Original Article
Therapeutic ultrasound protects HUVECs from ischemia/hypoxia-induced apoptosis via the PI3K-Akt pathway

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Abstract: Background: Previous studies have demonstrated that therapeutic ultrasound (TUS) ameliorates angiogenesis on ischemic hind limb animals and also promotes human umbilical vein endothelial cells (HUVECs) tube formation. Apoptosis plays a key role in post-ischemic angiogenesis pathogenesis. However, the mechanisms underlying the anti-apoptotic effects of TUS are not clear. Therefore we put forward the hypothesis that TUS might promote angiogenesis during ischemia/hypoxia (I/H) by decreasing apoptosis. Methods: We investigated the cytoprotective role of TUS and the underlying mechanisms in I/H-induced HUVEC apoptosis. HUVECs were treated under hypoxic serum-starved conditions for 36 h and then treated with or without TUS (9 minutes, 1 MHz, 0.3 W/cm²). The cell viability was examined by the CCK-8 assay, apoptosis cell rate was determined by TUNEL staining and flow cytometry assay. In addition, the mitochondrial-dependent apoptosis pathway was evaluated by the protein activity of Bax, Bcl-2 and Caspase-3. Results: 1) apoptosis could be induced by I/H in HUVECs. 2) TUS attenuates HUVECs cell apoptosis induced by I/H. 3) TUS inhibits the protein expression of apoptosis modulators and effectors that regulate the mitochondrial pathway of apoptosis in HUVECs. 4) TUS increases the phosphorylation of Akt, which demonstrates the activation of the phosphoinositide 3-kinase (PI3K)- serine/threonine kinase (Akt) signal pathway. Conclusions: The present study indicates that exposure to TUS exerts a protective effect against I/H-induced apoptosis among HUVECs and that this process is mediated through the mitochondrial-dependent intrinsic apoptotic pathway. We also confirm that the PI3K-Akt signal cascade may be taken part in the TUS effects on apoptosis.

Keywords: Therapeutic ultrasound, angiogenesis, hypoxia, apoptosis, human umbilical vein endothelial cells

Introduction
Peripheral artery disease (PAD) is a progressive atherosclerotic occlusive disease that causes insufficient blood flow to the lower extremities and can result in debilitating, activity-induced, pain even while walking short distances. About one third of PAD would develop to critical limb ischemia (CLI) when the arterial blood flow to the part or entire foot markedly reduced, in most cases as a result of progressive obstructive atherosclerosis [1]. The current management of PAD has three major therapeutic options: medical treatment, catheter intervention, and bypass surgery. According to a report of the American College of Cardiology Foundation/American Heart Association Task Force (ACCF/AHA) on practice guidelines, there will be approximately 500 to 1000 new cases of CLI every year within 1 million European/North American population, among which about 25% of the patients with CLI undergo primary foot amputation even in developed countries, and amputation seems to be the common first-line therapy in developing countries, where there is lack of specialized podiatry program [2]. It is
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therefore crucial to develop alternative therapeutic strategies for severe peripheral artery disease.

Therapeutic ultrasound (TUS) is commonly used for physiotherapy and can exert biological effects through either thermal or mechanical mechanisms. It has been demonstrated that TUS induces angiogenesis through the up-regulation of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF) in ischemic tissue and ameliorates blood perfusion in models of hind limb ischemia [3-5]. TUS contributes to post-ischemic angiogenesis by regulating not only cell proliferation but also apoptosis [6]. However, the molecular mechanisms underlying the anti-apoptotic effects of TUS have not been fully elucidated.

Apoptosis plays a key role in the pathogenesis of PAD due to the loss of endothelial cells. It has been reported that patients with PAD have higher rates of endothelial cell apoptosis than normal subjects [7]. Apoptosis is a highly regulated program of cell death and precipitated by sequential activation of cysteine proteases of the caspase family, in two distinct but converging pathways, the extrinsic and intrinsic pathway (also termed the ‘mitochondrial’ or ‘stress’ pathway) [8]. The intrinsic pathway activates when cytochrome c is released from damaged mitochondria in response to diverse stresses, including hypoxia, including hypoxia, ROS, oxidized low-density lipoprotein, and DNA damage [9, 10]. Many studies have demonstrated that the inhibition of apoptosis can prevent the development of CLI and improve post-ischemic angiogenesis [11].

It is known that serine/threonine kinase (Akt) regulates cell growth and survival. The survival effects of Akt are mediated by several anti-apoptotic and pro-apoptotic proteins, including B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase-3 [12]. The potential role of Akt in the anti-apoptotic effect of TUS has not been explored; it is unclear whether Akt plays a role in regulating HUVECs fate, and the downstream elements of the Akt signaling remain to be determined. To address these issues, we put forward the hypothesis that TUS can attenuate endothelial cell apoptosis. Our results suggested that TUS inhibits apoptosis through activation of the phosphatidylinositol-3-kinase (PI3K)-Akt signaling that modulates mitochondrial-dependent intrinsic apoptotic pathway and protects HUVECs against I/H injury.

Materials and methods

Cell culture

HUVECs were purchased from Clonetics (San Diego, CA, USA) and were cultivated in DMEM medium supplemented with 10% fetal bovine serum, 25 Mm glucose and antibiotics [13]. The cells were maintained in a humidified atmosphere incubator (HF 212 UV; Health Force Development Ltd, UK) with 5% CO₂ at 37°C, passaged regularly. Each condition was assessed in triplicate.

Ischemia/hypoxia (I/H) model

To induce apoptosis, the culture medium was replaced with serum-free DMEM, and HUVECs were transferred into a hypoxia chamber (Series II Water Jacket, Thermo Fisher Scientific Inc; USA) flushed with 1% O₂, 5% CO₂ and 94% N₂ at 37°C. To choose the appropriate time to induce apoptosis, HUVECs were cultured under the I/H conditions for 12, 24, or 36 h.

Therapeutic ultrasound stimulation

TUS were produced by a device with applicator designed and made by Institute of Acoustics, Tongji University (Shanghai, China). Ultrasound was delivered to cell culture plates with an energy flux density of 0.3 W/cm² at a frequency of 1.0 MHz. Common ultrasound gel pad was put on a circular ultrasound transducer (2.0 cm in diameter), and they were laid together under the base of the culture plate. After incubation under I/H conditions for 36 h, HUVECs exposed to 9 minutes TUS and then cultured the cells for 24 h under normal conditions. I/H control cells were routinely plated in wells furthest from those exposed to TUS, and there was no detectable TUS exposure at this site. Moreover, two groups incubated under normal conditions and treated with TUS (9 minutes, 1.0 MHz, 0.3 W/cm²) or without TUS were evaluated as another control.

Cell viability assay

HUVEC were seeded in 96-well plates at a density of 2 × 10³/well. Cells were exposed to nor-
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moxia or I/H and treated with 9 minutes TUS or no TUS. Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) was used to measure cell viability. Cells were incubated with 10 μl CCK-8 solution at 37°C for 4 h following the manufacturer’s instructions. Optical density (OD) values at 490 nm were obtained using a microplate reader (Multiskan MK, Thermo Fisher Scientific Inc; USA).

Transferase-mediated dUTP nick end labelling assay (TUNEL)

To identify apoptotic cells, TUNEL reaction was performed according to the manufacturer’s instructions (Roche Molecular Systems, Pleasanton, CA, USA). Briefly, HUVECs were fixed with 4% paraformaldehyde for 60 min at room temperature, washed thrice with PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and then rinsed with PBS. Cells were stained with 50 μl TUNEL reaction mixture at 37°C for 60 min, washed with PBS. HUVECs nuclei were stained for 10 min with Hoechst 33258. Images were acquired under a fluorescent microscope (IX-71; Olympus, Tokyo, Japan) with 12.8 M pixel recording digital color cooled camera (DP72; Olympus).

Cell apoptosis assay

Cell apoptosis was quantified using the Annexin V/propidium iodide (PI) detection kit (Beyotime, Shanghai, China) and analyzed by flow cytometry. Cells (2 × 10⁵/well) were plated in 6-well dishes and treated with 9 minutes TUS or no TUS exposed to I/H or normoxia. After treatment, collected cells were incubated in 400 μl binding buffer with 5 μl Annexin V-FITC and 5 μl PI in dark for 15 min at room temperature.

Western blotting analysis

Cell lysates of HUVEC treated with TUS or no TUS under I/H or normoxia were analyzed by western blotting using antibodies to β-actin, Bcl-2, Bax, caspase-3, Akt, p-Akt (Ser473), Erk1/2, p-Erk1/2 (Thr202/Tyr204), p38MAPK and p-p38MAPK (Thr180/Tyr182). The antibodies (1:500) listed above were purchased from Cell Signaling Technology (Beverly, CA, USA). Blots were reprobed with β-actin to confirm equal loading of cell lysate proteins. The intensity of protein bands was similar to previously described [14].

Statistical analysis

Statistical analysis was performed in SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Multi-group analysis of variances was carried out by one-way ANOVA test followed by post hoc tests. All experiments were repeated at least four times and the data are expressed as the mean ± SEM. P < 0.05 is considered to be statistically significant (*p < 0.05 or **p < 0.01, respectively, in the figures).

Results

TUS attenuates the apoptosis induced by ischemia/hypoxia in HUVECs

In order to determine the optimal time to induce cell apoptosis HUVECs were cultured under I/H conditions for 12 h, 24 h, or 36 h. As assayed by the CCK-8 assay (Figure 1A), results showed a time-dependent reduction in cell viability compared to the normoxia control (NC). HUVECs cell viability was decreased significant after incubation under I/H conditions for 36 h, which is a suitable degree of cell injury. Thus HUVEC treated under I/H conditions for 36 h were used in the follow-up experiments.

Compared to treatment with I/H, cell viability was strongly enhanced after TUS treatment (9 minutes, 1.0 MHz, 0.3 W/cm²) (Figure 1B). Furthermore, the apoptotic effect of I/H was further affirmed by I/H-induced increases in DNA fragmentation, which was demonstrated by TUNEL staining. As shown in Figure 1C and 1D, chromatin condensation was decreased in I/H plus TUS group compared with I/H group. Annexin-V/PI staining showed that I/H treatment for 36 h induced apparent early and late apoptosis in HUVECs. As shown in Figure 1F, the NC and I/H groups showed early and late
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A

Bcl-2

β-actin

B

Bax

β-actin

C

D

Cleaved Caspase-3

β-actin

Bcl-2/β-actin (Fold change)

Bax/β-actin (Fold change)

Cleaved Caspase-3/β-actin (Fold change)
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After treatment with TUS (1.0 MHz, 0.3 W/cm²), the apoptosis rate was significantly reduced, not only in NC plus TUS group, but also in I/H plus TUS group (Figure 1E). The results indicate that TUS could reduce the apoptosis induced by I/H in HUVECs.

**TUS suppresses the expression of intrinsic apoptosis molecules induced by I/H**

In order to explore the underling mechanism of anti-apoptotic effect of TUS in I/H-treated HUVECs. We detected the expression of key molecules (Bcl-2, Bax, and cleaved Caspase-3) in the intrinsic signaling pathways of apoptosis by western blot analysis. As shown in Figure 2A, Bcl-2 plays an anti-apoptosis effect in cell programmed death, and the protein abundance of Bcl-2 was prominently increased in the TUS group compared with the I/H group, whereas Bcl-2 protein level was increased in the NC and TUS co-treated group. Besides activation of anti-apoptotic protein, we also found that TUS decreased the expression of pro-apoptosis protein Bax compared to the I/H group (Figure 2B). Moreover, the Bcl-2/Bax ration was significantly increased in the TUS treatment group compared to the I/H group (Figure 2C).

**Figure 2.** TUS suppresses the expression of intrinsic apoptosis molecules induced by I/H. Western blotting analysis of Bcl-2, Bax, cleaved Caspase3 abundance in HUVECs. A, C: TUS promoted the expression of Bcl-2 protein and the Bcl-2/Bax ration, significantly. B, D: TUS treatment significantly suppressed the protein expression of Bax and cleaved Caspase3 induced by I/H. Data of Western blotting were represented as percentages of expression in NC group. Values are mean ± SEM; n = 5, *means p < 0.05, **means p < 0.01.

**Figure 3.** TUS's anti-apoptotic effects are mediated through PI3K-AKT-dependent signaling pathways. HUVECs were incubated in NC or in I/H culture conditions with or without TUS. A. Western blotting analysis of p-Akt, Akt, p-p38 MAPK, p38 MAPK, p-Erk1/2 and Erk1/2 abundance in HUVECs. B-D. The average data of p-Akt, p-p38 MAPK and p-Erk1/2 in HUVECs. Western blotting were represented as percentages of expression in NC group. Values are mean ± SEM; n = 5, *means p < 0.05, **means p < 0.01.
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Figure 4. Role of PI3K-Akt signaling cascade in TUS attenuates intrinsic apoptosis in HUVECs.

The PI3K-Akt signal pathway may be involved in the TUS effects on apoptosis

Given that Akt is linked to intrinsic apoptosis, and TUS inhibited the expression of Bax and cleaved Caspase-3 induced by I/H, we next examined whether TUS affects the PI3K-Akt mediated apoptotic signal pathway. Compared with the I/H group, TUS increased protein expression of phospho-Akt (Figure 3A and 3B). We next assessed whether the TUS-induced anti-apoptosis effect is due to the activation of other MAPK signal, such as the p38 MAPK and Erk1/2 signal cascades. Results showed that there were no significant differences in the phosphorylation of p38 MAPK and Erk1/2 between the TUS and I/H groups (Figure 3A, 3C and 3D), which indicate that the activation of PI3K-Akt cascades contributes to the anti-apoptosis activity of TUS at least partly.

Discussion

The most significant and novel of the findings discussed in this manuscript are that 1) TUS can attenuate I/H-induced apoptosis in HUVECs and that 2) anti-apoptotic effects of TUS are mediated through PI3K-Akt-dependent signal pathway.

Resistance to apoptosis is the important step in the process of angiogenesis in endothelial cells (ECs) and is regulated by a diverse of biochemical and microenvironment factors. ECs dysfunction due to vessel occlusion or rupture can cause decreased oxygen delivery and is a pathogenic driver in diabetic retinopathy, peripheral artery disease, and ischemic diseases [15]. In order to adapt to a hostile environment, ECs change the phenotype in ischemic diseases by activating a series of genes, including Bcl-2, Bax and Caspase-3. Considering that the resistance to apoptosis is vital characteristics of angiogenesis, improve cell apoptosis may be a promising treatment strategy for therapeutic angiogenesis [16].

As a new developing technology, TUS has achieved some good effects in a few studies, and made a tendency to expand the application gradually. Although Serizawa et.al have cerfified that TUS can improve the walking ability of patients with PAD and intermittent claudication, it is still not completely clear the precise underling mechanism [1]. Apoptosis plays a key role in ischemic diseases, but there are few reports on whether non-invasive TUS treatment can inhibit apoptosis in endothelial cells. Considering that the resistance to apoptosis is vital characteristics of angiogenesis, improve cell apoptosis may be a promising treatment strategy for therapeutic angiogenesis [17, 18].

It is found that apoptosis under hypoxic stress is mainly dependent on the activation of mitochondrial-dependent intrinsic apoptotic path-
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way in HUVECs [19]. The Bcl-2 family are the central regulators of the intrinsic apoptotic pathway and now includes both pro- and anti-apoptotic proteins, which form a complex network regulate cell fate [20]. Bax is a pro-apoptotic protein of the Bcl-2 family that targets the mitochondria causing the release of cytochrome c from mitochondria [21]. Otherwise, Bcl-2, an anti-apoptotic member of the Bcl-2 family, maintains the mitochondria membrane potential preventing the release of those apoptotic signaling molecules. Hypoxia decreases Bcl-2 expression in endothelial cells, and the expression ratio of Bcl-2 to Bax was reported to determine cell survival or death following an apoptotic stimulus [11]. The ultimate vulnerability of cells to apoptotic stimuli is determined by the relative ratio of various pro-apoptotic and anti-apoptotic members of the Bcl-2 family. In this study, we found that the expression levels of the pro-apoptotic protein Bax increased and the anti-apoptotic protein Bcl-2 expression decreased significantly after ischemia/hypoxic stress for 36 h. The apoptotic cell rate increased in TUS groups compared with the control groups, as indicated by increased Bax and Caspase-3 expression and reduced Bcl-2 and Bcl-2/ Bax ration.

It has been reported that the apoptosis induced by ischemia and hypoxia plays an important role in the development in PAD: the loss of endothelial cells causes low post-ischemic angiogenesis, which may induce PAD progress and even lead to CLI [22]. Recent studies have demonstrated that TUS improves tissue perfusion of ischemic tissue and post-ischemic angiogenesis in animal models of hind limb ischemia and also ameliorates part or entire foot ischemia in patients with severe PAD [1, 23]. In acute limb ischemia rabbit model, TUS preserved skeletal muscle viability and promoted post-ischemic angiogenesis by increasing tissue perfusion in the TUS group but not in the control group [23]. In our previous study, it was observed that TUS could normalize blood perfusion, decrease necrosis and promote angiogenesis in hind limb ischemia mice [5]. In the present study, we investigated whether TUS had any effect on endothelial cell apoptosis in vitro. We observed that TUS attenuated the cell death induced by I/H in HUVECs, the expression of Bcl-2 and Bcl-2/ Bax ration were significantly increased, and the expression of Bax and cleaved Caspase-3 were decreased. Taken together, our results suggest that TUS attenuates the mitochondrial-dependent intrinsic apoptotic pathway in HUVECs.

In order to further investigate the possible regulation mechanism by which TUS protects HUVECs from apoptosis in vitro, three important intracellular signal pathways were detected: the Akt, Erk1/2, and p38 MAPK pathways. We found that under the intervention of TUS, phospho-Akt increased significantly, nevertheless, phosphorylation of Erk1/2 and p38 MAPK had no obvious change, which indicates that the PI3K-Akt signal cascade may be involved in the effects of TUS on apoptosis at least partially. As we know, the PI3K-Akt signaling pathway is related to proliferation, growth, and apoptosis inhibition, playing an important role in maintaining cell survival [24]. The PI3K-Akt pathway is considered to be an important signaling pathway mediating survival signals in multiply cell types [12]. The PI3K-Akt pathway participates in numerous cellular processes by phosphorylating a diverse array of substrates: PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate, and then phosphorylation of the serine/threonine kinase Akt by phosphoinositide-dependent kinase 1 inhibits the formation of the pro-apoptotic proteins Bad and Bax, and, in the meantime, maintains the level of the anti-apoptotic protein Bcl-2. In this context, it has been pointed out that activation of PI3K-Akt pathway may be involved in the anti-apoptosis effects of TUS, at least partially, but more in-depth mechanistic research is needed.

Although it could be demonstrated that ultrasound application is effective in experimental hind limb ischemia and PAD patients, there also has some conflicting viewpoint for ultrasound. Several reports pointed out that ultrasound exposure can cause irreversible cell damage and may participate in ischemia-reperfusion injury, which is expected to result in basic functional loss of the endothelial cells. Given the lack of safety studies, a Japanese study evaluates the safety of TUS on the ischemic calf muscle and its effects on maximum walking distance, recovery time of tissue oxygenation index (TOI), and CT Angiography in PAD patients [1]. In another study that hind limb ischemic rats were treated with TUS on the
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adductor muscle. Fluorescence activated cell sorting analysis of the peripheral blood revealed high numbers of CD31/CD34-positive cells in the treatment group. Greater numbers of capillaries were found in the treated muscles. Blood perfusion and walking analysis increased markedly in the treatment group [25]. Moreover, TUS did not increase the apoptosis of endothelial cells in vivo, and there were no signs of inflammation after TUS treatment [26]. According to the results of the in vivo and in vitro observation, we believe that the TUS is a safe and effective therapy for ischemic diseases, especially for PAD.

There were a number of limitations that should be considered in the context of this study. First, the best strategy of TUS delivery remains to be elucidated. In our study, TUS was delivered to I/H HUVECs with an energy flux density of 0.3 W/cm² at a frequency of 1.0 MHz for 9 minutes. Future studies are needed to determine the best treatment strategy, including intervention time, acoustic energy and frequency. Second, although we found that the Akt pathway may be involved in the effects of TUS on apoptosis, but more detailed mechanistic analyses are required.

In conclusion, these experiments provide the first evidence that TUS exerts a protective effect against I/H-induced cell apoptotic and indicate that the anti-apoptotic effect of TUS may mediate by preventing the activation of the intrinsic apoptotic pathway. Furthermore, these effects are likely dependent on the PI3K-Akt signaling pathway (Figure 4). Together with further work on this topic, our observations may also lend insight that could assist in the development of TUS as a novel therapeutic strategy for ischemic diseases.

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Disclosure of conflict of interest

None.

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