Peripheral perfusion is impaired in patients with chronic heart failure (CHF) because of progressive peripheral vasoconstriction, which leads to increased resistance and cardiac afterload and exercise intolerance.\(^1,2\) The hemodynamic improvement produced by vasodilators, such as angiotensin-converting enzyme (ACE) inhibitors, provides great therapeutic benefit to these patients.\(^3\) Our previous study showed that taking a warm-water bath or a sauna, performed appropriately, also reduces cardiac preload and afterload, by inducing vasodilation of the systemic and pulmonary arteries and veins, in both patients with CHF and healthy subjects.\(^4\) We have investigated thermal vasodilation by sauna therapy, which is free of the effects of hydrostatic pressure, as a new nonpharmacologic therapy for patients with CHF, and have found that most patients had a good response to this therapy.\(^5\) We recently discovered that one mechanism by which sauna therapy improves the peripheral circulation is by enhancing endothelial function.\(^6,7\)

It has been previously reported that sauna therapy, a thermal therapy, improves the hemodynamics and clinical symptoms in patients with chronic heart failure and also improves endothelial function, which is impaired in such patients. The present study investigated whether the improvements observed with sauna therapy are through modulation of arterial endothelial nitric oxide synthase (eNOS) expression. Eight male Syrian golden hamsters underwent sauna therapy, using an experimental far infrared-ray dry sauna system, at 39°C for 15 min followed by 30°C for 20 min daily for 4 weeks. Control group hamsters were placed in the sauna system switched off at room temperature of 24°C for 35 min. Immunohistochemistry found greater amounts of the immunoreactive products of eNOS in the endothelial cells of the aorta and carotid, femoral and coronary arteries in the sauna group than in the control group. Western blot analysis also revealed that 4-week sauna therapy significantly increased eNOS expression in aortas by 50% in 4 series of independent experiments with an identical protocol (p<0.01). In reverse transcription polymerase chain reaction assay, the eNOS mRNA in aortas was greater in the sauna group than in controls, with a peak at 1-week of sauna therapy (approximately 40-fold increase). In conclusion, repeated thermal therapy upregulates eNOS expression in arterial endothelium. (\textit{Jpn Circ J} 2001; \textbf{65}: 434–438)

**Key Words:** Endothelial function; Gene expression; Nitric oxide; Sauna therapy; Vasodilation

### Methods

**Animals and Sauna Therapy**

Male Syrian golden hamsters (Japan SLC, Hamamatsu, Japan) underwent sauna therapy in an experimental far infrared-ray dry sauna system (Kyushu Olympia, Miyazaki, Japan) at 39°C for 15 min, and then at 30°C for 20 min. We had previously established that with this protocol the rectal temperature rises almost 1°C and remains elevated for at least 20 min, as shown in the clinical setting.\(^6\) All animals were allowed food and water ad libitum and maintained under controlled environmental conditions (24°C, 12-h light/dark cycles). The study was carried out in accordance with the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

**Experimental Protocol**

Hamsters in the sauna group had a sauna daily for 4 weeks, whereas those in the control group were placed in the sauna system switched off for 35 min (24°C). On the day after the last (28th) sauna, the hamsters were weighed, killed and aortas, carotid and femoral arteries, and hearts were collected for further analysis. Immunohistochemistry was performed as described above.

**Western Blot Analysis**

Western blot analysis was performed as described previously.\(^6\,7\) The eNOS protein expression in the aorta was detected using a rabbit polyclonal antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were probed with secondary antibodies conjugated to horseradish peroxidase, and the blots were visualized using enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK). The optical density of the bands was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

**Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay**

Total RNA was extracted from the aorta using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1000 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The PCR reactions were performed using the TaqMan Gene Expression Assays (Applied Biosystems) for eNOS (Hs00277006_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1), and the 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA levels were normalized to the GAPDH levels and then expressed as fold changes compared to the control group.
were harvested, rapidly frozen, and stored at -80°C. Four series of independent experiments were performed with the same protocol to quantify the eNOS expression by Western blot analysis.

Temperature and Hemodynamic Measurements

In a group of 5 additional hamsters, we measured the rectal temperature, using a Thermistor thermometer (Sibaura, Tokyo, Japan), and systolic and diastolic blood pressure (SBP and DBP) and heart rate, using a Millar catheter pressure transducer (Millar Instruments, Houston, TX, USA) cannulated into the right carotid artery, immediately after anesthesia with pentobarbital sodium (50 mg/kg ip). Hemodynamic parameters were recorded on a computer using the Mac Lab system (AD Instruments, Castle Hill, NSW, Australia).

Immunohistochemistry

The labeled streptavidin biotin method was performed using a Histfine kit (Nichirei, Tokyo, Japan). Briefly, cross-sections of arteries were incubated overnight with rabbit polyclonal eNOS antibodies at 4°C, diluted 1:1,000, and incubated with anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at room temperature (dilution 1:1,000) for 30 min. The bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-ray film. We confirmed that the amounts of proteins loaded on the gel were equal by Coomassie blue staining and quantified the densities of the bands by scanning densitometry using NIH image computer software (NIH, Bethesda, MD, USA).

RT-PCR Assay

Aortas were taken from each of 6 extra hamsters before the sauna therapy began and then on the day after 3-days', 1-week', 2-weeks', and 4-weeks' sauna therapy. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method described previously.15 To adjust the volume of the RNA sample, diethylpyrocarbonate-treated water was added to a total volume of 10 µl. Reactions were heated to 94°C for 150 s and then cycled as follows: 95°C for 1 min, and extension at 55°C for 1 min, and extension at 72°C for 1 min. The primer for eNOS corresponded to 5'-TACG-AGTCCTTTGAT-3' (antisense), and the primer for GAGCAGCAAATCCAC-3' (sense) and 5'-CAGGCTGCGTCACTGCTTGAGGTTATG-3' (antisense), used as a positive control, were synthesized according to sequences published previously.14,16,17 The primer for eNOS corresponded to 5'-TACGGCAGCAAATCCAC-3' (sense) and 5'-CAGGCTGCAGTCCCTTTTATGAT-3' (antisense), and the primer for -actin corresponded to 5'-GCATCCTCACCACCAATCCAC-3' (sense) and 5'-ACTCCTGATCATCCTGCTGTATGAT-3' (antisense). PCR was performed in a total volume of 50 µl containing 1 µl cDNA, 5 µl of 0.1 mol/L DTT, 5 µl of 2.5 mmol/L dNTPs, 0.5 µl of 200 U/µl reverse transcriptase (Gibco BRL) and 1 µl of ribonuclease inhibitor at 20 U/µl (Takara biochemicals) were added. Reactions were incubated at 37°C for 60 min and then at 70°C for 2 min. Then 5 µl of 5× reverse transcriptase buffer (250 mmol/L Tris-HCl at pH 8.3, 375 mmol/L KCl, and 15 mmol/L MgCl2; Gibco BRL, Grand Island, NY, USA), 2.5 µl of 0.1 mol/L DTT, 5 µl of 2.5 mmol/L dNTPs, 0.5 µl of 200 U/µl reverse transcriptase (Gibco BRL) and 1 µl of ribonuclease inhibitor at 20 U/µl (Takara biochemicals) were added. Reactions were incubated at 37°C for 60 min and then at 70°C for 2 min. Primer concentrations were adjusted according to sequences published previously16,17. The primer for eNOS corresponded to 5'-TACG-GAGCAGCAAATCCAC-3' (sense) and 5'-CAGGCTGCGTCACTGCTGTATGAT-3' (antisense), and the primer for -actin corresponded to 5'-GCATCCTCACCACCAATCCAC-3' (sense) and 5'-ACTCCTGATCATCCTGCTGTATGAT-3' (antisense). PCR was performed in a total volume of 50 µl containing 1 µl cDNA, 5 µl of 10×PCR buffer (20 mmol/L Tris-HCl at pH 8.0, 100 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5% Tween 0.5%, 200 U/µl reverse transcriptase (Gibco BRL) and 0.2 U/µl Taq polymerase. The mixed samples were heated to 94°C for 150 s and then cycled as follows: denaturation at 94°C for 60 s, primer annealing at 53°C for eNOS and 55°C for -actin for 1 min, and extension at 72°C for 1 min for 32 cycles. Final extension was at 72°C for

<table>
<thead>
<tr>
<th>Table 1 Effects of Sauna Therapy on Body and Heart Weight in Hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
</tr>
<tr>
<td>Series 1</td>
</tr>
<tr>
<td>Sauna</td>
</tr>
<tr>
<td>Series 2</td>
</tr>
<tr>
<td>Sauna</td>
</tr>
<tr>
<td>Series 3</td>
</tr>
<tr>
<td>Sauna</td>
</tr>
<tr>
<td>Series 4</td>
</tr>
<tr>
<td>Sauna</td>
</tr>
</tbody>
</table>

% gain, percentage of body weight gain; HW, whole heart weight; HW/BW, whole heart weight to body weight ratio. All values are given as mean±SD.
7 min. PCR products were subjected to electrophoresis on a 1% agarose gel and then stained with ethidium bromide. The expected size of the eNOS PCR product was 819 bp, and that of \( \beta\)-actin was 906 bp, as in a preliminary study, we had found that these PCR cycles were within the linear phase of amplification. The quantity of the product was in proportion to the amount of cDNA used. The densities of the bands of the PCR products were estimated using NIH image computer software.

**Statistical Analysis**

All values are given as the mean ± SD, and statistical significance was set at p<0.05. Unpaired t test was used for comparison between control and sauna group, and ANOVA was used for comparison of changes of hemodynamic parameters.

**Results**

**Body Weight, Temperature, and Hemodynamic Measurements**

There were no significant differences in the percentage of body weight gain and the ratio of whole heart weight to body weight between the 2 groups (Table 1). The rectal temperature of the hamsters rose by approximately 1°C following a 15-min 39°C sauna, and was maintained by a 20-min sauna at 30°C (Table 2). Heart rates did not show any change throughout the sauna nor between the first and 28th sauna. SBP and DBP did not change before or after the 1st sauna, but the post-sauna pressures were lower than the pre-sauna pressures at the 28th sauna (Table 2). Furthermore, the SBP and DBP before the 28th sauna were lower than those before the 1st sauna (Table 2).

**Immunohistochemistry**

Immunoreactive products of eNOS were identified in the endothelial cells of arteries, and immunoreactivity was stronger in hamsters given sauna therapy than in untreated controls. Only minimal amounts of immunoreactive product were detected in the media and adventitia of either group. Aorta in (A) controls, (B) the sauna group; Coronary artery in (C) controls and (D) the sauna group (×100).

**Western Blot Analysis**

Western blot analysis revealed that eNOS expression

---

**Table 2 Effects of Sauna Therapy on Hemodynamics in Hamsters**

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>During</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rectal temperature (°C)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st sauna</td>
<td>35.4±0.2</td>
<td>36.4±0.1*</td>
<td>36.2±0.1†</td>
</tr>
<tr>
<td>28th sauna</td>
<td>35.5±0.4</td>
<td>36.5±0.2†</td>
<td>36.4±0.2*</td>
</tr>
<tr>
<td><strong>Heart rate (beats/min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st sauna</td>
<td>353±17</td>
<td>364±29</td>
<td>349±18</td>
</tr>
<tr>
<td>28th sauna</td>
<td>335±31</td>
<td>336±39</td>
<td>323±25</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st sauna</td>
<td>124±4</td>
<td>123±3</td>
<td>117±16</td>
</tr>
<tr>
<td>28th sauna</td>
<td>114±6*</td>
<td>104±9†</td>
<td>92±7*‡</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st sauna</td>
<td>85±4</td>
<td>84±10</td>
<td>81±13</td>
</tr>
<tr>
<td>28th sauna</td>
<td>78±5*</td>
<td>70±10</td>
<td>63±6‡</td>
</tr>
</tbody>
</table>

Sauna therapy was performed in an experimental far infrared-ray dry sauna system at 39°C for 15 min, and then at 30°C for 20 min.

Before indicates before 39°C sauna; During, at the end of 15-min 39°C sauna; After, at the end of 20-min 30°C sauna. *p<0.05 and †p<0.01 vs 1st sauna, *p<0.05 and ‡p<0.01 vs before sauna. All values are given as mean±SD (n=5).
Thermal Therapy Upregulates eNOS

(135kDa) was greater in the particulate fraction of aortas from the sauna group than in that from the control group (Fig 2A). Quantitative densitometric analysis confirmed that eNOS protein was significantly greater in the sauna group than in the control group (109±10 vs 74±16 n=8, p<0.01, Fig 2B). There was no difference between the 2 groups in the eNOS expression in the cytosolic fraction.

**RT-PCR Assay**

We next examined the expression of eNOS mRNA using RT-PCR (Fig 3). The eNOS mRNA in aortas, expressed as a percentage of ß-actin, was greater in the sauna group than in the control group, with a peak at 1-week of sauna therapy. The amount of eNOS mRNA expression at 1-week of sauna therapy increased approximately 40-fold in comparison with that in control group by densitometric analysis.

The levels of RT-PCR products for eNOS were greater in the sauna group than in the control group. (B) Densitometric analysis revealed that eNOS expression in sauna-treated hamsters and untreated controls.

**Discussion**

The present study clarified that repeated sauna therapy upregulates eNOS protein and mRNA in the arterial endothelium by immunohistochemistry, Western blot analysis and RT-PCR assay. Several recent studies have established that the endothelium-dependent vasodilatory response is attenuated in CHF because of decreased NO production and increased degradation of NO. Patients with CHF have reduced cardiac output and decreased peripheral blood flow, resulting in a decrease in shear stress, and it is thought that these changes decrease NO production and downregulate eNOS. Smith et al have shown that eNOS protein is markedly reduced in the thoracic aorta of dogs with pacing-induced heart failure and similar results have been reported in rats with heart failure. The hemodynamic changes induced by sauna in thoracic aorta of dogs with pacing-induced heart failure have shown that eNOS protein is markedly reduced in the thoracic aorta.

This study was supported in part by a Scientific Research Grant (10858025) from the Ministry of Education, Science and Culture of Japan.

**References**


---

**Fig 2.** Western blot analysis. (A) eNOS expression was greater in the sauna group than in the control group. (B) Densitometric analysis of eNOS expression in sauna-treated hamsters and untreated controls. *p<0.05 vs control.

**Fig 3.** RT-PCR assay of eNOS and ß-actin. The eNOS mRNA expression in aortas increased by sauna therapy, whereas ß-actin mRNA expression did not change. The eNOS mRNA expression at 1-week of sauna therapy increased approximately 40-fold in comparison with that in the control group by densitometric analysis.


