Thermal Treatment Attenuates Neointimal Thickening With Enhanced Expression of Heat-Shock Protein 72 and Suppression of Oxidative Stress

Motoi Okada, MD; Naoyuki Hasebe, MD, PhD; Yoshiaki Aizawa, MD, PhD; Kazuma Izawa, MD; Jun-ichi Kawabe, MD, PhD; Kenjiro Kikuchi, MD, PhD

Background—The beneficial effects of thermal therapy have been reported in several cardiovascular diseases. However, it is unknown whether the thermal treatment has some beneficial roles against the development of atherosclerosis.

Methods and Results—The inflammatory arterial lesion was introduced by placement of a polyethylene cuff on femoral arteries of male Sprague-Dawley rats for 4 weeks. Thermal-treated group underwent daily bathing in 41°C hot water for 15 minutes. Neointimal thickening along with immunohistochemical expression of heat-shock proteins (HSPs), monocyte chemoattractant protein-1 (MCP-1), and NADPH oxidase were compared with those of a thermally untreated (Control) group. Morphometric analysis demonstrated a significant suppression of neointimal thickening in thermal-treated group compared with the Control group (intimal/medial area ratios, 0.01 ± 0.01 versus 0.31 ± 0.04, P < 0.01). Expression of MCP-1 and infiltration of ED-positive cells were enhanced in the adventitial layer of Control. More importantly, expression of HSP72 in media was enhanced by thermal treatment. Expression of p22-phox, the major membrane subunit of NADPH oxidase, and MCP-1 was augmented in cuff-injured adventitia of the Control but not the thermal-treated groups.

Conclusions—Thermal treatment significantly attenuated infiltration of inflammatory cells in adventitia and suppressed neointimal thickening in cuff-injured arteries with the enhancement of HSP72 expression and suppression of oxidative stress.

Key Words: heat-shock proteins • atherosclerosis • oxidative stress

In Japan, the traditional hot-spring cure is believed to improve peripheral circulation and perfusion of organs in various diseases. Imamura et al reported that repeated sauna treatments improved vascular endothelial function as well as cardiac function in chronic heart failure patients in Japan. It has been reported that thermal treatment enhances endothelial nitric oxide production, suppresses proliferation of vascular smooth muscle cells (VSMCs), and inhibits necrosis of VSMCs. However, it has not been clarified whether the thermal therapy affects development of atherosclerosis.

Reactive oxygen species (ROS) play a major role in atherogenesis and vascular inflammatory response, including inflammatory gene expression. Recent studies have shown that NADPH oxidase enhances vascular damage, and expression of p22-phox, an essential component of NADPH oxidase, is enhanced in human atherosclerotic lesions. The role of adventitia is extremely important in vascular function. Recent study shows that the largest source of ROS is NADPH oxidase from fibroblasts in adventitia. Heat-shock proteins (HSPs) are known as important endogenous cell-protective proteins induced in response to a wide variety of stresses. In particular, thermal treatment induced HSPs, suppressed macrophage activation, and attenuated cytokine production. Thermal treatment is protective against myocardial ischemia/reperfusion injury and is associated with proportional induction of HSP72 expression. Yamashita et al reported that thermal treatment increased antioxidative activity with relation to HSP72 in myocardium.

Surgically placed hollow polyethylene tubes around femoral arteries induce intimal thickening. This was considered to be a site prone to atherosclerosis. The advantage of this model is that normal and intima-bearing vessels can be obtained from the same animal. Furthermore, the cuff-induced intimal thickening occurs with minimal medial smooth muscle damage under an uninterrupted endothelial cell layer.

We investigated whether thermal treatment inhibits neointimal formation in cuff-injured rat femoral artery and cultured cells and what mechanisms are involved in it.

Methods

The animals used in this study were treated in accordance with the guidelines of the Ethics Committee on Laboratory Animals of Asahikawa Medical College.

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From the First Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan.

Correspondence to Naoyuki Hasebe, MD, First Department of Medicine, Asahikawa Medical College, 2-1-1-1, Midorigaoka-Higashi, Asahikawa 078-8510, Japan. E-mail haselove@asahikawa-med.ac.jp

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Cuff Implantation
We performed cuff implantation to cause intimal thickening as described previously. Briefly, male Sprague-Dawley rats (10 weeks old, 250 g; Charles River Co, Tokyo, Japan) were kept on a normal chow diet. All surgical procedures were performed under general anesthesia by sodium pentobarbital (40 mg/kg IP). The right femoral artery was surgically exposed and cleaned of connective tissue along a 10-mm length. A hollow polyethylene tube (length, 5 mm; external diameter along bore, 1.57 mm; internal diameter at ends, 1.14 mm; Intramedic PE160, Becton Dickinson) was loosely placed around the artery. Muscle, fat, and skin layers were sutured, and then, the animals were allowed to recover for 28 days.

Thermal Treatment
We operated on 20 rats randomly divided into 2 groups: a nontreated control group (n=10) and a thermal-treated group (n=10). In the thermal-treatment group, rats underwent bathing for 15 minutes in hot water every day. Water temperature was maintained between 40.5°C and 41.5°C. The average rectal temperature of rats was elevated from 35°C to 38°C at the end of the thermal treatment. The rats were then released from the bath and allowed to cool down. No deaths were observed during thermal treatment.

Tissue Harvesting
Four weeks after cuff injury, rats were irrigated from the left ventricle with PBS for 2 minutes and 4% paraformaldehyde for 2 minutes. Then, both femoral arteries were harvested, and arteries were cleaned of fat and connective tissue. Three ring segments 1 mm in width were cut out from the area covered by the cuff and from the sham-operated region of the left femoral artery as non–cuff-treated control samples. Ring segments were fixed in 4% paraformaldehyde for 16 hours at 4°C, 10% sucrose in PBS for 4 hours, 20% sucrose in PBS for 4 hours, and 20% sucrose and 5% glycerol in PBS for 1 hour and embedded in OCT compound.

All samples in the present study were classified into the following 4 groups: non–cuff- and non–thermal-treated group, C(−)T(−); cuff-treated but non–thermal-treated group, C(+)-T(−); non–cuff- but thermal-treated group, C(−)-T(+); and cuff- and thermal-treated group, C(+)-T(+).

Measurement of Neointimal Formation
Morphometric analysis was performed as described previously with minor modification. To measure thickness on intimal and medial areas of injured femoral arteries, 3 cross sections of each artery spaced at 1-mm intervals were stained with hematoxylin and eosin. The cross-sectional intimal and medial areas of each lesion in a given photomicrograph were determined with image analysis software (NIH IMAGE, NIH Research Service Branch). Then, the ratio of average intimal area to medial area was calculated for each artery. The histological measurements were performed by at least 2 people blinded to treatment.

Immunohistochemical Staining
Immunohistochemical staining was performed with the following primary antibodies: mouse monoclonal anti-ED-1, anti-ED-2, anti-ED-3 antibodies (Biomedicalals AG, 1:500); goat polyclonal anti-rat monocyte chemoattractant protein-1 (MCP-1) antibody (Santa Cruz Biotechnology, 1:100); goat polyclonal anti-rat p22-phox (Santa Cruz Biotechnology, 1:200); and goat monoclonal anti-mouse HSP72 antibody (Stressgen, 1:200). Secondary antibodies (Cruz Biotechnology, 1:200); goat polyclonal anti-rat p22-phox (Santa Cruz Biotechnology, 1:200); and goat monoclonal anti-mouse HSP72 antibody (Stressgen, 1:200) were used for immunostaining of monocytes/macrophages, MCP-1, NADPH oxidase, and HSP72, respectively. Control samples were incubated with a goat nonimmunoglobulin antibody at the same dilution as the experimental conditions.

Figure 1. Transverse sections of arteries 4 weeks after cuff injury (top, hematoxylin and eosin staining; magnification ×400). A, Cuff-treated but non–thermal-treated [C(+)-T(−)] group. B, Cuff-treated and thermal-treated [C(+)-T(+)] group. Neointimal thickening was apparently reduced in thermal-treated group. Arrowheads and arrows indicate internal and external elastic lamina, respectively. Bars=25 μm. Effect of thermal treatment on neointimal regression (bottom). C, Ratios of intimal to medial areas (I/M ratio) in C(+)-T(+) group were significantly smaller than those of C(+)-T(−) group. D, Open bars indicate medial area and closed bars indicate neointimal area in cuff-injured artery [C(+)] groups, both non–thermal-treated [T(−)] and thermal-treated [T(+)] groups. Area of neointima was significantly smaller in C(+)-T(+) group. Data represent mean±SEM (n=8 each). *P<0.01 vs C(+)-T(−).
Figure 2. Immunohistochemical stainings using anti-ED-1, ED-2, and ED-3 antibodies. A, Representative high-magnification photomicrographs (×200). Top, Cuff-injured but non-thermal-treated [C(+)]T(−)] and bottom, cuff-injured and thermal-treated [C(+)]T(+) arteries. Accumulation of ED-1, -2, and -3-positive cells in C(+)]T(−)] was marked, particularly around adventitia, but was apparently suppressed in C(+)]T(+). Bars=25 μm. B, ED-1−, -2−, and -3-positive nucleated cells were counted and averaged per 3 cross sections for each sample at a magnification of ×400 (n=4 each). Accumulation of ED-positive cells in adventitia was significantly suppressed in C(+)]T(+) group. Values are expressed as mean±SEM. *P<0.05 vs C(+)]T(−).

mune IgG. The sections were incubated with the biotinylated secondary antibody and visualized by avidin-biotin–conjugated methods. Nuclei were counterstained by 0.5% methyl green.

Immunofluorescence was performed with horseradish peroxidase–conjugated anti-goat IgG after primary antibody incubation and 30 minutes of amplification with biotinyl tyramide for 10 minutes and fluorescence visualization with avidin–Alexa Fluor 488 conjugate (Molecular Probes Inc) for 30 minutes at room temperature. Green fluorescence was observed with a 505-nm-long pass emission filter under 488-nm laser illumination with an Olympus IX70 fluorescence. Representative high-magnification photomicrographs (×100) were taken. A, Representative high-magnification photomicrographs (×200). Top, Cuff-injured but non-thermal-treated [C(+)]T(−)] and bottom, cuff-injured and thermal-treated [C(+)]T(+) arteries. MCP-1 expression in C(+)]T(−)] was marked, particularly around adventitia, but was apparently suppressed in C(+)]T(+). Bars=100 μm.

Figure 3. Immunohistochemical staining using anti-MCP-1 antibody. Representative high-magnification photomicrographs (×100) of (A) cuff-injured but non-thermal-treated [C(+)]T(−)] and (B) cuff-injured and thermal-treated [C(+)]T(+) arteries. MCP-1 expression in C(+)]T(−)] was marked, particularly around adventitia, but was apparently suppressed in C(+)]T(+). Bars=100 μm.

Statistical Analysis

All results are expressed as mean±SEM. Statistical comparisons were made by 1- or 2-way repeated-measures ANOVA with Bonferroni corrections and by paired t tests where appropriate, respectively. A probability value of P<0.05 was considered statistically significant.

Results

Inhibitory Effect of Thermal Treatment on Intimal Thickening

Four weeks after cuff injury, neointima was formed in the subendothelial layers in C(+)]T(−)] group (Figure 1A). Daily thermal treatment produced a marked reduction in intimal thickening in C(+)]T(+) group (Figure 1B). No neointimal thickening was observed in non-cuff-treated groups [C(−)]T(−)), C(−)]T(+)].

Results quantified by morphometric analysis are shown in Figure 1, C and D. Thermal treatment significantly inhibited intimal thickening, but morphologically, no remarkable differences were observed in the segment between the C(−)]T(−)) and C(−)]T(+) groups (Figure 1C). In contrast, the medial area was not affected by the thermal treatment (Figure 1D).

Localization of ED-Positive Cells

Immunohistochemical staining shows that the ED-1−, -2−, and -3-positive cells, which represent a marker for monocytes/macrophages, were infiltrated, particularly in the adventitia of cuff-injured arteries of the C(+)T(−) group (Figure 2A). However, all isoforms of ED-positive cells were less frequently observed in thermal-treated arteries of the C(+)T(+) group (Figure 2A). The number of ED-1−, -2−, and -3-positive cells was counted as 25.8±21.9, 156.3±15.2, and 190.5±29.3 (per 25 μm² of adventitia) in the C(+)T(−) group, respectively. Conversely, in the thermal-treated group, the ED-1−, -2−, and -3-positive cells

MCP-1 Concentrations

The MCP-1 concentrations of the culture media were determined with ELISA kits according to the manufacturer’s instructions (Amersham International Plc). The lower limits of detection were MCP-1, 51 pg/mL.

Cell Culture and Treatments

Fibroblasts were prepared from thoracic aorta of 5-week-old Sprague-Dawley rats by the explant technique. Fibroblasts were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS). Fibroblasts were grown to subconfluence and made quiescent by incubation in medium containing 0.2% FBS and 0.02% BSA. Fibroblasts were incubated in 35-mm culture dishes. Thermal treatment was performed by setting the culture dish on a hot plate (41°C) for 15 minutes. For all experiments, passages 6 to 9 of subcultured cells were used.
Immunohistochemical staining shows that the expression of HSP72 was not evident in non–thermal-treated arteries (Figure 4, A and B). In contrast, the expression of HSP72 was markedly enhanced in the adventitia and media of thermal-treated arteries (Figure 4, C and D). This thermal induction of HSP72 expression was not affected by cuff treatment. HSP72 expression in media of the thermal-treated artery was observed even after only 1 bathing, and its expression was observed continuously for at least 72 hours after bathing (data not shown).

Localization of HSP72
Immunohistochemical staining shows that p22-phox, a major component of NADPH oxidase, was detected in adventitia of cuff-injured arteries of the C(+)/T(−) group (Figure 5A). In contrast, we could hardly see the expression of p22-phox in thermal-treated arteries of the C(+)/H(+) group or non–cuff-treated arteries of the C(−)/T(+) group (Figure 5B).

Discussion
This study demonstrated for the first time that thermal treatment attenuated neointimal thickening in the cuff-injured artery model. Its major mechanisms appeared to be mediated by enhanced expression of HSP72 and suppression of oxidative stress.

Cuff injury produces a diffuse intimal thickening of artery that is similar to early lesions of atherosclerosis. Huth et al speculated that medial and adventitial hypoxia induced by closing of the vasa vasorum triggered the atherosclerotic process in this model. The exact mechanism of intimal thickening in this model remains unclear. Several potential factors have been suggested: response to inflammation, hypoxia resulting from obstruction of the vasa vasorum feeding the media, loss of the perivascular innervation, and changes in blood flow velocity even though the cuff is nonocclusive.

It has been generally accepted that adhesion and migration of monocytes/macrophages in the intima, rather than in the adventitia, play an important role in the initiation of atherosclerosis. Therefore, the cuff injury model, which has the primary lesion in adventitia, does not seem to fully represent the developmental process of atherosclerosis. We also appreciate the importance of inflammatory changes in the intima and have conducted previous studies using a balloon-injury model. We believe, however, that adventitial inflammation also plays an important role in the atherosclerotic process. Impairment of adventitial function is one of the potential initiators of atherosclerosis.

Infiltration of inflammatory cells is frequently observed in the adventitia of atherosclerotic arteries. Neutrophils and macrophages accumulated in the adventitia surrounding adipose tissues after angioplastic surgery. We found massively accumulated ED-1−, -2−, and -3+ positive cells at the adventitia of cuff-injured artery. More importantly, thermal treatment dramatically attenuated accumulation of ED-positive cells in adventitia.

Accumulated macrophages in the adventitia play an important role in the development of atherosclerotic lesions. Perivascular inflammatory cytokines induce infiltration of macrophages in the adventitia and facilitate neointimal formation. Miyata et al reported that accumulated macrophages moved from the adventitia toward the intima through...
Atherosclerosis emerges through the response to inflammatory stimulation. It takes a long time to develop the lesion. Cuff injury caused neointimal thickening through adventitial inflammation. It is well known that endothelial dysfunction or endothelial damage is one of the major mechanisms of progression of atherosclerosis. Neointimal formation progresses not only via adventitial inflammation. It is well known that endothelial dysfunction or endothelial damage is one of the major mechanisms of progression of atherosclerosis. Next, we confirmed a marked expression of HSP72 in the media of thermal-treated cuff-injured arteries. This intriguing fact may indicate a crucial role of HSP72 as a barrier against activation and migration of inflammatory cells into the intima. We supposed that the induced HSP72 preserved adventitial function, attenuated the medial VSMC migration, and suppressed inflammatory cell transfer into the media. Gorman et al reported a cross-linkage of HSP induction and ROS production leading to suppression of damage in cultured cells. Thermal treatment exclusively induced HSPs, suppressed oxidative stress, and potentially attenuated cellular damage in cuff-injured arteries.

Neschis et al demonstrated that thermal preconditioning inhibited neointimal formation in balloon-injured arteries. They applied hyperthermia only once to rats 8 hours before balloon injury and observed a significant reduction of intimal/medial area ratios after 14 and 90 days. In our preliminary study, 1 day of thermal preconditioning could not produce any significant difference in neointimal formation 2 weeks later in the cuff-injured artery. We also confirmed that enhanced expression of HSP72 did not last longer than 72 hours after a single thermal treatment (data not shown). Therefore, we elected a daily thermal treatment for 4 weeks to maintain enhanced HSP expression levels in cuff-injured artery. This suggests differences in the mechanisms of development of neointimal lesions and the role of HSP72 between cuff and balloon injured arteries.

Thermal treatment resulting in expression of HSPs is a potential intervention for the acquisition of arterial protection. Clinical application of this therapeutic concept is not difficult by taking baths. However, the interaction among HSP levels, ROS production, and progression of atherosclerosis is complex; thus, further investigation is required.

Atherosclerosis emerges through the response to inflammatory stimulation. It takes a long time to develop the lesion. Cuff injury caused neointimal thickening through adventitial dysfunction. However, neointimal formation progresses not only via adventitial inflammation. It is well known that endothelial dysfunction or endothelial damage is one of the major mechanisms of progression of atherosclerosis.
we would like to evaluate the long-term effect of adventitial inflammation and the effect of thermal treatment on endothelial function.

In conclusion, thermal treatment has a significant beneficial effect to protect vasculogenesis through the attenuation of accumulation of inflammatory cells in adventitia and suppresses neointimal thickening in cuff-injured arteries with the enhancement of HSP72 expression and suppression of oxidative stress.

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References


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