Non-Thermal Effect of Far-Infrared Activates Akt to Reduce Doxorubicin-Induced Apoptosis in Human Umbilical Vein Endothelial Cells

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Abstract

BACKGROUND: The adequacy of hemodialysis is greatly influenced by the long-term patency of vascular access. Far-infrared radiation (FIR) therapy has been proved to be effective in increasing blood flow and inhibiting inflammation of vascular access. However, decreased blood flow may induce apoptosis of endothelial cells. Apoptosis of endothelial cells may stimulate proliferation, migration, and dedifferentiation of vascular smooth muscle cells, thus causing neointimal formation. Ultimately, the vicious cycle of vascular stenosis persists. Our study is to investigate the biological effects of FIR on endothelial cells and elucidate the signaling pathway.

METHODS: Human umbilical vein endothelial cells (HUVEC) were pretreated with doxorubicin and then exposed to FIR. The doxorubicin-induced apoptosis in HUVECs was detected using TUNEL staining. The signals of cleaved caspase-3, caspase-8, caspase-9, and Bcl-2 were detected by Western blotting. The influence of FIR on Akt was analyzed using the phosphoinositide 3-kinase (PI3K) inhibitor. Both thermal and non-thermal effects of FIR were also monitored.

RESULTS: FIR exposure for 30 min reduced significantly doxorubicin-induced apoptosis in HUVECs. FIR exposure led to enhancement of phosphorylated-Akt expression in HUVECs. The quantity of cleaved caspase-3, caspase-8 and caspase-9 in doxorubicin-treated cells also reduced significantly after FIR exposure. The reduced expression of Bcl-2 was recovered after 30-min FIR exposure. The anti-apoptotic effect of FIR was abolished by Akt inhibitor. In addition, the temperature of culture medium exceeding 37°C attenuated the anti-apoptotic effect of FIR.

CONCLUSION: Our data suggest that FIR therapy inhibits the apoptosis of HUVEC via activation of Akt. The non-thermal effect of anti-apoptosis on endothelial cells may contribute to patency of vascular access.

KEY WORDS: Far-Infrared therapy, endothelial cell, apoptosis, vascular access, Akt

Introduction

Vascular endothelium health is very important for cardiovascular function. In hemodialysis patients, arteriovenous fistula (AVF) access dysfunction results in decreased blood flow, which may induce endothelial cell apoptosis (1-5). It has also been shown that dysregulation of endothelial cell apoptosis has a major...
regulatory effect on the establishment of the primordial vascular network, termed vasculogenesis in the embryo, causing severe hemorrhage and finally leading to embryonal death (6). Excess endothelial cell apoptosis may limit angiogenesis, thus leading to vessel regression. Therefore, inhibition of endothelial cell apoptosis can serve as a potential therapeutic target, especially in patients suffering from AVF dysfunction and vessel regression diseases.

Apoptosis is the process of programmed cell death that involves two major pathways. One is the death receptor-mediated pathway, which requires binding a ligand to a death receptor on the cell surface, including tumor necrosis factor (TNF) and Fas ligand receptors (7). The other is the intrinsic, mitochondrial-dependent pathway, which triggers the mitochondrial pathway and activation of caspase-3. In mammalian cells, a major caspase activation pathway is the cytochrome c-initiated pathway. In this pathway, various apoptotic stimuli cause cytochrome c release from mitochondria, which in turn induces a series of biochemical reactions, resulting in activating caspases to cause subsequent cell death (8). Cytochrome c release is known to be regulated by Bcl-2 family proteins, including Bcl-2 and Bcl-xL, which bind to the mitochondrial outer membrane and block cytochrome c efflux (9).

Far-infrared radiation (FIR) therapy has the potential to improve endothelial function and reduce the frequency of some vascular-related diseases. In recent years, a clinical study evaluated the effect of FIR therapy on 145 hemodialysis (HD) patients with a native AVF, and found that FIR therapy can improve inadequate access flow and survival of the AVF in HD patients through both its thermal and nonthermal effects (10). Lin et al. also demonstrated that FIR induced the expression of heme oxygenase-1 (HO-1), a potent anti-inflammatory agent, in human umbilical vein endothelial cells (HUVECs) (11). In human heme oxygenase-1 deficiency, the endothelial cell injury was enhanced by oxidative stress (12). Therefore, FIR therapy may contribute to inhibition of endothelium cell apoptosis.

In this study, we evaluated the biological effect of FIR on doxorubicin-induced apoptosis in HUVECs. FIR generation from a FIR emitter usually accompanies thermal transmission. To distinguish the FIR effect from the thermal effect, a FIR exposure system was carefully designed. By studying molecular mechanisms, we found that FIR inhibited doxorubicin-induced apoptosis in HUVECs via Akt signaling pathway.

**Materials and Methods**

**Cell Culture**

The HUVEC line was purchased from the Bio-

**FIR Activates Akt to Reduce Apoptosis**

**Apoptosis Detection**

FITC-annexin V/propidium iodide (PI) double staining was utilized to detect apoptosis induced by doxorubicin treatment. HUVECs, untreated or FIR-treated, were harvested and washed twice with ice-cold PBS, and specific binding of FITC-annexin V and staining with PI was performed with an apoptosis detection kit (BD Pharmingen), according to the manufacturer’s instructions. The cells were then analyzed by flow cytometry. Doxorubicin-mediated apoptosis in HUVECs was also detected by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) stain. TUNEL staining was conducted using a Cell Death Detection kit (Roche, Mannheim, Germany). To reveal total nuclei, the same slides were also stained with DAPI (4′,6-diamidino-2-phenylindole) (1 μg/mL) in PBS plus 0.5% 1,4-diazabicyclo[2,2,2]octane.

**Western Blot Analysis**

In total, 15 μg of HUVEC lysate proteins was applied to each lane and analyzed by Western blotting. Peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) (at a 1:5000 dilution) was used as the second antibody to detect primary antibodies by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA). The data of protein bands on Western blots were also quantitated with
QuantiScan software (Biosoft, Cambridge, United Kingdom).

**Statistics**

Student’s *t*-test was used in all statistical analyses. Distributions of continuous variables in groups were expressed as the means ± SD. A value of *P* < 0.05 was considered to indicate statistical significance.

**Results**

**FIR Reduces Doxorubicin-Induced Apoptosis in HUVECs**

To evaluate the biological effects of FIR, a ceramic FIR generator was chosen as the FIR source in this study, which generates electromagnetic wavelengths ranging from 3 to 25 μm.

The doxorubicin-induced apoptosis in HUVECs was detected using annexin V and PI staining. As shown in Fig. 1A, 30 nM of doxorubicin induced significant apoptosis but not necrosis in HUVECs. FIR exposure for 30 min inhibited doxorubicin-induced apoptosis in HUVECs. The doxorubicin-induced apoptosis was also detected using TUNEL staining. In dosage tests, HUVECs were treated with 30 nM doxorubicin for 30 min, exposed to FIR at 0.13 mW/cm² for the indicated periods, and then cultured at 37°C overnight. The level of apoptosis was revealed by TUNEL assays, and presented with the percentage of TUNEL positive cells for each treatment. Results are the means ± S.D. (*n* = 3). *P* < 0.05 compared with the group without doxorubicin treatment; # *P* < 0.05 compared with the doxorubicin alone group; C, untreated control.

![Fig. 1. The protective effect of FIR exposure on doxorubicin-induced apoptosis in HUVECs.](image)
FIR (min)

| FIR (0.13 mW/cm²) for 30, 60 or 90 min, and then cultured for 24 h. FIR exposure for 30 min reduced significantly doxorubicin-induced apoptosis in HUVECs (Fig. 1C). This reduction was not found in 60- and 90-min FIR exposure, revealing that FIR exposure under proper conditions protects HUVECs from doxorubicin-induced apoptosis.

We also monitored the influence of FIR exposure on the apoptotic signaling pathway caused by doxorubicin. The effect of FIR on doxorubicin-induced activation of caspases was evaluated by Western blotting using antibodies that recognize cleaved caspases. The quantity of cleaved caspase-3, caspase-8 and caspase-9 were greatly elevated in cells treated with 30 nM of doxorubicin for 16 h (Figs. 2, 3). FIR exposure for 30 min significantly reduced the quantity of cleaved caspase-3, caspase-8 and caspase-9 in doxorubicin-treated cells. We also monitored the influence of FIR on the expression of Bcl-2, an anti-apoptotic protein, in doxorubicin-treated HUVECs. Western blotting revealed that doxorubicin reduced the expression of Bcl-2, and this reduction was recovered by 30-min FIR exposure (Figs. 2, 3). However, these influences of FIR on the apoptotic signal proteins were not found in doxorubicin-treated HUVECs after FIR exposure for 60 and 90 min.

To understand the molecular mechanisms of the anti-apoptotic effect of FIR, we monitored the activation of Akt that has been established as an important intracellular signaling in regulating cell survival. As shown in Fig. 4, the phosphorylation of Akt was significantly induced by FIR exposure for 30 min, whether with or without doxorubicin treatment. When we pretreated HUVECs with wortmannin, an inhibitor for Akt signaling pathway, the anti-apoptotic effect of FIR exposure became insignificant (Fig. 5). Therefore, the phosphorylation of Akt plays a critical role in the anti-apoptotic effect of FIR exposure.

The thermal effect of FIR on the cell culture system was also examined in this study. With FIR
exposure, we detected the temperature of the 3-ml culture medium in a 6-cm plate at different time points. The temperature of the medium was increased by FIR exposure in a time-dependent manner (Fig. 6A). For example, the effective FIR intensity of 0.13 mW/cm² raised the temperature of the medium from 36.6°C to 38°C in 90 min. In doxorubicin-treated HUVECs, the anti-apoptotic effect of FIR was also inhibited by 38°C treatment for 30 min (Fig. 6B). There was no significant difference in apoptosis levels between 37°C and 38°C treatments without FIR. Therefore, a short-term change of temperature in the medium did not induce apoptosis in HUVEC. Taken together, the above results reveal the thermal effect of FIR exposure on inhibiting anti-apoptosis.

Discussion

Far-infrared radiation (FIR) therapy has been proved to be effective in increasing blood flow and inhibiting inflammation of vascular access. In our present study, FIR exerted inhibitory effect on vascular endothelial cell apoptosis, and induced Akt phosphorylation. Akt promotes cell survival and inhibits apoptosis by phosphorylating the Bcl-2 family. Our results demonstrate that the anti-apoptotic effect of FIR was blocked by Akt inhibitor. Thus, the salutary effect of FIR was mediated by the signaling pathway involving Akt activation.

Akt pathway plays a key role in vascular homeostasis. The effects of vascular endothelial growth factor on cell survival have been shown to be mediated by PI3K-Akt pathway (13). Several endothelial cell stimuli, including insulin-like growth factor-I, fluid shear stress, estrogen, reactive oxygen species, and corticosteroids, also activate PI3K-Akt signaling pathway. Early studies demonstrated that Akt can directly phosphorylate endothelial nitric oxide

Fig. 4. The influence of FIR on Akt phosphorylation in HUVECs. HUVECs were exposed to FIR for different periods as indicated, and then treated with or without 30 nM doxorubicin at 37°C for 30 min. Akt and phospho-Akt in HUVECs were detected using Western blotting assay with proper antibodies.

Fig. 5. The influence of wortmannin on the anti-apoptosis effect of FIR in HUVECs. Cells were treated with 30 nM doxorubicin with or without wortmannin (1 μM) for 30 min, exposed to FIR for 30 min as indicated, and then cultured at 37°C overnight. The level of apoptosis was revealed by TUNEL assays, and presented with the percentage of TUNEL positive cells for each treatment. Results are the means ± S.D. (n = 3).

Fig. 6. The influence of a thermal effect on the biologic effect of FIR in HUVECs. (A) The temperature of cultured medium influenced by FIR exposure. The temperature of 3 ml of culture medium in 6-cm culture dishes was detected after FIR exposure for the indicated periods. (B) The influence of a thermal effect on the anti-apoptosis effect of FIR. Cells were treated with 30 nM doxorubicin for 30 min, exposed to FIR at 37 or 38°C for 30 min as indicated, and then cultured at 37°C overnight. The level of apoptosis was revealed by TUNEL assays, and presented with the percentage of TUNEL positive cells for each treatment. Results are the means ± S.D. (n = 3).
synthase on serine 1179 and activate the enzyme; leading to nitric oxide (NO) production (14). It is well known that endothelial cell dysfunction is the culprit of many diseases, such as insulin resistance and coronary artery disease. FIR therapy is associated with phosphorylated-Akt and inhibition of endothelial cell apoptosis; both making it a potential therapeutic strategy for vascular access patency.

The maximal mitigating effect of FIR exposure at 0.13 mW/cm² on inhibiting apoptosis of endothelial cells was achieved at 30 min. FIR is heat-generated and the temperature of the culture medium increased with the exposure time. The anti-apoptotic effect of FIR declined when the temperature exceeded 38°C. There are some explanations to this deleterious thermal effect. First, heat induces TNF-α and oxidative stress, both promoting endothelial cell apoptosis (15, 16). Second, though there are possible mechanisms for increase in cell survival by HO-1, overexpression of HO-1 downregulates the expression of phosphorylated-Akt via a possible negative interaction on the Akt/NO pathway (17). Moreover, the heme degrading product, free iron, if not sequestered by ferritin, is a potent oxidant that can induce cell apoptosis. We hypothesize that the above mechanisms co-interact to overcome the anti-apoptotic effect of FIR.

There are some limitations in this study. First, unlike in physiologic vascular environment, the shear stress of blood flow may play an important role in cell homeostasis. Lin et al. (10) found that FIR therapy has the effect of improving blood flow. Our cellular study is not flow-dependent, which makes the experimental results difficult to apply to blood flow improvement. Second, AVF is not applied in doxorubicin infusion in normal clinical situations. Doxorubicin-mediated apoptosis in HUVECs may not reflect real clinical practice on AVF. The serum deprivation model may be more appropriate for the study of endothelium cell apoptosis in AVF. However, in this study, we try to focus on the influence of FIR therapy on the general apoptosis in vascular endothelium cells. This general apoptosis is mediated by oxidative stress, and exists in serum deprivation, vessel regression and other vascular diseases. Induction of oxidative stress-mediated apoptosis is an important cytotoxic mechanism of doxorubicin (18). We used doxorubicin to establish an in vitro HUVEC apoptosis model to study the protective effect of FIR. Therefore, the finding in this study can partially explain the beneficial effect of FIR therapy on vascular diseases.

In conclusion, our data suggest that FIR therapy inhibits the apoptosis of HUVEC via Akt activation. The non-thermal anti-apoptosis effect on endothelial cells may play a role in the protective effect of FIR on vascular function.

References