and mortality in male patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2002;166:680–5.
[23] Ernst E, Pecho E, Wirz P, Saradeth T. Regular sauna bathing and the incidence of common colds. Ann Med 1990;22:225–7.
[24] Masuda A, Nakazato M, Kihara T, Minagoe S, Tei C. Repeated thermal therapy diminishes appetite loss and subjective complaints in mildly depressed patients. Psychosomatic Med 2005;67:643–7.
[25] van Manen JG, Bindels PJ, Dekker FW, Ijzermans CJ, van der Zee JS, Schadé E. Risk of depression in patients with chronic obstructive pulmonary disease and its determinants. Thorax 2002;57:412–6.

Journal of Cardiology (2008) 52, 79–85

ELSEVIER

JOURNAL of CARDIOLOGY
Official Journal of the Japanese College of Cardiology
www.elsevier.com/locate/jjcc

ORIGINAL ARTICLE

# Beneficial effects of Waon therapy on patients with chronic heart failure: Results of a prospective multicenter study

**Masaaki Miyata (MD, FJCC)[a], Takashi Kihara (MD)[a], Takuro Kubozono (MD)[a], Yoshiyuki Ikeda (MD)[a], Takuro Shinsato (MD)[a], Toru Izumi (MD, FJCC)[b], Masunori Matsuzaki (MD, FJCC)[c], Tetsu Yamaguchi (MD, FJCC)[d], Hiroshi Kasanuki (MD, FJCC)[e], Hiroyuki Daida (MD, FJCC)[f], Masatoshi Nagayama (MD)[g], Kazuhiro Nishigami (MD)[h], Kumiko Hirata (MD)[i], Koichi Kihara (MD)[j], Chuwa Tei (MD, FJCC)[a,*]**

[a] *Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduated School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*
[b] *Department of Cardiology, Kitasato University School of Medicine, Sagamihara, Japan*
[c] *Division of Cardiology, Department of Medicine and Clinical Science, Graduate School of Medicine, Yamaguchi University, Ube, Japan*
[d] *Department of Cardiovascular Center Medicine, Toranomon Hospital, Tokyo, Japan*
[e] *Department of Cardiology, Tokyo Women's Medical University, Tokyo, Japan*
[f] *Department of Cardiovascular Medicine, Juntendo University, Tokyo, Japan*
[g] *Department of Cardiology, Sakakibara Heart Institute, Tokyo, Japan*
[h] *Division of Cardiology, Saiseikai Kumamoto Hospital Cardiovascular Center, Kumamoto, Japan*
[i] *Division of Cardiology, Higashisumiyoshi Morimoto Hospital, Osaka, Japan*
[j] *Division of Cardiology, Fujimoto Hayasuzu Hospital, Miyakonojo, Japan*

Received 17 June 2008; accepted 3 July 2008
Available online 27 August 2008

**KEYWORDS**
Waon therapy;
Heart failure;
Treatment

**Summary**
*Background:* We conducted a prospective multicenter case–control study to confirm the clinical efficacy and safety of Waon therapy on chronic heart failure (CHF).
*Methods:* Patients ($n = 188$) with CHF were treated with standard therapy for at least 1 week, and then were randomized to Waon therapy ($n = 112$) or a control group ($n = 76$). All patients continued conventional treatment for an additional 2 weeks.

* Corresponding author. Tel.: +81 99 275 5316; fax: +81 99 275 5322.
*E-mail addresses:* miyatam@m3.kufm.kagoshima-u.ac.jp (M. Miyata), tei@m.kufm.kagoshima-u.ac.jp (C. Tei).

0914-5087/$ – see front matter 
doi:10.1016/j.jjcc.2008.07.009

Natriuretic peptides;
Brain;
Non-pharmacological therapy

The Waon therapy group was treated daily with a far infrared-ray dry sauna at 60 °C for 15 min and then kept on bed rest with a blanket for 30 min for 2 weeks. Chest radiography, echocardiography, and plasma levels of brain natriuretic peptide (BNP) were measured before and 2 weeks after treatment.

*Results:* NYHA functional class significantly decreased after 2 weeks of treatment in both groups. Chest radiography also showed a significant decrease of the cardiothoracic ratio in both groups (Waon therapy: 57.2 ± 8.0% to 55.2 ± 8.0%, $p < 0.0001$; control: 57.0 ± 7.7% to 56.0 ± 7.1%, $p < 0.05$). Echocardiography demonstrated that left ventricular diastolic dimension (LVDd), left atrial dimension (LAD), and ejection fraction (EF) significantly improved in the Waon therapy group (LVDd: 60.6 ± 7.6 to 59.1 ± 8.4 mm, $p < 0.0001$; LAD: 45.4 ± 9.3 mm to 44.1 ± 9.4 mm, $p < 0.05$; EF: 31.6 ± 10.4% to 34.6 ± 10.6%, $p < 0.0001$), but not in the control group (LVDd: 58.4 ± 10.3 mm to 57.9 ± 10.4 mm; LAD: 46.3 ± 9.7 mm to 46.2 ± 10.1 mm; EF: 36.6 ± 14.1% to 37.3 ± 14.0%). The plasma concentration of BNP significantly decreased with Waon therapy, but not in the control group (Waon: 542 ± 508 pg/ml to 394 ± 410 pg/ml, $p < 0.001$; control: 440 ± 377 pg/ml to 358 ± 382 pg/ml).

*Conclusion:* Waon therapy is safe, improves clinical symptoms and cardiac function, and decreases cardiac size in CHF patients. Waon therapy is an innovative and promising therapy for patients with CHF.



## Introduction

Chronic heart failure (CHF) is a major and growing public health problem in Japan, as in other developed countries. Drugs that interfere with excessive activation of the rennin–angiotensin–aldosterone system can relieve the symptoms of heart failure in patients with a depressed ejection fraction (EF) by stabilizing and/or reversing cardiac remodeling. Thus, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), and β blockers have emerged as cornerstones of modern heart failure therapy for patients with a depressed EF [1]. Angiotensin II plays an important role in the pathogenesis of CHF, and many large clinical trials have demonstrated the benefits of ACE inhibitors [2,3], and ARBs [4–8] on the morbidity and mortality of CHF. However, the number of heart failure deaths has increased steadily despite advances in treatment, in part, because of increasing numbers of patients with CHF due to better treatment and salvage of patients with acute myocardial infarction earlier in life [9].

We developed a form of thermal therapy that differs from the traditional sauna [10], and have been investigating the effects of thermal therapy since 1989. We discovered that the new thermal therapy offers prominent beneficial effects for patients with CHF [10–13] and peripheral artery disease [14,15]. Thermal therapy at very high temperature was originally used to treat localized cancer. However, the therapy we developed to treat cardiovascular diseases is quite different, in that it consists of systemic soothing warmth that comfortably refreshes the mind and body. Therefore, we have changed the name from thermal, to "Waon" therapy, since "Waon" in Japanese means soothing warmth [16]. Waon therapy is defined as "therapy in which the entire body is warmed in an evenly heated chamber for 15 min at a temperature that soothes the mind and body, and after the deep-body temperature has increased by approximately 1.0–1.2 °C, the soothing warmth is sustained by maintaining the warmth at rest for an additional 30 min, with fluids supplied at the end to replace the loss from perspiration" [16]. We have reported that Waon therapy, the repeated use of a dry sauna at 60 °C, improves hemodynamics and ameliorates symptoms, suppresses ventricular arrhythmias, and improves vascular function in CHF patients [10–13]. We have already performed Waon therapy in several hundred CHF patients in our hospital without any severe adverse effects.

In order to expand the use of Waon therapy, we developed a movable and sitting-position sauna system, in which the temperature at the top and bottom of the chamber is uniformly maintained at the same temperature of 60 °C (Fig. 1). Using this sitting-position sauna system, we conducted a prospective multicenter case–control study to confirm the clinical effect and safety of Waon therapy on CHF at 10 different hospitals.

## Subjects and methods

### Subjects

Ten hospitals participated in this multicenter study: Kagoshima University Hospital, Kitasato University Hospital, Sakakibara Memorial Hospital, Yamaguchi University Hospital, Juntendo University Hospital, Tokyo Women's Medical University Hospital, Toranomon Hospital, Higashisumiyoshi Morimoto Hospital, Saiseikai Kumamoto Hospital, and Fujimoto Hayasuzu Hospital. We enrolled 188 patients with CHF, aged 26–94 years (mean age: 64.7 ± 13.7 years). 94 patients had idiopathic dilated cardiomyopathy, 45 had ischemic cardiomyopathy, 16 patients had valvular heart disease, and 33 patients had other heart disease (7 hypertensive heart disease, 10 hypertrophic cardiomyopathy, 4 dilated hypertrophic cardiomyopathy, 3 cardiac sarcoidosis, 3 restrictive cardiomyopathy, 2 atrial septal defect, 1 cardiac amyloidosis, 1 drug-induced cardiomyopathy, 1 alcoholic cardiomyopathy, and 1 left ventricular noncompaction).

Inclusion criteria were the presence of symptomatic CHF, left ventricular ejection fraction (LVEF) <50% on echocardiography, and New York Heart Association (NYHA) functional classes II–IV. Exclusion criteria were the presence of severe aortic stenosis, severe obstruction with hypertrophic obstructive cardiomyopathy, and high fever due to infectious disease. Informed consent was obtained from all of the patients before participation. This protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University.

### Design of the study protocol

All patients could receive any kind of medication for CHF and doctors also could change the medication during the study. The subjects were treated with conventional therapy for at least 1 week, and then were randomized to the Waon therapy group or a control group at each hospital. The patients in the Waon therapy group received thermal therapy daily, 5 days a week, for 2 weeks. The patients in the control group continued the conventional treatment for 2 more weeks.

### Waon therapy

Waon therapy uses a far infrared-ray dry sauna, which is evenly maintained at 60 °C and differs from traditional sauna. Waon therapy has an absence of hydration pressure, and was performed as previously reported [10]. Briefly, the patients were placed in a sitting-position in a 60 °C sauna system for 15 min, and then after leaving the sauna, they underwent bed rest with a blanket to keep them warm for an additional 30 min. All patients were weighed before and after the therapy, and oral hydration with water was used to compensate for weight lost due to perspiration. Waon therapy was performed once a day, 5 days a week for 2 weeks, for a total of 10 sessions. To rule out any acute effects of Waon therapy, all examinations were performed before the first treatment and on the next day after the last treatment.

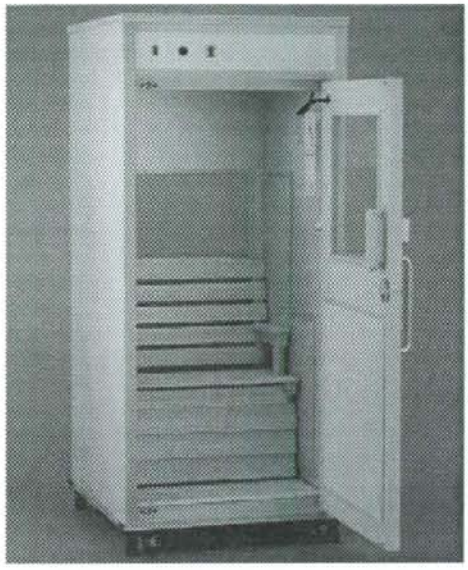

**Figure 1** Movable and sitting-position sauna system. The temperature at the top and bottom of the chamber is uniformly maintained at the same temperature of 60 °C.

## Measurements

### Physical examination

The blood pressure (BP), pulse rate, body weight, and body temperature were measured before and 2 weeks after treatment.

### Cardiac function

The clinical state of CHF was evaluated by NYHA functional class. Before and 2 weeks after treatment, the cardiothoracic ratio (CTR) was measured by chest radiography and left ventricular diastolic dimension (LVDd), left atrial dimension (LAD), and

**Table 1** Baseline clinical characteristics and changes in several variables

| | Waon therapy group ($n=112$) | | | Control group ($n=76$) | | | Comparison at baseline[a] |
|---|---|---|---|---|---|---|---|
| | Baseline | After 2 weeks | *p*-Value | Baseline | After 2 weeks | *p*-Value | *p*-Value |
| Age (years) | 63 ± 13 | | | 66 ± 14 | | | NS |
| Gender (male/female) | 74/38 | | | 51/25 | | | NS |
| DCM/ICM/VD/other disease | 62/29/7/14 | | | 32/16/9/19 | | | NS |
| NYHA functional class (average) | 2.61 ± 0.62 | 1.99 ± 0.60 | <0.0001 | 2.51 ± 0.62 | 2.23 ± 0.48 | <0.01 | NS |
| Body weight (kg) | 56.7 ± 11.8 | 55.9 ± 11.4 | <0.0001 | 54.6 ± 12.0 | 54.6 ± 12.5 | NS | NS |
| Heart rate (beats/min) | 74 ± 15 | 72 ± 13 | NS | 74 ± 13 | 71 ± 11 | NS | NS |
| Systolic BP (mm Hg) | 108 ± 21 | 104 ± 18 | <0.01 | 110 ± 21 | 106 ± 19 | <0.05 | NS |
| Diastolic BP (mm Hg) | 64 ± 12 | 62 ± 11 | <0.01 | 67 ± 12 | 65 ± 10 | <0.05 | NS |

DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; VD, valvular disease; NYHA, New York Heart Association; BP, blood pressure; and NS, not significant.
[a] Comparison with baseline values. Data are presented as the mean value ± S.D.

LVEF were evaluated by conventional echocardiography.

### Laboratory measurements

A fasting blood sample was obtained in the morning to measure the plasma concentrations of the brain natriuretic peptide (BNP) with radioimmunoassay, before and 2 weeks after treatment.

### Statistical analysis

All data are expressed as the mean value ± S.D. Differences in baseline characteristics were evaluated by a $\chi^2$ test and unpaired *t*-test. The data before and 2 weeks after treatment were compared using a paired *t*-test. A *p*-value of <0.05 was considered statistically significant.

## Results

### Baseline clinical characteristics

The baseline clinical characteristics are summarized in Table 1. There were no significant differences in age, gender, causative heart disease, NYHA functional class, body weight, heart rate, systolic BP (SBP), or diastolic BP (DBP) at baseline between the two groups.

### Clinical findings and physical examinations

During the study, none of the patients treated with Waon therapy had worsened clinical symptoms. The changes in the clinical findings and variables after 2 weeks are summarized in Table 1. NYHA functional class, SBP, and DBP significantly decreased in both groups. Body weight significantly decreased in the Waon therapy group, but not in the control group. There were no significant changes in heart rate in either group.

### Chest radiography and echocardiography

Fig. 2 shows the results of chest radiography and echocardiography. Chest radiography showed a significant decrease of the CTR after 2 weeks of treatment in both groups (Waon therapy group: 57.2 ± 8.0% to 55.2 ± 8.0%, $p<0.0001$; control group: 57.0 ± 7.7% to 56.0 ± 7.1%, $p<0.05$). In addition, echocardiography demonstrated that LVDd, LAD, and LVEF significantly improved after Waon therapy (LVDd: 60.6 ± 7.6 mm to 59.1 ± 8.4 mm, $p<0.0001$; LAD: 45.4 ± 9.3 mm to 44.1 ± 9.4 mm,

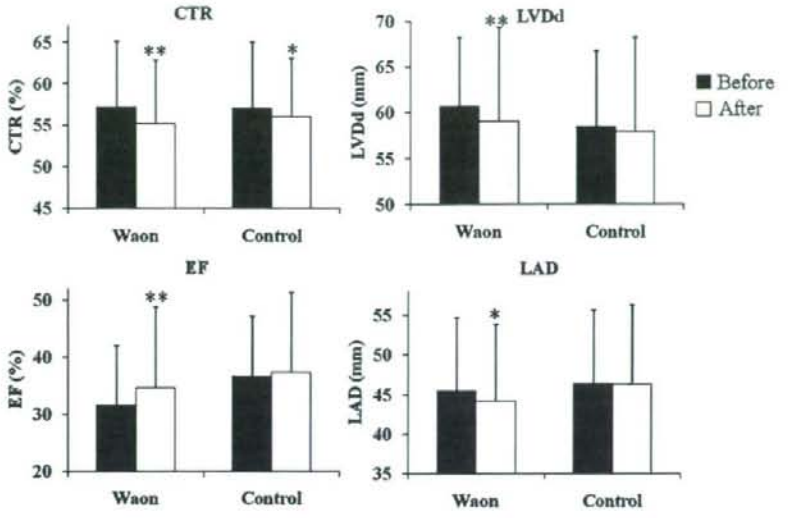


**Figure 2** Data from chest radiography and echocardiography. Chest radiography showed a significant decrease of the cardiothoracic ratio (CTR) after 2 weeks of treatment in both groups. Echocardiography demonstrated that left ventricular diastolic dimension (LVDd), left atrial dimension (LAD), and left ventricular ejection fraction (LVEF) significantly decreased after 2 weeks of Waon therapy, but did not change after 2 weeks of conventional therapy in the control group. $^{*}p < 0.05$ vs. baseline; $^{**}p < 0.0001$ vs. baseline. Closed bars show baseline and open bars indicate values after 2 weeks of treatment.

$p < 0.05$; LVEF: $31.6 \pm 10.4\%$ to $34.6 \pm 10.6\%$, $p < 0.0001$), but did not change in the control group (LVDd: $58.4 \pm 10.3$ mm to $57.9 \pm 10.4$ mm, not significant; LAD: $46.3 \pm 9.7$ mm to $46.2 \pm 10.1$ mm, not significant; LVEF: $36.6 \pm 14.1\%$ to $37.3 \pm 14.0\%$, not significant).

### Plasma levels of BNP

Fig. 3 shows the changes in plasma concentration of BNP. The plasma concentration of BNP significantly decreased after 2 weeks of Waon therapy, while it did not change in the control group (Waon therapy group: $542 \pm 508$ pg/ml to $394 \pm 410$ pg/ml, $p < 0.001$; control group: $440 \pm 377$ pg/ml to $358 \pm 382$ pg/ml, not significant).

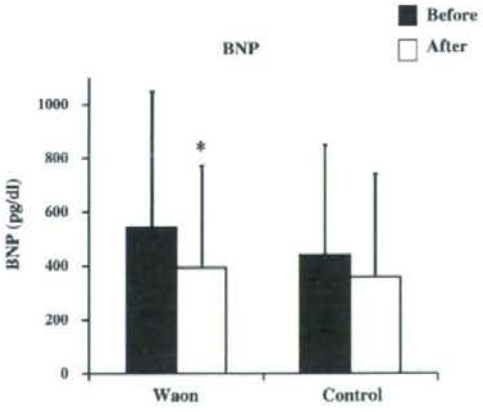


**Figure 3** Changes in plasma concentration of BNP. The plasma concentration of BNP significantly decreased after 2 weeks of Waon therapy, but did not change in the control group. $^{*}p < 0.001$ vs. baseline. Closed bars show baseline and open bars indicate values after 2 weeks of treatment.

## Discussion

We developed the sitting-position sauna system, and conducted a prospective multicenter case–control study to confirm the clinical efficacy and safety of Waon therapy on CHF at 10 hospitals. In this study, we confirmed that Waon therapy improved clinical symptoms and cardiac function evaluated by echocardiography and BNP concentrations, and decreased cardiac size on chest radiography and echocardiography after 2 weeks of Waon therapy in patients with CHF. We also demonstrated that our movable and sitting-position sauna system is effective and safe for patients with CHF.

Regarding the acute effect of Waon therapy, we have reported that 60 °C sauna therapy for 15 min improved acute hemodynamics in patients with CHF, including cardiac index, mean pulmonary wedge pressure, systemic and pulmonary resistance, and cardiac function [10]. Subsequently, we examined the chronic effects of repeated Waon therapy on clinical symptoms and cardiac function in patients with CHF and have reported that 4 weeks of Waon therapy significantly improved clinical symptoms, increased ejection fraction, and decreased cardiac size on the echocardiogram and chest X-ray [10,11]. Furthermore, we demonstrated that daily Waon therapy for 2 weeks decreased ventricular premature contractions and increased heart rate variability (SDNN, standard deviation of normal-to-normal beat interval) in patients with CHF, suggesting that Waon therapy decreased sympathetic nervous activity and improved ventricular arrhythmias [13].

We then investigated the vascular endothelial function to clarify the mechanisms of the effect of Waon therapy on CHF, since vascular endothelial function had been reported to be impaired in CHF. We have reported that 2 weeks of Waon therapy significantly reduced BNP concentrations and improved endothelial function in patients with CHF. There was a significant correlation between the change in %FMD (flow-mediated dilatation) and the percent improvement in BNP concentrations in the Waon therapy group [12].

In order to confirm the effect of Waon therapy on CHF and clarify its mechanism, we performed experimental studies using TO-2 cardiomyopathic hamsters with heart failure. We reported that the repeated Waon therapy improved survival in TO-2 cardiomyopathic hamsters with heart failure [17]. We clarified that one of the molecular mechanisms by which repeated Waon therapy improved endothelial function was an increase in mRNA and protein of endothelial nitric oxide synthase (eNOS) in Syrian golden hamsters [18] and TO-2 cardiomyopathic hamsters [19]. We believe that eNOS up-regulation induced by repeated Waon therapy is caused by an increase in cardiac output and blood flow, which in turn results in increased shear stress, although thermal stimulation might up-regulate arterial eNOS directly.

Compared to pharmacological vasodilator therapy and other non-pharmacological therapy, such as cardiac resynchronization therapy and physical therapy, there are several advantages of Waon therapy for CHF. First, it is quite safe and has no adverse effects. Second, it is less expensive and more cost-effective. Third, unlike physical therapy, patients who are elderly or have severe congestive heart failure, uncontrolled ventricular arrhythmias, and orthopedic limitations are not exempt from undergoing Waon therapy. Fourth, this treatment promotes mental and physical relaxation. Waon therapy may thus be a valuable adjunct to pharmacological or non-pharmacological intervention in the management of CHF.

We have treated many CHF patients with Waon therapy, and none of the patients so far has shown any deterioration in their condition. However, Waon therapy does not appear to be indicated for CHF patients with severe aortic stenosis or obstructive hypertrophic cardiomyopathy, because the pressure gradient might be increased during Waon therapy. Patients with infectious disease are also excluded from Waon therapy.

## Study limitations

We have already reported that repeated Waon therapy improved the prognosis of TO-2 cardiomyopathic hamsters with CHF, suggesting a new potential non-pharmacologic therapy for CHF [17]. The ultimate goal of treatment is the improvement of prognosis and quality of life. Therefore, we must evaluate the effect of Waon therapy on prognosis, as well as quality of life, in patients with CHF. We are conducting a prospective clinical randomized study to assess the impact of Waon therapy on the rate of cardiac death or re-hospitalization in patients with CHF.

## Conclusion

In this prospective multicenter study, we confirmed that Waon therapy is quite safe, improved clinical symptoms and cardiac function, and decreased cardiac size in patients with CHF. Therefore, Waon therapy is an innovative and promising therapy for patients with CHF.

## Acknowledgements

We appreciated Dr. Tsuyoshi Fukudome, Dr. Shoji Fujita, and Dr. So Kuwahata in Kagoshima University, Dr. Chiharu Noda in Kitasato University, Dr. Shigeki Kobayashi in Yamaguchi University, Dr. Katsuyuki Masaki in Juntendo University, Dr. Kazue Watanabe in Tokyo Women's Medical University, and Dr. Minoru Ohno in Toranomon Hospital for collecting data at each hospital.

## References


[1] Mann DL. Management of heart failure patients with reduced ejection fraction. In: Libby P, Bonow RO, Mann DL, Zipes DP, editors. Braunwald's heart disease: a textbook of cardiovascular medicine. 8th ed. Philadelphia: WB Saunders; 2008. p. 611–40.

[2] The CONSENSUS Trial Study Group.Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). N Engl J Med 1987;316:1429–35.

[3] The SOLVD Investigators.Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. N Engl J Med 1991;325:293–302.

[4] Cohn JN, Tognoni G, Valsartan Heart Failure Trial Investigators. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. N Engl J Med 2001;345:1667–75.

[5] Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, Michelson EL, et al. Effects of candesartan on mortality and morbidity in patients with chronic heart failure: the CHARM-overall programme. Lancet 2003;362:759–66.

[6] McMurray JJ, Ostergren J, Swedberg K, Granger CB, Held P, Michelson EL, et al. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function taking angiotensin-converting-enzyme inhibitors: the CHARM-added trial. Lancet 2003;362:767–71.

[7] Granger CB, McMurray JJ, Yusuf S, Held P, Michelson EL, Olofsson B, et al. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function intolerant to angiotensin-converting-enzyme inhibitors: the CHARM-alternative trial. Lancet 2003;362:772–6.

[8] Yusuf S, Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, et al. Effects of candesartan in patients with chronic heart failure and preserved left-ventricular ejection fraction: the CHARM-preserved Trial. Lancet 2003;362:777–81.

[9] Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al. American College of Cardiology; American Heart Association Task Force on Practice Guidelines; American College of Chest Physicians; International Society for Heart and Lung Transplantation; Heart Rhythm Society. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation 2005;112:e154–235.

[10] Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Toyama Y, et al. Acute hemodynamic improvement by thermal vasodilation in congestive heart failure. Circulation 1995;91:2582–90.

[11] Tei C, Tanaka N. Thermal vasodilation as a treatment of congestive heart failure: a novel approach. J Cardiol 1996;27:29–30.

[12] Kihara T, Biro S, Imamura M, Yoshifuku S, Takasaki K, Ikeda Y, et al. Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. J Am Coll Cardiol 2002;39:754–9.

[13] Kihara T, Biro S, Ikeda Y, Fukudome T, Shinsato T, Masuda A, et al. Effects of repeated sauna treatment on ventricular arrhythmias in patients with chronic heart failure. Circ J 2004;68:1146–51.

[14] Tei C, Shinsato T, Kihara T, Miyata M. Successful thermal therapy for end-stage peripheral artery disease. J Cardiol 2006;47:163–4.

[15] Tei C, Shinsato T, Miyata M, Kihara T, Hamasaki S. Waon therapy improves peripheral arterial disease. J Am Coll Cardiol 2007;50:2169–71.

[16] Tei C. Waon therapy: soothing warmth therapy. J Cardiol 2007;49:301–4.

[17] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Kihara T, Minagoe S, et al. Effect of repeated sauna therapy on survival in TO-2 cardiomyopathic hamsters with heart failure. Am J Cardiol 2002;90:343–5.

[18] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, et al. Repeated thermal therapy upregulates arterial endothelial nitric oxide synthase expression in Syrian golden hamsters. Jpn Circ J 2001;65:434–8.

[19] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, et al. Repeated sauna therapy increases arterial endothelial nitric oxide synthase expression and nitric oxide production in cardiomyopathic hamsters. Circ J 2005;69:722–9.

Journal of Cardiology (2009) xxx, xxx–xxx

ELSEVIER

JOURNAL of CARDIOLOGY
Official Journal of the Japanese College of Cardiology
www.elsevier.com/locate/jjcc

ORIGINAL ARTICLE

# Waon therapy improves the prognosis of patients with chronic heart failure

**Takashi Kihara (MD)[a], Masaaki Miyata (MD, FJCC)[a], Tsuyoshi Fukudome (MD)[a], Yoshiyuki Ikeda (MD)[a], Takuro Shinsato (MD)[a], Takuro Kubozono (MD)[a], Shoji Fujita (MD)[a], So Kuwahata (MD)[a], Shuichi Hamasaki (MD,FJCC)[a], Hiroyuki Torii (MD)[b], Soki Lee (MD,FJCC)[c], Hitoshi Toda (MD)[c], Chuwa Tei (MD,FJCC)[a,*]**

[a] *Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*
[b] *Department of Cardiology, Kagoshima City Medical Association Hospital, Kagoshima, Japan*
[c] *Department of Cardiology, Kagoshima City Hospital, Kagoshima, Japan*

Received 21 October 2008; received in revised form 6 November 2008; accepted 7 November 2008

**KEYWORDS**
Waon therapy;
Prognosis;
Heart failure

Summary
*Background:* We developed a Waon therapy (soothing warm therapy) and have previously reported that repeated Waon therapy improves hemodynamics, peripheral vascular function, arrhythmias, and clinical symptoms in patients with chronic heart failure (CHF). The aim of this study was to investigate the effect of Waon therapy on the prognosis of CHF patients.
*Patients and methods:* We studied 129 patients with CHF in NYHA functional class III or IV who were admitted to our hospital between January 1999 and March 2001. In the Waon therapy group, 64 patients were treated with a far infrared-ray dry sauna at 60 C for 15 min and then kept on bed rest with a blanket for 30 min. The patients were treated daily for 5 days during admission, and then at least twice a week after discharge. In the control group, 65 patients, matched for age, gender, and NYHA functional class, were treated with traditional CHF therapy. The follow-up time was scheduled for 5 years.
*Results:* Recent, complete follow-up data on each patient were obtained. The overall survival rate was 84.5% (Kaplan–Meier estimate). Twelve patients died in the control group and 8 patients died in the Waon therapy group at 60 months of follow-up.

* Corresponding author. Tel.: +81 99 275 5316; fax: +81 99 275 5322.
*E-mail address:* tei@m.kufm.kagoshima-u.ac.jp (C. Tei).

0914-5087/$ – see front matter 
doi:10.1016/j.jjcc.2008.11.005

Please cite this article in press as: Kihara T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol (2009), doi:10.1016/j.jjcc.2008.11.005

Cardiac events due to heart failure or cardiac death occurred in 68.7% of the control group but only 31.3% of the Waon therapy group ($P < 0.01$) at 60 months of follow-up. *Conclusion:* Waon therapy reduced cardiac events in patients with CHF. This therapy is a promising non-pharmacological treatment for CHF.


## Introduction

Recently, many researchers have reported that vasodilators, such as angiotensin-converting enzyme inhibitors [1], angiotensin receptor blockers [2], and beta-blockers [3], improve prognosis in patients with chronic heart failure (CHF). Furthermore, new technologies to treat CHF, such as cardiac rehabilitation, cardiac resynchronization therapy, left ventricular assist devices, and left ventricular reconstruction surgery, have been developed over the past decade. Despite advances in therapy for heart failure, improving clinical outcomes of patients with acute heart failure remains a challenge for physicians. Re-hospitalization within 60–90 days occurs in approximately 30% of patients with acute heart failure [4].

We have developed a form of thermal therapy, namely Waon therapy, which differs from the traditional sauna and is useful in the treatment of CHF. Waon therapy is defined as "therapy in which the entire body is warmed in an evenly heated chamber for 15 min at a temperature that soothes the mind and body, and after the deep-body temperature has increased by approximately 1.0–1.2 °C, the soothing warmth is sustained by maintaining the warmth at rest for an additional 30 min, with fluids supplied at the end to replace the loss from perspiration [5]." We have already reported that Waon therapy, the repeated use of a dry sauna at 60 °C, improves hemodynamics [6], ameliorates symptoms [7], suppresses ventricular arrhythmias [8], and improves vascular function [9] in CHF patients. Recently, in a prospective multicenter case–control study, we found that 2 weeks of Waon therapy improved clinical symptoms and cardiac function in CHF patients [10].

Furthermore, we reported that repeated Waon therapy improves survival in TO-2 cardiomyopathic hamsters with heart failure [11]. However, the effect of Waon therapy on prognosis in CHF patients has not yet been elucidated. Thus, the purpose of this study was to investigate the effect of Waon therapy on the prognosis of CHF patients.

## Methods

### Patients and study design

The study subjects included 129 CHF patients who were admitted to Kagoshima University Hospital, Kagoshima City Hospital, or Kagoshima City Medical Association Hospital between January 1999 and March 2001. All patients received traditional medications for CHF, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, beta-blockers, diuretics, and digitalis. None of these patients was implanted with a defibrillator device. Sixty-four patients were treated daily with Waon therapy for 5 days after admission, and Waon therapy was continued at least twice a week in an out-patient clinic after hospital discharge. The remaining 65 control patients, who were matched with the Waon therapy group for age, gender, and etiology and severity of CHF, continued medical therapy for CHF.

Clinical characteristics at discharge from the first admission were considered as the patient's baseline characteristics. Data on body mass index, heart rate, systolic blood pressure, and diastolic blood pressure were also measured at discharge from the first hospitalization. The baseline data also included the more recent data on the cardiothoracic ratio (CTR) measured by chest radiography and left ventricular diastolic dimension and left ventricular ejection fraction measured by two-dimensional echocardiography during the first admission.

All 129 patients were followed-up for 5 years, and cardiac events, such as cardiac death and re-hospitalization due to heart failure, were compared between the control and Waon therapy groups.

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University. Informed consent was obtained from all of the patients.

### Waon therapy

Waon therapy uses a far infrared-ray dry sauna, which is evenly maintained at 60 °C and differs

Please cite this article in press as: Kihara T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol (2009), doi:10.1016/j.jjcc.2008.11.005

**Table 1** Baseline clinical characteristics.

| | Waon therapy group | | Control group |
|---|---|---|---|
| | *P*-value | | |
| | ($n=64$) | ($n=65$) | |
| Age (years) | 61.9 ± 12.1 | 64.6 ± 9.2 | ns |
| Gender (M/F) | 40/24 | 42/23 | ns |
| DCM/ICM/Other disease | 39/16/9 | 45/13/7 | ns |
| NYHA functional class (average) | 2.6 ± 0.6 | 2.6 ± 0.5 | ns |
| Body mass index (kg/m$^2$) | 22.6 ± 3.0 | 21.9 ± 3.5 | ns |
| Heart rate (beats/min) | 74 ± 13 | 71 ± 9 | ns |
| Systolic BP (mmHg) | 112 ± 15 | 111 ± 17 | ns |
| Diastolic BP (mmHg) | 77 ± 69 | 70 ± 10 | ns |
| CTR (%) | 56.2 ± 5.7 | 54.8 ± 5.9 | ns |
| LVDd (mm) | 58.9 ± 11.7 | 59.0 ± 7.8 | ns |
| LVEF (%) | 38.5 ± 15.2 | 35.8 ± 10.9 | ns |
| AF (%) | 32.8 | 36.9 | ns |
| Medications | | | |
| ACE-I or ARB (%) | 68.8 | 64.6 | ns |
| Beta-blocker (%) | 60.9 | 56.9 | ns |
| Digitalis (%) | 39.1 | 49.2 | ns |
| Diuretics (%) | 73.4 | 83.1 | ns |
| Statin (%) | 18.8 | 13.8 | ns |

DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NYHA, New York Heart Association; BP, blood pressure; CTR, cardiothoracic ratio; LVDd, left ventricular diastolic dimension; LVEF, left ventricular ejection fraction; AF, atrial fibrillation; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ns, not significant.

from traditional sauna. Waon therapy has no hydration pressure and was performed as previously reported [6]. Briefly, the patients were placed in a supine or sitting position in a sauna system evenly maintained at 60 °C for 15 min, and then, they underwent bed rest with a blanket to keep them warm for an additional 30 min. All patients were weighed before and after the therapy, and oral hydration with water was used to compensate for weight lost due to perspiration.

## Statistical analyses

Data were analyzed using Stat View 4.0. All data are expressed as the mean ± SD. Differences in baseline characteristics were evaluated by the chi-square test or unpaired *t*-test. The cardiac event point was the time-to-the-first-event of combined cardiac death or re-hospitalization due to heart failure. Cardiac event curves were analyzed with Kaplan–Meier method, and the log-rank test was

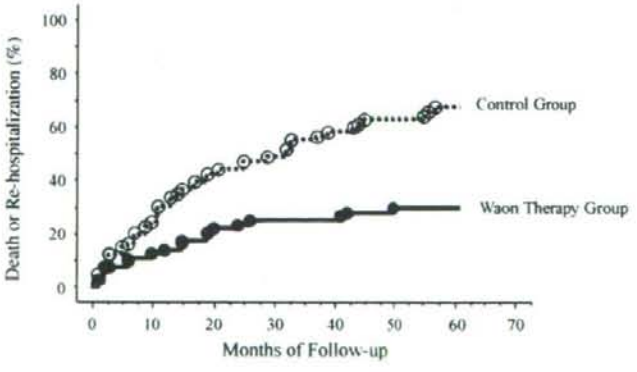


**Figure 1** Re-hospitalization due to heart failure or cardiac death rate was 68.7% in the control group compared to 31.3% in the Waon therapy group ($P<0.01$) at 60 months of follow-up.

Please cite this article in press as: Kihara T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol (2009), doi:10.1016/j.jjcc.2008.11.005

used to assess the differences between two groups. A value of $P < 0.05$ was considered statistically significant.

## Results

### Baseline patient characteristics

Baseline clinical characteristics in the control and Waon therapy groups are shown in Table 1. There were no significant differences in age, gender, or etiology and severity of CHF between the two groups. In addition, there were no significant differences in the use of CHF medications, such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, digitalis, diuretics, or statins between the two groups.

### Cardiac events

All 129 patients were followed-up for 5 years; there was no death due to non-cardiac events during this study, and the overall survival rate was 84.5%. Twelve patients died in the control group and 8 patients died in the Waon therapy group over 60 months of follow-up. Re-hospitalization due to worsening CHF occurred in 44 patients in the control group and 20 patients in the Waon therapy group.

The cardiac event rate, such as cardiac death or re-hospitalization due to heart failure was 68.7% in the control group and 31.3% in the Waon therapy group ($P < 0.01$) at 60 months of follow-up.

Kaplan–Meier analysis demonstrated that Waon therapy significantly reduced the cardiac event rate compared with the control group, and the reduction of cardiac events by Waon therapy was 38% at 60 months of follow-up (Fig. 1).

## Discussion

This retrospective follow-up study demonstrated that Waon therapy decreased cardiac death and re-hospitalization in patients with CHF over a 60-month follow-up period. Although we have already reported in an animal study that repeated Waon therapy improved survival in TO-2 cardiac hamsters with CHF [11], this is the first report to show the beneficial effect of Waon therapy on the long-term prognosis of CHF patients.

We have already reported that Waon therapy induced thermal vasodilation of the systemic and pulmonary arteries and veins, reduced cardiac preload and after-load, and improved hemodynamics and clinical symptoms in CHF patients [6]. In addition, we have reported that 4 weeks of Waon therapy significantly improved clinical symptoms, increased ejection fraction, and decreased cardiac size on echocardiography and chest radiography in CHF patients [7]. Recently, we confirmed the beneficial effects and safety of Waon therapy applied for 2 weeks in CHF patients in a prospective multicenter case–control study [10].

We previously demonstrated that Waon therapy improved not only cardiac function, but also endothelial function in patients with CHF. We have reported that 2 weeks of Waon therapy significantly reduced brain natriuretic peptide blood levels and improved flow-mediated vasodilation in CHF patients [9]. Furthermore, we have reported that Waon therapy for 2 weeks decreased ventricular premature contractions and increased heart rate variability in CHF patients [8], suggesting that Waon therapy decreased sympathetic nervous activity and improved ventricular arrhythmias.

In addition, Waon therapy improved vascular function in patients with coronary risk factors [12,13] or peripheral arterial disease [14,15] and improved exercise capacity in patients with chronic obstructive pulmonary disease [16].

Waon therapy improves cardiac and vascular function and reduces ventricular arrhythmias in CHF patients. We think that these beneficial effects of Waon therapy led to the reduction of cardiac events in CHF patients in the present study.

Furthermore, we reported that Waon therapy increased mRNA and protein expression of endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO) in Syrian golden hamsters [17] and TO-2 cardiomyopathic hamsters [18]. This up-regulation of eNOS and NO may play an important role in the beneficial effects of Waon therapy in CHF patients.

### Study limitation

This study was a retrospective study to investigate the effect of Waon therapy on the prognosis in patients with CHF. A further prospective randomized multicenter study is needed to clarify the beneficial effect of Waon therapy on the prognosis of CHF patients.

In addition, patients in the Waon therapy group went to the hospital at least twice per week. In contrast, patients in the control group went to the hospital once per month. Therefore, this difference in frequency of hospital visits may affect the result of this study.

Please cite this article in press as: Kihara T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol (2009), doi:10.1016/j.jjcc.2008.11.005

## Conclusion

In this retrospective follow-up study, we demonstrated that Waon therapy reduced cardiac events due to heart failure over a 60-month follow-up period. Therefore, this therapy is a promising non-pharmacological treatment for CHF.

## References

[1] The CONSENSUS Trial Study Group. Effects of enalapril on mortality in severe heart failure. N Engl J Med 1987;316:1429–35.

[2] Cohn JN, Tognoni G, Glazer RD, Spormann D, Hester A. Rationale and design of the Valsartan Heart Failure Trial: a large multinational trial to assess the effects of valsartan, an angiotensin-receptor blocker, on morbidity and mortality in chronic congestive heart failure. J Card Fail 1999;5:155–60.

[3] CIBIS Investigators and Committees. A randomized trial of beta-blockade in heart failure. Circulation 1994;90:1765–73.

[4] Mann DL. Management of heart failure patients with reduced ejection fraction. In: Libby P, Bonow RO, Mann DL, Zipes DP, editors. Braunwald's heart disease: a textbook of cardiovascular medicine. 8th ed. Philadelphia: WB Saunders; 2008. p. 611–40.

[5] Tei C. Waon therapy: soothing warmth therapy. J Cardiol 2007;49:301–4.

[6] Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Toyama Y, Tanaka N. Acute hemodynamic improvement by thermal vasodilation in congestive heart failure. Circulation 1995;91:2582–90.

[7] Tei C, Tanaka N. Thermal vasodilation as a treatment of congestive heart failure: a novel approach. J Cardiol 1996;27:29–30.

[8] Kihara T, Biro S, Ikeda Y, Fukudome T, Shinsato T, Masuda A, Miyata M, Hamasaki S, Otsuji Y, Minagoe S, Akiba S, Tei C. Effects of repeated sauna treatment on ventricular arrhythmias in patients with chronic heart failure. Circ J 2004;68:1146–51.

[9] Kihara T, Biro S, Imamura M, Yoshifuku S, Takasaki K, Ikeda Y, Otuji Y, Minagoe S, Toyama Y, Tei C. Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. J Am Coll Cardiol 2002;39:754–9.

[10] Miyata M, Kihara T, Kubozono T, Ikeda Y, Shinsato T, Izumi T, Matsuzaki M, Yamaguchi T, Kasanuki H, Daida H, Nagayama M, Nishigami K, Hirata K, Kihara K, Tei C. Beneficial effects of Waon therapy on patients with chronic heart failure: results of a prospective multicenter study. J Cardiol 2008;52:79–85.

[11] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Kihara T, Minagoe S, Tei C. Effect of repeated sauna therapy on survival in TO-2 cardiomyopathic hamsters with heart failure. Am J Cardiol 2002;90:343–5.

[12] Imamura M, Biro S, Kihara T, Yoshifuku S, Otsuji Y, Minagoe S, Toyama Y, Tei C. Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors. J Am Coll Cardiol 2001;38:1083–8.

[13] Biro S, Masuda A, Kihara T, Tei C. Clinical implications of thermal therapy in lifestyle-related diseases. Exp Biol Med 2003;228:1245–9.

[14] Tei C, Shinsato T, Kihara T, Miyata M. Successful thermal therapy for end-stage peripheral artery disease. J Cardiol 2006;47:163–4.

[15] Tei C, Shinsato T, Miyata M, Kihara T, Hamasaki S. Waon therapy improves peripheral artery disease. J Am Coll Cardiol 2007;50:2169–71.

[16] Umehara M, Yamaguchi A, Itakura S, Suenaga M, Sakaki Y, Nakashiki K, Miyata M, Tei C. Repeated Waon therapy improves pulmonary hypertension during exercise in patients with severe chronic obstructive pulmonary disease. J Cardiol 2008;51:106–13.

[17] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, Kihara T, Tei C. Repeated thermal therapy upregulates arterial endothelial nitric oxide synthase expression in Syrian golden hamsters. Jpn Circ J 2001;65:434–8.

[18] Ikeda Y, Biro S, Kamogawa Y, Yoshifuku S, Eto H, Orihara K, Yu B, Kihara T, Miyata M, Hamasaki S, Otsuji Y, Minagoe S, Tei C. Repeated sauna therapy increases arterial endothelial nitric oxide synthase expression and nitric oxide production in cardiomyopathic hamsters. Circ J 2005;69:722–9.



Please cite this article in press as: Kihara T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol (2009), doi:10.1016/j.jjcc.2008.11.005

*Original Article*

# The Long-Term Effect of Angiotensin II Type 1a Receptor Deficiency on Hypercholesterolemia-Induced Atherosclerosis

Hideyuki ETO[1], Masaaki MIYATA[1], Takahiro SHIRASAWA[1], Yuichi AKASAKI[1], Narisato HAMADA[1], Aya NAGAKI[1], Koji ORIHARA[1], Sadatoshi BIRO[1], and Chuwa TEI[1]

**Angiotensin II type 1 receptor may contribute to atherogenesis by facilitating the proliferative and inflammatory response to hypercholesterolemia. In the present study, we investigated the long-term effect of angiotensin II type 1a receptor (AT1a) deficiency on hypercholesterolemia-induced atherosclerosis by the use of AT1a-knockout (AT1a-KO) mice and apolipoprotein E–knockout (apoE-KO) mice. AT1a-KO were crossed with apoE-KO, generating double-knockout (D-KO) mice. Mice were fed a standard diet and analyzed at 25- or 60-weeks-old. The quantification of atherosclerotic volume in the aortic root revealed that the atherosclerotic lesions of D-KO mice were significantly smaller than those of apoE-KO mice at 25-week-old ($0.81\pm0.16$ mm$^2$ *vs.* $1.05\pm0.21$ mm$^2$, $p<0.001$) and at 60-week-old ($0.89\pm0.11$ mm$^2$ *vs.* $2.44\pm0.28$ mm$^2$, $p<0.001$). Surprisingly, there was no significant difference in atherosclerotic lesion size of D-KO mice at 25- and 60-week-old, suggesting that AT1a deficiency completely protected against the age-related progression of atherosclerosis. The amounts of collagen and elastin, the expression of p22phox, serum amyloid P (SAP), matrix metalloproteinase (MMP)-2, and MMP-9, and the number of apoptotic cells of D-KO mice were lower than those of apoE-KO mice. Furthermore, we confirmed that the expression of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 decreased in cultured vascular smooth muscle cells from D-KO mice compared with those of apoE-KO mice. In conclusion, AT1a deficiency reduces atherosclerotic lesion size of apoE-KO mice and protects against the age-related progression of atherosclerosis. Reduction of oxidative stress, apoptosis, and MMP expression in atherosclerotic lesions by AT1a deficiency may contribute to plaque size. (*Hypertens Res* 2008; 31: 1631–1642)**

***Key Words***: **angiotensin, apoptosis, atherosclerosis, hypercholesterolemia, oxidative stress**

## Introduction

Angiotensin II (Ang II) is a potent vasoconstrictor, and, apart from its effects on blood pressure, it is strongly implicated in the pathogenesis of atherosclerosis. Oxidative stress and inflammation, triggered by Ang II, are involved in the initiation and progression of atherosclerosis (*1–3*). Ang II elicits production of superoxide anion, a reactive oxygen species (ROS), from arterial endothelial cells (EC) and smooth muscle cells (SMC) (*4*). Ang II can also increase expression of proinflammatry cytokines such as interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 in arterial SMC and the leukocyte adhesion molecule, VCAM-1, in EC (*5–7*).

Angiotensin II type 1 receptor (AT1) may contribute to atherogenesis by facilitating the proliferative and inflammatory response to hypercholesterolemia. AT1 blockers (ARB) and angiotensin-converting enzyme (ACE) inhibitors normal-

From the [1]Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, Kagoshima, Japan.

Address for Reprints: Masaaki Miyata, M.D., Ph.D., Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8–35–1 Sakuragaoka, Kagoshima 890–8520, Japan. E-mail: miyatam@m3.kufm.kagoshima-u.ac.jp

Received March 11, 2008; Accepted in revised form April 11, 2008.

ize oxidative stress and endothelial dysfunction, and they reduce the progression of atherosclerosis (*8*, *9*). Several studies using various animal models have shown that blocking the actions of Ang II by ACE inhibitors or ARB is effective in preventing the progression of atherosclerosis (*9*). Recently, it was reported that AT1 deficiency significantly reduced hypercholesterolemia-induced atherosclerosis in apolipoprotein E (apoE)-knockout (apoE-KO) mice at 19-weeks-old (*10*) or in low density lipoprotein (LDL) receptor deficient mice at 20-weeks-old (*11*). However, the long-term effect of angiotensin II type 1a receptor (AT1a) deficiency on hypercholesterolemia-induced atherosclerosis has not been reported. AT1-KO mice did not show any atherosclerotic lesion themselves. Therefore, AT1a-KO mice were crossed with apoE-KO mice in order to generate double-knockout (D-KO) mice. We compared the atherosclerotic lesion and expression of factors that are related to plaque vulnerability between the apoE-KO and D-KO mice at 25- and 60-week-old. Furthermore, to address the role of AT1 in atherogenesis, we performed cell culture experiments using vascular smooth muscle cells (VSMCs) explanted from the medial layer of aortas from the D-KO or apoE-KO mice.

## Methods

### Generation of AT1a$^{-/-}$/ApoE$^{-/-}$ D-KO Mice

ApoE-KO mice on a C57BL/6 background were donated by Dr. Jan L Breslow (Rockefeller University, New York, USA), and AT1a-KO mice on a C57BL/6 background were obtained from Tanabe Seiyaku Co. Ltd. (Osaka, Japan). ApoE-KO mice were crossed with AT1a-KO mice, and heterozygous knockout mice were crossed until homozygous D-KO mice were obtained. Genotypes for apoE and AT1a were determined by polymerase chain reaction amplification of DNA isolated from the tail. These mice were fed a standard diet, and water was available ad libitum. The study protocol was approved by the ethics committee of the Graduate School of Medicine, Kagoshima University. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Measurements of Heart Rate, Blood Pressure, Cholesterol, and 8-Isoprostane

D-KO and apoE-KO mice were compared in this study. Before sacrifice, the heart rate and blood pressure of conscious restrained mice were measured using a tail-cuff system. At the age of 25 or 60 weeks, mice were anesthesized with pentobarbital (80 μg/kg, i.p.) after measuring body weight, and blood was drawn from the left ventricle of anesthesized mice. The mean blood pressure was calculated from the systolic and diastolic blood pressure. Plasma total- and high density lipoprotein (HDL)-cholesterol were measured with an enzymatic kit (Cat.#DR-2100, DR-2210, Kainos Laboratories, Inc., Tokyo, Japan).

To clarify the production of oxidative stress, 8-isoprostane (a plasma indicator of oxidative stress) was determined using plasma from the apoE-KO and D-KO mice using an 8-Isoprostane EIA Kit (Cat.#516351, Cayman Chemical, Ann Arbor, USA) as described previously (*12*).

### Genotyping by PCR

AT1a genotyping used the following primers: antisense, 5′-ATCCTAAAGATGTCATCATTTC-3′ and sense, 5′-ATG GATTTTGAACAGTGTTG-3′. Resultant wild-type and deficient allele bands were 770 bp and 1,134 bp, respectively (data not shown). ApoE genotyping used the following primers: antisense, 5′-GCCTAGCCGAGGGAGAGCCG-3′ and sense, 5′-GCCGCCCCGACTGCATCT-3′. Resultant wild-type and deficient allele bands were 155 bp and 245 bp, respectively (data not shown).

### Tissue Preparation and Lesion Assessment

Animals were anesthesized with pentobarbital (80 μg/kg, i.p.) and perfused with phosphate-buffered saline (pH 7.4) followed by 10% neutral-buffered formalin through a catheter placed in the left ventricle. The heart with aortic root was fixed in 10% neutral-buffered formalin, embedded in O.C.T. compound (O.C.T.; Sakura Finetechnical Co., Tokyo, Japan) and frozen at −80°C. Frozen sections were cut into 10-μm sections and fixed to glass slides. The slides were stained with oil red O. All sections were examined under a microscope, and the lipid-staining aorta and total area of the histological sections were measured (*13*).

For en face preparations, the aorta was dissected from the aortic valve to the iliac bifurcation and fixed in 10% neutral-buffered formalin. The aorta was opened longitudinally and pinned on a board. To identify atherosclerotic lesions, the aorta was stained with oil red O. It has been reported that the extent of atherosclerosis in the entire aorta (expressed as percent of surface area) reflects the extent of atherosclerosis in the aortic root (expressed as average lesion area per section) of apoE-KO mice and LDL receptor deficient mice (*14*).

For histological analysis of the extracellular matrix (ECM) and immunohistochemical staining, the hearts and aortas of mice were carefully removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Azan staining for collagen and Victoria blue staining for elastin were carried out to assess matrix production.

### Immunohistochemical Analysis

Immunohistochemical staining was carried out on paraffin-embedded sections as described previously (*15*). After deparaffinization and hydration of specimens, endogenous peroxidase activity was blocked, and the specimens were fixed by

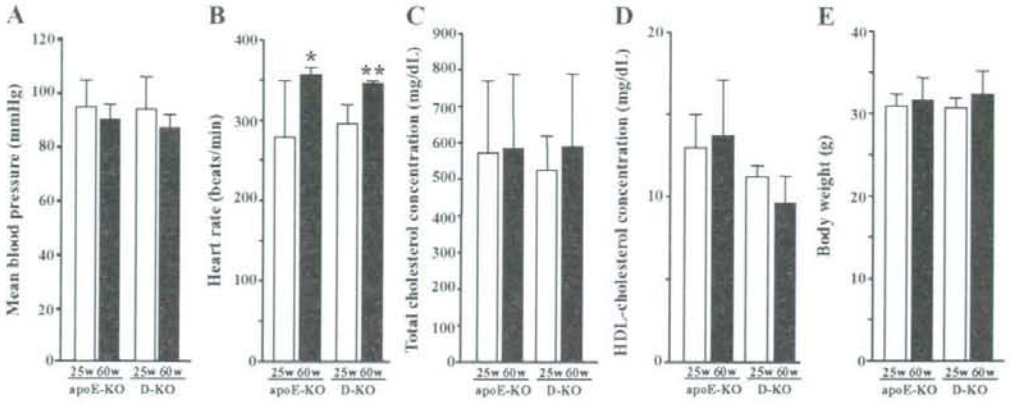


**Fig. 1.** *Hemodynamic parameters, body weight, and cholesterol concentration. There was no significant difference in mean blood pressure (A), heart rate (B), total cholesterol concentration (C), HDL-cholesterol concentration (D), or body weight (E) between apoE-KO and D-KO mice at both 25- and 60-week-old. There was no significant difference in mean blood pressure, total- and HDL-cholesterol concentration, or body weight between 25- and 60-week-old in both apoE-KO and D-KO mice. However, heart rate at 60-week-old was significantly higher than that at 25-week-old. *$p < 0.05$ vs. 25-week-old apoE-KO mice, **$p < 0.05$ vs. 25-week-old D-KO mice.*

immersion in 0.3% $H_2O_2$ in methanol for 20 min. Immunohistochemical staining was performed with a goat polyclonal antibody against human muscle actin (Cat.#sc-1615, Santa Cruz Biotechnology, Santa Cruz, USA), a goat polyclonal antibody against human CD-64 (Cat.#sc-7642, Santa Cruz Biotechnology), a goat polyclonal antibody against human p22phox (Cat.#sc-11712, Santa Cruz Biotechnology), a goat polyclonal antibody against mouse serum amyloid P (SAP; Cat.#sc-18312, Santa Cruz Biotechnology), a goat polyclonal antibody against human matrix metalloproteinase (MMP)-2 (Cat.#sc-6838, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human MMP-9 (Cat.#sc-10737, Santa Cruz Biotechnology), a goat polyclonal antibody against human angiotensin II type 2 receptor (AT2, Cat.#sc-7420, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human activated caspase-3 (Cat.#557035, Santa Cruz Biotechnology), and a goat polyclonal antibody against human apoptosis inducing factor (AIF; Cat.#sc-9416, Santa Cruz Biotechnology) using the labeled streptavidin biotin complex method (Cat.#424141, 424151, Simple-stain MAX-PO kit, Nichirei, Tokyo, Japan). After blocking with 10% rabbit or goat serum, slides were incubated overnight with a primary antibody at 4°C in a moisture chamber. Slides were washed with Tris-buffered saline (TBS) and incubated with a biotinylated secondary antibody at room temperature for 30 min. After washing with TBS, slides were incubated with streptavidin at room temperature for 30 min and visualized with 3,3′-diaminobenzidine.

## Cell Experiments

Primary VSMCs were explanted from the medial layer of apoE-KO or D-KO aortas as described previously (*16*). They were cultured in 75 $cm^2$ flasks at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$, and the medium was changed every 3 d. The growth medium consisted of Dulbecco's modified Eagle Medium (D-MEM; Cat.#31600-034, Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Cat.#12303-500M, SAFC Biosciences, Wicklow, Ireland), 100 units/mL penicillin, and 100 mg/mL streptomycin (Cat.#15140-122, Invitrogen). VSMCs used for experiments were from the third to the fifth passages.

Reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously (*17*). Total RNA for use in RT-PCR was isolated using the Mini RNA isolation kit (Cat.#R1005, ZYMO RESEARCH, Orange, USA). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed with random hexamers using SuperScript II RT (Cat.#18064-022, Invitrogen). The transcribed cDNA was amplified by PCR with specific primers for procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, MMP-9, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Two specific primer pairs corresponding to published sequences were used to amplify procollagen α1(I) (5′-CAGCCGCTTCACCTACAGC-3′ and 5′-AATCACTGT CTTGCCCCAGG-3′) (*18*), procollagen α1(III) (5′-TCCAACTGCTCCTACTCGCC-3′ and 5′-GAGGGCCTG GATCTCCCTT-3′) (*18*), tropoelastin (5′-GGTGCGGTG GTTCCTCAGCCTGG-3′ and 5′-GGGCCTTGAGATACC

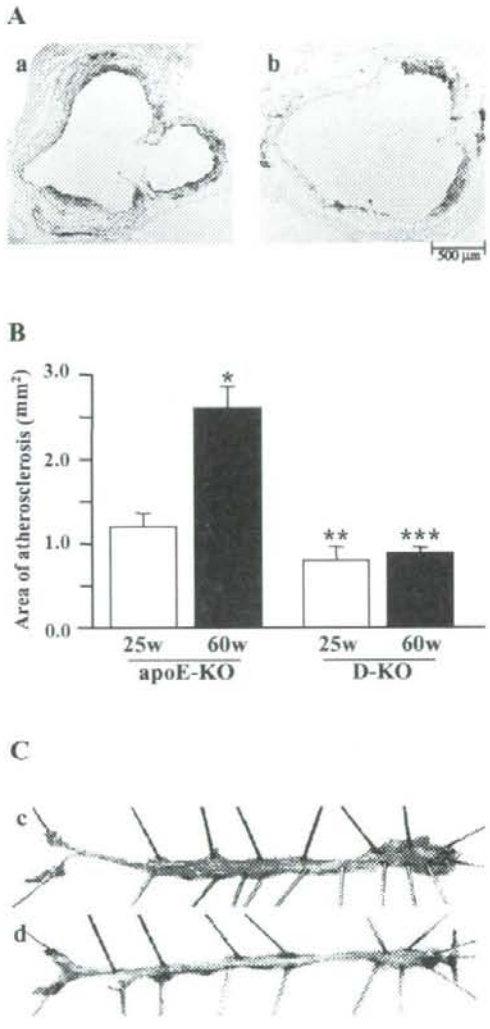


**Fig. 2.** *Atherosclerotic lesions. A: Representative oil red O stained cross-sections of the aortic root of apoE-KO mice (a) and D-KO mice (b) at 60-week-old. B: Quantification of atherosclerotic volume in aortic root. Atherosclerotic lesions in D-KO mice were significantly smaller than those in apoE-KO mice at 25- and 60-week-old. *$p < 0.001$ vs. 25-week-old apoE-KO mice, **$p < 0.001$ vs. 25-week-old apoE-KO mice, ***$p < 0.001$ vs. 60-week-old apoE-KO mice. C: Oil red O stained en face preparation of longitudinally opened aortas of a 60-week-old apoE-KO mouse (c) and a D-KO mouse (d).*

CCAGTG-3′) (*19*), p22phox (5′-TGGGCGGCTGCT TATGGT-3′ and 5′-GTTTGTGTGCCTGCTGGAGT-3′) (*20*), SAP (5′-CTCAGACAGACCTCAATCAG-3′ and 5′-TCAGCAATACCAGAGGAGGA-3′) (*21*), MMP-2 (5′-ACCCAGATGTGGCCAACTAC-3′ and 5′-TACTTT TAAGGCCCGAGCAA-3′) (*22*), MMP-9 (5′-ATGATG GAGGAGAAGCAGTC-3′ and 5′-AGGTGAAGGGAA AGTGACAT-3′) (*23*), and GAPDH (5′-CAGGAATTCGGT GAAGGTCGGAGTCAAGGG-3′ and 5′-AGTGGATCC GGTCATGAGTCCTCCCAGGAT-3′) (*24*). The PCR amplification protocol included 35 cycles of denaturing, annealing, and elongation with *Taq* polymerase (Cat.#R001A, TAKARA BIO Inc., Shiga, Japan). Equal amounts of PCR products were subjected to electrophoresis through 1.5% agarose gels and visualized with ethidium bromide. The quantification of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, MMP-9, and GAPDH mRNA was analyzed by real-time PCR using LightCycler FastStart DNA Master SYBR Green I Kit (Cat.#3003230, Roche Diagnostics K.K., Tokyo, Japan) as described previously (*25*). GAPDH expression was used as a reference for quantification of the respective mRNAs.

### Western Blotting

For Western blotting of collagen type I and elastin, proteins were extracted from apoE-KO and D-KO aortas as reported previously (*17*). The aorta was ground to a fine powder under liquid nitrogen and incubated in ice cold 0.1% Triton lysis solution (10 mmol/L HEPES [pH 7.4], 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, 50 mmol/L NaCl, 100 mmol/L $Na_3VO_4$, 0.1% Triton X-100, 500 mmol/L PMSF, and 10 mg/mL leupeptin) for 30 min.

For Western blotting of p22phox, SAP, MMP-2, MMP-9, activated caspase-3, and AIF, cytoplasmic and nuclear proteins were extracted from cultured VSMCs of apoE-KO and D-KO using the Protein and RNA Isolation System (Cat.#1921, Ambion Inc., Austin, USA).

Insoluble matter was removed by centrifugation, and the protein concentration was measured by a bicinchoninic acid assay (PIERCE Biotechnology Inc., Rockford, USA). Western blotting was performed with a NuPAGE™ Electrophoresis System (Cat.#NPO322BOX, Invitrogen) as reported previously (*17*). Briefly, 10-μg protein samples were resuspended in reduced sample buffer, electrophoresed on a 4–12% Bis-Tris gel (Invitrogen) with MOPS running buffer, blotted to nitrocellulose membrane, and sequentially probed with a goat polyclonal antibody against human collagen type I (Cat.#sc-27954, Santa Cruz Biotechnology), a goat polyclonal antibody against elastin (Cat.#sc-17581, Santa Cruz Biotechnology), a goat polyclonal antibody against human p22phox (Cat.#sc-11712, Santa Cruz Biotechnology), a goat polyclonal antibody against mouse SAP (Cat.#sc-18312, Santa Cruz Biotechnology), a goat polyclonal antibody

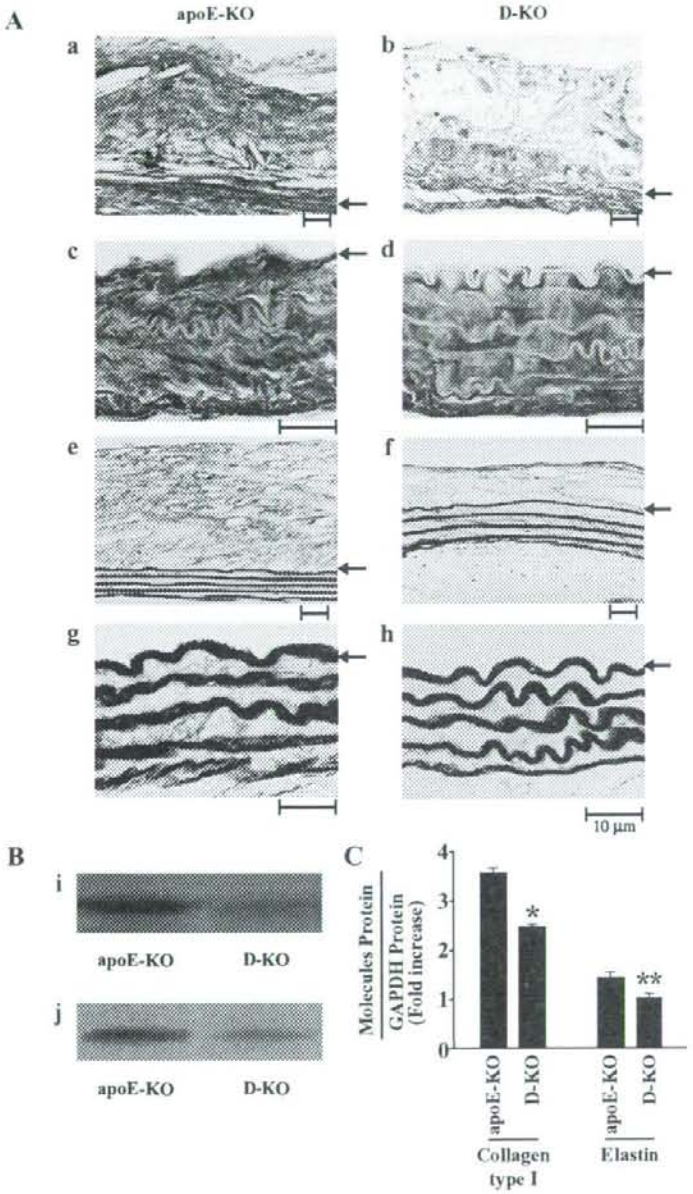


**Fig. 3.** *Extracellular matrix. A: Extracellular matrix was analyzed in atherosclerotic lesions of the aortic root in apoE-KO (a, e) and D-KO mice (b, f) at 60 weeks of age. The amount of collagen detected by Azan staining (b, d) and the amount of elastin detected by Victoria blue staining (f, h) in atheromas or non-atherosclerotic aortas of D-KO mice were less than those of apoE-KO mice. The arrows indicate internal elastic lamina. B: Western blotting for collagen type I (i) and elastin (j) of aorta. C: Densitometry of Western blots for collagen type I and elastin protein of aorta. The amounts of collagen type I and elastin proteins of D-KO mice were less than those of apoE-KO mice.* $^{*}p < 0.01$ *vs. apoE-KO mice,* $^{**}p < 0.05$ *vs. apoE-KO mice.*

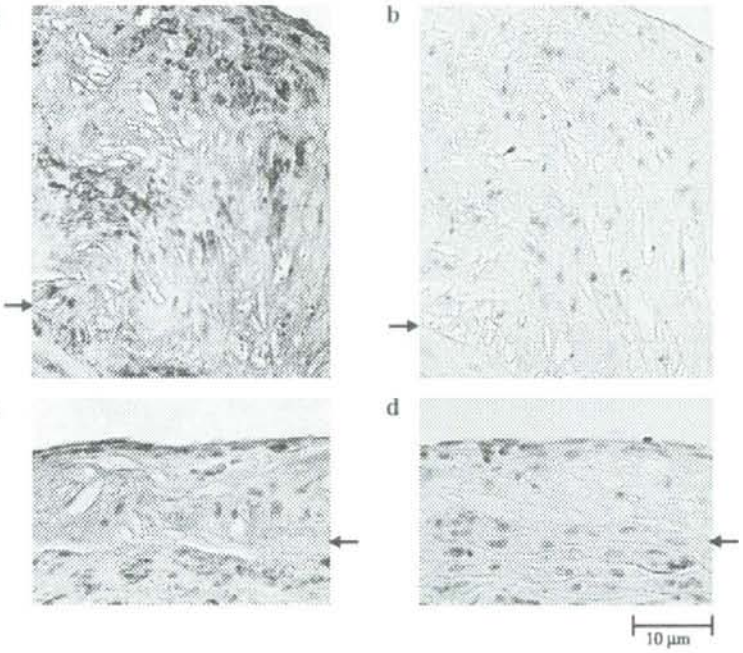


**Fig. 4.** *Immunohistochemical staining for muscle actin (a, c) and CD-64 (b, d) of apoE-KO (a, b) and D-KO mice (c, d) at 60 weeks of age. Muscle actin is a marker for VSMCs, and CD-64 is a marker for macrophages. The immunoreactivity of muscle actin was detected in apoE-KO and D-KO at 60-week-old, but the immunoreactivity of CD-64 was only slightly apparent. The arrows indicate internal elastic lamina.*

against human MMP-2 (Cat.#sc-6838, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human MMP-9 (Cat.#sc-10737, Santa Cruz Biotechnology), a rabbit polyclonal antibody against human activated caspase-3 (Cat.#557035, Santa Cruz Biotechnology), and a goat polyclonal antibody against human AIF (Cat.#sc-9416, Santa Cruz Biotechnology). Either horseradish peroxidase–conjugated rabbit anti-goat antibody (Santa Cruz Biotechnology) or donkey anti-rabbit antibody (Santa Cruz Biotechnology) was then added, and the secondary antibody was detected by autoradiography using enhanced chemiluminescence (Cat.#RPN2132, ECL Plus, GE Healthcare UK Ltd., Little Chalfont, UK). GAPDH expression was used as a reference for quantification of the respective proteins.

### Statistical Analysis

All calculated data are presented as the mean±SD. Statistical significance was evaluated using unpaired Student's *t*-test for comparisons between two groups. A probability value of $p<0.05$ was considered statistically significant.

## Results

### Hemodynamic Parameters, Body Weight, Cholesterol Concentration, and 8-Isoprostane

At both 25- and 60-week-old, there were no significant differences in mean blood pressure, heart rate, total- and HDL-cholesterol concentrations, or body weight between the apoE-KO and D-KO mice. In both apoE-KO and D-KO mice, although there was no significant difference in mean blood pressure, total- and HDL-cholesterol concentrations, or body weight between 25- and 60-week-old, the heart rate at 60-week-old was significantly higher than in those at 25-week-old (Fig. 1).

The plasma concentration of 8-isoprostane in the D-KO mice was significantly lower than that in the apoE-KO mice at 60-week-old (135.7±18.1 *vs.* 230.7±36.0 pg/mL, $p<0.05$).

### Atherosclerosis and Extracellular Matrix

We analyzed atherosclerotic lesions in aortic roots and ECM production in both the aortic root and aorta. Figure 2A shows

representative oil red O stained cross-sections of the aortic root of apoE-KO and D-KO at 60-week-old. Figure 2C demonstrates oil red O stained en face preparations of longitudinally opened aortas of 60-week-old apoE-KO and D-KO mice. The atherosclerotic area of D-KO mice was smaller than of apoE-KO mice. The quantification of atherosclerotic volume in the aortic root revealed that the atherosclerotic lesion of D-KO mice was significantly smaller than that of apoE-KO mice at 25-week-old ($0.81\pm0.16$ *vs.* $1.05\pm0.21$ mm$^2$, $p<0.001$) and at 60-week-old ($0.89\pm0.11$ *vs.* $2.44\pm0.28$ mm$^2$, $p<0.001$). Surprisingly, there was no significant difference in atherosclerotic lesion size of D-KO mice at 25- and 60-week-old, and AT1a deficiency completely protected against the age-related progression of atherosclerosis (Fig. 2B).

We analyzed the ECM in atherosclerotic lesions of the aortic root. Azan staining for collagen and Victoria blue staining for elastin were carried out to assess matrix production. Expression of collagen type I and elastin proteins in aorta was quantitatively analyzed by Western blotting. The amount of collagen and elastin in atheromas of D-KO mice was less than that of apoE-KO mice (Fig. 3).

### Immunohistochemistry

In order to analyze factors that are related to plaque vulnerability, immunohistochemical staining was performed using primary antibodies against muscle actin, CD-64, p22phox, SAP, MMP-2, MMP-9, or AT2. Muscle actin is a marker for VSMCs, and CD-64 is a marker for macrophages. p22phox is an essential component of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, which causes oxidative stress. SAP is highly inducible during the acute-phase response in mice and is a marker for inflammation. MMP-2 and MMP-9 may be co-related with plaque stability in atherosclerotic lesions.

The immunoreactivity of muscle actin was detected in atherosclerotic lesions of apoE-KO and D-KO mice at 60-week-old, but the immunoreactivity of CD-64 was only slightly apparent (Fig. 4). Thus, the majority of cells in the atherosclerotic lesions in old mice were found to be VSMCs, not macrophages. The immunoreactivities of p22phox, SAP, MMP-2, and MMP-9 in atherosclerotic lesions of D-KO mice were lower than those of apoE-KO mice (Fig. 5a–h). Furthermore, using immunohistochemistry, we analyzed the expression of AT2, which can act as an antagonist of AT1 responses such as blood pressure, vascular reactivity, and apoptosis. We hypothesized that the absence of AT1 may lead to a compensatory up-regulation of AT2. However, AT2 expression in atherosclerotic lesions of D-KO mice did not differ from those of apoE-KO mice (Fig. 5i, j).

### Cell Experiments

To confirm the precise effect of AT1 on the expression of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 in VSMCs, we performed cell culture experiments using VSMCs explanted from the medial layer of aortas from apoE-KO or D-KO mice. mRNA expression of these molecules was evaluated by RT-PCR and real-time PCR. Decreases in mRNA expression of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 were found in VSMCs of D-KO mice compared with apoE-KO mice (Fig. 6A, B).

We also performed Western blotting to analyze the protein expression of p22phox, SAP, MMP-2, and MMP-9 in cultured VSMCs. The level of protein expression of these molecules decreased in D-KO mice compared with apoE-KO mice (Fig. 6C).

### Apoptosis

We analyzed apoptosis in atherosclerotic lesions of D-KO and apoE-KO mice at 60-week-old by immunohistochemistry. The expression of activated caspase-3 and AIF in atherosclelotic lesions decreased in D-KO mice compared with apoE-KO mice. Moreover, expressions of activated caspase-3 and AIF of cultured VSMCs from D-KO mice were lower than those from the apoE-KO mice (Fig. 7).

## Discussion

AT1 may contribute to atherogenesis by facilitating proliferative and inflammatory responses to hypercholesterolemia. To clarify the long-term effect of AT1 in atherogenesis, AT1a-KO mice were crossed with apoE-KO mice, resulting in D-KO mice. Although there was no significant difference in blood pressure or total- and HDL-cholesterol concentrations between D-KO and apoE-KO mice, the atherosclerotic lesions of D-KO mice were significantly smaller than those of apoE-KO mice. The amount of collagen and elastin, the expression of p22phox, SAP, MMP-2 and MMP-9, and the number of apoptotic cells in atherosclerotic lesions of D-KO mice were lower than those of apoE-KO mice. However, AT2 expression in atherosclerotic lesions of D-KO mice did not differ from AT2 expression in the lesions of apoE-KO mice. Furthermore, we confirmed that the expression of procollagen α1(I), procollagen α1(III), tropoelastin, p22phox, SAP, MMP-2, and MMP-9 decreased in cultured VSMCs of D-KO mice compared with apoE-KO mice.

Ang II activates NAD(P)H oxidase, which is a major source of ROS production by vascular cells (*26*). Oxidative stress initiates several processes involved in atherogenesis, including expression of adhesion molecules, stimulation of VSMC proliferation and migration, apoptosis in the endothelium, oxidation of lipids, activation of matrix metalloproteinases, and altered vasomotor activity (*27, 28*). We confirmed that AT1 deficiency reduced the plasma concentration of 8-isoprostane.

Vascular NAD(P)H oxidase consists of a cytochrome b558,