These results suggest that exercise training plus calorie restriction might cause synergistic protection against cognitive decline via up-regulation of BDNF in the hippocampus of SHRSP.

It has already demonstrated that exercise training cause the protection against cognitive decline via up-regulation of BDNF in the hippocampus [7, 8]. The results obtained in the present study were compatible to these previous studies. A previous study indicates that superoxide induces down-regulation of BDNF via phosphorylation of cAMP response element binding protein [17]. We have demonstrated that angiotensin II type 1 receptor-induced superoxide is increased in the brain of SHRSP [18], and exercise training reduces superoxide in the brain of SHRSP [14]. Several previous studies have demonstrated that the exercise training inhibits the brain renin-angiotensin system including angiotensin converting enzyme (ACE), ACE2, angiotensin II, angiotensin- (1-7), and their receptors [19-21]. Furthermore, one of the important activating factors of brain renin-angiotensin system is the inflammatory cascade [22], and exercise training is known to lower the inflammatory substances in the brain of rats [23]. We consider that the exercise training-induced anti-inflammation, anti-oxidant, and inhibition of brain renin-angiotensin system cause the up-regulation of BDNF in the hippocampus, which contribute to the protection against cognitive decline.

Interestingly, in the present study, exercise training plus calorie restriction improved the cognitive performance and increases BDNF in the hippocampus to a greater extent than exercise training alone in spite of the similar depressor effects, whereas calorie restriction alone did not cause such effects. We consider that these results involve two findings. First, exercise training-induced protection against cognitive decline is independent of its depressor effect. Second, exercise training plus calorie restriction causes synergistic

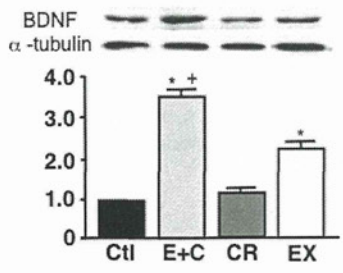

Figure 2. Expression of BDNF in the hippocampus in each group. BDNF / α-tubulin expression was expressed relative to that in Ctl, which was assingned a value of 1. *P<0.05 versus Ctl, +P<0.05 in E+C versus EX, n=5 for each. Abbreviations; Ctl, control; E+C. exercise training+calorie restriction; CR, calorie restriction; EX, exercise training.

protection effect against cognitive decline. Previously, we have demonstrated that exercise training inhibits sympathetic nervous system activation via reduction of oxidative stress in the brain of SHRSP [14]. Furthermore, we also have demonstrated that calorie restriction inhibits sympathetic nervous system activation via reduction of oxidative stress in the brain of dietary-induced obesity rats [15]. These previous results suggest that exercise training or calorie restriction could affect the brain. Although the mechanism in which calorie restriction inhibits oxidative stress in the brain could not been determined in the present study, we hypothesize that calorie restriction may improve adipocytes, inhibit central renin-angiotensin system, directly inhibit oxidative stress in the brain. Circulating angiotensin II acts at circumventricular organs to subsequently activate complex pathways, including those using central angiotensin II as a neurotransmitter, to increases sympathetic outflow [24]. We consider that calorie restriction also reduce oxidative stress in the hippocampus through these mechanisms, and causes the synergistic effect to exercise training. However, it is necessary to do further examination.

To determine the cognitive function, we performed Morris water maze test in the present study, instead of the shuttle avoidance test so that we could focus on hippocampus function. A spatial working memory task, such as Morris water maze test, depends on hippocampus function [25, 26]. Moreover, we used SHRSP as a hypertension and cerebrovascular disease model, and examined the cognitive function by only Morris water maze test. We must do further examination with regard to other cognitive functions in other models, such as Alzheimer, diabetic, and aging.

There are several study limitations in the present study. First, we did not determine the strength and the physiological benefits of the exercise training, such as body weights, lactate level and maximum O_2 consumption. Second, we did not check the calorie restriction-induced changes in metabolism. We did not clarify the cause-and-effect between exercise training/calorie restriction and cognitive function due to these limitations. We have to perform the further studies.

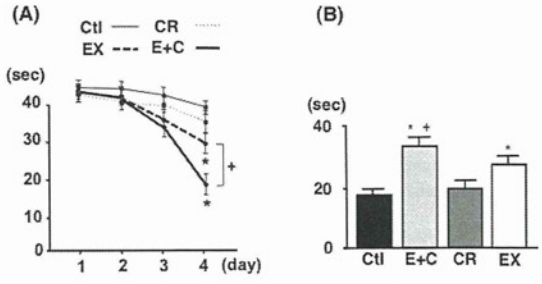

Figure 3. (A) Escape latency time in each group, (B)Time in the target quadrant in each group. *P<0.05 versus Ctl, +P<0.05 in E+C versus EX, n=5 for each. Abbreviations; Ctl, control; E+C. exercise training+calorie restriction; CR, calorie restriction; EX, exercise training.

V. Conclusion

Exercise training plus calorie restriction causes synergistic protective effect against cognitive decline via up-regulation of BDNF in the hippocampus of SHRSP. These results indicate that both exercise training and calorie restriction should be done to the patients with hypertension for the protection against cognitive decline in addition to the pharmacological therapy.

APPENDIX

None.

ACKNOWLEDGMENT

THIS WORK WAS SUPPORTED BY A GRANT-IN-AID FOR SCIENTIFIC RESEARCH FROM THE JAPAN SOCIETY FOR THE PROMOTION OF SCIENCE (22790709).

REFERENCES

[1] Berr C, Balansard B, Arnaud J, Roussel AM, Alperovitch A, "Cognitive decline is associated with systemic oxidative stress: the EVA study. Etude du Vieillissement Arterial," *J Am Geriatr Soc*, 2000, vol. 48, pp. 1285-1291.

[2] Sato H, Takahashi T, Sumitani K, Takatsu H, Urano S, "Glucocorticoid generates ROS to induce oxidative injury in the hippocampus, leading to impairment of cognitive function of rats," *J Clin Biochem Nutr*, 2010, vol. 47, pp. 224-232.

[3] Mancias M, Dwornik A, Ziemlinska E, Fehr S, Schachner M, Czarkowska-Bauch J, Skup M, "Locomotor exercise alters expression of pro-brain-derived neurotrophic factor, brain-derived neurotrophic factor and its receptor TrkB in the spinal cord of adult rats," *Eur J Neurosci*, 2007, vol. 25, pp. 2425-2444.

[4] Zacchigna S, Lambrechts D, Carmeliet P, "Neurovascular signaling defects in neurodegeneration. *Nat Rev*, 2008, vol. 9, pp. 169-181.

[5] Lee TH, Yang JT, Kato H, Wu JH, "Hypertension downregulates the expression of brain-derived neurotrophic factor in the ischemic-vulnerable hippocampal CA1 and cortical areas after carotid artery occlusion," *Brain Res*, 2006, vol. 1116, pp. 31-38.

[6] Beck T, Lindholm D, Castren E, Wree A, "Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus," *J Cereb Blood Flow Metab*, 1994, vol. 14, pp. 689-692.

[7] Liu YF, Chen HI, Wu CL, Kuo YM, Yu L, Juang AM, Wu FS, Chuang JI, Jen CJ, "Differential effects of treadmill running and wheel running on spatial or aversive learning and memory: roles of amygdalar brain-derived neurotrophic factor and synaptotagmin I," *J Physiol*, 2009, vol. 586, pp. 3221-3231.

[8] Kim H, Heo HI, Kim DH, Ko IG, Lee SS, Kim SE, Kim TW, Ji ES, Kim JD, Shin MS, Choi YW, Kim CJ, "Treadmill exercise and methylphenidate ameliorate symptoms of attention deficit/hyperactivity disorder through enhancing dopamine synthesis and brain-derived neurotrophic factor expression in spontaneously hypertensive rats," *Neurosci Lett*, 2011, vol. 504, pp. 35-39.

[9] Gomez-Pinilla F, Vaynman S, Ying Z, "Brain-derived neurotrophic factor functions as a metabotrophin to mediate the effects of exercise on cognition," *Eur J Neurosci*, 2008, vol. 28, pp. 2278-2287.

[10] Wu P, Shen Q, Dong S, Xu Z, Tsien JZ, Hu Y, "Calorie restriction ameliorates neurodegenerative phenotypes in forebrain-specific presenilin-1 and presenilin-2 double knockout mice," *Neurobiol Aging*, 2008, vol. 29, pp.1502-1511.

[11] Yilmaz N, Vural H, Yilmaz M, Sutcu R, Sirmali R, Hicyilmaz H, Delibas N, "Calorie restriction modulates hippocampal NMDA receptors in diet-induced obese rats," *J Recept Signal Transduct Res*, 2011, vol. 31, pp. 214-219.

[12] Martin CK, Anton SD, han H, York-Crowe E, Redman LM, Ravussin E, Williamson DA, "Examination of cognitive function during six months of calorie restriction: results of a randomized controlled study," *Rejuvenation Res*, 2007, vol. 10, pp. 179-190.

[13] Kimura S, Saito H, Minami M, Togashi H, Nakamura N, Nemoto M, Parvez HS, "Pathogenesis of vascular dementia in stroke-prone spontaneously hypertensive rats," *Toxicology*, 2000, vol. 153, pp. 167-178.

[14] Kishi T, Hirooka Y, Katsuki M, Sunagawa K, "Exercise training inhibits sympathetic nerve activity via blockade of AT_1 receptor in the rostral ventrolateral medulla of hypertensive rats," *Clin Exp Hypertens*, to be published.

[15] Kishi T, Hirooka Y, Ogawa K, Konno S, Sunagawa K, "Calorie restriction inhibits sympathetic nerve activity via anti-oxidant effect in the rostral ventrolateral medulla of obesity-induced hypertensive rats," *Clin Exp Hypertens*, 2011, vol. 33, pp. 245-250.

[16] Morris R, "Development of a water-maze procedure for studying spatial learning in the rat," *J Neurosci Methods*, 1984, vol. 11, pp. 47-60.

[17] Chan SH, Wu CW, Chang AY, Hsu KS, Chan JY, "Transcriptional upregulation of brain-derived neurotrophic factor in rostral ventrolateral medulla by angiotensin II: significant in superoxide homeostasis and neural regulation of arterial pressure," *Circ Res*, 2010, vol. 107, pp. 1127-1139.

[18] Kishi T, Hirooka Y, Konno S, Ogawa K, Sunagawa K, "Angiotensin II type 1 receptor-activated caspase-3 through ras/mitogen-activated protein kinase-extracellular signal-regulated kinase in the rostral ventrolateral medulla is involved in sympathoexcitation in stroke-prone spontaneously hypertensive rats," *Hypertension*, 2010, vol. 55, pp. 291-297.

[19] Mousa TM, Liu D, Cornish KG, Zucker IH, "Exercise training enhances baroreflex sensitivity by an angiotensin II-dependent mechanism in chronic heart failure," *J Appl Physiol*, 2008, vol. 104, pp. 616-624.

[20] Kar S, Gao L, Zucker IH, "Exercise training normalizes ACE and ACE2 in the brain of rabbits with pacing-induced heart failure," *J Appl Physiol*, 2010, vol. 108, pp. 923-932.

[21] Felix JVC, Michelini LC, "Training-induced pressure fall in spontaneously hypertensive rats is associated with reduced angiotensinogen mRNA expression within the nucleus tractus solitarii," *Hypertension*, 2007, vol. 50, pp. 780-785.

[22] Chennaoui M, Drogou C, Gomez-Merino D, "Effects of physical training on IL-1β, IL-6 and IL-1ra concentrations in various brain areas of the rat," *Eur Cytokine Netw*, 2008, vol. 19, pp. 8-14.

[23] Francis J, Chu Y, Johnson AK, Weiss RM, Felder RB, "Acute myocardial infarction induces hypothalamic cytokine synthesis," *Am J Physiol*, 2004, vol. 286, pp. H2264-H2271.

[24] Ferguson AV, Washburn DL, "Angiotensin II: a peptidergic neurotransmitter in central autonomic pathways," *Prog Neurobiol*, 1998, vol. 54, pp. 169-192.

[25] Moser MB, Trommald M, Andersen P, "An Increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses," *Proc Natl Acad Sci U S A*, 1994, vol. 91, pp. 12673-12675.

[26] McHugh TJ, Blum KI, Tsien JZ, Tonegawa S, Wilson MA, "Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice," *Cell*, 1996, vol. 87, pp. 1339-1349.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Leg Heating Using Far Infra-red Radiation in Patients with Chronic Heart Failure Acutely Improves the Hemodynamics, Vascular Endothelial Function, and Oxidative Stress

Shujiro Inoue[1], Masao Takemoto[1], Akiko Chishaki[2], Tomomi Ide[1], Mari Nishizaka[1], Mami Miyazono[2], Hiroyuki Sawatari[2] and Kenji Sunagawa[1]

Abstract

Background Systemic thermal therapy (STT) has been associated with beneficial effects in patients with chronic heart failure (CHF). The fact, however, that it requires a dedicated as well as spacious facility and trained personnel makes it difficult to practice in the daily care of patients with CHF.

Objective The aim of this study was to determine whether the leg thermal therapy (LTT) has a positive impact similar to that of STT in patients with CHF.

Methods and Results Twenty patients with CHF (57±17 years old, left ventricular ejection fraction=30±10%) received LTT (45℃) for 20 minutes. Immediately after the treatment, the core temperature had increased (+0.3±0.3℃) ($p<0.01$). While the LTT had no significant effects on the heart rate, systolic arterial pressure, and diastolic blood pressure, it increased the cardiac output (mixed venous oxygen saturation; +2±3%) and decrease the pulmonary capillary wedge pressure (-2±2 mmHg). The LTT significantly improved the flow-mediated vasodilatation (FMD) from 4.8±2.6 to 7.1±3.6%, the antioxidative markers, thiol from 4.0±0.7 to 4.5±0.9 μmoL/g, and the marker of oxidative deoxyribonucleic acid (DNA) damage, urine 8-hydroxy-2'deoxyguanosine (8OHdG) from 100 to 82±3%, respectively ($p<0.05$). No patient had any adverse effects associated with LTT.

Conclusion LTT acutely improved FMD, and oxidative stress in patients with CHF. Although the long-term effect of LTT remains to be investigated, its practicality which is comparable to that of STT would make it an attractive therapeutic strategy for patients with CHF.

Key words: heart failure, thermal therapy, endothelial function, oxidative stress

(Intern Med 51: 2263-2270, 2012)
(DOI: 10.2169/internalmedicine.51.7115)

Introduction

Systemic thermal therapy (STT), so-called Sauna or warm water insertion therapy (1), which is considered to be one of the thermal vasodilatation therapies and has been applied to many healthy people for centuries, has been gathering attention from various medical fields. There have been reviews of the physiologic effects, benefits and risks of sauna bathing (2, 3). It seems that the risks of sauna have been emphasized in people without chronic heart failure (CHF) (4), and thus many patients with CHF do avoid sauna because of those risks or adverse effects. However, sauna bathing may be a beneficial therapeutic option for patients with hypertension, CHF, or coronary artery disease (5-8). The common mechanism of the action might be improvement in vascular endothelial function which results in a reduced cardiac preload and afterload (2).

Tei and colleagues have introduced a supervised dry sauna at 60℃ and have shown that hyperthermia appeared

[1]Department of Cardiovascular Medicine, Kyushu University Hospital, Japan and [2]Department of Health Sciences, Kyushu University Graduate School of Medical Sciences, Japan
Received for publication December 16, 2011; Accepted for publication April 1, 2012
Correspondence to Dr. Masao Takemoto, matakemo@cardiol.med.kyushu-u.ac.jp

Table 1. Patient Characteristics

Number of patients	20
Male : Female	13 : 7
Underlying heart disease	
DCM	10
HHD	1
HCM	3
ICM	2
SAR	2
AMY	2
NYHA functional class	2.8 ± 0.6
Left ventricular ejection fraction (%)	30 ± 10
Brain natriuretic peptide (pg/dL)	611 ± 607
Peak oxygen consumption (mL/kg/min)	16 ± 4
Basic heart rhythm	
sinus rhythm	19
atrial fibrillation	1
Cardiac resynchronized therapy	2

DCM: dilated cardiomyopathy, HHD: hypertensive heart disease, ICM: ischemic cardiomyopathy, SAR: Sarcoidosis, AMY: Amyloidosis, NYHA: New York Heart Association

to improve left ventricular function and vascular activity in people with CHF (5-9). In addition, many people, even patients with CHF, have found sauna bathing pleasurable and relaxing (9). Thermal therapy may be an alternative or adjuvant treatment for patients with CHF which provides not only cardiovascular benefits but also relaxation and pleasure.

Currently thermal vasodilatation therapies have limited use because they need certain facilities and supervisors for sauna treatment. Because of this limitation sauna therapy cannot be performed at home and patients who would benefit from the sauna therapy would have to be limited to hospitals or special places capable of providing such facilities. If we can expand the possibility of thermal therapy as to be performed at patients' homes, then it would really be an alternative or adjuvant therapy for CHF. Thus, we looked into the possibility of leg thermal therapy (LTT) as one of the thermal vasodilatation therapies and studied its general effects on cardiovascular dynamics and on other factors closely related to CHF.

Materials and Methods

Study population and laboratory analysis (Table 1)

Twenty patients with CHF (13 males and 7 females with a mean age of 57±17 years) underwent LTT at our hospital. To be included into the study the patients had to have a history of congestive heart failure with New York Heart Association (NYHA) functional class II to III symptoms. Ten of them had dilated cardiomyopathy, 1 hypertensive heart disease, 3 hypertrophic cardiomyopathy (dilated phase) and 2 ischemic heart disease, cardiac sarcoidosis, or cardiac amyloidosis. The basic heart rhythm of 19 patients was sinus rhythm, and that of only 1 patient was atrial fibrillation. Two of them underwent implantation of cardiac resynchronization therapy with intrinsic p wave and paced QRS wave. All patients' left ventricular ejection fraction by echocardiography was less than 50%. All patients received appropriate medical therapy for their CHF including angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 receptor antagonists, beta-adrenergic receptor antagonists, and diuretics. Their medications were not changed during the study. All patients were in a stable clinical condition for at least 1-month before study entry. The study protocol was approved by the Ethics Committee of the Faculty of Medicine in Kyushu University, and written informed consent was obtained from all patients before the study.

Protocol of the LTT

All patients were placed in a supine position on a bed in a temperature-controlled room at 25℃. In order to exclude the effect of simply rest, they took bed rest for at least 30 minutes. After evaluation of flow-mediated vasodilatation (FMD) as described below, they received leg heating with far infra-red radiation (Leghot®, Fujika Co., Ltd., Tokyo, Japan) at 45℃ for 20 minutes, and then, remained in bed at rest with a blanket to keep them warm for an additional 30 minutes (Fig. 1). In order to exclude the possibility of the benefits of such a bed rest without LTT on CHF, we evaluated the FMD after bed rest for 50 minutes without LTT (n= 5).

Evaluation of the hemodynamic parameters

The hemodynamic data including the pulmonary capillary wedge pressure (PCWP), mixed venous oxygen saturation (SvO_2) which is an indicator of the cardiac output, and right atrial pressure were monitored by a Swan-Ganz catheter (Edwards Lifesciences Co., Ltd., Tokyo, Japan) inserted into the right jugular vein, and the heart rate and arterial blood pressure (Life Scope LT, Nihon Cohden Co., Ltd., Tokyo, Japan) were measured before and after the LTT (Fig. 1). The body core temperature was measured by a Swan-Ganz catheter placed in the pulmonary artery and skin temperature sensors (CoretempCM-210, TERUMO Corporation, Tokyo, Japan) placed on the forehead and chest. The amount of sweating was evaluated from the body weight measurements before and after the LTT. All the patients drank 300 milliliters of water to compensate for the loss of weight before and during the LTT.

Heart rate variability (HRV) analysis

The HRV was analyzed using a commercial software program (NI Labview, National Instruments Corporation, Tokyo, Japan) as previously described (10). The following frequency-domain measurements were assessed: 1) low frequency; LF (0.05 to 0.15 Hz), 2) high frequency; HF (0.15 to 0.50 Hz), and 3) LF/HF ratio. The LF power reflected the sympathetic and parasympathetic modulation of the heart rate, whereas the HF power mainly reflected the vagal modulation (11). The LF and HF measurements were reported as their natural logs (ln). The data were also analyzed by correcting the LF and HF components for the total power (0.0 to 1.0 Hz). The frequency-domain measurements were

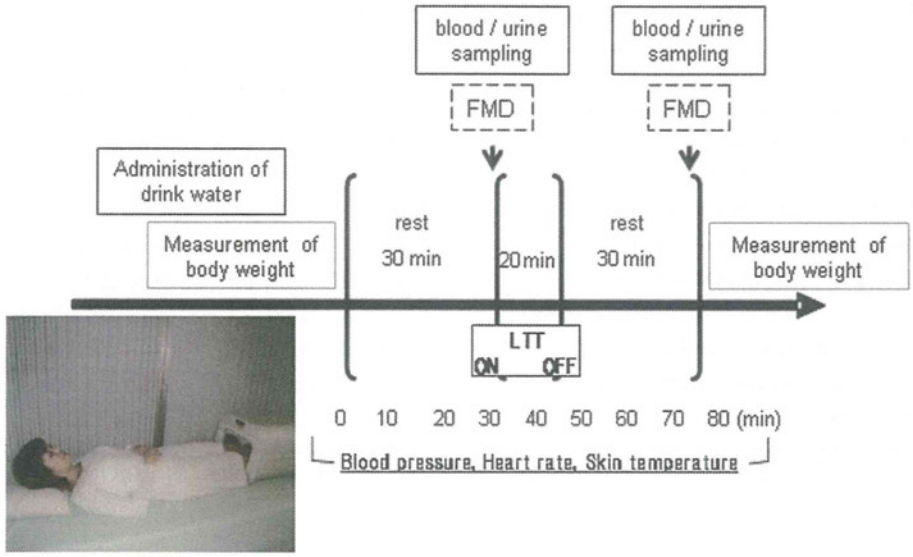

Figure 1. Setup of the leg thermal therapy for patient with chronic heart failure. LTT and FMD indicate leg thermal therapy and flow-mediated vasodilation, respectively.

examined for 5 minutes before and after the LTT.

Laboratory measurements

30 minutes following complete bed rest before the LTT and after the LTT, blood and urine samples were obtained to evaluate the serum level of the neurohormonal factors including the serum human atrial natriuretic peptide (hANP), brain natriuretic peptide (BNP), ACE activity, plasminogen activator inhibitor-1 (PAI-1), highly sensitive C-reactive protein (hs-CRP), and urine catecholamines including norepinephrine (NEP), epinephrine (EP), and dopamine (DOPA). The serum hANP and BNP were measured with a radioimmunoassay. The serum ACE activity was determined using a fluorometric assay. The hs-CRP was measured with a clinically validated high-sensitivity assay. The plasminogen activator inhibitor (PAI-1) was measured with enzyme-linked immunosorbent assays (ELISA). The urine catecholamines were measured with high-performance liquid chromatography. The degree of lipid peroxidation was determined in the blood sample through biochemical assay of thiobarbituric acid-reactive substances (TBARS) (12, 13). The plasma was mixed with 0.1 mol/L H_2SO_4 and 1% phosphotungstic acid, and the mixture was centrifuged. The sediment was suspended in distilled water, 1% thiobarbituric acid, and 0.1% butylated hydroxytoluene. The reaction mixture was then heated at 100℃ for 60 minutes in an oil bath. After the mixture was cooled with tap water, it was extracted with n-butanol and centrifuged at 1,600 g for 15 minutes. The fluorescence intensity of the organic phase was measured by use of a spectrofluorometer with a wavelength of 515-nm excitation and 553-nm emission. Malondialdehyde standards (Sigma Chemical Co., St. Louis, MO, USA) were included with each assay batch, and plasma TBARS were expressed as nanomoles per milliliter of plasma in reference to these standards. Urine samples were centrifuged at 2,000 rpm for 10 minutes and the supernatants were used for assay. Concentration of 8-OHdG in each urine sample was determined by using a competitive enzyme-linked immunosorbent assay kit (8-OHdG check, Japan Institute for the Control of Aging, Nagoya, Japan). Each value was corrected by urinary creatinine measured by a colorimetric assay kit based on color reactions between creatinine and picrate (Sigma). Concentration of urinary 8-OHdG was calculated as ng/mg of creatinine. Plasma thiols were assayed according to the method described by Ellman (14). Ellman's reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Wako Pure Chemical Industries, Inc., Osaka, Japan) was added and samples were incubated at 37℃ for 15 minutes. The absorbance was read at 412 nm against a reagent blank. Results were calculated using a molar extinction coefficient of yellow anion (ε=13,600). Glutathione peroxidase (GPx) activities were examined by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of Glutathione (GSH) reductase, which catalyzes the reduction of oxidized GSH formed by GPx. Both samples and reference cuvettes contained 0.1 M Tris-HCl, pH 7.4, 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM 2-({2-[bis (carboxymethyl)amino]ethyl}(carboxymethyl)amino)acetic acid (EDTA), 2 mM GSH, and 1 unit of GSH reductase in a total volume of 1 mL. An aliquot of each enzyme was added to the sample cuvette only. The reaction mixture was preincubated at 37℃ for 2 minutes, after which the reaction was started by the addition of peroxide to both cuvettes. The oxidation of NADPH was followed at 340 nm at 37℃, and activity was expressed as micromoles of NADPH oxidized per minute. The serum vascular endothelial growth factor (VEGF) was measured using Quantikine sandwich (ELISA; R&D Systems, Minneapolis, MN, USA) (15).

Assessment of the endothelial function

The endothelial-dependent vascular reactivity was indexed by a direct assessment of the brachial artery FMD. Changes

Table 2. Hemodynamic Parameters and Heart Rate Variability

	Baseline	After LTT	changes from baseline of the parameters
Body core temperature (℃)	36.2 ± 0.4	36.5 ± 0.4	+0.3± 0.3*
Amount of sweating (ml)			222 ± 174
Heart rate (beats per minute)	74 ± 22	70 ± 18	-3 ± 6
Systolic arterial pressure (mmHg)	88 ± 10	90 ± 17	+2.3 ±11
Diastolic arterial pressure (mmHg)	52 ± 9	52 ± 15	+0.4 ± 12
Pulmonary capillary wedge pressure (mmHg)	26.0 ± 11.0	23.7 ± 8.7	-2.3 ± 1.9 (n=3)
Right atrial pressure (mmHg)	10.0 ± 3.6	9.7 ± 3.5	-0.3 ± 1.2 (n=3)
Mixed venous oxygen saturation (%)	60.3 ± 4.5	62.0 ± 7.2	+2.0 ± 3.0 (n=3)
Heart Rate Variability (LF / HF)	1.1 ± 0.9	1.0 ± 0.7	-0.14± 0.5

n=20, * p<0.01 compared with before the leg thermal therapy, HF: High Frequency, LF: Low Frequency

in the brachial artery diameter during reactive hyperemia were measured by high-resolution ultrasound (Philips iE33, Philips Electronics Japan Co., Ltd., Tokyo, Japan) as previously described in detail (16, 17). Briefly, after the 30 minutes rest in the supine position before and after the LTT, an FMD measurement was performed with a 7.5-MHz linear-array ultrasound probe (Philips iE33L11-3, Philips Electronics Japan Co., Ltd., Tokyo, Japan). Increased blood flow was induced by a blood pressure cuff placed around the forearm, with a 5-minute inflation at 50 mmHg above the subject's systolic blood pressure, followed by rapid deflation. Baseline images before the cuff inflation and then for 2 minutes after the cuff deflation were recorded. The arterial diameter was measured in the end-diastolic phase from the recordings. The imaging and analysis were performed by a single observer blinded to the subject's identity. Measurements were taken from the anterior to posterior interface between the media and adventitia. Every 5 cardiac cycles of measurement from 60 to 120 seconds for the baseline and from 30 to 240 seconds after the cuff deflation (hyperemia) were taken. For the reactive hyperemia response, the measurements with the 5 largest diameters were averaged, and the percent increase from baseline was determined as the % FMD. Endothelium-independent vasodilation was evaluated after the sublingual administration of 0.4 mg of nitroglycerin, an exogenous nitric oxide (NO) donor. The brachial diameter and blood pressure were measured before and 3 to 4 minutes after the nitroglycerin administration.

Statistical analysis

The numerical results are expressed in the text as the mean ± standard deviation. Statistical analysis was performed using a Student *t* test or two-way Analysis of Variance (ANOVA) for the comparison of 2 groups. A value of $p<0.05$ was considered to indicate statistical significance.

Results

Study population and laboratory analysis (Table 1)

Before the LTT, the mean NYHA functional class was 2.8±0.6 and the left ventricular ejection fraction (LVEF), assessed by echocardiography, was decreased to 30±10%, with an increased serum BNP, measured in a stable clinical condition, which was increased to 611±607 pg/dL. Lastly, peak oxygen consumption (peak VO_2), measured by cardiopulmonary exercise test, was 16±4 mL/kg/min. These findings indicate that all patients had mild to moderate CHF.

Hemodynamic parameters and the HRV (Table 2)

The LTT steadily elevated not only systemic body temperature but also the body core temperature by 0.3±0.3℃ compared with before ($p<0.01$), and that elevation continued for at least 30 minutes after the leg heating was terminated. The amount of sweating was 222±174 milliliters during the LTT. The LTT comparably decreased the heart rate (-3±6 beats per minute), systemic arterial pressure (-2.3±11 mmHg), and PCWP (-2.3±1.9 mmHg), and increased the SvO_2 (+2±3%) compared with before the LTT, respectively. Although the cardiac amyloidosis and sarcoidosis are potentially at high risk of hypotension, LTT was safely performed for these patients without any adverse effects. The LTT did not significantly change the LF/HF ratio compared with before the LTT.

Neurohormonal factors and oxidative stress markers

The treatment with the LTT did not affect the neurohormonal factors, as measured by the change in the hANP (102±37%), ACE (94±11%), PAI-1 (102±32%), and hs-CRP (103±11%) serum levels (Fig. 2A). The LTT tended to decrease the urine catecholamines including the NEP (90± 37%), EP (91±33%), and dopamine (DOPA) (94±41%) but the decrease was not statistically significant (Fig. 2A). On the other hand, the LTT significantly increased the serum antioxidative stress marker, Thiol (4.0±0.7 vs. 4.5±0.9

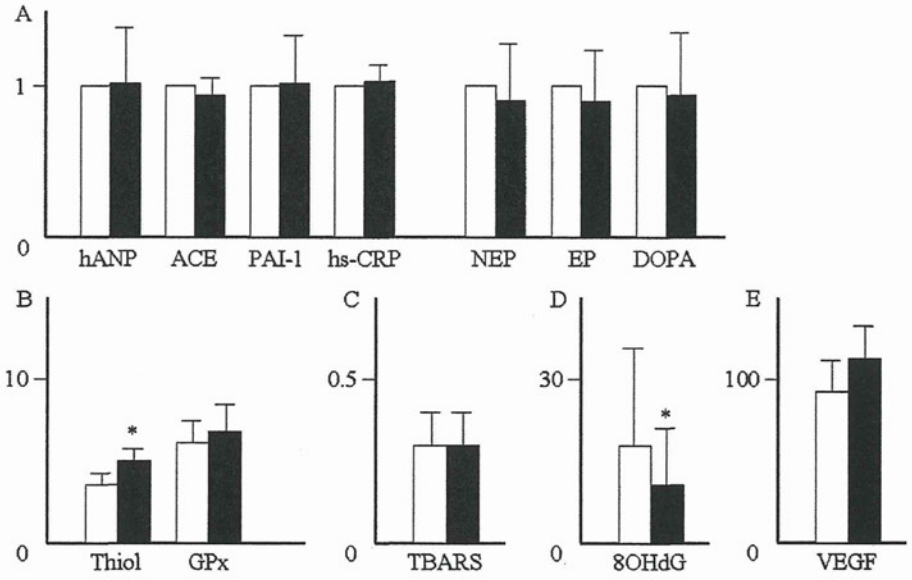

Figure 2. The leg thermal therapy (LTT) did not affect the neurohormonal factors including the serum human atrial natriuretic peptide (hANP), angiotensin-converting enzyme (ACE), plasminogen activator inhibitor (PAI)-1, highly sensitive C reactive protein (hs-CRP), and urine norepinephrine (NEP), epinephrine (EP), and dopamine (DOPA) levels (A). The LTT significantly increased the serum antioxidative stress marker, sulfhydryl content (Thiol) and significantly decreased the urine 8-hydroxy-2'deoxyguanosine (8OHdG) (D), but not the serum Glutathione peroxidase (GPx) (B) and the thiobarbituric acid-reactive substances (TBARS) (C). The LTT tended to increase vascular endothelial growth factor (VEGF) (E). The open and closed bars indicate the levels before and after the LTT, respectively. *p<0.05 vs. before LTT.

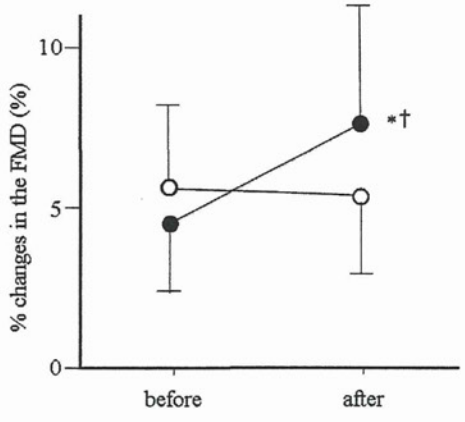

Figure 3. The leg thermal therapy (LTT) improved the % change in the flow-mediated vasodilation (FMD). The open and closed circle indicate the levels without and with the LTT, respectively.*p<0.05 vs. before the LTT. †p<0.05 vs. without the LTT.

μmoL/g, p<0.05), and tended to increase the antioxidant enzyme, GPx (6.1±1.3 vs. 6.8±1.6 unit/g) (Fig. 2B). The LTT did not affect the plasma oxidative stress marker, the thiobarbituric acid-reactive substances (TBARS) (0.3±0.1 vs. 0.3±0.1 nmoL/mL) (Fig. 2C). The LTT significantly decreased the urine 8OHdG (82±3%) (Fig. 2D). The LTT tended to increase serum VEGF, but it was not statistically significant (95±10 vs. 109±12 pg/mL) (Fig. 2E).

Effect on the endothelial function

At baseline, the brachial artery diameter increased in all subjects in response to the reactive hyperemia, however, that increase was much less, compared to the reported normal range (17). For all subjects, the mean FMD after the LTT (7.1±3.6%) significantly increased compared to that before (4.8±2.6%, p<0.01, Fig. 3). These significant improvements in the FMD with the LTT were similar to those with STT as previously described (6). The baseline diameter of the studied artery was not affected by the LTT. Also, the nitroglycerin-induced vasodilation was similar before and after the LTT. The bed rest for 50 minutes without LTT did not affect FMD (5.6±2.4% vs. 5.3±2.4%).

Discussion

Despite the major advances in the pharmacologic, medical devices, and surgical treatment of CHF, the mortality and morbidity still remain high (18). Thus, the search for effective and safe modalities continues. One approach that has attracted attention is STT, which utilizes relatively small increases in the core body temperature intermittently for therapeutic purposes. Previous reports revealed that a significant improvement using STT was noted across many CHF-related parameters, including the endothelial function (5, 6), hemodynamics (19), cardiac geometry (20), neurohormonal markers (6), oxidative stress (21, 22), quality of life and

prognosis (7, 23).

The precise mechanisms of the beneficial effects of STT have not been fully clarified, yet we speculated that the reduction in the preload and afterload of the heart due to thermal vasodilatation, and the elevation in the core body temperature were two essential factors. Further, we presumed that patients with CHF might benefit from partial body warming if it could raise the core body temperature and if it could cause thermal venous vasodilatation sufficient to reduce the venous return and peripheral arterial vasodilatation which would lead to a reduction in the arterial resistance. If we could achieve a similar effect as STT by warming the lower extremities, then it would benefit the patients with moderate to severe CHF who have to stay in bed. In addition, the type of facilities for warming the patients would be simpler than those for systemic warming with a sauna bath, which would definitely have a financial benefit. For example, an electrical warming blanket might be of some use in partial body warming, which many hospitals are surely equipped with.

We decided to apply the use of a far infra-red radiant heater to the patients' legs for the following reasons. First, it is made for use in the home and has a safety qualification; SG: Safety Goods mark from the Consumer Product Safety Association (CPSA), Japan. Also, far infra-red radiant heaters are more efficient than heaters using heat conduction. Secondly, there was a report on the LTT, which proved that it increases the core body temperature (24).

The effects of partial body warming from LTT were smaller than those reported for STT, and the rise in the core body temperature was 0.3 degrees on average, whereas STT induced a 1℃ rise in the core body temperature. However, it still brought about favorable changes in the systemic arterial pressure, heart rate (HR), PCWP, and SvO_2. This suggests that patients with CHF may benefit from partial body warming though the efficacy of the LTT may fall short of that of STT. However, the side effects of partial thermal therapy may be fewer than those for STT, because the heat is applied to only a part of the body. The temperature during the experiment was set to 45 degrees which was comparatively low for a sauna bath. We did not experience any adverse effects of the LTT throughout the experiment. Moreover, the LTT improved the oxidative stress and endothelial function which is equivalent to that for STT as previously described (6).

Endothelial function and oxidative stress

Some studies have demonstrated that endothelial function decreases in patients with CHF (25, 26), and the mechanism has been proposed to be a decreased NO bioavailability associated with increased NADPH oxidase, which is an important source of oxidative stress-derived superoxide generation (27-29). Another study demonstrated that STT-induced attenuation of vascular densities was associated with the upregulation of endothelial NO synthesis and VEGF expression in the noninfarcted myocardium (30). In the present study, we demonstrated that the LTT increased the antioxidative stress marker, Thiol, and, moreover, the antioxidant enzyme, GPx, and serum VEGF tended to increase. Furthermore, the marker of oxidative DNA damage, urine 8OHdG, significantly decreased after the LTT. These findings may indicate that the LTT decreased the oxidative stress contributing to the improvement in the NO bioavailability and upregulation of the VEGF, and could finally improve the endothelial function. A recent clinical study showed that an impaired FMD was a proven independent strong predictor of an adverse outcome and poor prognosis in patients with CHF (27). Thus, the LTT may improve the clinical outcome and prognosis in patients with CHF.

LTT and vagal modulation

In this study, we considered several possible mechanisms for the decrease in the HR ($p=0.52$) and systolic arterial pressure, but not significant change. One was the reduction in the venous return to the right atrium (RA). It is well known that an increase in the venous return to the heart causes an increase in the HR and cardiac output as the result of a stretched RA causing increased sinus node activity and a Bainbridge reflex (31). Further, the reduction in the venous return may cause a reduction in those activities resulting in a decrease in the HR and cardiac output, and ultimately the systemic arterial pressure, if decreased, would not be adequate to activate the sympathetic nerves to cancel those changes. Another possible mechanism is the activation of the vagal nerve. Despite being statistically insignificant, the LTT tended to decrease the LF/HF ratio. This indicated that the LTT may cause vagal nerve activation. There have been reports on somato-autonomic reflexes in which the effects of acupuncture on the lower extremities were described to cause a decrease in the HR (32). Local heat therapy might cause some similar effects as acupuncture in the same place. Further investigation will be necessary.

In addition to the direct effect of the LTT, there may be indirect effects of the LTT. Higashi et al. revealed that thermal therapy attenuated psychological stress (22). We think this may lead to parasympathetic activation, which may explain our findings. Activation of the vagal nerves is good for patients with CHF. Vagal nerve stimulation markedly improved the long-term survival of CHF rats through the prevention of pumping failure and cardiac remodeling (33). Further, it has been reported that vagal nerve activation-induced vasodilation is mediated by NO (34), and the LTT may benefit patients with CHF via vagal nerve activation which may lead to an improvement in the endothelial function and prevent the development of CHF.

Comparison LTT and STT

STT, and the rise in the core body temperature was 0.4 degrees on average, whereas STT caused about 1 degree rise in the core body temperature. However, improvement of % FMD was almost the same level (from 4% to 6%) (5). These results indicate that the elevation of body core tem-

perature by 0.4℃ may be sufficient to improve the impaired endothelial function, which is an independent strong predictor of an adverse outcome and poor prognosis in patients with CHF (27). In view of these findings, LTT may be in no way inferior to STT.

Clinical implications

All patients completed this study without any LTT-associated adverse effects including worsened clinical symptoms, skin burns, hypotension, dehydration, or arrhythmias. The acute improvement in the FMD with LTT was similar to that of STT as previously described (6). Moreover, since the LTT does not require a dedicated spacious facility or any trained personnel, the LTT may easily and repeatedly be performed at a low cost anytime or anywhere, and would be applicable for any patient with CHF such as those that are bedridden.

Limitations of this study

The limitation of this study is the assessment of a relatively small number of patients and with many different causes of heart failure. In the present study, we evaluated only the acute effects, not long term effects, of the LTT, and performed the LTT in patients with mild to moderate CHF who were in NYHA functional class II or III (mean NYHA class = 2.8±0.6), and in a stable clinical condition for at least 1 month before the study entry. Whether our results can safely be extrapolated to patients with severe CHF and a greatly reduced LVEF should be determined in further studies. A recent case report showed that there was a similar beneficial effect of appendicular thermal therapy in patients with a more severe form of heart failure (22), further supporting our hypothesis.

Conclusion

LTT acutely improves the hemodynamics, vascular endothelial function, and oxidative stress in patients with CHF. LTT may be an effective, safe and attractive therapeutic strategy for patients with CHF. Supporting data from large clinical trials, however, are needed before such recommendations can be made. We plan to examine whether additional long-term LTT further decreases the oxidative stress, activates the vagal nerves, and improves the endothelial function and hemodynamic factors contributing to an improvement in the clinical status and prognosis of patients with CHF.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

We thank Yoshihiro Murayama, Yuko Hata, Shinobu Arai, and Akiko Sugiyama for their technical assistance with the leg thermal therapy, and John Martin for his linguistic assistance with this paper. This study was supported by the Grants-in-Aid for Scientific Research (#19590821 to Takemoto M. and #18390586 to Chishaki A.) from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

References

1. Gruner Svealv B, Cider A, Tang MS, Angwald E, Kardassis D, Andersson B. Benefit of warm water immersion on biventricular function in patients with chronic heart failure. Cardiovasc Ultrasound **7**: 33, 2009.
2. Frishman WH, Grattan JG, Mamtani R. Alternative and complementary medical approaches in the prevention and treatment of cardiovascular disease. Curr Probl Cardiol **30**: 383-459, 2005.
3. Eren B, Fedakar R, Turkmen N, Akan O. Deaths in the Turkish hamam (hot bath). Bratisl Lek Listy **110**: 697-700, 2009.
4. Heckmann JG, Rauch C, Seidler S, Dutsch M, Kasper B. Sauna stroke syndrome. J Stroke Cerebrovasc Dis **14**: 138-139, 2005.
5. Imamura M, Biro S, Kihara T, et al. Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors. J Am Coll Cardiol **38**: 1083-1088, 2001.
6. Kihara T, Biro S, Imamura M, et al. Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. J Am Coll Cardiol **39**: 754-759, 2002.
7. Kihara T, Miyata M, Fukudome T, et al. Waon therapy improves the prognosis of patients with chronic heart failure. J Cardiol **53**: 214-218, 2009.
8. Miyata M, Kihara T, Kubozono T, et al. Beneficial effects of Waon therapy on patients with chronic heart failure: Results of a prospective multicenter study. J Cardiol **52**: 79-85, 2008.
9. Basford JR, Oh JK, Allison TG, et al. Safety, acceptance, and physiologic effects of sauna bathing in people with chronic heart failure: a pilot report. Arch Phys Med Rehabil **90**: 173-177, 2009.
10. Singh N, Mironov D, Armstrong PW, Ross AM, Langer A. Heart rate variability assessment early after acute myocardial infarction. Pathophysiological and prognostic correlates. GUSTO ECG Substudy Investigators. Global Utilization of Streptokinase and TPA for Occluded Arteries. Circulation **93**: 1388-1395, 1996.
11. Tiainen M, Parikka HJ, Makijarvi MA, Takkunen OS, Sarna SJ, Roine RO. Arrhythmias and heart rate variability during and after therapeutic hypothermia for cardiac arrest. Crit Care Med **37**: 403-409, 2009.
12. Shiomi T, Tsutsui H, Matsusaka H, et al. Overexpression of glutathione peroxidase prevents left ventricular remodeling and failure after myocardial infarction in mice. Circulation **109**: 544-549, 2004.
13. Ellman G, Lysko H. A precise method for the determination of whole blood and plasma sulfhydryl groups. Anal Biochem **93**: 98-102, 1979.
14. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys **82**: 70-77, 1959.
15. Gupta PK, Prabhakar S, Sharma S, Anand A. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. J Neuroinflammation **8**: 47, 2011.
16. Nishizaka MK, Zaman MA, Green SA, Renfroe KY, Calhoun DA. Impaired endothelium-dependent flow-mediated vasodilation in hypertensive subjects with hyperaldosteronism. Circulation **109**: 2857-2861, 2004.
17. Corretti MC, Anderson TJ, Benjamin EJ, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol **39**: 257-265, 2002.
18. Owan TE, Hodge DO, Herges RM, Jacobsen SJ, Roger VL, Redfield MM. Trends in prevalence and outcome of heart failure with preserved ejection fraction. N Engl J Med **355**: 251-259, 2006.
19. Tei C, Horikiri Y, Park JC, et al. Acute hemodynamic improve-

ment by thermal vasodilation in congestive heart failure. Circulation **91**: 2582-2590, 1995.

20. Tei C, Tanaka N. Thermal vasodilation as a treatment of congestive heart failure: a novel approach. J Cardiol **27**: 29-30, 1996.

21. Masuda A, Miyata M, Kihara T, Minagoe S, Tei C. Repeated sauna therapy reduces urinary 8-epi-prostaglandin F(2alpha). Jpn Heart J **45**: 297-303, 2004.

22. Higashi H, Komamura K, Oda N, et al. Experience of appendicular thermal therapy applied to a patient with a left ventricular assist device awaiting heart transplantation. J Cardiol **53**: 301-305, 2009.

23. Ikeda Y, Biro S, Kamogawa Y, et al. Effect of repeated sauna therapy on survival in TO-2 cardiomyopathic hamsters with heart failure. Am J Cardiol **90**: 343-345, 2002.

24. Liao WC, Landis CA, Lentz MJ, Chiu MJ. Effect of foot bathing on distal-proximal skin temperature gradient in elders. Int J Nurs Stud **42**: 717-722, 2005.

25. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM. Endothelium-dependent vasodilation is attenuated in patients with heart failure. Circulation **84**: 1589-1596, 1991.

26. Katz SD, Biasucci L, Sabba C, et al. Impaired endothelium-mediated vasodilation in the peripheral vasculature of patients with congestive heart failure. J Am Coll Cardiol **19**: 918-925, 1992.

27. Meyer B, Mortl D, Strecker K, et al. Flow-mediated vasodilation predicts outcome in patients with chronic heart failure: comparison with B-type natriuretic peptide. J Am Coll Cardiol **46**: 1011-1018, 2005.

28. Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. Br Heart J **65**: 245-248, 1991.

29. Dworakowski R, Walker S, Momin A, et al. Reduced nicotinamide adenine dinucleotide phosphate oxidase-derived superoxide and vascular endothelial dysfunction in human heart failure. J Am Coll Cardiol **51**: 1349-1356, 2008.

30. Sobajima M, Nozawa T, Shida T, et al. Repeated sauna therapy attenuates ventricular remodeling after myocardial infarction in rats by increasing coronary vascularity of noninfarcted myocardium. Am J Physiol Heart Circ Physiol **301**: H548-H554, 2011.

31. Bergstrom RM, Hakumaki MO, Sarajas HS. Heart rate in relation to sympathetic efferentation associated with the Bainbridge reflex in dogs. Acta Physiol Scand **83**: 571-573, 1971.

32. Noguchi E. Mechanism of reflex regulation of the gastroduodenal function by acupuncture. Evid Based Complement Alternat Med **5**: 251-256, 2008.

33. Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation **109**: 120-124, 2004.

34. Zhao G, Shen W, Xu X, Ochoa M, Bernstein R, Hintze TH. Selective impairment of vagally mediated, nitric oxide-dependent coronary vasodilation in conscious dogs after pacing-induced heart failure. Circulation **91**: 2655-2663, 1995.

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Hypertension and Circulatory Control

Reduction of Nitric Oxide-Mediated γ-Amino Butyric Acid Release in Rostral Ventrolateral Medulla Is Involved in Superoxide-Induced Sympathoexcitation of Hypertensive Rats

Keisuke Shinohara, MD; Yoshitaka Hirooka, MD, PhD;
Takuya Kishi, MD, PhD; Kenji Sunagawa, MD, PhD

Background: The rostral ventrolateral medulla (RVLM) in the brainstem is responsible for regulation of the sympathetic nervous system. In the RVLM, nitric oxide (NO)-mediated γ-amino butyric acid (GABA) is a major sympathoinhibitory amino acid neurotransmitter and superoxide is a major sympathoexcitatory factor. In this study, we investigated whether or not NO-mediated GABA release is involved in superoxide-induced sympathoexcitation in the RVLM of hypertensive rats.

Methods and Results: For our model hypertensive rats with sympathoexcitation, we used stroke-prone spontaneously hypertensive rats (SHRSP). GABA levels in the RVLM were measured by in vivo microdialysis. Microinjection of tempol, a superoxide scavenger, into the RVLM decreased arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) with an increase in GABA release in the RVLM. Microinjection of N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, into the RVLM increased AP, HR, and RSNA with a decrease in GABA release in the RVLM. Prior microinjection of L-NMMA into the RVLM attenuated the tempol-induced changes in AP, HR, RSNA, and GABA release in the RVLM. Microinjection of bicuculline, a GABA receptor blocker, into the RVLM attenuated the tempol- and L-NMMA-induced changes in AP, HR, and RSNA.

Conclusions: The findings suggest that reduction of NO-mediated GABA release in the RVLM is partly involved in superoxide-induced sympathoexcitation of SHRSP. (*Circ J* 2012; **76:** 2814–2821)

Key Words: Amino acids; Brain; Nitric oxide; Oxidative stress; Sympathetic nervous system

Activation of the sympathetic nervous system (SNS) has an important role in the pathogenesis of hypertension[1] and central mechanisms are crucially involved in sympathetic hyperactivity.[1,2] The rostral ventrolateral medulla (RVLM) in the brainstem contains the pre-sympathetic neurons that maintain baseline sympathetic tone,[2,3] and previous studies have demonstrated that nitric oxide (NO) in the RVLM inhibits the activation of the SNS.[4–7] The sympathoinhibitory effect of NO in the RVLM is considered to be reduced in spontaneously hypertensive rats (SHR).[8,9] Furthermore, γ-amino butyric acid (GABA) is a major inhibitory neurotransmitter[10] and GABA receptors in the RVLM have been demonstrated.[11,12] Activating the $GABA_A$ receptor inhibits the activity of the RVLM neurons,[10,13] and it has been reported that, in SHR, there is a GABAergic disinhibition of neuronal activity in the RVLM.[9,10,14–16] NO is an important mediator in the autonomic nuclei, such as RVLM, paraventricular nucleus (PVN), and nucleus tractus solitarii,[4,7,17–22] for cardiovascular regulation, and acting on presynaptic terminals to increase vesicular GABA release.[17,20,21] NO has been shown to increase the release of GABA in the PVN and decrease arterial pressure (AP), although the release of excitatory amino acids in the PVN was also increased by NO.[22] We also have demonstrated that increased NO production by overexpression of endothelial NO synthase (eNOS) caused GABAergic inhibition in the RVLM.[7,9]

Many previous studies in experimental animal models of hypertension have indicated that superoxide in the brain con-

Received March 25, 2012; revised manuscript received July 26, 2012; accepted August 7, 2012; released online September 5, 2012 Time for primary review: 22 days

Department of Cardiovascular Medicine (K. Shinohara, K. Sunagawa), Department of Advanced Cardiovascular Regulation and Therapeutics (Y.H.), and Department of Advanced Therapeutics for Cardiovascular Diseases (T.K.), Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Mailing address: Yoshitaka Hirooka, MD, PhD, Department of Advanced Cardiovascular Regulation and Therapeutics, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: hyoshi@cardiol.med.kyushu-u.ac.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-12-0399

tributes to the activation of the SNS, thereby increasing AP.[23–31] It has already been determined that superoxide causes sympathoexcitation in the RVLM.[23,26–28] Although several mechanisms of superoxide-induced sympathoexcitation have already been determined,[23] the aim of the present study was to determine whether or not NO-mediated GABA release is involved in superoxide-induced sympathoexcitation in the RVLM of hypertensive rats with sympathoexcitation, which has not been fully clarified. For this purpose, we used stroke-prone SHR (SHRSP), and infused either 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol), a superoxide dismutase (SOD) mimetic, or N^G-monomethyl-L-arginine (L-NMMA), an NO synthase (NOS) inhibitor, into the RVLM. GABA levels in the RVLM of SHRSP were measured before and during infusion of each drug. Furthermore, bicuculline was also injected into the RVLM to confirm the effects of GABA on superoxide-induced sympathoexcitation and NO-induced sympathoinhibition in the RVLM.

Methods

Animals and General Procedures

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at the Kyushu University Graduate School of Medical Sciences and conducted according to the Guidelines for Animal Experiments of Kyushu University. Experiments were performed on male SHRSP and Wistar-Kyoto rats (WKY) (280–340 g, 14–18 weeks old; SLC Japan, Hamamatsu, Japan). Rats were initially anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal followed by 20 $mg \cdot kg^{-1} \cdot h^{-1}$ intravenous infusion). A catheter was inserted into the femoral artery to record mean AP (MAP) and heart rate (HR), and another catheter was inserted into the femoral vein to allow for intravenous drug injections. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. The left renal nerve was exposed with a left retroperitoneal flank incision. Stainless steel bipolar electrodes were placed beneath the renal nerve to record multifiber renal sympathetic nerve activity (RSNA).[32,33] The rats were placed in a stereotaxic frame with incisor bar and the dorsal surface of the medulla was surgically exposed to allow for positioning of the microinjection pipettes into the RVLM (with the pipette angled rostrally 18°, 1.8 mm lateral, 3.5 mm below the calamus scriptorius), as described previously.[7,33] After identification of the RVLM by monitoring the response to an injection of a small dose of L-glutamate,[6–9,27,33] microinjection or microinfusion studies with in vivo microdialysis were performed. At the end of each experiment, microinjection of the vehicle solution containing Evans blue dye (100 nl) was made into the RVLM injection or infusion site, using the same coordinates as for the drug injections. The animal was then killed by an overdose of sodium pentobarbital, and the brain removed and placed in 10% formalin for at least 48 h. Subsequently, 100-μm thick coronal sections of the brainstem were cut on a microtome. The labeled sites of microinjection were identified by examining the sections using a microscope.

Measurement of GABA Levels by In Vivo Microdialysis

A microdialysis probe with juxtapositional infusion cannula (MI-C-I-12–01FEP; Eicom, Kyoto, Japan) was inserted in one (unilateral) of the RVLM. The RVLM was perfused with Ringer solution (140 mmol/L NaCl, 4 mmol/L KCl, 1.26 mmol/L $CaCl_2$, and 1.15 mmol/L $MgCl_2$, pH 7.4) at a constant flow rate of 3 μl/min through a microdialysis probe. To measure GABA levels, the perfused dialysates were collected every 5 min, and GABA levels were measured by high-performance liquid chromatography with an electrochemical detector (HTEC-500, Eicom, Kyoto, Japan).[7,34] GABA levels were quantitated by averaging 2 consecutive dialysate samples, which were obtained at approximately ≥1 h after starting the brain perfusion with Ringer's solution.

Experimental Protocols

(1) To confirm the role of superoxide in the RVLM in the regulation of AP, HR, and sympathetic nerve activity, tempol (1 nmol in 100 nl) was acutely microinjected into both (bilateral) RVLM of SHRSP and WKY. The dose of tempol was chosen because there is a dose-response relationship between different doses of tempol (0.01, 0.1, and 1 nmol) and effects on MAP, as determined in a previous study,[27] and was used for subsequent microinjection experiments.

(2) To explore the role of superoxide in the RVLM in the regulation of the GABA release, tempol (0.01, 0.1, or 1 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the juxtapositional infusion cannula attached to the microdialysis probe, while recording AP, HR, and RSNA, and collecting the perfused dialysates. We chose the dose of tempol as 1 nmol/min for subsequent infusion experiments, because of the apparent sympathoinhibitory response to it.

(3) To explore the role of endogenous NO in the RVLM in the regulation of AP, HR, RSNA, and the GABA release, L-NMMA (10 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the juxtapositional infusion cannula. This dose of L-NMMA was chosen because the increases in MAP induced by infusion of 100 nmol (10 nmol/min for 10 min) and 1 μmol (100 nmol/min for 10 min) in the unilateral RVLM did not different (data not shown), and we considered that a total infusion of 100 nmol (10 nmol/min for 10 min) L-NMMA into the unilateral RVLM would be sufficient to inhibit the effects of NO in the RVLM.

(4) To explore the role of endogenous NO in the RVLM in superoxide-induced changes, tempol (1 nmol/50 nl/min) was infused for 10 min into the unilateral RVLM of SHRSP through the cannula following the infusion of L-NMMA into the ipsilateral RVLM (10 nmol/50 nl/min for 10 min).

(5) To confirm that changes in AP, HR, and RSNA caused by infusion of tempol or L-NMMA for 10 min were the result of an increase or decrease in GABA release, bicuculline (200 pmol in 100 nl) was acute microinjected into the bilateral RVLM of SHRSP followed by acute microinjection of tempol (1 nmol in 100 nl) or L-NMMA (10 nmol in 100 nl). The dose of bicuculline was chosen because of the results in previous studies.[7,9] The dose of L-NMMA was chosen because we confirmed the pressor and sympathoexcitatory responses by infusion of 10 nmol/min L-NMMA in the unilateral RVLM at 1 min after the initiation.

(6) To investigate whether glutamatergic excitatory inputs into the RVLM are involved in superoxide-induced sympathoexcitation, a glutamate receptor antagonist, kynurenic acid (2.7 nmol in 100 nl) was acutely microinjected into the bilateral RVLM of SHRSP, followed by acute microinjection of tempol (1 nmol in 100 nl). This dose of kynurenic acid was chosen because of the results in previous studies.[7,35,36] Tempol was acutely microinjected at 20–30 min after the injection of kynurenic acid, because we confirmed that the decreases in AP and RSNA occurred rapidly and reached a peak value within 15–20 min after the acute microinjection of kynurenic acid into the bilateral RVLM, lasting for 40 min in the preliminary experiments.

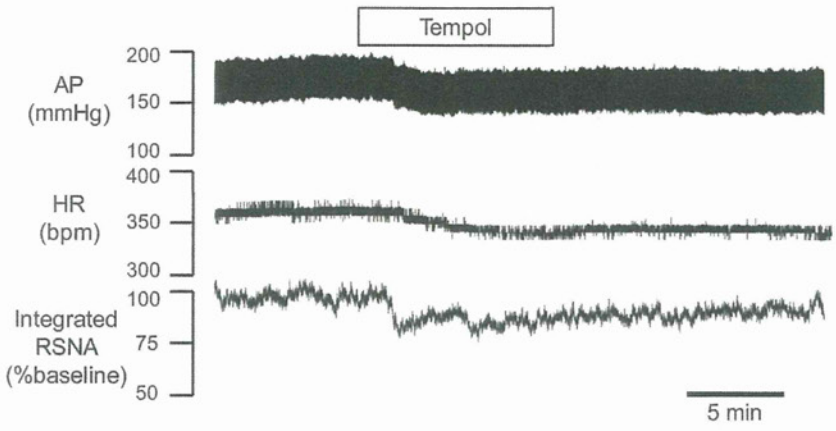

Figure 1. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. bpm indicates beats/min.

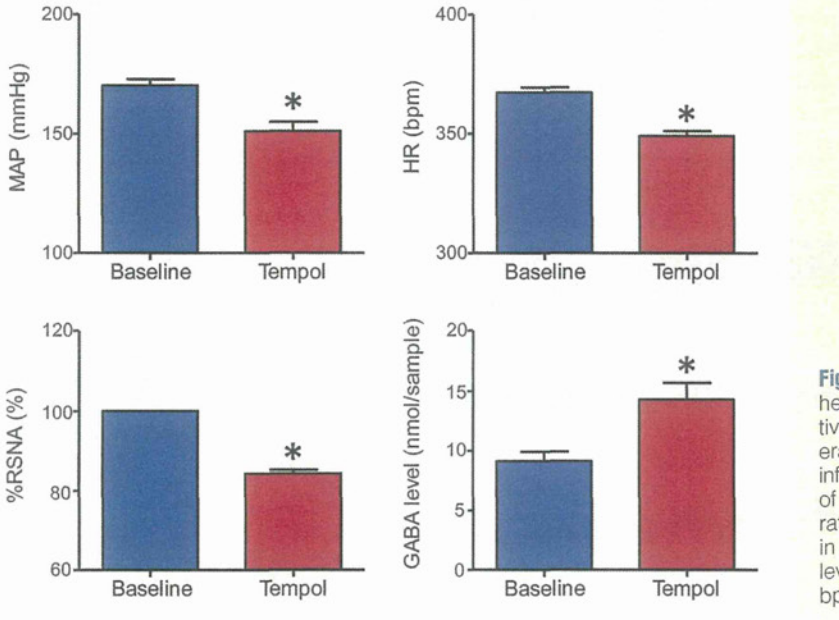

Figure 2. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the unilateral rostral ventrolateral medulla (RVLM) to infusion of tempol (1 nmol/50 nl/min for 10 min) of stroke-prone spontaneously hypertensive rats. Group data of tempol-induced changes in mean AP (MAP), HR, RSNA, and GABA levels in the RVLM. *P<0.01 (n=5 for each). bpm indicates beats/min.

Statistical Analysis

All values are expressed as the mean±SEM. A paired t-test was used to compare the changes in MAP, HR, RSNA, and GABA values with a few exceptions. An unpaired t-test was used to compare the baselines and changes in MAP, HR, and RSNA between SHRSP and WKY, and to compare the changes in MAP, HR, RSNA, and GABA values between the infusion of tempol and L-NMMA plus tempol during the experiments. Values of P<0.05 were considered significant.

Results

Effects of Tempol in the RVLM of SHRSP and WKY

Basal MAP and HR were significantly higher in SHRSP than in WKY (182±2 vs. 101±3 mmHg, 365±3 vs. 305±3 beats/min, P<0.01, n=5 for each). Acute microinjection of tempol into the bilateral RVLM decreased MAP, HR, and RSNA (ΔMAP, −33±8 mmHg; ΔHR, −29±5 beats/min; ΔRSNA %baseline, −19±2 %; n=5) in SHRSP, but not in WKY (ΔMAP, −4±1 mmHg; ΔHR, −3±1 beats/min; ΔRSNA %baseline, −3±1 %; n=5). The magnitude of the decreases in these variables was significantly greater in SHRSP than in WKY (P<0.01).

Effects of Tempol on GABA Levels in the RVLM of SHRSP

Infusion of tempol for 10 min into the unilateral RVLM decreased MAP (−19±2 mmHg from baseline 170±3 mmHg, n=5), HR (−18±2 beats/min from baseline 367±2 beats/min, n=5), and RSNA (−16±1%, n=5), and increased the level of GABA in the dialysates (5.2±1.0 nmol/sample from baseline 9.1±0.8 nmol/sample, n=5) in SHRSP (Figures 1,2).

Effects of L-NMMA in the RVLM of SHRSP

Infusion of L-NMMA for 10 min into the unilateral RVLM increased MAP (9±1 mmHg from baseline 169±2 mmHg, n=5), HR (15±1 beats/min from baseline 367±4 beats/min, n=5), and RSNA (5±1 %, n=5), and decreased the level of GABA in the dialysates (−1.1±0.2 nmol/sample from baseline 10.6±1.4 nmol/sample, n=5) in SHRSP (Figure 3).

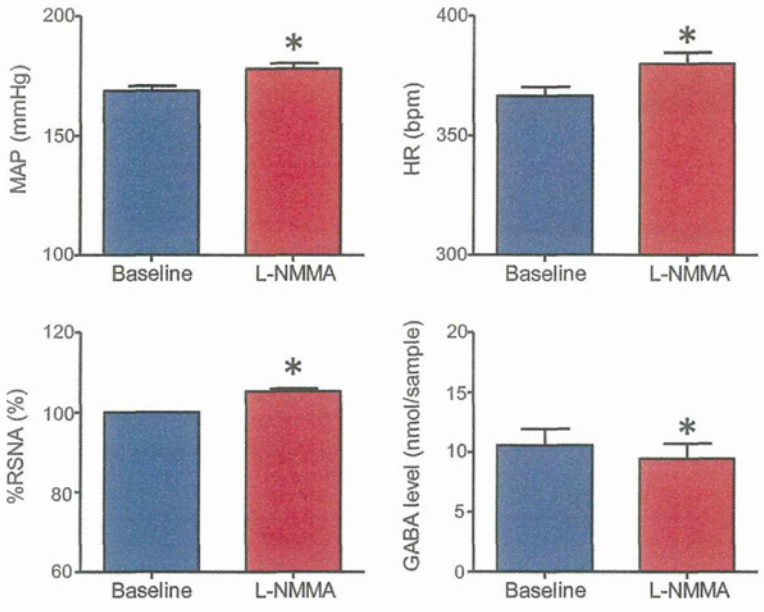

Figure 3. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the rostral ventrolateral medulla (RVLM) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats. Group data of L-NMMA-induced changes in mean AP (MAP), HR, RSNA, and GABA levels in the RVLM. *P<0.01 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

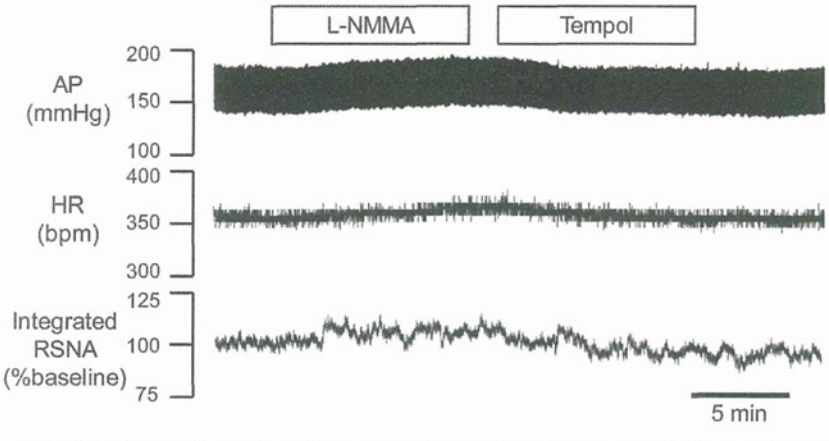

Figure 4. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) and subsequent tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

Effects of L-NMMA on the Responses to Tempol in RVLM of SHRSP

Following the infusion of L-NMMA for 10 min into the unilateral RVLM, infusion of tempol for 10 min into the ipsilateral RVLM decreased MAP (−11±1 mmHg from baseline 176±2 mmHg, n=5), HR (−11±2 beats/min from baseline 377±5 beats/min, n=5), and RSNA (−8±1 %, n=5), and increased the level of GABA in the dialysates (2.3±0.5 nmol/sample from baseline 9.5±1.3 nmol/sample, n=5) in SHRSP (Figures 4,5). The tempol-induced changes in these variables were significantly attenuated by prior infusion of L-NMMA (Figure 5). Although prior infusion of L-NMMA changed the basal values before the infusion of tempol, the percentage changes from baseline induced by tempol were also significantly attenuated by L-NMMA.

Effects of Bicuculline on the Responses to Tempol or L-NMMA in the RVLM of SHRSP

Prior acute microinjection of bicuculline into the bilateral RVLM of SHRSP attenuated the tempol-induced depressor and sympathoinhibitory responses and L-NMMA-induced pressor and sympathoexcitatory responses (Figure 6). Although prior acute microinjection of bicuculline changed the basal values before acute microinjection of tempol or L-NMMA, the percentage changes from baseline induced by tempol or L-NMMA were also significantly attenuated by bicuculline.

Effects of Kynurenic Acid on the Responses to Tempol in the RVLM of SHRSP

Acute microinjection of kynurenic acid into the bilateral RVLM significantly decreased MAP (−58±5 mmHg from baseline 182±4 mmHg, n=5), HR (−37±6 beats/min from baseline 361±3 beats/min, n=5), and RSNA (−25±3 %, n=5) in SHRSP. The depressor and sympathoinhibitory responses caused by the acute microinjection of tempol into the bilateral RVLM were unchanged between before and after acute microinjection of kynurenic acid (Figure 7). Although kynurenic acid changed the basal values before acute microinjection of tempol, the percentage changes from baseline induced by tempol were also unchanged.

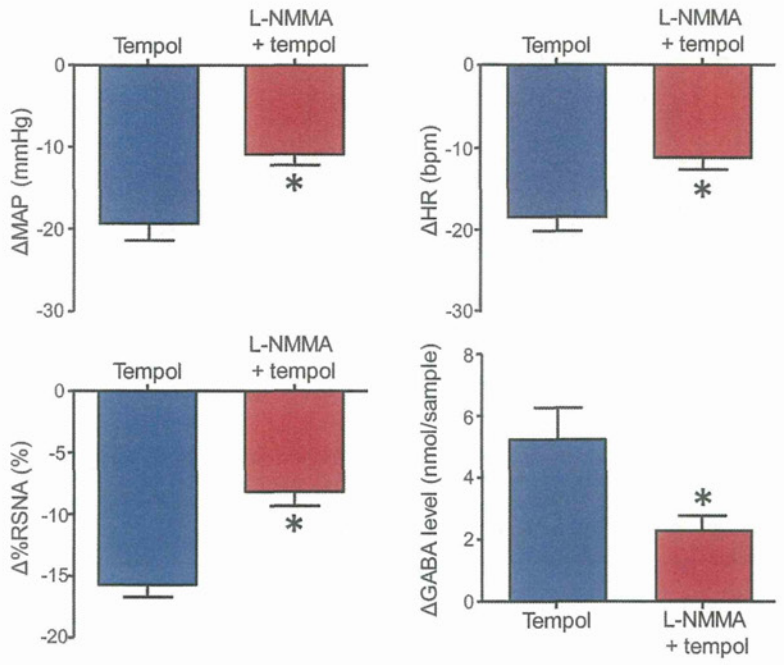

Figure 5. Effect of inhibition of nitric oxide (NO) production by L-NMMA (10 nmol/50 nl/min for 10 min) in the rostral ventrolateral medulla (RVLM) on the responses to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats (SHRSP). Prior infusion of L-NMMA attenuated the tempol-induced changes in mean arterial pressure (MAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the RVLM of SHRSP. *P<0.05 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

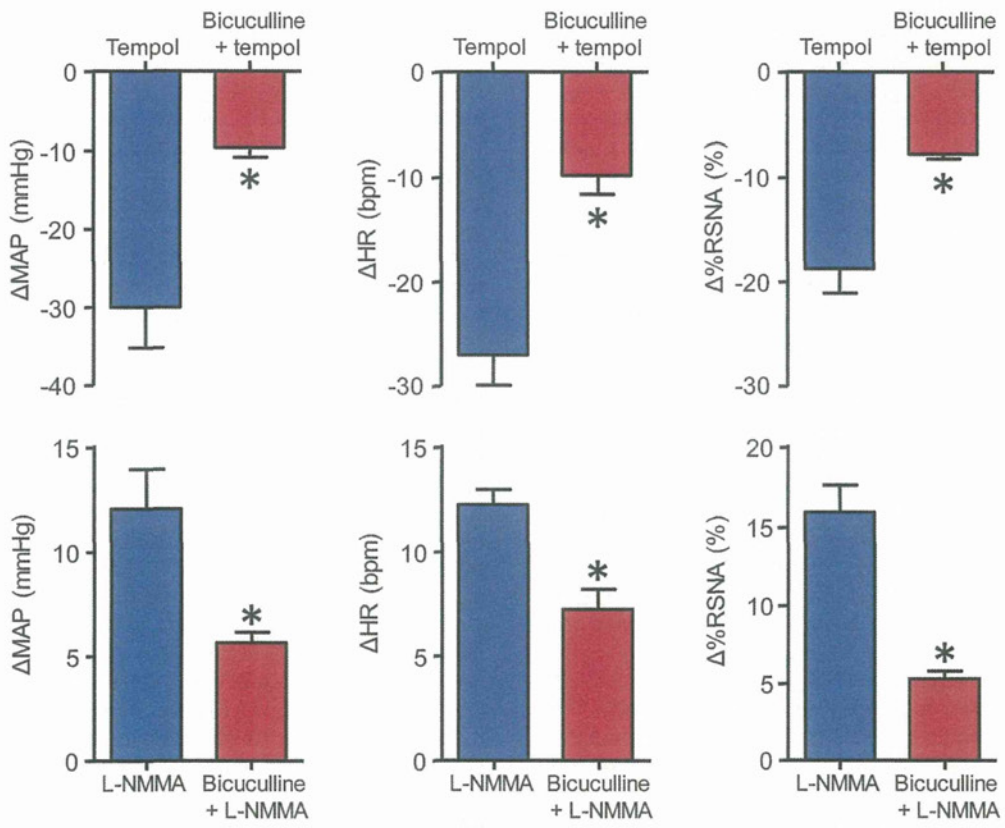

Figure 6. Effect of bicuculline (200 pmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) or L-NMMA (10 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior microinjection of bicuculline attenuated the tempol-induced and L-NMMA-induced changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA). *P<0.05 (n=5 for each). L-NMMA, N^G-monomethyl-L-arginine. bpm indicates beats/min.

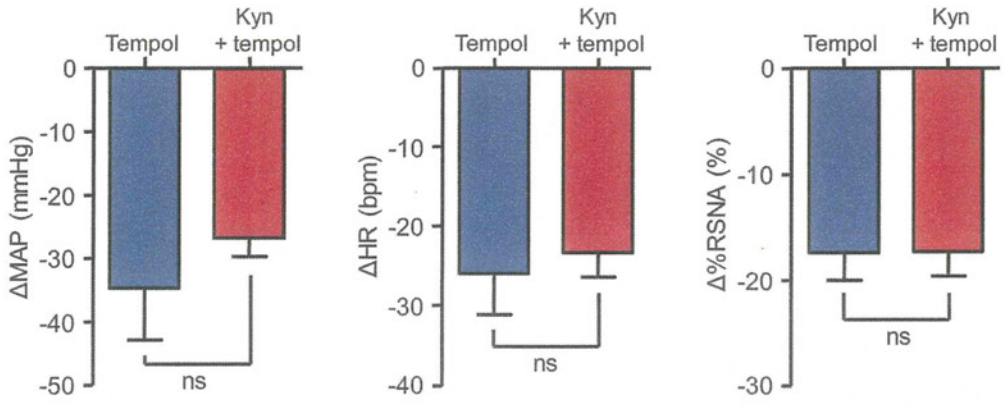

Figure 7. Effect of kynurenic acid (2.7 nmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior acute microinjection of kynurenic acid did not affect the tempol-induced changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) (n=5 for each). kyn, kynurenic acid; ns, not significant. bpm indicates beats/min.

Discussion

We have demonstrated 3 major findings. First, infusion of tempol into the RVLM increased GABA release in the RVLM with sympathoinhibition in SHRSP, and these responses were attenuated by prior infusion of bicuculline into the RVLM. Second, infusion of L-NMMA into the RVLM decreased GABA release in the RVLM with sympathoexcitation in SHRSP. Third, prior infusion of L-NMMA into the RVLM attenuated the tempol-induced increase in GABA release with sympathoinhibition in SHRSP. In particular, we were able to detect changes in GABA release using a microdialysis technique. Thus, in the present study, we provide the first evidence that superoxide inhibits NO-mediated GABA release in the RVLM of SHRSP, thereby increasing the activity of the SNS.

Many previous studies have indicated the microinjection of tempol, widely used as an antioxidant agent,[37] into the RVLM causes sympathoinhibition in animal models of hypertension, probably via reduction of superoxide.[26,27,38–40] We also confirmed that microinjection of tempol into the RVLM resulted in sympathoinhibition in SHRSP, but not in WKY, in the present study. In addition, we also showed that prior injection of bicuculline into the RVLM attenuated the tempol-induced decrease in MAP and HR with sympathoinhibition. These results suggest that the increase in GABA release in the RVLM caused by a reduction of superoxide is functionally relevant to sympathoinhibition. Moreover, in the present study, we confirmed that NO-mediated GABAergic inputs into the RVLM are involved in tonic inhibition of the SNS in SHRSP, consistent with the results from previous studies in normotensive rats.[7,41,42] NO acts on presynaptic terminals to increase vesicular GABA release,[17,20,21] although postsynaptic inhibitory effects of NO on neuronal firing have also been reported.[17,43]

In the present study, we also demonstrated that prior infusion of L-NMMA into the RVLM attenuated the tempol-induced increase in GABA release with sympathoinhibition in the RVLM of SHRSP. Although we did not measure superoxide production in the RVLM of SHRSP, these results suggest that a reduction of NO-mediated GABA release in the RVLM is involved in sympathoexcitation, probably induced by superoxide in SHRSP, because tempol is as effective as native SOD in preventing superoxide production.[37] It has been demonstrated that superoxide reacts with and inactivates NO and thereby modulates its bioavailability,[17,44] and that superoxide in the RVLM of hypertensive rats is increased compared with normotensive rats.[23,26–28] In addition, previous reports indicate that, in the RVLM, the modulatory effect of NO on GABA release and the interaction between superoxide and NO are respectively involved in the pathogenesis of hypertension in hypertensive rats.[9,26] Moreover, our findings suggest that superoxide suppresses NO-mediated GABAergic inhibition in the RVLM of SHRSP.

The RVLM is known to receive both excitatory and inhibitory inputs,[2,45] and glutamate is the major excitatory neurotransmitter. In the present study, tempol-induced sympathoinhibition was attenuated by prior injection of bicuculline, but not kynurenic acid, into the RVLM of SHRSP. Although we did not measure glutamate levels in the RVLM of SHRSP, these results suggest that GABAergic disinhibition might contribute to superoxide-induced sympathoexcitaion in SHRSP. Furthermore, the majority of GABAergic neuronal terminals in the RVLM come from the caudal ventrolateral medulla (CVLM) and inhibit the neuronal excitability of RVLM neurons.[13,15,45,46] It has also been reported that inhibition of the CVLM or blockade of its GABAergic inhibitory inputs to the RVLM by injection of bicuculline into the RVLM caused a smaller pressor response in SHR than in WKY.[10,14–16] In the present study, we focused only on superoxide and NO-mediated GABA release in the RVLM, because the effects of GABA in the RVLM are mainly caused by GABAergic inputs from the CVLM into the RVLM. However, further examination is necessary to clarify the relationship between the RVLM and CVLM in the pathway of superoxide-NO-GABA-sympathetic nerve activity. It would be important to investigate this issue further, because abnormal activation of the SNS is the target of treatments for various cardiovascular diseases.[47–49]

Study Limitations

First, we used L-NMMA, a non-selective inhibitor of all NOS isoforms (neuronal, endothelial, and inducible) and we could not explore the role of each isoform. These NOS isoforms generate superoxide depending on the availability of L-argi-

nine and tetrahydrobiopterine, a co-factor of NOS.[17,50] Second, the NO concentration and superoxide levels in the RVLM remain to be determined. It is still difficult to measure NO concentration in vivo,[51] causing it to be difficult to investigate the role of NO, and we could not conclude whether NO production itself or NO-mediated GABA release is reduced in the RVLM of SHRSP. Further studies are needed to develop a quantitative understanding of NO and superoxide. Third, we did the examinations in SHRSP only, not WKY. However, the aim of the present study was to investigate the further mechanisms by which superoxide in the RVLM causes sympathoexcitation in SHRSP. We speculate that infusion of tempol into the RVLM will not change GABA levels in the RVLM of WKY, because our recent previous study demonstrated that chronic inhibition of superoxide did not change the MAP and RSNA responses to microinjection of bicuculline into the RVLM of WKY.[36]

Conclusions

Our results suggest that a reduction of NO-mediated GABA release is partly involved in superoxide-induced sympathoexcitation in the RVLM of SHRSP. Superoxide-induced reduction of GABA release in the RVLM may play an important role in the mechanism of sympathoexcitation in SHRSP, and NO-mediated GABA release in the RVLM should be more of a focus in studies of the central mechanisms of sympathoexcitation, in addition to the direct action of superoxide in the brain.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B193290231, 22790709, and B24390198) and, in part, a Kimura Memorial Foundation Research Grant.

Disclosures

Conflict of Interest: None.

References

1. Guyenet PG. The sympathetic control of blood pressure. *Nat Rev Neurosci* 2006; **7:** 335–346.
2. Dampney RA. Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 1994; **74:** 323–364.
3. Dampney RA, Horiuchi J, Tagawa T, Fontes MA, Potts PD, Polson JW. Medullary and supramedullary mechanisms regulating sympathetic vasomotor tone. *Acta Physiol Scand* 2003; **177:** 209–218.
4. Tseng CJ, Liu HY, Lin HC, Ger LP, Tung CS, Yen MH. Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 1996; **27:** 36–42.
5. Zanzinger J, Czachurski J, Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol* 1995; **268:** R958–R962.
6. Kagiyama S, Tsuchihashi T, Abe I, Fujishima M. Cardiovascular effects of nitric oxide in the rostral ventrolateral medulla of rats. *Brain Res* 1997; **757:** 155–158.
7. Kishi T, Hirooka Y, Sakai K, Shigematsu H, Shimokawa H, Takeshita A. Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release. *Hypertension* 2001; **38:** 896–901.
8. Kagiyama S, Tsuchihashi T, Abe I, Fujishima M. Enhanced depressor responses to nitric oxide in the rostral ventrolateral medulla of spontaneously hypertensive rats. *Hypertension* 1998; **31:** 1030–1034.
9. Kishi T, Hirooka Y, Ito K, Sakai K, Shimokawa H, Takeshita A. Cardiovascular effects of overexpression of endothelial nitric oxide synthase in the rostral ventrolateral medulla in stroke-prone spontaneously hypertensive rats. *Hypertension* 2002; **39:** 264–268.
10. Smith JK, Barron KW. GABAergic responses in ventrolateral medulla in spontaneously hypertensive rats. *Am J Physiol* 1990; **258:** R450–R456.
11. Bowery NG, Hudson AL, Price GW. $GABA_A$ and $GABA_B$ receptors binding sites distribution in the rat central nervous system. *Neuroscience* 1987; **20:** 365–383.
12. Masuda N, Ootsuka Y, Terui N. Neurons in the caudal ventrolateral medulla mediate the somato-sympathetic inhibitory reflex response via GABA receptors in the rostral ventrolateral medulla. *J Auton Nerv Syst* 1992; **40:** 91–98.
13. Peng YJ, Gong QL, Li P. GABA(A) receptors in the rostral ventrolateral medulla mediate the depressor response induced by stimulation of the greater splanchnic nerve afferent fibers in rats. *Neurosci Lett* 1998; **249:** 95–98.
14. Smith JK, Barron KW. Cardiovascular effects of L-glutamate and tetrodotoxin microinjected into the rostral and caudal ventrolateral medulla in normotensive and spontaneously hypertensive rats. *Brain Res* 1990; **506:** 1–8.
15. Chalmers J, Arnolda L, Kapoor V, Llewellyn-Smith I, Minson J, Pilowsky PM. Amino acid neurotransmitters in the central control of blood pressure and in experimental hypertension. *J Hypertens Suppl* 1992; **10:** S27–S37.
16. Muratani H, Ferrario CM, Averill DB. Ventrolateral medulla in spontaneously hyperensive rats: Role of angiotensin II. *Am J Physiol* 1993; **264:** R388–R395.
17. Garthwaite J. Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci* 2008; **27:** 2783–2802.
18. Talman WT, Dragon DN. Transmission of arterial baroreflex signals depends on neuronal nitric oxide synthase. *Hypertension* 2004; **43:** 820–824.
19. Talman WT. NO and central cardiovascular control: A simple molecule with a complex story. *Hypertension* 2006; **48:** 552–554.
20. Li DP, Chen SR, Pan HL. Nitric oxide inhibits spinally projecting paraventricular neurons through potentiation of presynaptic GABA release. *J Neurophysiol* 2002; **88:** 2664–2674.
21. Li DP, Chen SR, Finnegan TF, Pan HL. Signaling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus. *J Physiol* 2004; **554:** 100–110.
22. Horn T, Smith PM, McLauglin BE, Bauce L, Marks GS, Pittman QJ, et al. Nitric oxide actions in paraventricular nucleus: Cardiovascular and neurochemical implications. *Am J Physiol* 1994; **266:** R306–R313.
23. Hirooka Y, Sagara Y, Kishi T, Sunagawa K. Oxidative stress and central cardiovascular regulation: Pathogenesis of hypertension and therapeutic aspects. *Circ J* 2010; **74:** 827–835.
24. Peterson JR, Sharma RV, Davisson RL. Reactive oxygen species in the neuropathogenesis of hypertension. *Curr Hypertens Rep* 2006; **8:** 232–241.
25. Chan SH, Tai MH, Li CY, Chan JY. Reduction in molecular synthesis or enzyme activity of superoxide dismutases and catalase contributes to oxidative stress and neurogenic hypertension in spontaneously hypertensive rats. *Free Radic Biol Med* 2006; **40:** 2028–2039.
26. Tai MH, Wang LL, Wu KL, Chan JY. Increased superoxide anion in rostral ventrolateral medulla contributes to hypertension in spontaneously hypertensive rats via interactions with nitric oxide. *Free Radic Biol Med* 2005; **38:** 450–462.
27. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 2004; **109:** 2357–2362.
28. Kishi T, Hirooka Y. Sympathoinhibitory effects of atorvastatin in hypertension. *Circ J* 2010; **74:** 2552–2553.
29. Zimmerman MC, Davisson RL. Redox signaling in central neural regulation of cardiovascular function. *Prog Biophys Mol Biol* 2004; **84:** 125–149.
30. Zimmerman MC, Larzatigues E, Shrama RV, Davisson RL. Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. *Circ Res* 2004; **95:** 210–216.
31. Campos RR, Bergamschi CT. Neurotransmission alterations in central cardiovascular control in experimental hypertension. *Curr Hypertens Rev* 2006; **2:** 193–198.
32. Ito K, Hirooka Y, Sakai K, Kishi T, Kaibuchi K, Shimokawa H, et al. Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: Possible involvement in neural mechanisms of hypertension. *Circ Res* 2003; **92:** 1337–1343.
33. Matsukawa R, Hirooka Y, Nishihara M, Ito K, Sunagawa K. Neuregulin-1/ErbB signaling in rostral ventrolateral medulla is involved in blood pressure regulation as an antihypertensive system. *J Hypertens* 2011; **29:** 1735–1742.
34. Matsuo I, Hirooka Y, Hironaga K, Eshima K, Shigematsu H, Shihara M, et al. Glutamate release via NO production evoked by NMDA in the NTS enhances hypotension and bradycardia in vivo. *Am J Physiol* 2001; **280:** R1285–R1291.

35. Ito S, Komatsu K, Tsukamoto K, Sved AF. Excitatory amino acids in the rostral ventrolateral medulla support blood pressure in spontaneously hypertensive rats. *Hypertension* 2000; **35:** 413–417.
36. Nishihara M, Hirooka Y, Matsukawa R, Kishi T, Sunagawa K. Oxidative stress in the rostral ventrolateral medulla modulates excitatory and inhibitory inputs in spontaneously hypertensive rats. *J Hypertens* 2012; **30:** 97–106.
37. Wilcox CS. Effects of tempol and redox-cycling nitroxides in models of oxidative stress. *Pharmacol Ther* 2010; **126:** 119–145.
38. Dugaich AP, Oliveira-Sales EB, Abreu NP, Boim MA, Bergamaschi CT, Campos RR. Role of the rostral ventrolateral medulla in the arterial hypertension in chronic renal failure. *Int J Hypertens* 2011; **2010:** 219358.
39. Chan SH, Wu CW, Chang AY, Hsu KS, Chan JY. Transcriptional upregulation of brain-derived neurotrophic factor in rostral ventrolateral medulla by angiotensin II-induced hypertension. *Am J Hypertens* 2009; **22:** 484–492.
40. Chan SH, Wu CA, Wu KL, Ho YH, Chang AY, Chan JY. Transcriptional upregulation of mitochondrial uncoupling protein 2 protects against oxidative stress-associated neurogenic hypertension. *Circ Res* 2009; **105:** 886–896.
41. Riediger T, Giannini P, Erguven E, Lutz T. Nitric oxide directly inhibits ghrelin-activated neurons of the arcuate nucleus. *Brain Res* 2006; **1125:** 37–45.
42. Nauli SM, Pearce WJ, Amer A, Maher TJ, Ally A. Effects of nitric oxide and GABA interaction within ventrolateral medulla on cardiovascular responses during static muscle contraction. *Brain Res* 2001; **922:** 234–242.
43. Sun C, Sellers KW, Sumners C, Raizada MK. NAD(P)H oxidase inhibition attenuates neuronal chronotropic actions of angiotensin II. *Circ Res* 2005; **96:** 659–666.
44. Ago T, Kuroda J, Kamouchi M, Sadoshima J, Kitazono T. Pathophysiological roles of NADPH oxidase/nox family proteins in the vascular system: Review and perspective. *Circ J* 2011; **75:** 1791–1800.
45. Amano M, Kubo T. Involvement of both GABA(A) and GABA(B) receptors in tonic inhibitory control of blood pressure at the rostral ventrolateral medulla of the rats. *Naunyn Schmiedebergs Arch Pharmacol* 1993; **348:** 146–153.
46. Suzuki T, Takayama K, Miura M. Distribution and projection of the medullary cardiovascular control neurons containing glutamate, glutamic acid decarboxylase, tyrosine hydroxylase and phenylethanolamine N-methyltransferase in rats. *Neurosci Res* 1997; **28:** 289–290.
47. Kishi T, Sunagawa K. Combination therapy of atorvastatin and amlodipine inhibits sympathetic nervous system activation and improves cognitive function in hypertensive rats. *Circ J* 2012; **76:** 1934–1941.
48. Yoshihisa A, Suzuki S, Miyata M, Yamaki T, Sugimoto K, Kunii H, et al. 'A single night' beneficial effects of adaptive servo-ventilation on cardiac overload, sympathetic nervous activity, and myocardial damage in patients with chronic heart failure and sleep-disordered breathing. *Circ J* 2012; **76:** 2153–2158.
49. Tsutamoto T, Sakai H, Ibe K, Yamaji M, Kawahara C, Nakae I, et al. Effect of atorvastatin vs. rosuvastatin on cardiac sympathetic nerve activity in non-diabetic patients with dilated cardiomyopathy. *Circ J* 2011; **75:** 2160–2196.
50. Murphy S, Gibson CL. Nitric oxide, ischaemia and brain inflammation. *Biochem Soc Trans* 2007; **35:** 1133–1137.
51. Hall CN, Garthwaite J. What is the real physiological NO concentration in vivo? *Nitric Oxide* 2009; **21:** 92–103.

Journal of Cardiology 60 (2012) 489–494

Contents lists available at SciVerse ScienceDirect

Journal of Cardiology

journal homepage: www.elsevier.com/locate/jjcc

Original article

Telmisartan protects against cognitive decline via up-regulation of brain-derived neurotrophic factor/tropomyosin-related kinase B in hippocampus of hypertensive rats

Takuya Kishi (MD PhD)[a,*], Yoshitaka Hirooka (MD PhD)[b], Kenji Sunagawa (MD PhD)[c]

[a] Department of Advanced Therapeutics for Cardiovascular Diseases, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
[b] Department of Advanced Cardiovascular Regulation and Therapeutics, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
[c] Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

ARTICLE INFO

Article history:
Received 10 April 2012
Received in revised form 24 May 2012
Accepted 20 June 2012
Available online 2 September 2012

Keywords:
Hypertension
Cognitive function
Brain
Angiotensin receptor blocker

ABSTRACT

Background and purpose: Cognitive decline may occur as a result of hypertension, and is dependent on the function of hippocampus. Brain-derived neurotrophic factor (BDNF) mediated by angiotensin II-induced oxidative stress protects against cell death in hippocampus. Angiotensin II receptor blocker (ARB), candesartan, activates BDNF in the hippocampus. Furthermore, peroxisome proliferator-activated receptor (PPAR)-gamma activation in the brain prevents brain damage. Telmisartan, a unique ARB with PPAR-gamma stimulating activity, protects against cognitive decline partly because of PPAR-gamma activation. The aim of the present study was to determine whether telmisartan protects against cognitive decline via up-regulation of BDNF and its receptor tropomyosin-related kinase B (TrkB) in the hippocampus of hypertensive rats, partly because of PPAR-gamma activation.

Methods and results: We divided stroke-prone spontaneously hypertensive rats (SHRSPs), as hypertensive and vascular dementia model rats, into five groups, telmisartan-treated (TLM), TLM + GW9662, a PPAR-gamma inhibitor, -treated (T + G), GW9662-treated (GW), TLM + ANA-12, a TrkB antagonist, -treated (T + A), and vehicle-treated SHRSPs (VEH). After the treatment for 28 days, systolic blood pressure did not change in all groups. However, BDNF expression in the hippocampus was significantly higher in TLM than in VEH to a greater extent than in T + G. Cognitive performance was significantly higher in TLM than in VEH to a greater extent than in T + G, and was not different between T + A, GW, and VEH.

Conclusion: Telmisartan protects against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus of SHRSPs, partly because of PPAR-gamma activation independent of blood pressure-lowering effect.

Introduction

One of the important organ damages related to hypertension is cognitive decline. In the brain, angiotensin II contributes to the physiological regulation of many different functions, including cerebral circulation, integrity of the blood–brain barrier, central sympathetic activity, hormonal production and release, response to stress, behavior, and cognition [1–5]. In the treatments for hypertension, angiotensin II type1 receptor (AT_1R) blockers (ARB) are widely used [6]. A previous clinical study demonstrated that antihypertensive drugs that act via the renin–angiotensin system have potential in preventing, delaying, or decelerating the onset and progression of cognitive decline in hypertensive patients [7]. In the treatments for hypertension, cognition should be focused as a target of the antihypertensive treatment. Among ARBs, telmisartan has a beneficial effect in rats treated with repeated cerebral ischemia [8,9], Alzheimer model [10,11], diabetic model [12], and coronary plaque vulnerability [13]. However, no benefit was found in cognitive performance after administration of telmisartan after stroke [14]. In ONTARGET and TRANSCEND, telmisartan did not provide positive effects on cognitive function [15]. The mechanisms of the protection against cognitive decline in cerebral ischemia by telmisartan should be discussed further. Telmisartan is a unique ARB with a partial peroxisome proliferator-activated receptor (PPAR)-gamma agonistic property in its antihypertensive effect [16]. Anti-inflammatory and anti-oxidant effects of telmisartan that were exerted in part by PPAR-gamma activation, but not its

* Corresponding author at: Department of Advanced Therapeutics for Cardiovascular Diseases, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5360; fax: +81 92 642 5374.
E-mail address: tkishi@cardiol.med.kyushu-u.ac.jp (T. Kishi).

0914-5087/$ – see front matter © 2012 Japanese College of Cardiology. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jjcc.2012.08.004

blood pressure-lowering effect, have protective roles against cognitive decline in cerebral ischemia [8,9]. PPAR-gamma activation is reported to reduce oxidative stress and inflammatory response in the vasculature and adipose tissue [17], and PPAR-gamma activation in the brain has been reported to prevent brain damage via anti-inflammatory effects in neurons [18].

Previous studies have suggested that the underlying mechanisms of the beneficial effect of ARBs in stroke may not only be the consequence of improved hemodynamics and vascular function, but may also involve a blood pressure-independent element of neuroprotection [19–22]. In the brain, brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) are known to be involved in the protective mechanisms against stress and cell death as an antioxidant [23–26]. Angiotensin II induces superoxide-dependent down-regulation of BDNF via phosphorylation of cAMP response element binding protein [27]. Candesartan at sub-hypotensive and renin–angiotensin system blocking dose affords neuroprotection after focal ischemia, associated with increased activity of BDNF [28]. Telmisartan improves memory impairment and reduces neural apoptosis in hippocampus via a PPAR-gamma-dependent anti-apoptotic mechanism in rats with repeated cerebral ischemia [8]. However, it has not been determined whether telmisartan has protective effects on cognitive decline via up-regulation of BDNF/TrkB in the hippocampus.

Combined with these previous studies, we had the hypothesis that the beneficial effects of telmisartan on cognition are not only because of its established effect of antihypertensive and systemic blockade of AT_1R but also because of the benefits on BDNF in the hippocampus via PPAR-gamma agonistic effect in hypertension. The aim of the present study was to determine whether telmisartan protects against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus of stroke-prone spontaneously hypertensive rats (SHRSPs) as hypertensive and vascular dementia model rats [29], partly because of PPAR-gamma activation. Previous studies have demonstrated that ARBs have benefits on brain damage and vascular inflammation in SHRSPs [30–32], as well as organ damage in spontaneously hypertensive rats [33]. Telmisartan also has anti-oxidant effects in vasculature [34] and brain [35] of SHRSPs. We divided SHRSPs into five groups, telmisartan-treated (TLM), TLM + GW9662, a PPAR-gamma antagonist, -treated (T + G), GW9662-treated (GW), TLM + N-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl) amino] carbonyl] phenyl]-benzothiophene-2-carboxamide (ANA-12), a TrkB antagonist, -treated (T + A), and vehicle-treated SHRSPs (VEH). Cognitive function was assessed by the Morris water maze test, which has been widely used as a test of spatial memory and cognition [36].

Methods

Animals

This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male SHRSPs (12–14 weeks), weighing 350–425 g and fed standard feed were used (SLC Japan, Hamamatsu, Japan). They were housed individually in a temperature-controlled room (22–23 °C) with a 12-h/12-h light-dark cycle (lights on at 7:00 AM). We divided SHRSPs into 5 groups: TLM, T + G, T + A, GW, and VEH ($n = 5$ for each). Systolic blood pressure and heart rate were measured daily using the tail-cuff method (BP-98 A; Softron, Tokyo, Japan).

Oral administration of drugs

SHRSPs were treated for 4 weeks. TLM group was administered telmisartan (1 mg/kg/day, Sigma Aldrich, St. Louis, MO, USA). GW group was administered GW9662 (1 mg/kg/day, Sigma Aldrich). T + G group was administered telmisartan (1 mg/kg/day) plus GW9662 (1 mg/kg/day). T + A group was administered telmisartan (1 mg/kg/day) plus ANA-12 (0.5 mg/kg/day, Sigma Aldrich). VEH group was administered 0.5% methylcellulose. All drugs were dissolved in 0.5% methylcellulose and administered by gastric gavage every day. The dose of telmisartan was selected as a low dose and non-depressor dose [37,38]. The dose of GW9662 was according to the previous studies examining the partial effect of telmisartan on PPAR-gamma activation [9,37]. The dose of ANA-12 was determined to blockade BDNF according to a previous study [39].

Western blotting analysis

To obtain the hippocampus tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with PBS (150 mol/L NaCl, 3 mmol/L KCl, and 5 nmol/L phosphate; pH 7.4, 4 °C). The brains were removed quickly, and the hippocampus tissues obtained according to a rat brain atlas were homogenized and sonicated in a lysing buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, and 1 mmol/L phenylmethanesulfonyl fluoride. The tissue lysate was centrifuged at 6000 rpm for 5 min at 4 °C with a microcentrifuge. The lysate was collected, and protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). An aliquot of 20 μg of protein from each sample was separated on 12% SDS-polyacrylamide gel. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore, Billarica, MA, USA). Membranes were incubated for 2 h with a rabbit polyclonal antiserum against BDNF (1:1000; Abcam, Cambridge, UK) or α-tubulin (1:1000; Cell Signaling, Danvers, MA, USA). Membranes were then washed and incubated with a horseradish peroxidase–conjugated horse anti-mouse IgG antibody (1:10,000) for 40 min. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (plus Western blotting detection kit; GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and was expressed as the ratio to α-tubulin protein.

Analysis of cognitive function

Spatial learning and memory function of the rats were investigated with the Morris water maze test in a circular pool filled with water at a temperature of 25.0 ± 1 °C [36]. In the hidden platform test, a transparent platform was submerged 1 cm below the water level. Swimming paths were tracked with a camera fixed on the ceiling of the room and stored in a computer. All the procedures of the Morris water maze were performed for 7 days. A pre-training session was carried out at day 0, in which animals were given 60 s free swimming without the platform. In the hidden-platform test for 4 days, the rats were given 2 trials (1 session) on day 1 and 4 trials (2 sessions) per day on days 2, 3, and 4. The initial trial interval was about 30 min and the inter-session interval was 2 h. During each trial, the rats were released from four pseudo-randomly assigned starting points and allowed to swim for 60 s. After mounting the platform, the rats were allowed to remain there for 15 s, and were then placed in the home cage until the start of the next trial. If a rat was unable to find the platform within 60 s, it was guided to the platform and allowed to rest on the platform for 15 s. Probe trials were performed at day 5. In the probe trial, the hidden platform was removed and the rats was released from the right quadrant and allowed to swim freely for 60 s. The time spent in the target