

## Repeated Sauna Therapy Increases Arterial Endothelial Nitric Oxide Synthase Expression and Nitric Oxide Production in Cardiomyopathic Hamsters

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**Background** Vascular endothelial dysfunction is involved in the pathophysiology of chronic heart failure (CHF). It has been reported that sauna therapy, which allows thermal vasodilation, improves vascular endothelial dysfunction in patients with CHF. The present study investigates the mechanisms through which sauna therapy improves endothelial dysfunction induced by CHF.

**Methods and Results** Normal control and male TO-2 cardiomyopathic hamsters were used. Thirty-week-old TO-2 hamsters were treated daily with an experimental far infrared-ray dry sauna system for 15 min at 39°C followed by 20 min at 30°C. This procedure raised the rectal temperatures by about 1°C. Arterial endothelial nitric oxide (NO) synthase (eNOS) mRNA and protein expressions were examined, and serum concentrations of nitrate were measured. The expression of eNOS mRNA in the aortas of normal controls did not change, whereas those of the TO-2 hamsters decreased with age. Four weeks of sauna therapy significantly increased eNOS mRNA expression in the aortas of TO-2 hamsters compared with those that did not undergo sauna therapy. Sauna therapy also upregulated aortic eNOS protein expression. Serum nitrate concentrations of the TO-2 hamsters were increased by 4 weeks of sauna therapy compared with those that did not undergo sauna.

**Conclusion** Repeated sauna therapy increases eNOS expression and NO production in cardiomyopathic hamsters with heart failure. (Circ J 2005; 69: 722–729)

**Key Words:** Arteries; Cardiomyopathy; Endothelium; Heart failure; Nitric oxide

In patients with chronic heart failure (CHF), vascular resistance is increased through the activation of neurohumoral systems, such as the sympathetic nervous, renin-angiotensin and endothelin systems.<sup>1</sup> Vascular endothelial function, which is mainly represented by endothelium-dependent vasodilatory function through the production of vasodilators derived from the endothelium such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factors, is impaired in CHF and affects clinical symptoms.<sup>2–5</sup> Impaired vascular endothelial function leads to increased vascular tone and vascular remodeling, which reduces peripheral blood flow and oxygen delivery to the skeletal muscles, followed by progressive exercise intolerance and clinical symptoms.<sup>2–5</sup> Therapies that improve endothelial function, such as angiotensin-converting enzyme inhibitors and regular exercise, improve the clinical symptoms and prognosis in CHF.<sup>6–9</sup>

We reported that sauna therapy at 60°C induces vasodilation of the systemic and pulmonary arteries and veins, reduces cardiac preload and afterload and improves hemo-

dynamics, clinical symptoms and cardiac arrhythmia in patients with CHF.<sup>10–12</sup> We also clarified that the beneficial effects of sauna therapy for CHF are caused by improved vascular endothelial function and the normalizing of the neurohormonal systems.<sup>13</sup> Furthermore, we showed that repeated sauna therapy improved the survival of cardiomyopathic hamsters with CHF.<sup>14</sup> Vascular endothelial dysfunction in CHF is mainly due to decreased NO production induced by decreased levels of endothelial NO synthase (eNOS) expression and increased oxidative stress.<sup>15–17</sup> We have already reported that repeated sauna therapy increased arterial eNOS protein and mRNA expressions from the normal levels in healthy hamsters.<sup>18</sup> However, it is important to determine whether repeated sauna therapy could increase eNOS expression downregulated by CHF. The present study aims to investigate whether repeated sauna therapy modulates eNOS expression and NO production in CHF.

### Methods

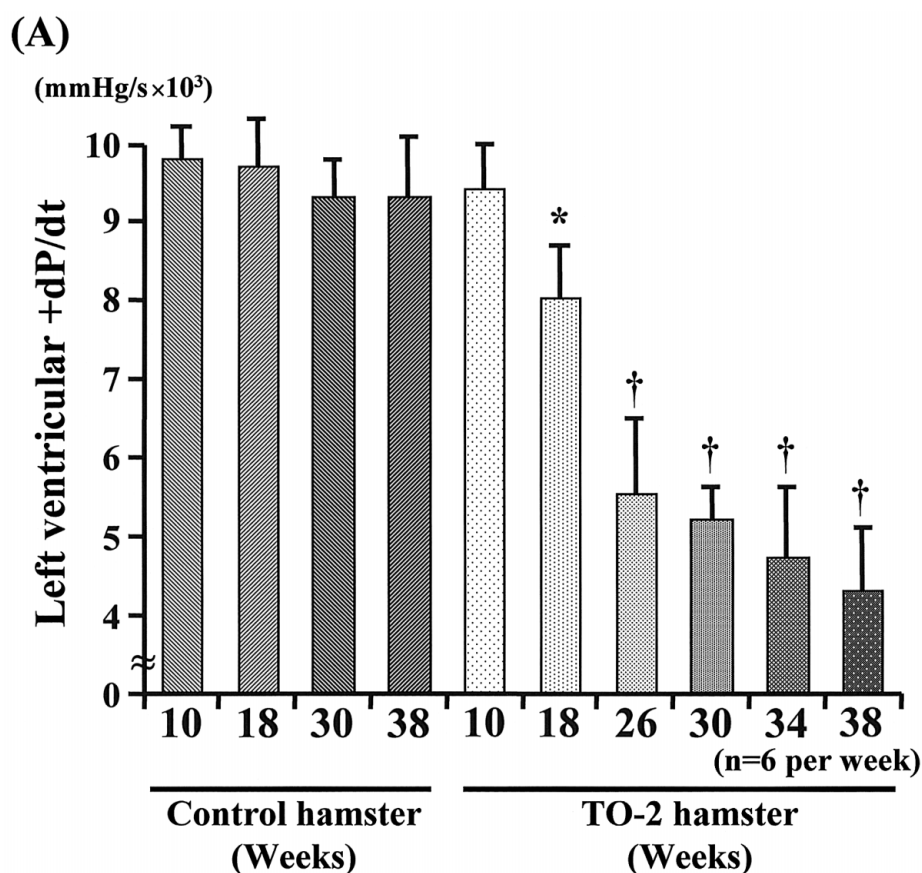
#### Animals

We used male TO-2 cardiomyopathic hamsters (Bio Breeders, Fitchburg, MA, USA) as a model of clinical dilated cardiomyopathy. These animals develop CHF (characterized by symptoms such as general edema and pleural effusion) at around 30 weeks of age and die within a year.<sup>19,20</sup> Male Syrian golden hamsters (Japan SLC, Hamamatsu, Japan) served as normal controls. All animals

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**(B)**

	Control hamster				TO-2 hamster					
	10W	18W	30W	38W	10W	18W	26W	30W	34W	38W
No.1	10.2	10.4	9.9	10.2	10.1	8.8	6.6	5.7	5.7	5.3
No.2	10.1	10.4	9.8	10.0	10.0	8.7	6.4	5.4	5.3	4.9
No.3	10.0	9.5	9.3	9.4	9.5	8.4	5.9	5.2	4.8	4.4
No.4	9.6	9.4	9.2	9.1	9.4	7.8	4.9	5.1	4.6	4.2
No.5	9.5	9.3	9.0	8.7	8.9	7.3	4.6	4.8	4.1	3.7
No.6	9.3	9.1	8.7	8.2	8.7	7.2	4.4	4.7	3.5	3.2

Fig 1. Hemodynamic changes in TO-2 and control hamsters. (A) Left ventricular +dP/dt of TO-2 hamsters gradually decreased with age, while those of controls did not change until 38 weeks, \* $p < 0.01$  and † $p < 0.001$  vs 10-week control. (B) The raw data for left ventricular +dP/dt.

were allowed access to food and water ad libitum and were maintained under controlled environmental conditions (24°C, 12-h light/dark cycles). This study proceeded in accordance with the Guide for Animal Experimentation at the Faculty of Medicine, Kagoshima University.

#### Hemodynamic Measurements

We estimated the progression of cardiac dysfunction in TO-2 hamsters using a Millar catheter pressure transducer (Millar Instruments, Houston, TX, USA) cannulated into the right carotid artery as described.<sup>18</sup> We measured left ventricular (LV) +dP/dt in 10, 18, 26, 30, 34 and 38 week-old TO-2 hamsters (n=36) and in 10, 18, 30 and 38 week-old control hamsters (n=24, n=6 per week).

After measuring LV +dP/dt, the TO-2 and control hamsters were killed immediately. We then measured eNOS

mRNA expression in the excised aortas.

#### Sauna Therapy

Sauna therapy was performed using an experimental far infrared-ray dry sauna system at 39°C for 15 min followed by 30°C for 20 min.<sup>18</sup> Core temperatures were increased by about 1°C and remained elevated for about 20 min, as shown, in a clinical setting.<sup>10–13</sup> Under these conditions, the sympathetic nervous system is not stimulated, the increase in oxygen consumption is only 0.3 METs and patients feel comfortable.<sup>10</sup>

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

To estimate the aortic eNOS mRNA expression in TO-2 and control hamsters, we performed RT-PCR as de-

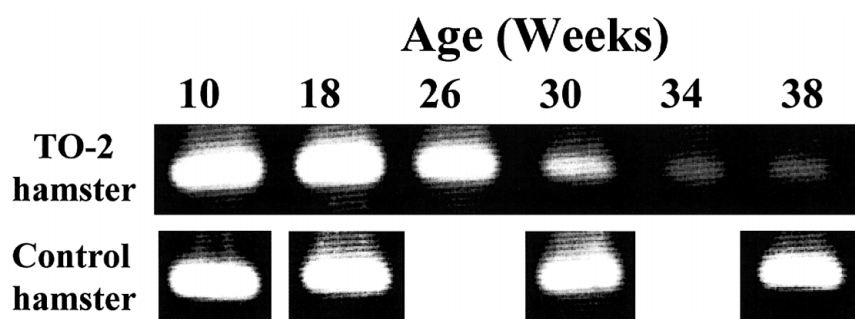


Fig 2. Representative bands of RT-PCR assay in aortas of TO-2 and control hamsters. Expression of eNOS mRNA in control aortas did not change until 38 weeks, whereas those of TO-2 decreased with age.

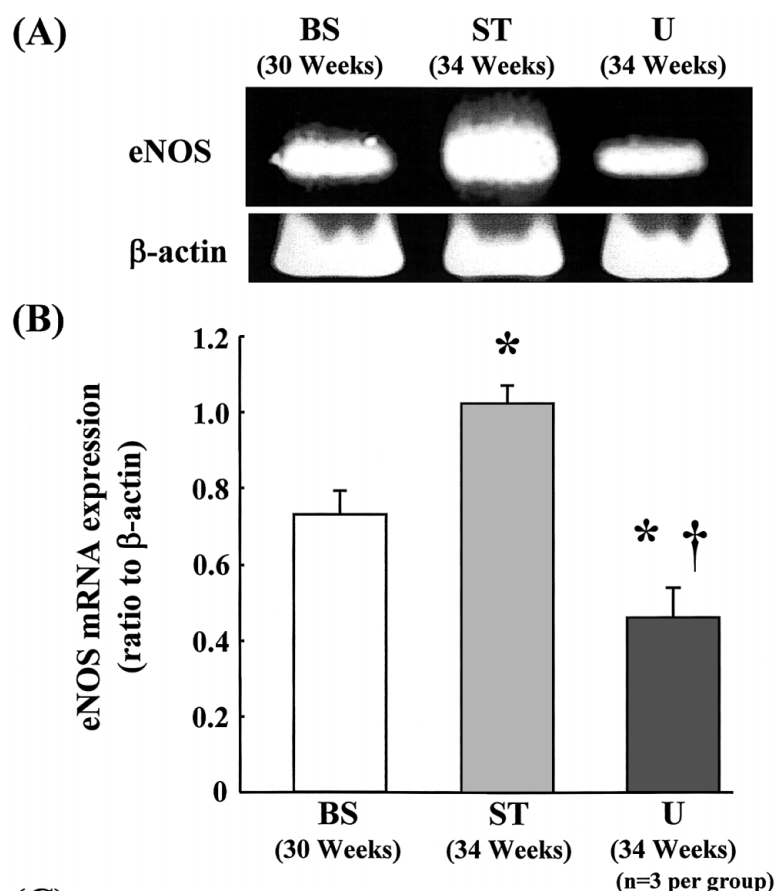


Fig 3. Effects of sauna therapy on eNOS mRNA expression assessed by RT-PCR assay. (A) Expression of eNOS mRNA in aortas pooled from 3 TO-2 hamsters significantly increased after 4 weeks of sauna therapy, whereas that of the U group decreased. (B) Densitometric analysis of eNOS mRNA expression in aortas of TO-2 hamsters. The densitometric values of eNOS mRNA were expressed as a ratio to  $\beta$ -actin. \* $p < 0.01$  vs BS and  $^{\dagger}p < 0.01$  vs ST ( $n = 3$  per group). BS, before sauna; U, untreated; ST, sauna treated. (C) Raw data from densitometric values of eNOS mRNA.

scribed!<sup>18</sup> Primers for eNOS and the internal control  $\beta$ -actin were synthesized according to published sequences<sup>21,22</sup> The primer for eNOS corresponded to 5'-GGGCTCCCTC-CTTCCGGCTGCCACC-3' (sense) and 5'-GGATCCCT-GGAAAAGGCGGTGAGG-3' (antisense), and the primer for  $\beta$ -actin corresponded to 5'-GCATCCTCACCTGAA-GTACCCCA-3' (sense) and 5'-ACTCGTCATACTCCT-GCTTGCTGAT-3' (antisense). Amplification by PCR proceeded as follows: 32 cycles of denaturation at 94°C for 60s, primer annealing at 60°C and extension at 72°C for 1 min each. The predicted sizes of the eNOS and  $\beta$ -actin PCR products are 254 and 906bp, respectively. We esti-

mated the density of PCR product bands using NIH image computer software (NIH, Bethesda, MD, USA) and the density of PCR product bands of eNOS were normalized to the density of  $\beta$ -actin.

#### Immunohistochemistry

The labeled streptavidin biotin method was performed using a Histfine kit (Nichirei, Tokyo, Japan) for immunohistochemistry as previously described!<sup>18</sup> Briefly, cross-sections of aortas were incubated overnight with rabbit polyclonal antibodies (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) specific for eNOS and inducible NO synthase



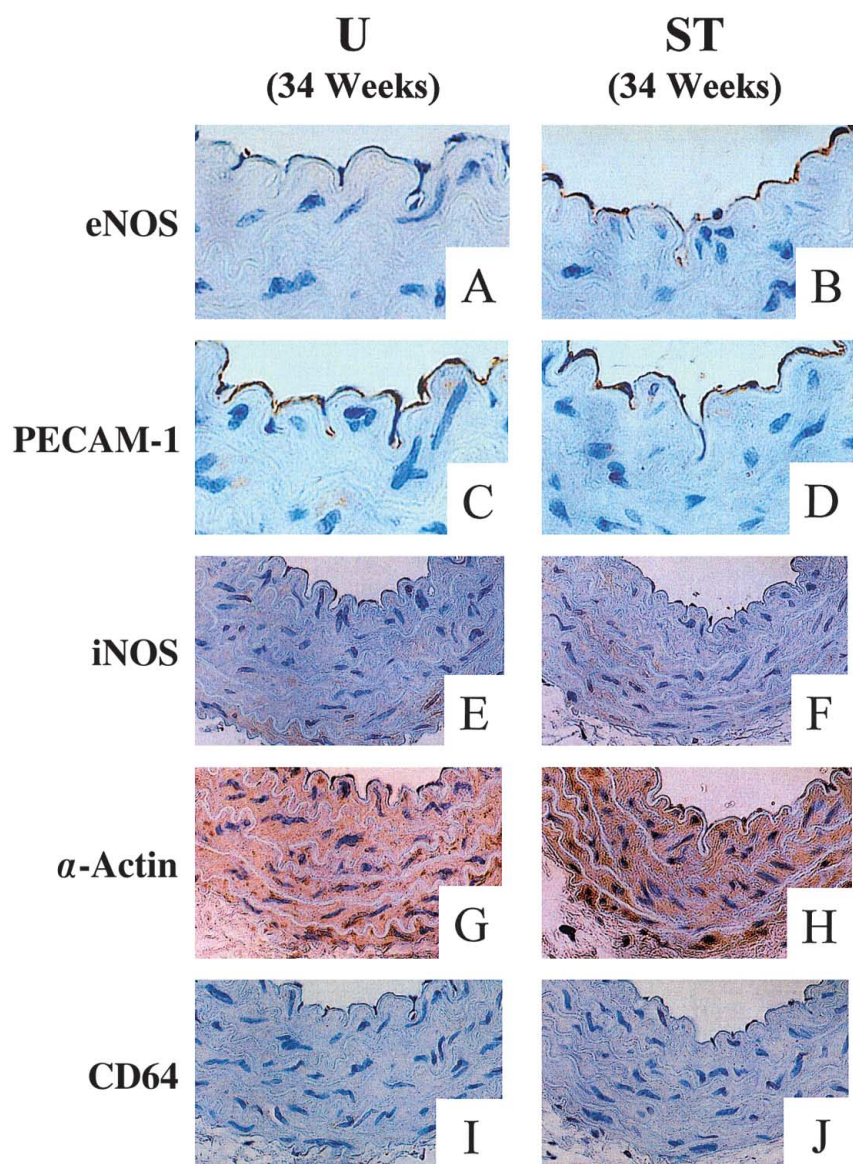


Fig 4. Effects of sauna therapy on the expression of eNOS and iNOS assessed using immunohistochemistry. The immunoreactivity of eNOS was stronger in TO-2 hamsters treated with a 4-week sauna therapy (B) than in the U group (A), and these cells were identified as endothelial cells with a polyclonal antibody against PECAM-1 in successive sections (C and D). (×400). However there was no difference in the immunoreactive products of iNOS between the U (E) and the ST group (F). The cells slightly expressing iNOS were identified as smooth muscle cells with a polyclonal antibody against  $\alpha$ -actin in successive sections (G and H). Macrophages, which can also express iNOS, were not detected in either the inner or medial layer (I and J). (×200). BS, before sauna; U, untreated; ST, sauna treated.

(iNOS), which produces NO that are related to cytotoxic activity in response to cytokine activation<sup>23,24</sup> For cell identification, antibodies against PECAM-1 for endothelial cells,  $\alpha$ -actin for smooth muscle cells and CD64 for macrophages were used (Santa Cruz). The specimens were then incubated with biotinylated anti-rabbit IgG. They were developed with diaminobenzidine and counterstained with hematoxylin.

#### Western Blotting

Western blotting proceeded as described previously<sup>18</sup> In brief, 10  $\mu$ g of protein samples from particulate fractions of TO-2 aortas were detected with the NuPAGE Electrophoresis System (NOVEX, San Diego, CA, USA) using rabbit polyclonal eNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We confirmed that the amounts of proteins loaded on the gel were equal using Coomassie blue staining. The band density was determined using densitometry using NIH image software.

#### Griess Reagent Assay

We performed the Griess reaction to assay the serum

concentrations of nitrate in TO-2 hamsters. The serum samples were deproteinized using ultrafiltration through a 10kDa micropore membrane (Millipore Corporation, Bedford, MA, USA). The results of the assay were determined using a nitrate colorimetric kit (Bio Dynamics Laboratory) as described<sup>25</sup>

#### Experimental Protocol

Seventy-two 30-week-old TO-2 hamsters were divided into before sauna (BS), sauna-treated (ST) and untreated (U) groups (n=24 per group). Hamsters in the ST group underwent a daily sauna for 4 weeks. TO-2 hamsters in the BS group did not undergo any treatment and were killed at 30 weeks of age. Those in the ST and U groups were killed at 34 weeks of age on the day after the last sauna treatment. At that time, the aortas were excised for RT-PCR assay, immunohistochemistry and Western blotting, and serum samples were taken for the Griess reaction. In order to evaluate the eNOS mRNA or protein, material from individual hamsters was insufficient to perform RT-PCR assay or Western blotting. Therefore, we pooled the mRNA or protein sample from 3 hamsters for an experiment and repeated

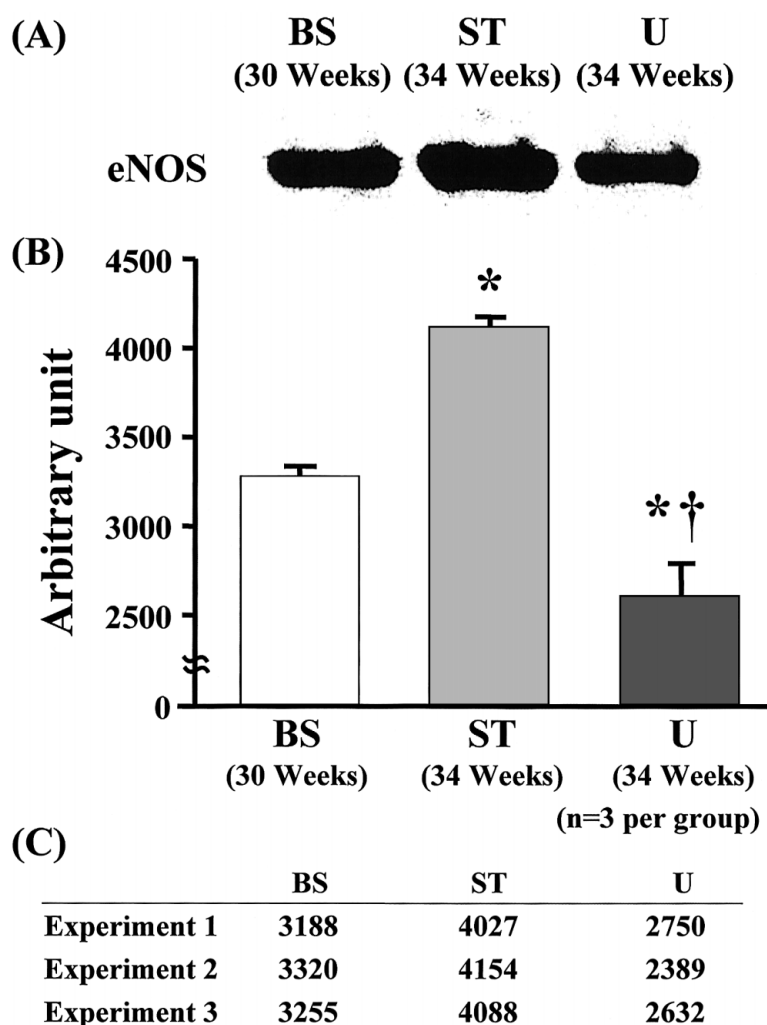


Fig 5. Effects of sauna therapy on eNOS protein expression as assessed using Western blotting. (A) The expression of eNOS protein in aortas pooled from 3 TO-2 hamsters significantly increased after 4 weeks of sauna therapy, while that of the U group decreased. (B) Densitometric analysis of eNOS protein expression in the aortas of TO-2 hamsters. \* $p < 0.01$  vs BS and † $p < 0.01$  vs ST ( $n = 3$  per group). (C) Raw data from the densitometric values of eNOS protein. BS, before sauna; U, untreated; ST, sauna treated.

the same experiment 3 times. Therefore we used total 27 hamsters for 3 groups for Western blotting and another 27 for RT-PCR assay. All samples were rapidly frozen and stored at  $-80^{\circ}\text{C}$ .

#### Statistical Analysis

All values are given as means  $\pm$  SD, and statistical significance was established at  $p < 0.05$ . Data were compared by ANOVA.

## Results

#### Hemodynamic Measurements

Fig 1 shows that the LV  $+dP/dt$  of TO-2 gradually decreased with age, whereas that of controls did not change until 38 weeks (controls: 10-weeks,  $9.8 \pm 0.4$ ; 18-weeks,  $9.7 \pm 0.6$ ; 30-weeks,  $9.3 \pm 0.5$ ; 38-weeks,  $9.3 \pm 0.8$ ; all NS, TO-2: 10-weeks,  $9.4 \pm 0.6$  NS 18-weeks,  $8.0 \pm 0.7$  ( $p < 0.01$ ); 26-weeks,  $5.5 \pm 1.0$  ( $p < 0.001$ ); 30-weeks,  $5.2 \pm 0.4$  ( $p < 0.001$ ); 34-weeks,  $4.7 \pm 0.8$  ( $p < 0.001$ ); 38-weeks,  $4.3 \pm 0.8$  mmHg/s  $\times 10^3$  ( $p < 0.001$ ) vs 10-week control,  $n = 6$  per week).

#### RT-PCR Assay for eNOS mRNA Expression

Fig 2 shows representative RT-PCR results. The expression of eNOS mRNA in control aortas did not change until 38 weeks, but those of TO-2 decreased with age. Densitometric analysis of RT-PCR showed that eNOS mRNA

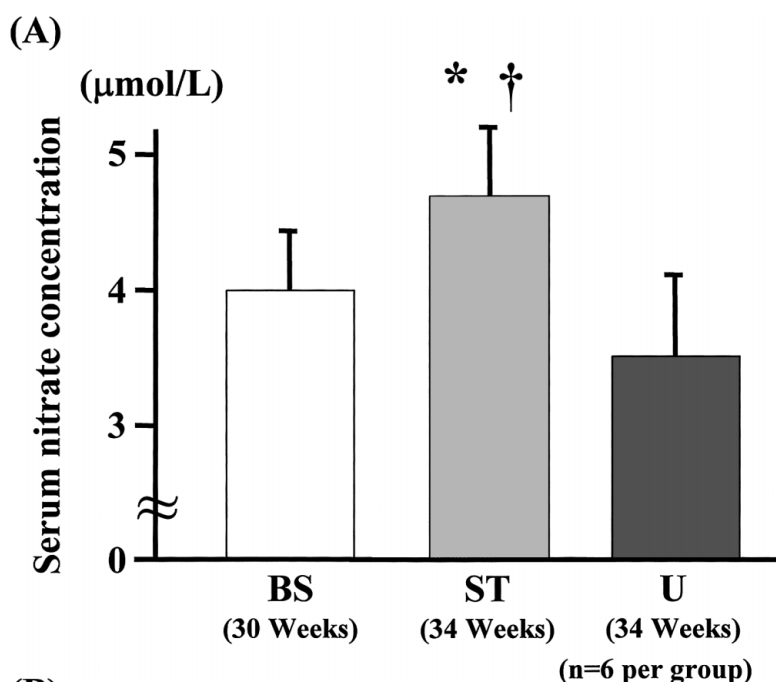
expression by the aorta decreased by 16%, 28%, 42%, 63% and 67% in 18-, 26-, 30-, 34- and 38-week-old TO-2 hamsters compared with those at 10 weeks of age.

#### Effects of Repeated Sauna Therapy on eNOS mRNA Expression

Fig 3A shows representative RT-PCR bands of eNOS mRNA expression in aortas pooled from 3 TO-2 hamsters. Four weeks of sauna therapy significantly increased eNOS mRNA expression in TO-2 hamsters compared with that of untreated TO-2 hamsters. The levels of RT-PCR products for  $\beta$ -actin did not differ between untreated groups and those that underwent 4 weeks of sauna therapy. Three series of independent experiments were performed with the same protocol to quantify the eNOS mRNA expression (Densitometric analysis (ratio to  $\beta$ -actin): BS,  $0.73 \pm 0.04$ ; ST,  $1.02 \pm 0.02$ ; U,  $0.46 \pm 0.06$ ; BS vs ST,  $p < 0.01$ ; ST vs U,  $p < 0.01$ ; BS vs U,  $p < 0.01$ ;  $n = 3$  per group; Fig 3B).

#### Immunohistochemistry

Four-week sauna therapy increased the immunoreactivities of eNOS compared to the U group (Fig 4A,B). These cells were identified as endothelial cells with a polyclonal antibody against PECAM-1 in successive sections (Fig 4C,D). In contrast, there was no difference in the iNOS between the ST and U group (Fig 4E,F). The cells slightly expressing iNOS were identified as smooth muscle



(B)

	BS	ST	U
No. 1	3.20	4.87	4.33
No. 2	4.18	4.77	3.20
No. 3	4.13	4.04	3.74
No. 4	4.13	4.18	3.79
No. 5	4.43	5.46	3.35
No. 6	3.79	4.63	2.51

Fig 6. Effects of sauna therapy on NO production as assessed using the Griess reaction. Serum nitrate concentrations significantly increased after 4 weeks of sauna therapy compared with those of the U group. \* $p < 0.05$  vs BS and † $p < 0.01$  vs U (n=6 per group). (C) Raw data from serum nitrate concentrations. BS, before sauna; U, untreated; ST, sauna treated.

cells with a polyclonal antibody against  $\alpha$ -actin in successive sections (Fig 4G,H). Macrophages that can also express iNOS were not detected in either the inner or medial layer (Fig 4I,J).

#### Effects of Repeated Sauna Therapy on eNOS Protein Expression

Fig 5A shows representative Western blotting bands of eNOS protein expression from aortas pooled from 3 TO-2 hamsters. Western blots showed that 4 weeks of sauna therapy upregulated eNOS protein expression by TO-2 hamsters compared with that by untreated TO-2 hamsters. Three series of independent experiments were performed with the same protocol to quantify the eNOS protein expression (Densitometric analysis: BS,  $3,250 \pm 70$ ; ST,  $4,090 \pm 60$ ; U,  $2,590 \pm 180$  arbitrary units; BS vs ST,  $p < 0.01$ ; ST vs U,  $p < 0.01$ ; BS vs U,  $p < 0.01$ ; n=3 per group; Fig 5B).

#### Effects of Repeated Sauna Therapy on NO Production

The serum nitrate concentrations of TO-2 hamsters were also increased by 4 weeks of sauna therapy compared with those without sauna (BS,  $3.98 \pm 0.43$ ; ST,  $4.66 \pm 0.51$ ; U,  $3.49 \pm 0.62$  mmol/L; BS vs ST,  $p < 0.05$ ; ST vs U,  $p < 0.01$ ; n=6 per group) (Fig 6).

## Discussion

The present study clarified that arterial eNOS expression

decreases with increasing cardiac dysfunction in TO-2 cardiomyopathic hamsters and that repeated sauna therapy upregulates arterial eNOS mRNA expression, arterial eNOS protein expression and NO production. We suggest that eNOS upregulation is one mechanism through which repeated sauna therapy improves endothelial function in CHF.

Vascular endothelial function, which is mainly represented by the endothelium-dependent vasodilatory response and is attenuated in CHF, is one of the most important factors affecting clinical symptoms.<sup>2-5</sup> Vascular endothelial dysfunction in CHF is also considered the result of decreased NO production induced by decreased eNOS expression and/or increased degradation by oxidative stress.<sup>2-5,15-17</sup> The NO radical is produced by the activation of 3 types of NO synthase (NOS), neuronal NOS, iNOS and eNOS. The NO derived from eNOS regulates vascular function, including the vascular relaxation and inhibition of smooth muscle proliferation, platelet aggregation and leukocyte adhesion to the endothelium.<sup>23,24</sup> Smith et al have shown that eNOS mRNA and protein are significantly reduced in the thoracic aorta of dogs at 4 weeks after pacing-induced heart failure.<sup>15</sup> However, it is still controversial as to whether NO production is augmented or deteriorated in patients with CHF. Katz et al have investigated the impaired NO production in heart failure and a similar result was supported by other reports.<sup>26-28</sup> In contrast, several reports demonstrated the increase of



NO production<sup>29–31</sup> If NO production is increased in heart failure, NO might be produced by the increase of iNOS, which is upregulated by several cytokines due to development of heart failure. However, the precise mechanisms have remained a matter for further investigation. In the present study, we demonstrated that NO production and eNOS expression decreased in TO-2 cardiomyopathic hamsters; an animal model of dilated cardiomyopathy.

One of the most important factors regulating arterial eNOS expression and its activation to produce NO is shear stress.<sup>32,33</sup> Patients with CHF have reduced cardiac output and decreased peripheral blood flow, resulting in decreased shear stress, which might downregulate eNOS expression and decrease NO production. We showed that eNOS expression decreased with the degree of heart failure, namely a decrease in +LV dP/dt. Therefore, we speculate that the downregulation of arterial eNOS expression in TO-2 hamsters is largely due to decreased shear stress. Another possibility is the involvement of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which downregulates eNOS expression.<sup>17</sup> It is also possible that thermal stimulation might directly upregulate arterial eNOS, because a recent study has shown that heat increases eNOS expression.<sup>34</sup>

We observed here that repeated sauna therapy upregulated arterial eNOS expression and increased NO production. In the present study, we could not clarify the precise mechanisms through which repeated sauna therapy upregulates eNOS expression. Several factors, including shear stress,<sup>32,33</sup> hypoxia<sup>35</sup> and hydrogen peroxide, upregulate eNOS expression.<sup>36</sup> Our previous clinical studies showed that sauna therapy increases the cardiac index, both in patients with CHF and in healthy individuals, and increases peripheral blood flow in healthy subjects.<sup>10,37</sup> These hemodynamic changes increase the amount of shear stress in vessels, which induces an increase in eNOS expression. Another possibility is that repeated sauna therapy decreases the serum level of TNF- $\alpha$ . However, it may be unlikely, because we found that plasma levels of TNF- $\alpha$  in patients with CHF did not change after 2 weeks of sauna therapy, although endothelial function assessed by flow-mediated dilatation (FMD) improved in our clinical study.<sup>13</sup>

Sauna therapy upregulates eNOS and increases NO production, possibly through increased shear stress that is improved by increased cardiac output and blood flow. Repeated sauna therapy increases shear stress. Increased eNOS and shear stress may increase NO production. In the present study, we found that the serum concentration of nitrate, which is metabolic product of NO, increased after 4 weeks of sauna therapy (Fig 6). However, serum nitrate could come from several potential sources aside from eNOS, including dietary sources and iNOS. We collected blood samples from hamsters after a 12-h fast, therefore we think that dietary sources had little influence on the serum nitrate concentration. Concerning the influence of iNOS, we observed that there was no difference in iNOS protein expression of aortas between sauna-treated and untreated TO-2 hamsters as assessed using immunohistochemistry (Fig 4). Macrophages, which can produce excessive NO through iNOS, were not detected in our model because the subjects had intact arteries, but not vessels with atherosclerosis or injury. Therefore, we think that the increase of serum nitrate from sauna therapy was probably derived from the increase in eNOS. In the present study, we could not examine the bioavailability of NO using physiological methods, such as vascular reactivity. However, we have al-

ready clarified that repeated sauna therapy improves vascular reactivity as assessed by FMD in clinical study.<sup>13,37</sup>

The increased NO production in vessels caused by repeated sauna therapy might decrease cardiac afterload, increase cardiac output and coronary flow and generally improve systemic hemodynamics, endothelial function and cardiac function. Our previous clinical study showed that repeated sauna therapy improves hemodynamics and impaired endothelial function, reduces clinical symptoms and cardiac arrhythmia and decreases brain natriuretic peptide concentrations in patients with CHF.<sup>11–13</sup> These results suggest that the improvement of endothelial function may be a new target in the treatment of CHF.

The sauna therapy did not appear to cause the hamsters stress. Their heart rates did not change and body weight did not differ between ST and U hamsters.<sup>18</sup> Hamsters exposed to the sauna remained calm, with behavior that was quite similar to that of the untreated hamsters that were placed in the switched-off sauna system. None of the hamsters died during or immediately after sauna therapy throughout the present study. Furthermore, we reported that sauna therapy improves the prognosis in TO-2 cardiomyopathic hamsters with CHF.<sup>14</sup>

In conclusion, arterial eNOS expression decreased with the development of cardiac dysfunction in CHF. Repeated sauna therapy increased arterial eNOS expression and NO production, which is one mechanism through which repeated exposure to heat improves endothelial function.

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