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

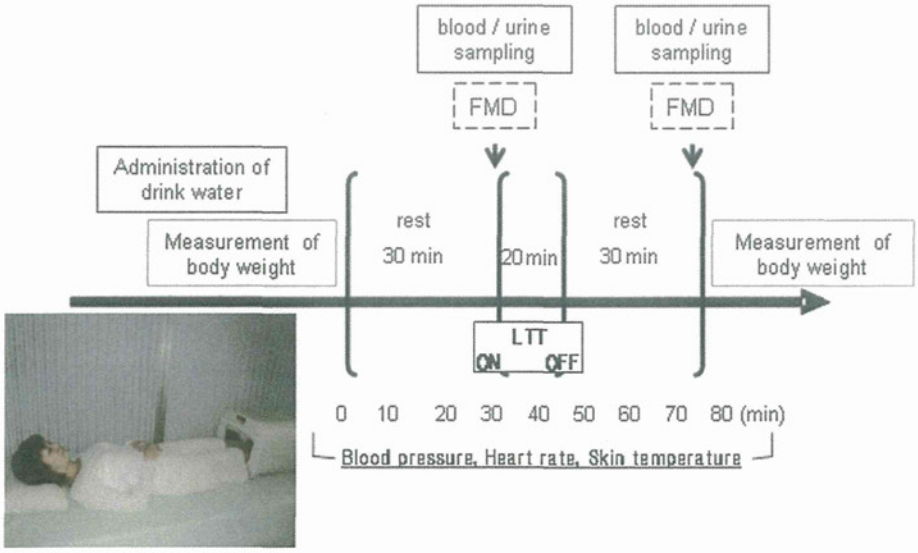

Figure 1. Setup of the leg thermal therapy for patient with chronic heart failure. LTT and FMD indicate leg thermal therapy and flow-mediated vasodilation, respectively.

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 (°C)	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

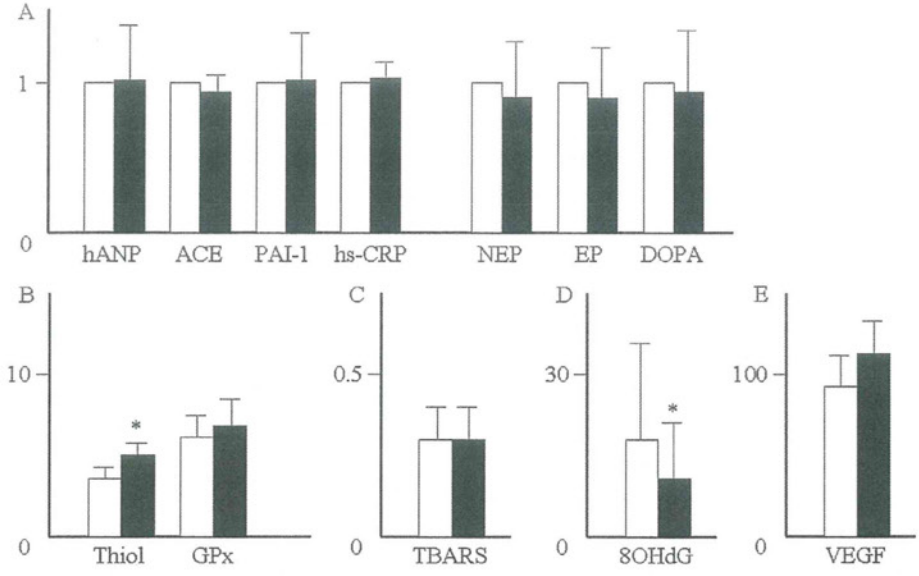

Figure 2. **The leg thermal therapy (LTT) did not affect the neurohormonal factors including the serum human atrial natriuretic peptide (hANP), angiotensin-converting enzyme (ACE), plasminogen activator inhibitor (PAI)-1, highly sensitive C reactive protein (hs-CRP), and urine norepinephrine (NEP), epinephrine (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.**

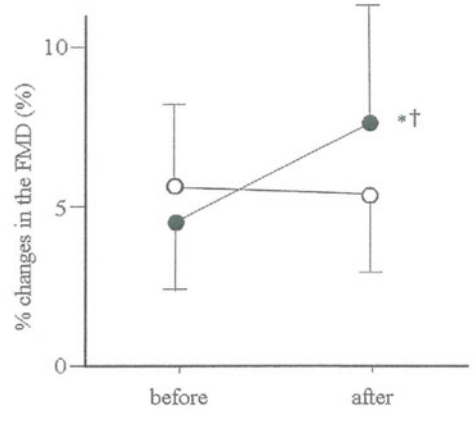

Figure 3. **The leg thermal therapy (LTT) improved the % change in the flow-mediated vasodilation (FMD). The open and closed circle indicate the levels without and with the LTT, respectively.*p<0.05 vs. before the LTT. †p<0.05 vs. without the LTT.**

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).

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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

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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-

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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.

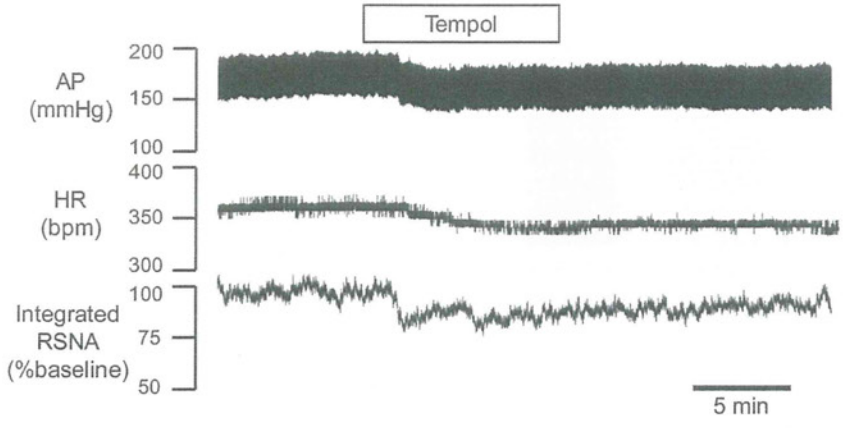

Figure 1. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats. bpm indicates beats/min.

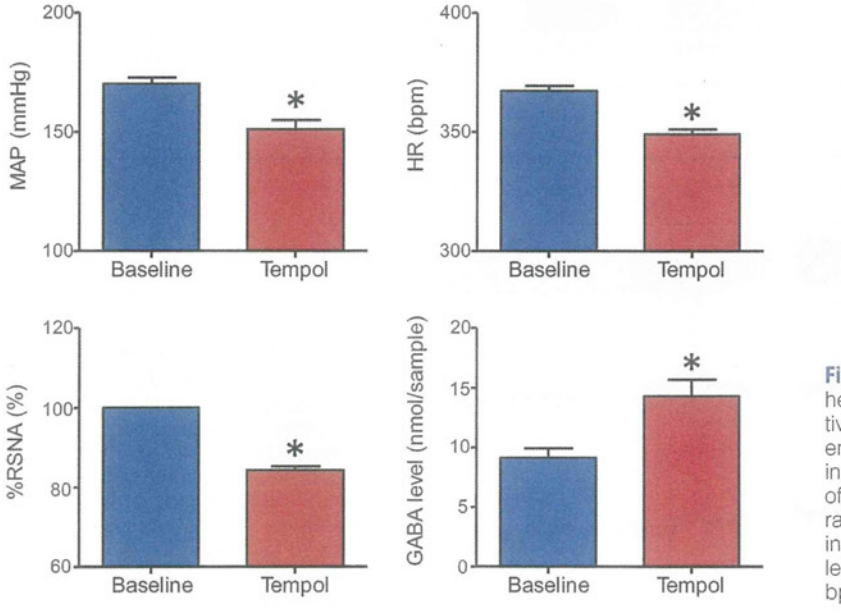

Figure 2. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the unilateral rostral ventrolateral medulla (RVLM) to infusion of tempol (1 nmol/50 nl/min for 10 min) of stroke-prone spontaneously hypertensive rats. Group data of tempol-induced 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).

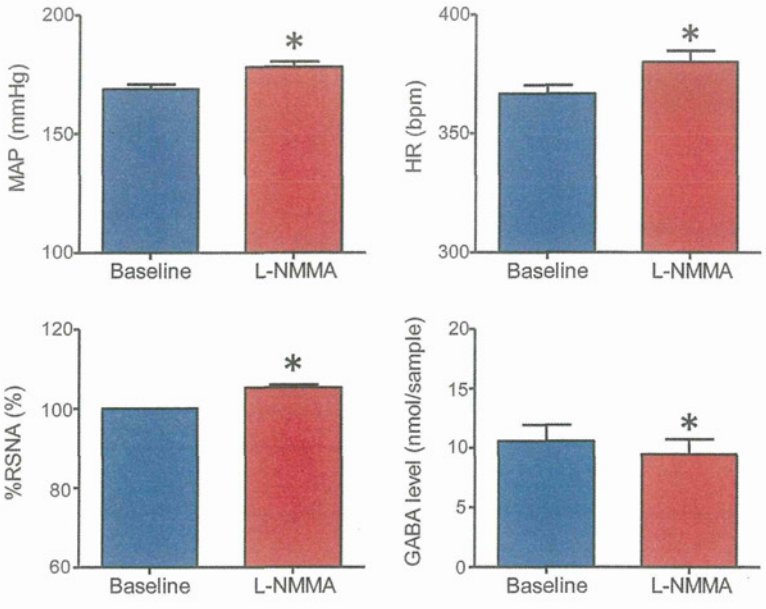

Figure 3. Response of arterial pressure (AP), heart rate (HR), renal sympathetic nerve activity (RSNA), and GABA levels in the rostral ventrolateral medulla (RVLM) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats. Group data 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.

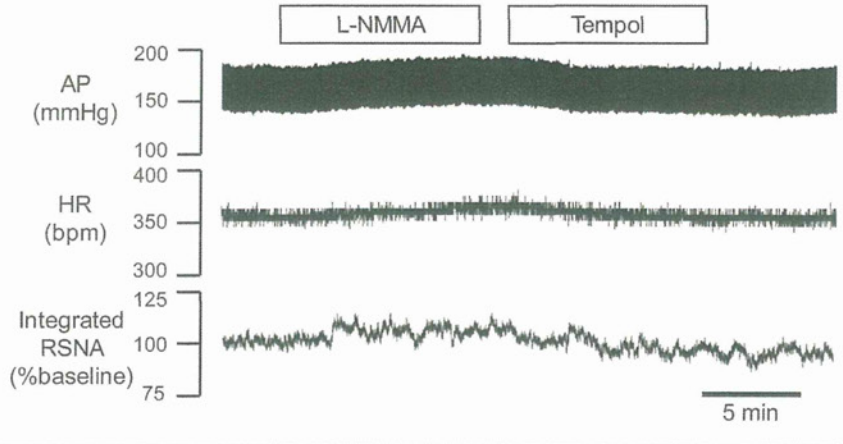

Figure 4. Raw data of the response of arterial pressure (AP), heart rate (HR), and renal sympathetic nerve activity (RSNA) to infusion of L-NMMA (10 nmol/50 nl/min for 10 min) and subsequent tempol (1 nmol/50 nl/min for 10 min) into the unilateral rostral ventrolateral medulla of stroke-prone spontaneously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

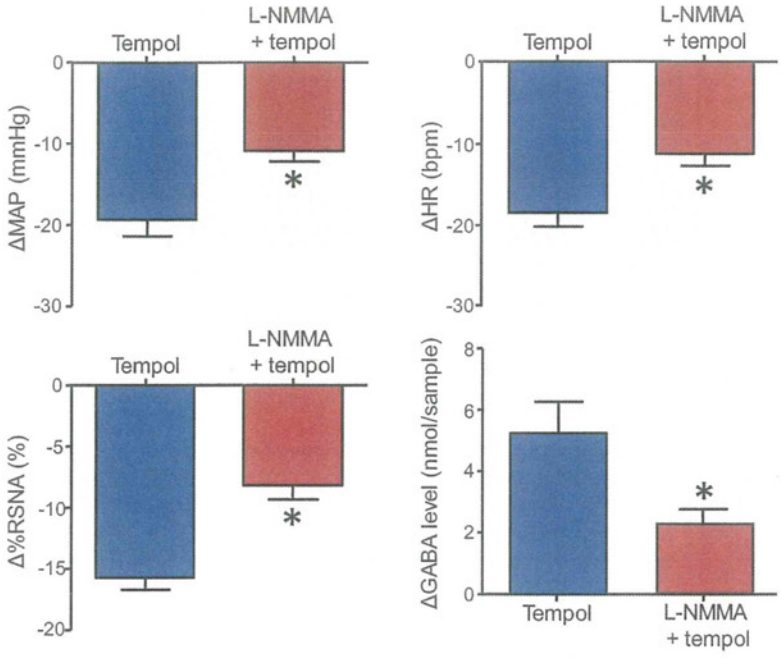

Figure 5. Effect of inhibition of nitric oxide (NO) production by L-NMMA (10 nmol/50 nl/min for 10 min) in the rostral ventrolateral medulla (RVLM) on the responses to infusion of tempol (1 nmol/50 nl/min for 10 min) into the unilateral RVLM of stroke-prone spontaneously hypertensive rats (SHRSP). Prior 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.

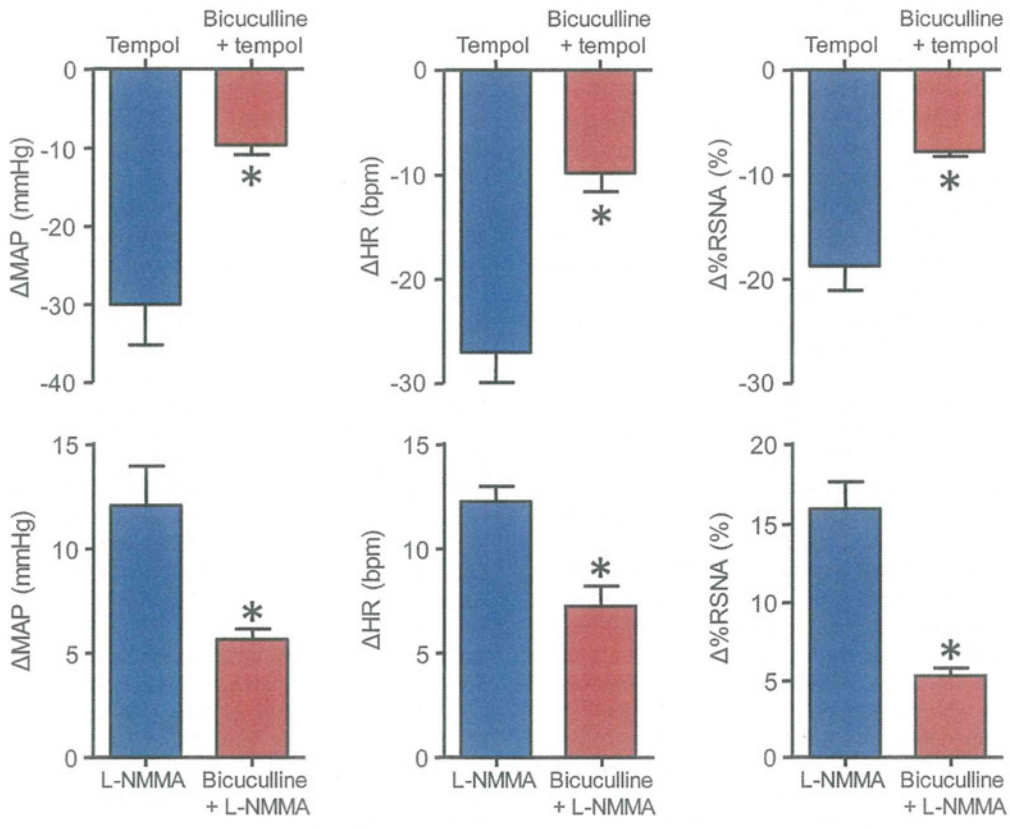

Figure 6. Effect of bicuculline (200 pmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) or L-NMMA (10 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior microinjection of bicuculline attenuated the tempol-induced 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.

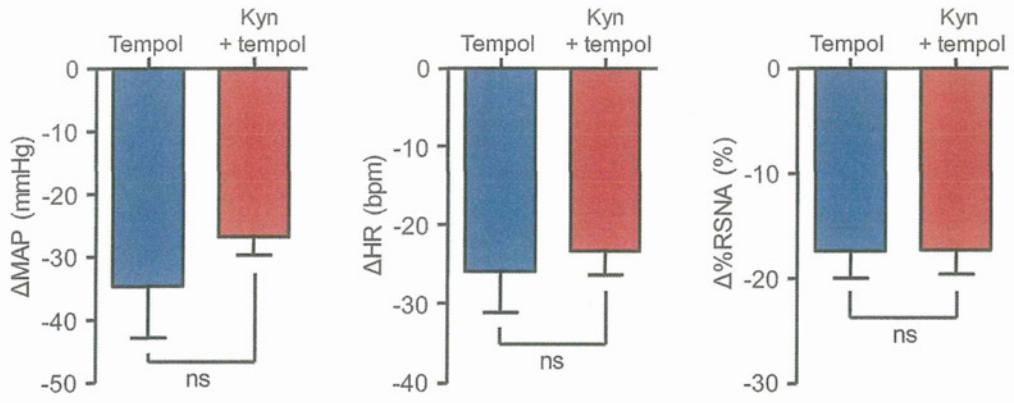

Figure 7. Effect of kynurenic acid (2.7 nmol) in the rostral ventrolateral medulla (RVLM) on the responses to acute microinjection of tempol (1 nmol) into the bilateral RVLM of stroke-prone spontaneously hypertensive rats. Prior acute microinjection of kynurenic acid did not affect the tempol-induced 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

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Disclosures

Conflict of Interest: None.

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Original article

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

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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

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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

Table 1
Physiological data.

	VEH	TLM	T+G	T+A	GW
SBP (mmHg)	240 ± 28	228 ± 17	229 ± 16	231 ± 19	243 ± 21
HR (bpm)	338 ± 30	331 ± 26	340 ± 29	343 ± 30	329 ± 37
BW (g)	282 ± 15	280 ± 14	288 ± 17	276 ± 19	291 ± 22
Calorie intake (Kcal/day)	77 ± 5	74 ± 8	72 ± 4	78 ± 6	74 ± 9
Water intake (ml/day)	32 ± 4	29 ± 4	30 ± 5	30 ± 3	28 ± 5

Data are expressed as the mean ± SEM.
SBP, systolic blood pressure; HR, heart rate; BW, body weight; VEH, vehicle; TLM, telmisartan; T+G, telmisartan+GW9662; T+A, telmisartan+ANA-12; GW, GW9662; $n=5$ for each.

quadrant, where the platform had been located during training, and the time spent in the other quadrants were measured. In the visible-platform test which was performed at day 6, the platform was elevated above the water surface and placed in a different position.

Statistical analysis

All values are expressed as mean ± SEM. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. ANOVA was used to compare all the parameters in all groups. Differences were considered to be statistically significant at a p-value of <0.05.

Results

Physiological data

Systolic blood pressure and heart rate were not changed in TLM, T+G, GW, T+A, and VEH after the treatments (Table 1). Body weight, dairy calorie intake, and water intake were also not different in all groups (Table 1).

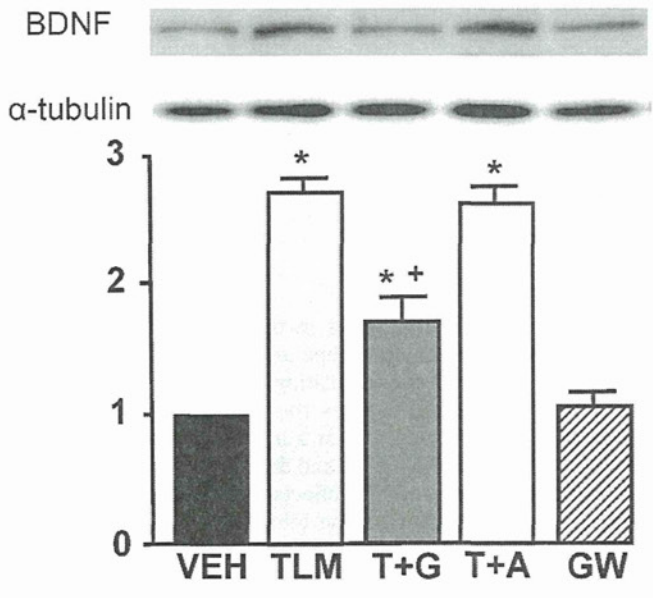

Fig. 1. Expression of BDNF in the hippocampus in each group. BDNF/α-tubulin expression was expressed relative to that in VEH which was assigned a value of 1. *$p<0.05$ versus VEH, +$p<0.05$ in T+G versus TLM, $n=5$ for each. BDNF, brain-derived neurotrophic factor; VEH, vehicle; TLM, telmisartan; T+G, telmisartan+GW9662; T+A, telmisartan+N-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl) amino] carbonyl] phenyl]-benzothiophene-2-carboxamide (ANA-12); GW, GW9662.

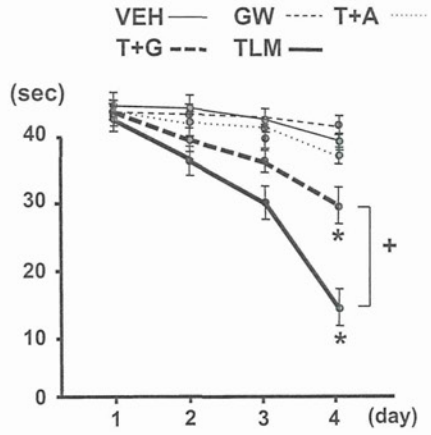

Fig. 2. Escape latency in the hidden platform test of Morris water maze. *$p<0.05$ versus VEH, +$p<0.05$ in T+G versus TLM, $n=5$ for each. VEH, vehicle; TLM, telmisartan; T+G, telmisartan+GW9662; T+A, telmisartan+N-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl) amino] carbonyl] phenyl]-benzothiophene-2-carboxamide (ANA-12); GW, GW9662.

Expression of BDNF in the hippocampus

The expression of BDNF in the hippocampus was significantly higher in TLM than in VEH (Fig. 1). The up-regulation of BDNF in the hippocampus in TLM was attenuated in T+G, but not in T+A (Fig. 1). However, the expression of BDNF in the hippocampus was not different between GW and VEH (Fig. 1).

Morris water maze test

In the hidden platform test, escape latency was significantly lower in TLM than in VEH to a greater extent than in T+G (Fig. 2), and was not different between in VEH, GW, and T+A (Fig. 2). In the probe test, TLM resulted in significantly more time in the target quadrant as compared with VEH, GW, and T+A to a greater extent than in T+G (Fig. 3). In the visible platform test, there were no significant differences in escape latency among all of the groups.

Discussion

In the present study, we have demonstrated two major findings. First, telmisartan has a protective effect on the cognitive decline via up-regulation of BDNF/TrkB in the hippocampus of SHRSPs without depressor effect. Second, co-administration of a PPAR-gamma antagonist with telmisartan partially attenuated the telmisartan-mediated protective effect on the cognitive decline. These results suggest that telmisartan has a possibility of protective effect against cognitive decline via activation of BDNF/TrkB through blockade of AT_1R and part activation of PPAR-gamma in the hippocampus of SHRSPs independent of blood pressure-lowering effect.

In the hippocampus, BDNF protects against ischemic cell damage [32]. Angiotensin II blocks long-term potentiation in the

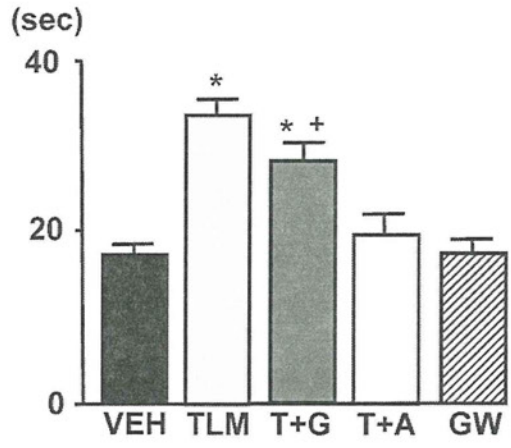

Fig. 3. Time in the target quadrant of the probe test of Morris water maze. $^{*}p < 0.05$ versus VEH, $^{+}p < 0.05$ in T+G versus TLM, $n = 5$ for each. VEH, vehicle; TLM, telmisartan; T+G, telmisartan+GW9662; T+A, telmisartan+N-[2-[[(hexahydro-2-oxo-1H-azepin-3-yl) amino] carbonyl] phenyl]-benzothiophene-2-carboxamide (ANA-12); GW, GW9662.

hippocampus [40–44], and induces superoxide-dependent down regulation of BDNF [27]. In the present study, low-dose telmisartan caused the protective effect against cognitive decline with the increase in BDNF expression in hippocampus of SHRSPs, and the effects were attenuated by TrkB antagonist. These results suggest that telmisartan has a protective effect on the cognitive decline via up-regulation of BDNF/TrkB in the hippocampus of SHRSPs without a depressor effect. Among ARBs, candesartan at sub-hypotensive and renin–angiotensin system blocking dose affords neuroprotection after focal ischemia, associated with increased activity of the BDNF [28]. Interestingly, ramipril at sub-hypotensive, hypotensive, and renin–angiotensin system blocking doses showed no significant neuroprotective effects [28]. Oxidative stress and/or antioxidant deficiency cause cognitive decline [45], and oxidative stress in hippocampus impairs cognitive function [46]. Combining the previous studies with our results in the present study, we consider that the telmisartan-induced up-regulation of BDNF/TrkB is caused by the blockade of AT_1R-induced superoxide in the hippocampus, and that ARBs have a potential to be preferable agents for the treatment of hypertension with the protection against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus.

We also demonstrated that, in the present study, telmisartan-induced protection against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus was partially attenuated by co-administration of PPAR-gamma antagonist with telmisartan. In a previous study, low-dose telmisartan without depressor effect protected against focal brain ischemia partly through activation of PPAR-gamma as well as AT_1R blockade [12]. 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]. In other studies, co-administration of PPAR-gamma antagonist had no effect on the losartan-mediated reduction in ischemic area [8,12]. Our results are comparable with those previous studies, and suggest that telmisartan could exert protective effects against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus through AT_1R blockade and partly PPAR-gamma stimulation. Interestingly, in the present study, PPAR-gamma antagonist alone did not change cognitive performance and the expression of BDNF in the hippocampus. There is a possibility that AT_1R blockade has a synergistic effect of PPAR-gamma activation. If so, ARB with partial PPAR-gamma agonist, telmisartan, has a potential to be a preferable agent for the treatment of hypertension with the protection against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus.

The protective effect against cognitive decline is not specific in telmisartan among ARBs. Candesartan has a positive effect on cognitive decline in hypertensive patients [47] or diabetic model [48], and also significantly reduced the incidence and progression of dementia [49]. In SHRSPs, candesartan improves hippocampal CA1 neuron cell reduction, and superoxide production in the hippocampus [50]. In the brain, AT_1R-induced superoxide decreases BDNF [27]. Both telmisartan and candesartan are reported to reduce oxidative stress via blockade of AT_1R in the brain [51–53]. Although candesartan was not examined in the present study, we consider that the protective effect against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus is also caused by candesartan, not only telmisartan among ARBs, through the blockade of AT_1R in the hippocampus. However, the change in permeability of the blood–brain barrier by ARBs has not been well assessed to date. Ischemic brain damage enhances blood–brain barrier permeability and penetration of ARBs into the brain, and blood–brain barrier is disrupted in SHRSPs [54,55]. Telmisartan is expected to readily shift to organs compared with other ARBs, due to its high lipid solubility [56,57]. Moreover, telmisartan is a unique ARB with a partial PPAR-gamma agonistic property [16]. From the results obtained in the present study, AT_1R blockade with PPAR-gamma agonist is considered to be preferable to the protection against cognitive decline via up-regulation of BDNF/TrkB in the hippocampus.

Although the present study could demonstrate a beneficial effect of low-dose telmisartan on cognitive function, depressor dose of telmisartan could not provide positive effect on cognition in previous clinical studies [14,15]. This discrepancy could not be due to the difference in the dose of telmisartan, because the beneficial effects in the present study were obtained with the low and not depressor dose of telmisartan. We could not fully clarify the reasons of the discrepancy in the present study. We used the Morris water maze test in SHRSPs to evaluate cognitive function instead of the shuttle avoidance test. A spatial working memory task, such as Morris water maze test, depends on hippocampus function [58,59]. Because we focused on cognitive performance via BDNF/TrkB in the hippocampus of SHRSPs, we used the Morris water maze test. However, it has not been determined whether other cognitive function tests could obtain similar beneficial effects in other models, such as Alzheimer, diabetes, or cardiovascular disease models. We consider that the cognitive decline in cardiovascular diseases has various clinical backgrounds, and that multi-targeted therapy by combination of agents is necessary to protect against cognitive decline. In these aspects, AT_1R blockade with PPAR-gamma agonist, telmisartan, might be considered to be preferable among ARBs.

Limitations

There are several limitations in the present study. First, we could not determine the dose dependency of telmisartan and not demonstrate the direct data indicating that telmisartan penetrates blood–brain barrier and reaches the hippocampus. Telmisartan used in the present study was at a low and not depressor dose, and we consider that the higher and depressor dose of telmisartan would provide more beneficial effects. It is necessary in a further study to determine whether the telmisartan-induced depressor effect is synergistic to the present results or not, and to measure the concentration of telmisartan in the hippocampus. Second, we did not quantify superoxide in the hippocampus, and did not determine whether telmisartan reduced superoxide in the hippocampus. Furthermore, we examined only cognitive function and BDNF expression in the hippocampus in the present study, and we did not examine the brain damage in the other areas and vascular inflammation. Previously many studies have already demonstrated

that ARBs could prevent brain damage [5,8,10–12] and vascular inflammation [30–32]. Telmisartan also has benefits in SHRSP [34], and anti-oxidant effects in the brain [35,51]. Because of these previous studies, we consider that the benefits of ARBs on brain damage and vascular inflammation are established, and focused on only cognitive function and BDNF expression in the hippocampus in the present study. Third, we did not perform histochemical experiments to determine the expression of PPAR-gamma and changes in CA1 neuron in the hippocampus, and performed only pharmacological inhibition of PPAR-gamma or BDNF/TrkB in the hippocampus. Although previous studies suggested the expression of PPAR-gamma in the hippocampus of cerebral-ischemia models [8,60] and GW9662 or ANA-12 have been used as reasonable agents to inhibit PPAR-gamma or TrkB [8,9,37,39,61], It would strengthen the results of the present study to determine the expression of PPAR-gamma and changes in CA1 neuron in the hippocampus and to do the specific PPAR-gamma or BDNF/TrkB-targeting methods (such as gene transfer methods) locally in the hippocampus.

Conclusion

Telmisartan has a possibility of protective effect against cognitive decline via activation of BDNF/TrkB through blockade of AT_1R and part activation of PPAR-gamma in the hippocampus of SHRSPs independent of blood pressure-lowering effect, which might not be a class effect of ARBs. These results could provide a new aspect that telmisartan may be more effective to prevent cognitive decline compared with other ARBs, and might contribute to improve quality of life in hypertensive patients.

Conflict of interest

None.

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