Far Infrared Therapy Inhibits Vascular Endothelial Inflammation via the Induction of Heme Oxygenase-1

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Objective—Survival of arteriovenous fistulas (AVFs) in hemodialysis patients is associated with both far infrared (FIR) therapy and length polymorphisms of the heme oxygenase-1 (HO-1) promoter. In this study, we evaluated whether there is an interaction between FIR radiation and HO-1 in regulating vascular inflammation.

Methods and Results—Treatment of cultured human umbilical vein endothelial cells (ECs) with FIR radiation stimulated HO-1 protein, mRNA, and promoter activity. HO-1 induction was dependent on the activation of the antioxidant responsive element/NF-E2-related factor-2 complex, and was likely a consequence of heat stress. FIR radiation also inhibited tumor necrosis factor (TNF)-α–mediated expression of E-selectin, vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, monocyte chemoattractant protein-1, interleukin-8, and the cytokine-mediated adhesion of monocytes to ECs. The antiinflammatory action of FIR was mimicked by bilirubin, and was reversed by the HO inhibitor, tin protoporphyrin-IX, or by the selective knockdown of HO-1. Finally, the antiinflammatory effect of FIR was also observed in patients undergoing hemodialysis.

Conclusions—These results demonstrate that FIR therapy exerts a potent antiinflammatory effect via the induction of HO-1. The ability of FIR therapy to inhibit inflammation may play a critical role in preserving blood flow and patency of AVFs in hemodialysis patients. (***Arterioscler Thromb Vasc Biol*. 2008;28:739-745**)

Key Words: endothelium ■ far infrared therapy ■ inflammation ■ leukocyte adhesion

A well-functioning vascular access is necessary for achieving adequate hemodialysis (HD) in patients with end stage renal failure. Approximately 17% to 25% of HD patient hospitalizations in the United States arise from vascular access complications, with an annual cost of more than 1 billion US dollars, which continues to grow.[1] Nearly 85% of vascular access failures arise from thromboses, more than 80% of which are associated with stenoses,[2] which usually presents with inadequate blood flow.[3,4] The causative factors of vascular access stenosis include small diameter of artery and vein, surgical technique, previous venipunctures, hemodynamic stress, and the presence of accessory veins.[5] Moreover, many factors leading to endothelial dysfunction, such as oxidative stress, hyperhomocysteinemia, platelet activation, and inflammation are associated with arteriovenous fistula (AVF) failure.[5]

The histological features of vascular access stenosis comprise vascular inflammation, intimal hyperplasia, and excessive accumulation of extracellular matrix.[5] Despite these findings, the cause of stenoses remains unknown in a significant proportion of HD patients. This interindividual variation may relate to differences in the genetic background that may influence susceptibility to vascular inflammation and neointima formation after endothelial and smooth muscle injury.[6,7] Consistent with this notion, AVF survival was reported to be associated with specific genetic polymorphisms of transforming growth factor (TGF)-β1[8] and methylene tetrahydrofolate reductase.[9] In addition, we recently identified a length polymorphism in the heme oxygenase-1 (HO-1) gene that is associated with patency of AVFs.[10] HO-1 catalyzes the oxidative degradation of heme into equimolar amounts of biliverdin, free iron, and carbon monoxide (CO) (see references 11–14). Biliverdin is subsequently metabolized to bilirubin by the enzyme biliverdin reductase. Induction of HO-1 elicits potent antiinflammatory, antiproliferative, antithrombotic, and antioxidant effects in the circulation via the generation of CO and bilirubin. Interestingly, we found that a long guanidine thymidine dinucleotide repeat [$(GT)_n \geq 30$] in the HO-1 promoter, which is linked to impaired inducibility,[11] is associated with a higher frequency of access failure and diminished patency of AVFs in HD patients, suggesting a critical role for HO-1 in governing the survival of AVFs.[10]

Original received July 24, 2007; final version accepted January 3, 2008.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.107.160085**

Far infrared (FIR) radiation is an invisible electromagnetic wave with a characteristic wavelength between 5.6 and 1000 μm that can be perceived as heat by thermo-receptors in the skin.[15,16] Recent studies indicate that FIR therapy exerts beneficial effects in the cardiovascular system. FIR radiation improves ventricular arrhythmias and endothelial function in patients with heart disease.[17,18] In addition, FIR radiation promotes microvascular blood flow and angiogenesis in various animal models.[19,20] Moreover, we recently demonstrated that FIR therapy improves access flow and patency of AVFs in HD patients.[21] However, the mechanism by which FIR radiation exerts these favorable effects is not known.

In the present study, we investigated the effect of FIR radiation on HO-1 gene expression and inflammation in human endothelial cells (ECs), In particular, we determined whether FIR radiation modulates the expression of inflammatory proteins and the adhesion of monocytes to the surface of ECs, and the potential involvement of HO-1 in mediating the antiinflammatory action of FIR therapy. In addition, we explored the effect of FIR therapy on circulating inflammatory markers in patients after a single HD session.

Methods

Human umbilical vein ECs (HUVECs) were exposed to FIR radiation using a WS TY101 FIR emitter (WS Far Infrared Medical Technology Co Ltd). This device generates electromagnetic waves with wavelengths between 3 and 25 μm (peak 5 to 6 μm). The radiator was set at a height of 25 cm above the bottom of the tissue culture plates and cells were exposed to FIR radiation for various times (0 to 40 minutes). The effect of FIR radiation on HO-1 or NF-E2-related factor-2 (Nrf2) protein and mRNA expression were quantified by Western and Northern blotting, respectively, whereas the action of FIR radiation on HO-1 promoter activity was determined using wild-type and mutant HO-1 promoter/firefly luciferase constructs. Vascular inflammation was assessed by measuring the expression of endothelial adhesion receptor molecules, the endothelial production of chemokines, and the adhesion of monocytes to HUVECs. Finally, the antiinflammatory effect of FIR was also investigated in patients undergoing HD. For complete details of Material and Methods please see Supplemental Materials and Methods (available online at http://atvb.ahajournals.org).

Results

FIR Therapy Stimulates HO-1 Expression via the Nrf2/ARE Complex in Human ECs

Treatment of HUVECs with FIR radiation for 40 minutes stimulated a time-dependent increase in HO-1 protein. A significant increase in HO-1 protein was observed after 2 hours of FIR radiation and protein levels returned to basal levels by 48 hours (Figure 1A). The induction of HO-1 protein by FIR radiation was also dependent on the duration of FIR exposure. Increasing the duration of FIR exposure from 10 to 40 minutes resulted in a progressive increase in HO-1 protein (Figure 1B). In addition, application of FIR radiation for 40 minutes stimulated a time-dependent increase in HO-1 mRNA beginning 1 hour after exposure (Figure 1C). The induction of HO-1 mRNA by FIR radiation was also dependent on the exposure interval (Figure 1D). Application of FIR radiation for 40 minutes had no effect on cell viability (data not shown) and did not stimulate the production of reactive oxygen species (please see supplemental Figure I). However, administration of FIR radiation for 40 minutes

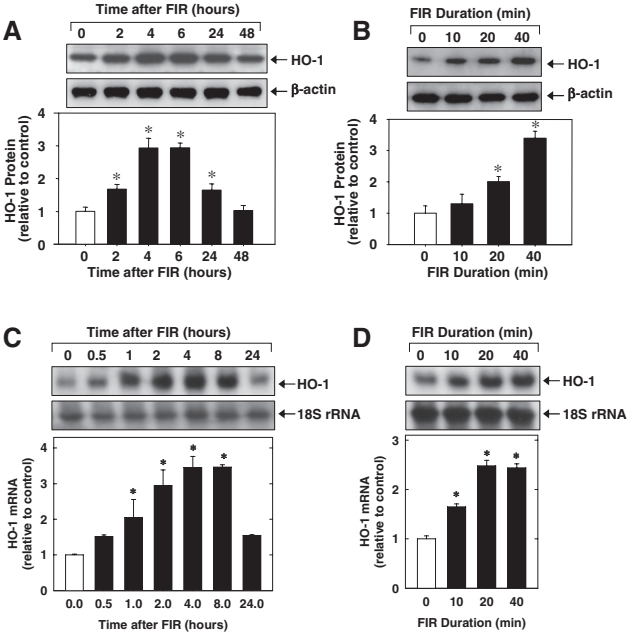

Figure 1. FIR radiation for 40 minutes stimulates HO-1 protein (A) and mRNA (C) expression in HUVECs. Duration-dependent effect of FIR radiation on HO-1 protein (B) and mRNA (D) expression in HUVECs 6 hours after FIR exposure. Results are means±SD (n=3 to 5). *Statistically significant effect of FIR therapy.

resulted in a significant elevation in temperature of the culture media (36.8±0.3°C in control media versus 39.6±0.4°C immediately after FIR radiation; n=3). Interestingly, raising the temperature from 36.8 to 39.6°C also evoked a significant increase in HO-1 expression in HUVECs (please see supplemental Figure IIA).

To determine whether the increased expression of HO-1 in response to FIR therapy involves the transcriptional activation of the gene, HUVECs were transiently transfected with a wild-type HO-1 promoter construct and promoter activity was monitored. Treatment of HUVECs with FIR therapy for 40 minutes stimulated more than a 2-fold increase in HO-1 promoter (E1) activity (Figure 2A). However, mutation of the ARE (M739) markedly attenuated basal HO-1 promoter activity and abolished the response to FIR radiation, suggesting that FIR exposure activates HO-1 transcription via the ARE. Because the transcription factor Nrf2 appears crucial for ARE-mediated gene expression, we determined whether Nrf2 was involved. Indeed, transfection of HUVECs with a dominant-negative Nrf2 mutant (dnNrf2) suppressed basal activity by more than 50% and blocked the induction of HO-1 promoter activity by FIR (Figure 2A). Furthermore, 40 minutes of FIR therapy resulted in a rapid time-dependent increase in Nrf2 protein (Figure 2B). A significant increase in Nrf2 protein was observed only 30 minutes after FIR exposure and Nrf2 levels progressively increased for up to 6 hours after FIR exposure. In addition, the induction of Nrf2 protein expression was dependent on the duration of FIR exposure, with longer exposures demonstrating a progressive increase in Nrf2 protein (Figure 2C). Interestingly, raising the temperature of the culture media from 36.8 to 39.6°C also stimulated

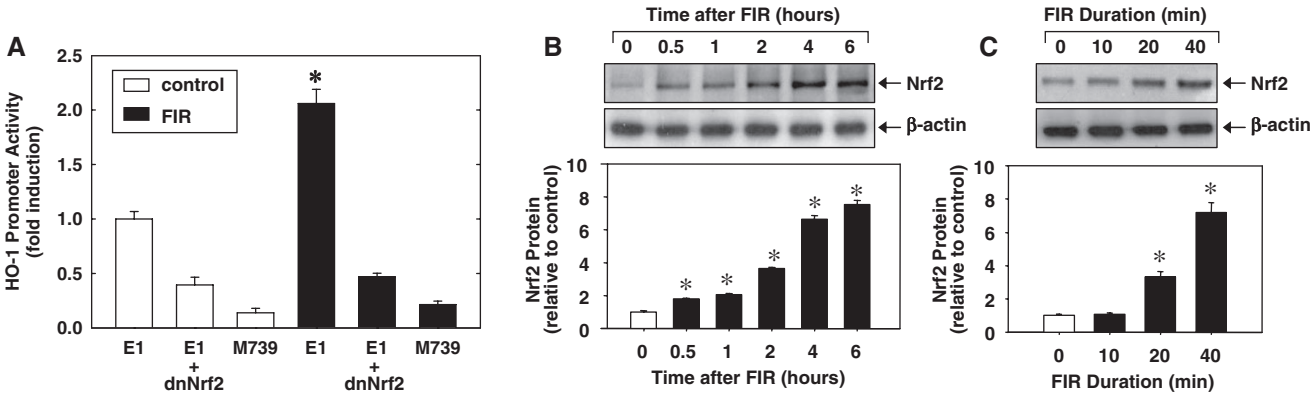

Figure 2. FIR radiation for 40 minutes stimulates HO-1 promoter activity in HUVECs 6 hours after FIR exposure (A). FIR radiation for 40 minutes stimulates Nrf2 protein expression (B). Duration-dependent effect of FIR radiation on Nrf2 protein expression (C). Results are means±SD (n=3 to 6). *Statistically significant effect of FIR therapy.

a time-dependent increase in Nrf2 protein (please see supplemental Figure IIB).

FIR Therapy Inhibits TNF-α-Stimulated Inflammatory Protein Expression

Treatment of HUVECs with TNF-α (100 ng/mL) stimulated a time-dependent increase in the expression of the adhesion molecules: E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 (Figure 3A). Application of FIR therapy for 40 minutes suppressed the cytokine-mediated induction of the adhesion molecules (Figure 3A). Optimal inhibition of adhesion molecules by FIR was observed at 4 hours for E-selectin, at 6 hours for VCAM-1, and at 24 hours for ICAM-1. The effect of FIR therapy duration on individual adhesion proteins was examined at their optimally inhibited times and revealed that protein expression for all 3 adhesion molecules was maximally inhibited by 40 minutes of FIR radiation (Figure 3B). In addition, treatment of HUVECs with TNF-α (100 ng/mL) for 6 hours stimulated an increase in the production of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) that was blocked by FIR therapy in a duration-dependent manner (Figure 3C and 3D).

FIR Therapy Suppresses TNF-α-Stimulated VCAM-1 Protein Expression and Monocyte Adhesion to Human ECs via the Induction of HO-1

In subsequent experiments, we explored whether the induction of HO-1 is involved in mediating the inhibitory effects of FIR therapy on adhesive protein expression. Because FIR radiation markedly inhibited VCAM-1 expression at nearly all time points studied, we focused on this particular adhesion molecule. Consistent with our earlier results, we found that FIR therapy for 40 minutes markedly suppressed the TNF-α (100 ng/mL for 6 hours)-mediated induction of VCAM-1 (please see supplemental Figure III). However, treatment of HUVECs with the HO inhibitor SnPP (10 or 20 μmol/L) reversed the inhibitory effect of FIR radiation in a concentration-dependent manner. Significantly, SnPP did not induce the expression of VCAM-1 in the absence of TNF-α and slightly, but not significantly, increased the induction of VCAM-1 by TNF-α.

The physiological significance of FIR therapy mediated inhibition of HUVEC adhesion molecule expression was examined by determining the effect of FIR radiation on monocyte adhesion. FIR therapy, SnPP, HO-1 siRNA, or the control nontargeting oligonucleotide minimally affected monocyte adhesion in the absence of TNF-α. Treatment of HUVECs with TNF-α (100 ng/mL) for 6 hours stimulated a nearly 3-fold increase in monocyte adhesion which was potentiated by SnPP (20 μmol/L) or by treating HUVECs with the HO-1 siRNA (0.1 μmol/L) for 2 days but unaffected by the nontargeting siRNA (NT siRNA; 0.1 μmol/L) (Figure 4A). Application of FIR therapy for 40 minutes significantly blocked cytokine-stimulated monocyte adhesion to HUVECs (Figure 4A). Similarly, the TNF-α-mediated adhesion of monocytes to human aortic endothelial cells (HAECs) was blocked by FIR radiation, indicating that the antiinflammatory effect of FIR was not unique to venous endothelium (please see supplemental Figure IV). However, treatment of ECs with SnPP (20 μmol/L) or pretreatment of cells with the HO-1 siRNA (0.1 μmol/L) fully reversed the inhibitory effect of FIR radiation (Figure 4A). In contrast, the NT siRNA (0.1 μmol/L) failed to block the antiadhesive effect of FIR therapy. Figure 4B shows relative HO-1 protein expression in HUVECs exposed to various treatment regimens. In comparison with control cells, HO-1 expression was elevated approximately 3-fold in HUVECs treated with FIR radiation for 40 minutes and 2-fold in cells treated with TNF-α (100 ng/mL for 6 hours). Treatment of cells with the combination of FIR and TNF-α resulted in an additive increase (approximately 4-fold) in HO-1 protein. In addition, treatment of HUVECs with the HO-1 siRNA (0.1 μmol/L) suppressed the increase in HO-1 protein expression evoked by the combined treatment of cells with FIR radiation and TNF-α. In contrast, the NT siRNA (0.1 μmol/L) had no effect on HO-1 expression in response to FIR therapy and TNF-α. The HO-1 siRNA also abolished the basal expression of HO-1 whereas the NT siRNA had no effect on HO-1 expression, confirming the selectivity of our HO-1 knockdown approach.

Exogenous Administration of Bilirubin Mimics the Anti-inflammatory Action of FIR Therapy

We also determined which of the HO-1 products mediates the anti-inflammatory action of HO-1. Incubation of HUVECs

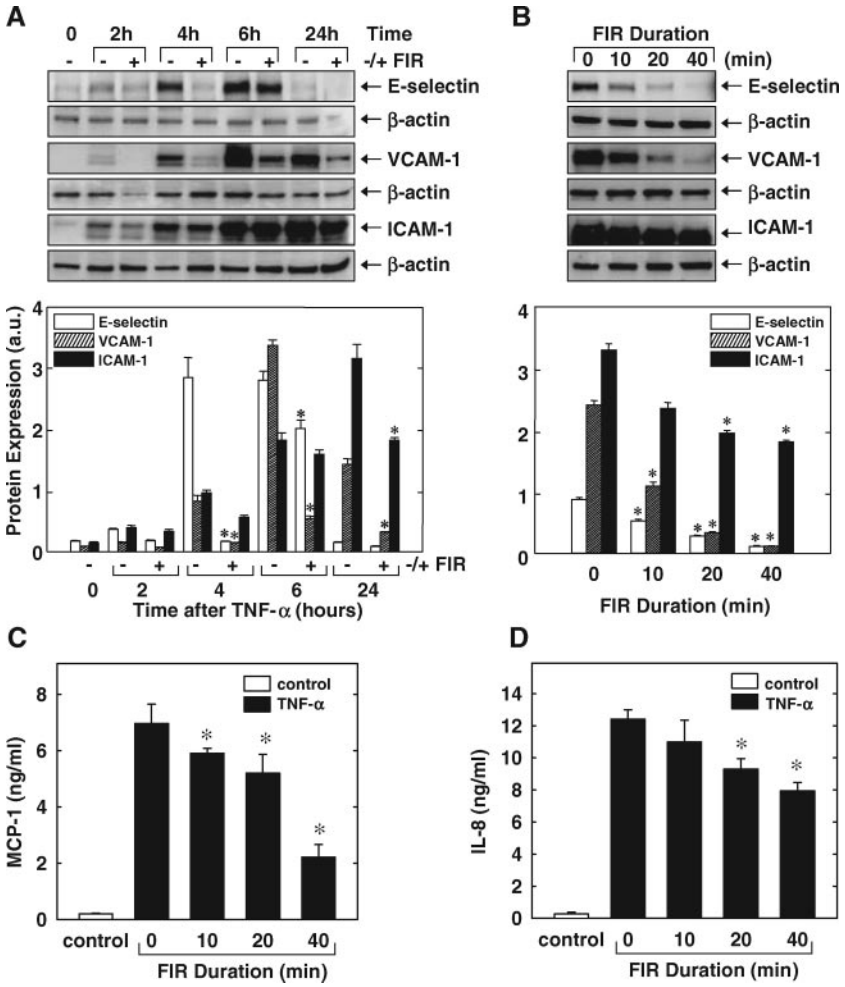

Figure 3. FIR radiation for 40 minutes inhibits TNF-α (100 ng/mL)-stimulated adhesion receptor expression in HUVECs (A). Duration-dependent effect of FIR radiation on adhesion receptor expression (B). FIR radiation inhibits TNF-α (100 ng/mL for 6 hours)-stimulated MCP-1 (C) and IL-8 (D) production. Results are means±SD (n=3 to 4). *Statistically significant effect of FIR radiation.

with bilirubin (BV; 20 μmol/L) significantly suppressed TNF-α (100 ng/mL for 6 hours)-stimulated monocyte adhesion (Figure 4C). In contrast, the addition of CO (500 ppm) or free iron (Fe; 20 μmol/L) failed to block cytokine-mediated monocyte adhesion (Figure 4C). In the absence of TNF-α, the HO-1 products had no effect on monocyte adhesion (data not shown).

FIR Therapy Exerts an Anti-inflammatory Effect in HD Patients

Finally, we examined whether FIR radiation could also evoke an anti-inflammatory effect in vivo. A prospective, randomized, controlled clinical trial involving 20 Chinese HD patients was conducted. Patient characteristics were as follows: 13 males and 7 females, average age of 66.5±9.1 years, HD duration of 91.2±63.8 months. The distribution of the underlying cause of end stage renal disease for these patients includes diabetes mellitus for 8, chronic glomerulonephritis for 6, hypertension for 4, IgA nephropathy for 1, and unknown for 1 patient.

Circulating levels of inflammatory markers before and after a single HD session as well as changes in inflammatory marker concentration are shown in the Table. Consistent with previous reports showing that HD can provoke an inflammatory response,[22–24] a significant increase in hypersensitive C-reactive protein (hsCRP), soluble ICAM-1 (sICAM-1), and soluble VCAM-1 (sVCAM-1) was observed in patients after a single HD session that lasted for 4 hours. However, significant elevations in only sICAM-1 and sVCAM-1 were noted in patients that were exposed to 40 minutes of FIR therapy. Interestingly, the concentration of sVCAM-1 after HD was significantly lower in patients treated with FIR therapy. Moreover, the incremental change in serum concentration of all 3 inflammatory markers after a single HD session with FIR therapy was significantly lower than that without FIR.

Discussion

In the present study, we identified FIR therapy as a novel inducer of HO-1 gene expression in human vascular endothelium. The induction of HO-1 is dependent on the duration of FIR exposure and requires the activation of the Nrf2-ARE signaling pathway. In addition, we found that FIR therapy inhibits the expression of proinflammatory adhesion receptors and chemoattractant molecules in human ECs, and blocks the adhesion of monocytes to ECs. Moreover, we found that

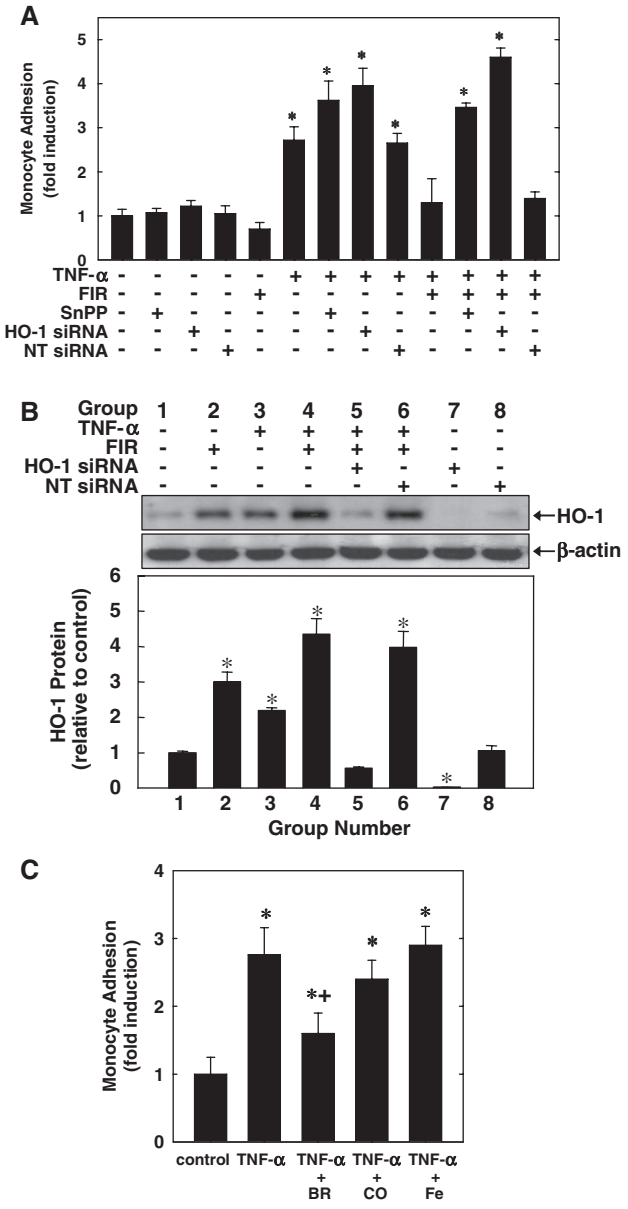

Figure 4. Monocyte adhesion (A, C) and HO-1 protein (B) in HUVECs treated with HO-1 or NT siRNA, FIR radiation, or untreated HUVECs exposed to TNF-α in the presence of SnPP, BR, CO, or Fe. Results are means±SD (n=3 to 6). *Statistically significant effect vs untreated cells. †Statistically significant effect of BR.

FIR therapy blunts the increase in circulating inflammatory markers in patients after a single HD session. These potent anti-inflammatory effects of FIR therapy are dependent on the induction of HO-1 and are mimicked by the exogenous administration of bilirubin. Thus, the ability of FIR therapy to inhibit inflammation via the HO-1–catalyzed production of bilirubin may provide an important mechanism by which FIR radiation promotes the survival of AVFs in HD patients.

Treatment of ECs with FIR radiation results in a time-dependent increase in HO-1 protein and mRNA expression that correlates with the duration of FIR exposure. Transient transfection experiments indicate that FIR radiation stimulates the transcriptional activation of the HO-1 gene. The

Table. Serum Concentrations of Inflammatory Markers for 20 HD Patients Before and After a Single HD Session With or Without FIR Therapy

	HD Session Without FIR	HD Session With FIR
hsCRP BHD, mg/L	4.10±4.07	4.63±4.32
hsCRP AHD, mg/L	4.34±4.26*	3.98±2.93
Δ (AHD-BHD) hsCRP, mg/L	0.24±0.43	−0.65±1.73†
sICAM-1 BHD, ng/mL	690±225	728±218
sICAM-1 AHD, ng/mL	886±281*	823±320*
Δ (AHD-BHD) sICAM, ng/mL	196±128	95±190†
sVCAM-1 BHD, ng/mL	1135±664	1164±676
sVCAM-1 AHD, ng/mL	1461±716*	1243±667*†
Δ (AHD-BHD) sVCAM-1, ng/mL	326±249	79±107†

hsCRP indicates hypersensitive C-reactive protein; BHD, before hemodialysis; AHD, after hemodialysis; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1.

Values are expressed as the mean±SD.

*Statistically significant effect of HD.

†Statistically significant effect of FIR therapy.

induction of HO-1 by FIR radiation is likely thermally-mediated. FIR radiation results in a modest degree of heat stress, and application of a similar amount of heat is sufficient to induce HO-1 expression in vascular endothelium. Interestingly, the activation of the HO-1 gene by FIR radiation does not occur via classical heat shock responsive elements because these elements are inactive in most mammalian species, including the mouse and human HO-1 gene.[25] Instead, we found that the induction of HO-1 requires the presence of AREs in the HO-1 promoter because mutation of these sites abolishes the stimulation of promoter activity by FIR radiation. Although many transcription factors are capable of binding to the ARE, Nrf2 appears to play a predominant role in ARE-dependent HO-1 expression.[25,26] In support of this, we found that FIR radiation stimulates the expression of Nrf2 in a manner that precedes the rise of HO-1 mRNA. In addition, we observed that transient transfection of ECs with a dominant-negative mutant of Nrf2 abrogates the activation of HO-1 promoter activity by FIR radiation. The mechanism by which FIR radiation activates Nrf2 is not known. Activation of Nrf2 is regulated by the cytosolic protein Keap1 that negatively modulates the nuclear translocation of Nrf2 and facilitates the degradation of Nrf2 via the proteasome. Interestingly, heat has been shown to inhibit proteasomal activity,[27] which can lead to the activation of Nrf2.[28] Thus, it is possible that FIR radiation activates Nrf2 via a thermally-induced decrease in proteasomal function. Consistent with this notion, we found that a similar degree of heat stress that is evoked by FIR radiation is able to increase Nrf2 protein levels.

Emerging evidence from numerous laboratories indicate that FIR therapy exerts beneficial effects in the cardiovascular system.[17–21,29,30] In the present study, we extend these findings to show that FIR radiation exerts a potent anti-inflammatory effect on vascular endothelium. We observed that FIR radiation inhibits the TNF-α-mediated expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin,

as well as the chemoattractants MCP-1 and IL-8. The expression of adhesion receptors and chemoattractant molecules is crucial in recruiting inflammatory cells to sites of active inflammation and therefore important for influencing the outcome of the inflammatory reaction. Each adhesion molecule plays a different role in modulating leukocyte-endothelial interactions. Whereas E-selectin is deemed to participate in the initial phases of rolling and attachment of leukocytes to ECs, VCAM is mainly responsible for establishing firm leukocyte adhesion to ECs, and ICAM functions to promote the transmigration of the leukocytes to the extravascular space.[31] Our finding that FIR radiation inhibits the expression of all 3 adhesive proteins, MCP-1, and IL-8 indicates the FIR therapy may modulate multiple components of the inflammatory response. The physiological significance of FIR radiation-mediated inhibition of adhesion molecule expression is underscored by our finding the FIR therapy completely suppresses the cytokine-mediated adhesion of monocytes to cultured human ECs. Interestingly, the intensity and duration of FIR radiation (40 minutes) used to inhibit endothelial adhesion molecule expression and monocyte adhesion is similar to what we recently used to improve access blood flow and patency of AVFs in hemodialysis patients.[21] Moreover, in the present study, we found that HD-induced inflammatory stress is reduced in patients treated with FIR therapy. Circulating levels of sVCAM-1 were significantly lower after a single HD session in patients exposed to FIR radiation. In addition, the increments in hsCRP, sICAM-1, and sVCAM-1 after a single HD session are reduced in patients treated with FIR therapy. Given that stenotic AVFs are associated with marked inflammatory activity consisting of abundant leukocyte infiltration and augmented ICAM-1 and VCAM-1 expression,[5,32] the ability of FIR radiation to exert potent anti-inflammatory effects may contribute to the beneficial effects observed with FIR therapy in HD patients.

The anti-inflammatory effect of FIR radiation is mediated via the induction of HO-1. We found that pharmacological inhibition of HO-1 activity completely reverses the ability of FIR radiation to suppress cytokine-mediated VCAM-1 expression. Furthermore, inhibition of HO-1 activity abolishes the antiadhesive action of FIR radiation. Similarly, the selective knockdown of HO-1 expression by HO-1 siRNA prevents the FIR radiation-mediated induction of HO-1 protein and the inhibition of monocyte adhesion. Interestingly, TNF-α also stimulates HO-1 protein expression. Moreover, the cytokine-mediated adhesion of monocytes is potentiated by inhibiting HO-1 expression or activity, suggesting that the induction of HO-1 functions in a negative feedback manner to limit cytokine-stimulated monocyte adhesion. Finally, the anti-inflammatory effect of HO-1 is likely mediated via the release of bilirubin because the exogenous administration of bilirubin mimics the antiadhesive action of HO-1 whereas the other HO-1 products had no effect on monocyte adhesion. Our finding the FIR therapy inhibits the expression of EC adhesion molecules and monocyte adhesion to endothelium via the HO-1–derived formation of bilirubin is consistent with studies demonstrating that overexpression of HO-1 or the exogenous application of bilirubin exerts an anti-inflammatory effect by blocking the activation of the transcription factor NF-κB, which is strictly required for cytokine-mediated induction of endothelial adhesion receptors.[33–36]

The ability of FIR radiation to stimulate vascular HO-1 gene expression may contribute to its therapeutic effect in maintaining the patency of AVFs in HD patients.[10] Aside from its potent antiinflammatory action, HO-1 may also preserve blood flow through the AVF by inhibiting vascular smooth muscle cell (VSMC) proliferation, platelet aggregation, and vasospasm (see references[12–14]). Furthermore, HO-1 can stimulate EC regrowth at sites of endothelial injury which further inhibits vascular stenosis by reestablishing a nonthrombogenic surface and retaining the underlying vascular smooth muscle cells in a quiescent state. Thus, the induction of HO-1 by FIR may promote the viability of AVFs by counteracting many of the causative factors that lead to AVF failure.

The induction of HO-1 may also contribute to other vasoprotective effects associated with FIR therapy. In particular, the ability of nonablative infrared laser therapy to inhibit neointimal hyperplasia after percutaneous coronary angioplasty in hypercholesterolemic rabbits[29] may also occur through HO-1 because this enzyme protects against intimal thickening in various animal models of arterial injury.[12–14] In addition, the induction of HO-1 may contribute to the antioxidant effect of FIR.[30] This latter effect may be particular relevant for uremic patients which suffer from excessive oxidative stress that is further aggravated by dialysis.[37]

In conclusion, the present study demonstrates that FIR therapy induces HO-1 gene expression in human ECs via the activation of the Nrf2/ARE complex. In addition, this study found that the induction of HO-1 contributes to the ability of FIR therapy to inhibit the expression of EC adhesion molecules and the adhesion of monocytes to vascular endothelium. The ability of FIR therapy to inhibit endothelial inflammation through the induction of HO-1 may play a critical role in maintaining the patency of AVFs in HD patients.

Sources of Funding

This work was supported by grants from the Taipei Veterans General Hospital (V95-ER2-003, V96-ER2-004, V96-B2-009), the National Science Council of Taiwan (NSC95-2314-B-075-070, NSC96-2314-B-010-045), and the National Institutes of Health of the USA (NIH R01 HL59976, NIH R01 HL74966, and NIH R01 HL62467).

Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Far Infrared Therapy Inhibits Vascular Endothelial Inflammation via the Induction of Heme Oxygenase-1

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Arterioscler Thromb Vasc Biol. 2008;28:739-745; originally published online January 17, 2008; doi: 10.1161/ATVBAHA.107.160085

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

SUPPLEMENTARY MATERIALS AND METHODS

Materials

SDS, EDTA, HEPES, Tes, streptomycin, penicillin, gelatin, M199 medium, iron, and minimum essential medium were purchased from Sigma Chemical (St. Louis, MO); tin protoporphyrin-IX and bilirubin were from Frontier Scientific Porphyrin Products (Logan, UT); a polyclonal HO-1 antibody was from StressGen Biotechnologies Inc. (Victoria, Canada); antibodies against Nrf2, vascular cell adhesion molecular-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and β-actin were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA); human recombinant TNFα was from R & D Systems (Minneapolis, MN); 5-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was from Invitrogen (Carlsbad, CA); γ-[^{32}P]ATP (3000 Ci/mmol) and [^{3}H]thymidine (90 Ci/mmol) were from NEN-Dupont (Boston, MA); [^{32}P]UTP (400 Ci/mmol) from Amersham (Arlington Heights, IL). HO-1 siRNA (sense:5'-AUGCUGAGUUCAUGAGGAAUU-3', antisense:5'-PUUCCUCAUGAACUCAGCAUUU-3') and a non-targeting siRNA (5'-AAUGGAAGACCACUCCCACUC-3') were purchased from Dharmacon Inc (Lafayette, CO).

Cell Culture

Human umbilical vein ECs (HUVEC) or human aortic ECs (HAEC) were purchased from Clonetics Corporation (Walkersville, MD) and serially cultured on gelatin-coated dishes and propagated in M199 medium supplemented with 20% bovine calf serum, 2 mM L-glutamine, 50 μg/ml endothelial cell growth factor, 90 μg/ml heparin, and 100 U/ml of penicillin and streptomycin. The cells were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C in plastic flasks and used at passage numbers 7 through 12. The human monocytic cell line U937

(American Type Culture Collection, Manassas, VA) was grown in suspension culture in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 2mM L-glutamine, 1mM sodium pyruvate, 10% fetal bovine serum, 4.5 g/L glucose, and 100 U/ml penicillin and streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C.

Far-infrared (FIR) Therapy

FIR radiation was administered using a WS^{TM} TY101 FIR emitter (WS Far Infrared Medical Technology Co., Ltd., Taipei, Taiwan). The electrified ceramic plates of this emitter generate the electromagnetic waves with the wavelengths in the range between 3 and 25 μm (a peak between 5 and 6 μm). The top radiator was set at a height of 25 cm above the bottom of the tissue culture plates and the cells were exposed to FIR radiation for various times (0-40 minutes).

Western Blotting

Cells were lysed in sample buffer (125 mM Tris [pH 6.8], 12.5% glycerol, 2% SDS, 50 mM sodium fluoride, and trace bromophenol blue) and proteins separated by SDS-PAGE. Following transfer to nitrocellulose membrane, blots were blocked with PBS and nonfat milk (5%) and then incubated with antibodies directed against HO-1 (1:1,500), Nrf2 (1:200), VCAM-1 (1:300), ICAM-1 (1:500), E-selectin (1:50) or β-actin (1:200). Membranes were then washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-mouse, or donkey anti-goat antibody and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL). The expression of HO-1 was quantified by scanning densitometry and normalized with respect to β-actin.

Northern Blotting

Total RNA (30 μg) was loaded onto 1.2% agarose gels, fractionated by electrophoresis, and blot transferred to Gene Screen Plus membranes (Perkin Elmer Life Sciences). Membranes were prehybridized for 4 hours at 68°C at in rapid hybridization buffer (Amersham) and then incubated overnight at 68°C in hybridization buffer containing [^{32}P]DNA probes (1 x 10^8 cpm) for HO-1 or 18S mRNA. DNA probes were generated by RT-PCR and labeled with [^{32}P]dCTP using a random priming kit (Amersham, Arlington Heights, IL). Following hybridization, membranes were washed, exposed to X-ray film at -70°C, and HO-1 expression quantified by scanning densitometry and normalized with respect to β-actin.

HO-1 Promoter Analysis

HO-1 promoter activity was determined using HO-1 promoter/firefly luciferase constructs (1 μg/ml) containing the wild type enhancer (E1) coupled to a minimum HO-1 promoter (E1) as well as the mutant enhancer (M739) that had its three antioxidant responsive element (ARE) core sequences mutated. In some experiments, a plasmid expressing a dominant-negative Nrf2 mutant (dnNrf2; 1 μg/ml) that had its transactivation domain deleted was used. All these constructs were generously provided by Dr. Jawed Alam at the Ochsner Clinic Foundation, New Orleans, LA. A plasmid encoding *Renilla* luciferase (hR*luc*/TK]-*Renilla* luciferase; 0.02 μg/ml) was included in all samples to control for transfection efficiency. ECs were transfected using lipofectamine, incubated for 48 hours, and then exposed to 40 minutes of FIR therapy. After an additional 6 hours incubation, ECs were collected, lysed, and luciferase activity measured using the Promega Dual-Light assay system and a Glomax luminometer (Promega, Madison, WI). Firefly luciferase activity was normalized with respect to *Renilla* luciferase

activity, and this ratio was expressed as fold induction over control cells.

Reactive Oxygen Species (ROS) Measurement

The cell permeable probe CM-H_2DCFDA was used to assess ROS production. The dye (5μM) was preloaded to cells grown on glass coverslips and incubated for 30 minutes at 37°C. The cells were then washed with PBS and either exposed to FIR radiation or H_2O_2 (200μM) for 40 minutes. Cell fluorescence intensity was assessed using a Bio-Rad Radiance 2000 confocal system coupled to an inverted IX70 microscope at 200x using excitation and emission wavelengths of 480nm and 520nm, respectively.

Monocyte Chemoattractant Protein-1 (MCP-1) and Interleukin-8 (IL-8) Measurement

The concentration of the chemoattractants MCP-1 and IL-8 were quantified in the culture media using sandwich ELISAs (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Monocyte Adhesion

U937 cells (1×10^6cells/ml) were labeled with [^{3}H]thymidine (1 μCi/ml for 24 hour) and layered onto endothelial cell monolayers that had been pretreated with TNFα (100ng/ml) for 6 hours in the presence or absence of pre-treatment with FIR and/or SnPP, HO-1 siRNA, or a control non-targeting oligonucleotide. Following a one hour incubation, nonadherent monocytes were removed and radioactivity associated with adherent cells was quantified by scintillation spectrometry after lysis with 0.2% SDS/0.2 N NaOH.

CO Exposure

ECs were exposed to CO via a previously described environmental chamber. Gas from stock tanks containing 1% CO in air and 5% CO_2 in air were combined in a stainless steel mixing cyclinder and delivered into a humidified 37°C environmental chamber at a rate of 1 L/min. A CO analyzer (Interscan, Chatsworth, CA) was used to continuously measure CO levels in the chamber.

Statistical analyses

Data management and statistical analysis were done using the SPSS statistical software (version 11.0; USA). Distributions of continuous variables in groups were expressed as mean ± SD and compared by one-way analysis of variance followed by the least significant different method for post hoc multiple comparisons of the means. The level of statistical significance was taken as *P* less than 0.05.

Clinical Study

Patients were recruited from the Taipei Veterans General Hospital who met the following criteria: (*1*) are receiving 4 hours of maintenance hemodialysis (HD) therapy three times weekly for at least 6 months, (*2*) are using a native AVF as the present vascular access for more than 6 months, without interventions within the last 3 months, (3) are without fever or clinical signs of active infection, and (4) creation of AVF by cardiovascular surgeons in our hospital with the standardized surgical procedures of venous end-to-arterial side anastomosis in the upper extremity. All patients were dialyzed three times weekly on standard bicarbonate dialysate bath

(38 mEq/L HCO_3, 3.0 mEq/L Ca^{++}, 2.0 mEq/L K^+), using the volumetric-controlled dialysis delivery system under constant dialysate flow at 500 ml/min. Patients were anticoagulated by means of systemic heparin, without change of the individual bolus or maintenance dose throughout the study. This prospective, randomized, and controlled study was based on the Helsinki Declaration [edition 6, revised 2000] and was approved by the Institutional Research Board of Taipei Veterans General Hospital.

Blood samples were taken at two different time points: immediately before and after HD. A week later, blood samples were taken again immediately before HD, then 40 minutes of FIR therapy was given during HD, and finally blood samples were taken immediately after HD. The blood samples were analysed for the concentrations of (1) soluble (s)ICAM-1 and VCAM-1 by specific enzyme-linked immunosorbent assay (ELISA) (Diaclone, Besançon, France) and (2) high sensitivity C-reactive protein (hsCRP) by the IMMAGE® Immunochemistry Systems CRPH reagent with a Beckman Coulter nephelometer (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. The sensitivity is 0.1 ng/mL, 0.6 ng/mL, and 0.2mg/L for sICAM-1, sVCAM-1, and hsCRP, respectively.

Data management and statistical analysis were done using the SPSS statistical software (version 11.0; USA). Distributions of continuous variables in groups were expressed as mean ± SEM and compared by paired *t*- test. All data have been tested for normal distribution before using paired *t*-tests. A statistically significant value was *P* less than 0.05.

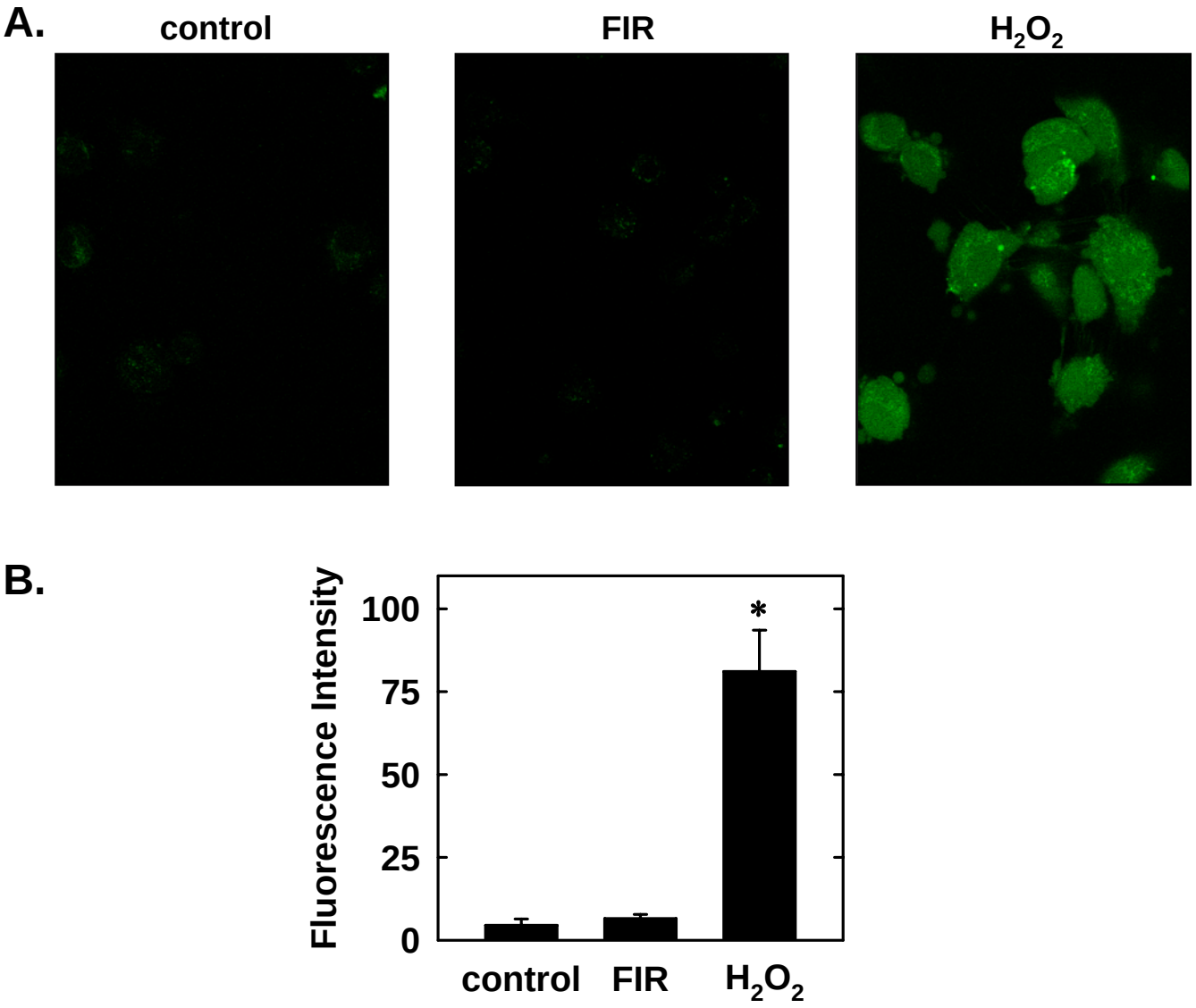

Figure I. FIR therapy fails to stimulate the production of reactive oxygen species (ROS). A. Representative images of the fluorescence of the ROS-sensitive dye CM-H_2DCFDA in HUVEC after exposure to FIR radiation or H_2O_2 (200μM) for 40 minutes. B. Quantification of CM-H_2DCFDA fluorescence intensity in response to FIR radiation or H_2O_2 (200μM) for 40 minutes. Results are means ± SD (n=6). *Statistically significant effect of H_2O_2.

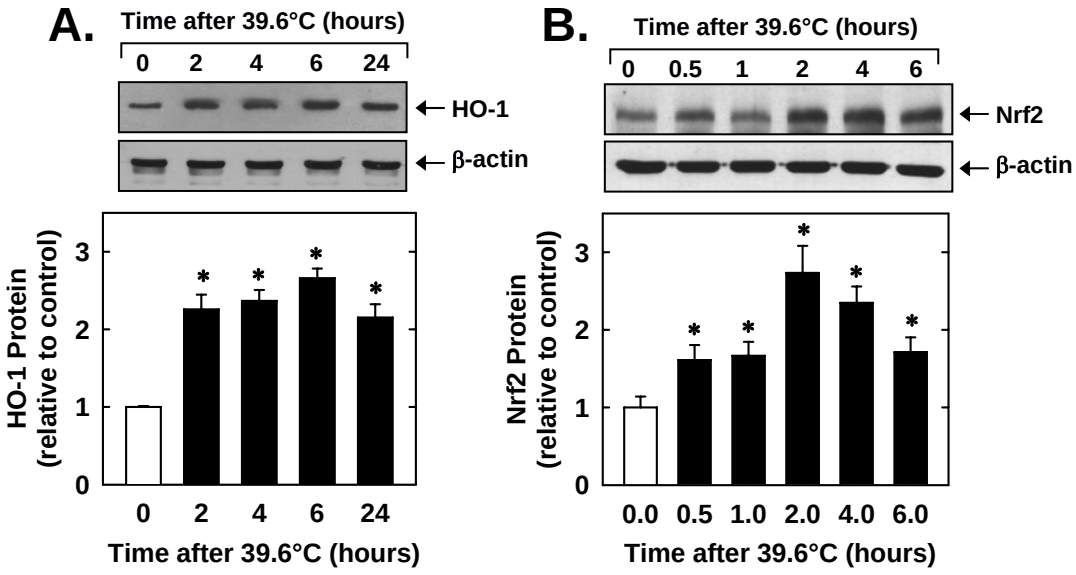

Figure II. Heat stress stimulates HO-1 (A) and Nrf2 (B) protein expression in HUVEC. The temperature of the culture media was raised from 36.8 to 39.6°C for 40 minutes and the expression of HO-1 and Nrf2 protein determined at various times (0-24 hours) after heat stress. Results are means ± SD (n=3). *Statistically significant effect of heat stress.

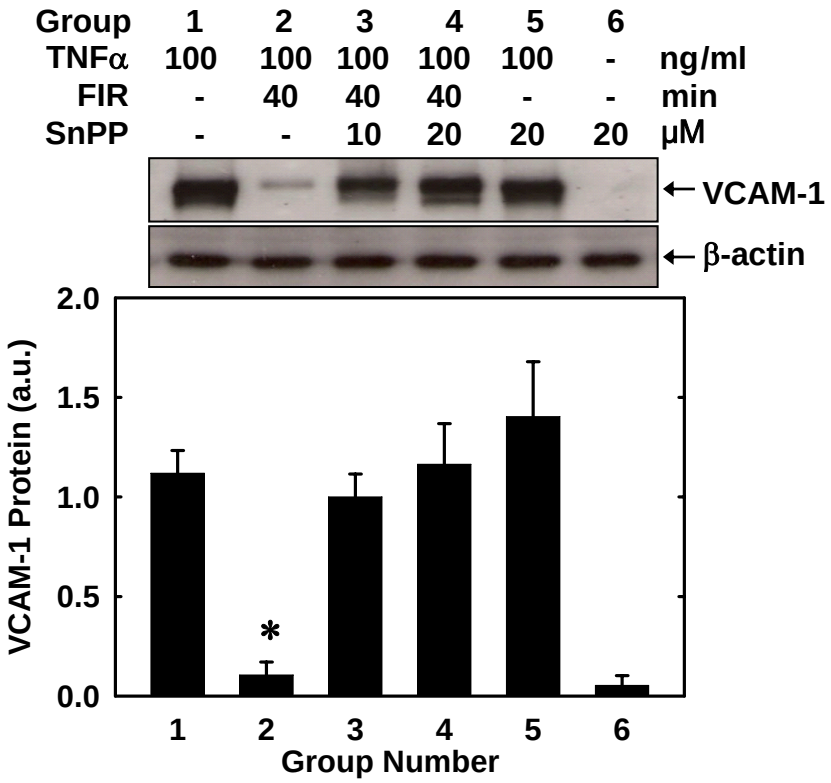

Figure III. FIR therapy inhibits TNFα-stimulated VCAM-1 expression in a HO-1-dependent manner in HUVEC. Cells were treated with TNFα (100ng/ml), FIR radiation for 40 minutes, and/or tin protoporphyrin (SnPP; 10 or 20μM) for 6 hours. VCAM-1 Protein expression was analyzed by western blotting, quantified by laser densitometry, Expressed in arbitrary units (a.u.), and normalized with respect to β-actin. Results are means ± SEM (n=3). *Statistically significant effect of FIR radiation.

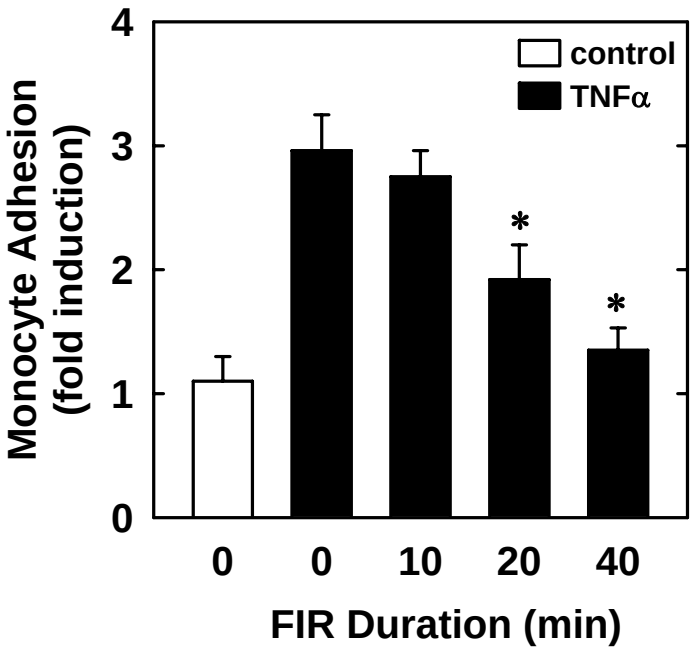

Figure IV. FIR therapy inhibits TNFα-stimulated monocyte adhesion to human aortic endothelial cells (HAEC). HAEC were treated with FIR radiation for various times (0-40 minutes) and then exposed to TNFα (100ng/ml) for 6 hours before the adhesion assay. Monocyte adhesion was quantified by scintillation spectroscopy and normalized with respect to control, untreated HAEC. Results are means ± SD (n=6). *Statistically significant effect of FIR radiation.