting with total Smad3 as internal control. (b) TGF- β in the supernatant was detected by ELISA. The results were presented as times of control, and the data were from three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$ versus Ang II group

phosphorylation of Smad3 in the condition that miR-29a was silencing [Figure 4a], indicating the possibility for other mechanisms than miR-29a.

DISCUSSION

For the first time, this study shows that low decibel infrasound acts as a suppressor of cardiac fibroblasts, which involved miR-29a-regulated TGF- β /Smad3 signaling. This conclusion was based on the following findings: (1) Ang II-enhanced proliferation and collagen synthesis of cardiac fibroblasts were alleviated by the exposure to low decibel infrasound. (2) The elevation of miR-29a induced by Ang II was suppressed by low decibel infrasound, and the levels of TGF- β as well as phosphorylation status of Smad3 were inversely altered with the expression of miR-29a. (3) Silence of miR-29a increased the levels of TGF- β and phosphorylation of Smad3 resulting in decreasing effects of infrasound. These findings indicate that low decibel infrasound might be a useful protector of the heart against fibrosis.

It is well known that cardiac fibrosis is a key process responsible for the end-stage heart failure under hypertension and coronary heart disease.^[6,11,12] Cardiac fibroblasts, which are the main source of collagen, play critical role in the process of cardiac fibrosis. Zhang *et al.*^[6] reported that miR-29b as a therapeutic agent for Ang II-induced cardiac fibrosis by targeting TGF- β /Smad3 signaling. Similarly, this study reveals that infrasound causes both functional and signaling changes in the cardiac fibroblasts. Importantly, miR-29a is necessary for the action of infrasound, which was testified by siRNA interference. Downregulation of the miR-29 family including miR-29a, b, and c has been shown to be associated with the pathogenesis of heart disease.^[13-15] We suggest that the loss of miR-29a in the process of cardiac fibrosis can be alleviated by low decibel infrasound. Our previous work showed that infrasound of 5 Hz at 130 dB induced cardiac injuries.^[1-4] However, this study gives evidence that infrasound on 4–20 Hz at 79.75–86.11 dB has cardiac protecting effects. It is notable that both the sound frequency and intensity are key factors. Thus, it is necessary to optimize the parameters of infrasound in the clinical application. We speculated that infrasound below 90 dB at 4–20 Hz causes only light but not intense resonance of the cells. To the cell, light resonance can be as beneficial physical exercise, which might be inducing signaling changes. The current exposure is just a presupposed possible therapeutic dose, which needs further evaluation. This study gives clues regarding the protecting effects of infrasound at the current dose. In the future, infrasound treatment with more doses of infrasound is worthy of investigation.

Taken together, low decibel infrasound inhibits Ang II-stimulated cardiac fibroblasts by reactivating miR-29a targeting the TGF- β /Smad3 pathway. It suggests that low decibel infrasound might act as a novel and effective strategy in the prevention and cure of cardiac disorders associated with fibrosis.

Financial support and sponsorship

This research was supported by the National Natural Science Foundation of China (Grant Nos. 81260293, 81671648, and 81470438) and by the scientific research program of Shaanxi provincial (Grant No. 15JK1617 and 2015SF119).

Conflicts of interest

There are no conflicts of interest.

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