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Far-infrared radiation acutely increases nitric oxide production by increasing Ca^{2+} mobilization and Ca^{2+}/calmodulin-dependent protein kinase II-mediated phosphorylation of endothelial nitric oxide synthase at serine 1179

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ABSTRACT

Repeated thermal therapy manifested by far-infrared (FIR) radiation improves vascular function in both patients and mouse model with coronary heart disease, but its underlying mechanism is not fully understood. Using FIR as a thermal therapy agent, we investigate the molecular mechanism of its effect on endothelial nitric oxide synthase (eNOS) activity and NO production. FIR increased the phosphorylation of eNOS at serine 1179 (eNOS-Ser^{1179}) in a time-dependent manner (up to 40 min of FIR radiation) in bovine aortic endothelial cells (BAEC) without alterations in eNOS expression. This increase was accompanied by increases in NO production and intracellular Ca^{2+} levels. Treatment with KN-93, a selective inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and H-89, a protein kinase A inhibitor, inhibited FIR radiation-stimulated eNOS-Ser^{1179} phosphorylation. FIR radiation itself also increased the temperature of culture medium. As transient receptors potential vanilloid (TRPV) ion channels are known to be temperature-sensitive calcium channels, we explore whether TRPV channels mediate these observed effects. Reverse transcription-PCR assay revealed two TRPV isoforms in BAEC, TRPV2 and TRPV4. Although ruthenium red, a pan-TRPV inhibitor, completely reversed the observed effect of FIR radiation, a partial attenuation (~20%) was found in cells treated with Tranilast, TRPV2 inhibitor. However, ectopic expression of siRNA of TRPV2 showed no significant alteration in FIR radiation-stimulated eNOS-Ser^{1179} phosphorylation. This study suggests that FIR radiation increases NO production via increasing CaMKII-mediated eNOS-Ser^{1179} phosphorylation but TRPV channels may not be involved in this pathway. Our results may provide the molecular mechanism by which FIR radiation improves endothelial function.

1. Introduction

Far-infrared (FIR) radiation is an invisible electromagnetic wave with 3–1000 μm defined by the International Commission on Illumination [1]. FIR radiation transfers energy to the human body and manifests a wide variety of biological effects. These effects may be attributable that FIR radiation, via its specific range of frequency, activates the important molecules, such as water molecule in the human body, responsible for diverse biological effects. Several studies showed that FIR radiation has been reported for a long time to exert beneficial effects in cardiovascular systems [2]. For example, FIR irradiation decreased the vascular endothelial inflammation which was mediated by induction of heme oxygenase-1 [3]. Furthermore, repeated thermal therapy manifested by FIR also greatly improved impaired vascular endothelial function [4] and ventricular arrhythmias [5] in patients with chronic heart failure, and increased angiogenesis in a hindlimb ischemic mouse model. Later, increased eNOS expression was reported to be involved in one of the mechanisms underlying thermal therapy (thus FIR as well)-stimulated endothelial function and angiogenesis. However, a detailed molecular mechanism has not been elucidated.

Abbreviations: FIR, far-infrared; eNOS, endothelial nitric oxide synthase; eNOS-Ser^{1179}, eNOS at serine 1179; EC, endothelial cell(s); NO, nitric oxide; AMPK, AMP-activated protein kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PKA, protein kinase A; BAEC, bovine aortic EC; HUVEC, human umbilical vein EC; siRNA, small interference RNA; TRP, transient receptors potential; TRPV, TRP vanilloid; TRPM, TRP melastatin; TRPA, TRP ankyrin.

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Endothelial nitric oxide synthase (eNOS) is the major source of NO production in endothelial cells (EC). Dysregulation of eNOS is thought to contribute to the pathogenesis of cardiovascular diseases such as atherosclerosis and hypertension [6,7]. eNOS is mainly regulated at the level of phosphorylation [8]. Several specific sites of phosphorylation have been identified, among which eNOS-Ser^{1179} (bovine sequence) is the most studied. Phosphorylation of eNOS-Ser^{1179} increases NO production, mediated by several protein kinases, including Akt [9,10], AMP-activated protein kinase (AMPK) [11], Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [12], protein kinase A (PKA) [13], and checkpoint kinase 1 [14]. The role of these protein kinases as signaling molecules for eNOS-Ser^{1179} phosphorylation is dependent on several stimuli including vascular endothelial growth factor (VEGF), bradykinin, shear stress, troglitazone, and UV irradiation [9–11,13,14]. In particular, it is well known that the increase in intracellular Ca^{2+} levels plays an important role in stimulating eNOS-Ser^{1179} phosphorylation and subsequent NO production through the reversible formation of the Ca^{2+}/calmodulin complex.

In this study, we investigate whether FIR increases NO production by activating a signaling axis in intracellular Ca^{2+} mobilization–CaMKII activation–eNOS-Ser^{1179} phosphorylation in BAEC.

2. Materials and methods

2.1. Materials

LY294002 (Akt inhibitor), Compound C (AMPK inhibitor), KN-93 (CaMKII inhibitor), BAPTA-AM (Ca^{2+} chelator), and ruthenium red (a pan-TRPV inhibitor) were purchased from Calbiochem (Darmstadt, Germany). Tranilast (an inhibitor of transient receptors potential vanilloid 2 (TRPV2) ion channels) and RN1734 (TRPV4 inhibitor) were purchased from A.G. Scientific (San Diego, CA) and Tocris Bioscience (Ellisville, MO), respectively. EGTA (extracellular Ca^{2+} chelator) and L-NAME (NOS inhibitor) were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against eNOS, p-eNOS-Ser^{1179}, p-CaMKII-Thr^{286}, and tubulin were purchased from Transduction Laboratories (Lexington, KY), Cell Signaling Technology (Boston, MA) and AbFrontier (Seoul, Korea), respectively. Minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS), penicillin–streptomycin antibiotics, L-glutamine, and trypsin–EDTA solution obtained from Gibco-BRL (Gaithersburg, MD). All other chemicals were of the purest analytical grade.

2.2. Cell culture, FIR irradiation, and drug treatments

BAEC were isolated and maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO_2 as described [15]. BAEC grown to 80% confluence were subjected to FIR radiation with wavelength between 6 and 20 μm using a ceramic FIR radiation generator, an S-O.T.M 9H FIR radiator (Saeik Medical Co Ltd, Bucheon, Korea). The radiator was set at a height of 30 cm above the bottom of culture plates, and the cells were exposed to FIR radiation at room temperature for the indicated times (0, 10, 20, 30, and 40 min). In some experiments, cells were pretreated for 1 h with 10 μM of Compound C, LY294002 or KN-93 in fresh MEM containing 0.5% NCS.

2.3. Transfection

Small interference RNA (siRNA) oligonucleotide designed against TRPV2 was synthesized as follows: 5′-ACU CAG UGC UGG AGA UCA UUU-3′ (Dharmacon Research Inc, Lafayette, CO). A non-specific siRNA oligonucleotide (Cat. No. D-001810-01) was also obtained for a control experiment. BAEC grown to 80% confluence in 60 mm culture dishes were transfected with 100 nM of each siRNA oligonucleotide using DharmaFECT (Dharmacon Research Inc.) according to manufacturer's instructions. After incubation for 5 h at 37 °C, DharmaFECT mixtures were washed out and the cells were further incubated in MEM containing 5% NCS for 24 h before FIR radiation.

2.4. Western blot analysis

For Western blot analysis, cells were treated without or with FIR radiation, washed with ice-cold DPBS and then lysed in lysis buffer, as previously described [9]. Protein concentrations were then determined using a BCA protein assay kit (Sigma–Aldrich). Equal quantities of protein (20 μg) were separated on SDS–PAGE under reducing conditions, after which they were electrophoretically transferred onto the nitrocellulose membranes. The blots were then probed with appropriate antibodies, followed by a corresponding secondary antibody, and finally developed using ECL reagents (Amersham, Buckinghamshire, UK).

2.5. Intracellular calcium measurement

Intracellular Ca^{2+} was detected by Fluo-4 AM (Invitrogen, Carlsbad, CA), an intracellular Ca^{2+} indicator, according to manufacturer's protocol. Briefly, BAEC grown on coverslips were treated without or with FIR radiation for the indicated times, and cells were then treated with 1 μM of Fluo-4 AM and fixed with 4% (wt/vol) paraformaldehyde. Images of intracellular Ca^{2+} were photographed using a confocal microscope (LSM5 Pascall, Carl ZEISS).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA from BAEC or human umbilical vein EC (HUVEC) were extracted using TRIzol reagent (Gibco-BRL, Gaithersberg, MD), as described previously [16]. cDNA was synthesized from total RNA using Superscript II reverse transcriptase and oligo-(dT)12–18 primer (Invitrogen), according to the manufacturer's instructions. The primer pairs for TRPV, TRP melastatin 8 (TRPM8), or TRP ankyrin 1 (TRPA1) were as follows: bovine TRPV1-F 5′-TGA CTC TGT GTC GGT CGA GT-3′ and bovine TRPV1-R 5′-GTG TTC CAG GTG GTC CAG TT-3′; bovine TRPV2-F 5′-TAC TAC ATG CGT GGC TTC CA-3′ and bovine TRPV2-R 5′-GAG ATG GCT TTC TGC AGC TT-3′; bovine TRPV3-F 5′-GAC ATC ACC TCG CAG GAC TC-3′ and bovine TRPV3-R 5′-GGC GAA CTT CTT CCA CTT CA-3′; bovine TRPV4-F 5′-CAA CTT GAA GGT GTG CGA TG-3′ and bovine TRPV4-R 5-TGG TTC CAG TGA GAC CAG TTC-3′; bovine TRPM8-F 5′-ATT CAC ATT TTC ACG GTC AGC-3′ and bovine TRPM8-R 5′-ACC TGG TCG TTG TTT TCC TG-3′; bovine TRPA1-F 5′-TCT CGT GGC TTT TGG ACT CT-3′ and bovine TRPA1-R 5′-TTT CAT GGG GGC AAA AGA TA-3′; bovine 18S rRNA-F 5′-GTT GGT GGA GCG ATT TGT CT-3′ and bovine 18S rRNA-R 5′-GGC CTC ACT AAA CCA TCC AA-3′; human TRPV1-F 5′-CTG TGC CGT TTC ATG TTT GT-3′ and human TRPV1-R 5′-TCT CCT GTG CGA TCT TGT TG-3′; human TRPV2-F 5′-TGT TGC CTA CCA TCA GCC TA-3′ and human TRPV2-R 5′-GTA GAT GCC TGT GTG CTG GA-3′; human TRPV3-F 5′-GGA AGA AGT TTG CCA AGC AC-3′ and human TRPV3-R 5′-GCA GGC GAG GTA CTC TTT GT-3′; human TRPV4-F 5′-TGT CCT GGT GAT CGT CTC AG-3′ and human TRPV4-R 5′-AAC AGG TCC AGG AGG AAG GT-3′; human TRPM8-F 5′-ATT CCG TTC GGT CAT CTA CG-3′ and human TRPM8-R 5′-GAA GGG GAA GGG GAT ATT GA-3′; human TRPA1-F 5′-GGA TCA GAA ATC CAC CAT CG-3′ and human TRPA1-R 5′-TGT GTT TTT GCC TTG ACT GC-3′; human 18S rRNA-F 5′-GCC GTT CTT AGT TGG TGG AG-3′ and human 18S rRNA-R 5′-GGG ACT TAA TCA ACG CAA GC-3′. PCR condition was one cycle at 94 °C for 5 min,