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Exercise Training Causes Sympathoinhibition through Antioxidant Effect in the Rostral Ventrolateral Medulla of Hypertensive Rats

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Abstract

Exercise training normalizes sympathetic outflow in hypertension and chronic heart failure. The aim of this study was to determine whether the exercise training inhibits sympathetic nerve activity (SNA) via reduction of oxidative stress through blocked angiotensin II type 1 receptor (AT_1R) in rostral ventrolateral medulla (RVLM). We divided stroke-prone spontaneously hypertensive rats (SHRSP) into SHRSP with exercised training (SHRSP-EX) and control (SHRSP-C). SNA and oxidative stress in the RVLM were significantly lower in SHRSP-EX than in SHRSP-C. These results suggest that exercise training inhibits SNA via reduction of oxidative stress through blocked AT_1R in the RVLM of hypertension.

Keywords: exercise training, hypertension, sympathetic nerve activity, oxidative stress, brain

INTRODUCTION

Exercise training has been shown to be beneficial for patients with hypertension (1) and heart failure (2). Previous studies have suggested that exercise training reduced the activity of sympathetic nervous system (SNS) in heart failure (3–5). Furthermore, it has been demonstrated that exercise training enhanced baroreflex sensitivity in heart failure (5–7). The activity of the SNS is determined by the brain (1,8). However, the changes in the brain caused by exercise training and the mechanisms by which exercise training causes the sympathoinhibition in hypertension have not been fully determined.

Rostral ventrolateral medulla (RVLM) in the brainstem is the vasomotor center that determines basal activity of the SNS, and the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone (8,9). Several previous reports have demonstrated that oxidative stress in the RVLM produced by angiotensin II type 1 receptor (AT_1R) increases the activity of the SNS (10–14) and that nitric oxide in the RVLM decreases the activity of the SNS in hypertension (15–17). Our other reports have suggested that the imbalance between oxidative stress and nitric oxide in the brain causes cardiovascular diseases (18–20). However, it has not been determined whether the exercise training decreases the activity of the SNS via antioxidant in the RVLM through the blocked AT_1R in the RVLM of hypertensive rats or not.

Therefore, the aims of this study were to investigate the effects of the exercise training on the activity of the SNS and oxidative stress in the RVLM of hypertensive rats. To determine these aims, we measured mean arterial pressure (MAP), heart rate (HR), urinary norepinephrine excretion as a parameter of the activity of the SNS, baroreflex sensitivity, and oxidative stress in the RVLM of the hypertensive and normotensive rats with exercise training. To inhibit the oxidative stress in the RVLM locally, we microinjected tempol, a superoxide dismutase mimetic, into the RVLM of the hypertensive and normotensive rats with exercise training. Furthermore, to activate the AT_1R in the RVLM, we microinjected exogenous angiotensin II into the RVLM of the hypertensive and normotensive rats with exercise training.

MATERIALS AND METHODS

Animals

This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University

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Graduate School of Medical Sciences and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male stroke-prone spontaneously hypertensive (SHRSP)/Izm rats and age-matched Wistar-Kyoto rats (WKY; 12- to 14-wk-old) that fed standard feed were used. Stroke-prone spontaneously hypertensive rats and WKY were divided randomly into two groups: SHRSP with exercise training for 28 days (SHRSP-EX) and control group (SHRSP-C) and WKY with exercise training for 28 days (WKY-EX) and control group (WKY-C), respectively.

Radio-Telemetry Monitoring of Blood Pressure and HR

The UA-10 telemetry system (Data Science International, Dallas, TX, USA) was used to measure MAP and HR, as described previously (10,11,16).

Measurement of Urinary Norepinephrine Excretion

We measured the urinary norepinephrine excretion in SHRSP-EX, SHRSP-C, WKY-EX, and WKY-C at day 28 after the exercise training as a parameter of the activity of the SNS, as described previously (10,11,16,21).

Measurement of Conscious Baroreflex Sensitivity by Spontaneous Sequence Method

We measured baroreflex sensitivity by using spontaneous sequence method as previously described (11,22). A rest period of about 10 minutes was obtained for all rats to allow for the stabilization of blood pressure or HR. After the analysis of hemodynamic recordings obtained for about 5 minutes from telemetry system, we selected all the sequences of three or more successive heart beats in which there was a concordant increase (up sequence) or decrease (down sequence) in arterial systolic blood pressure and peak-to-peak systolic blood pressure interval change. A linear regression was applied to each of the sequences, and an average regression slope was calculated for the sequences. This slope represents the cardiac baroreflex sensitivity. The threshold values for including beat-to-beat systolic blood pressure and its interval changes in a sequence are set at 1 mm Hg and 2 milliseconds, respectively.

Exercise Training

Stroke-prone spontaneously hypertensive rats with exercise training for 28 days and WKY-EX were submitted to a maximal exercise test on the treadmill (20° angle, 10 m/min for 30 min) every day for 28 days.

Measurement of the Levels of Thiobarbituric Acid-Reactive Substances in the RVLM

To obtain the RVLM tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with phosphate-buffered saline (150 mol/L NaCl, 3 mmol/L KCl, and 5 nmol/L phosphate; pH = 7.4, 4°C). The brains were removed quickly, and sections of 1-mm-thick were obtained with a cryostat at -7 ± 1°C. The RVLM was defined according to a rat brain atlas as described previously (10–12) and obtained by a punch-out technique. The RVLM tissues were homogenized in KCl (1.15%; pH = 7.4), sodium dodecyl sulfate (0.4%), and acetic acid (7.5%) adjusted to pH = 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 minutes, followed by heating to 100°C for 60 minutes. After cooling, the mixture was extracted with distilled water and *n*-butanolpyridine (15:1) and centrifuged at 1600 × *g* for 10 minutes. The absorbance of the organic phase was measured at 532 nm. The amount of thiobarbituric acid-reactive substances (TBARS) was determined by absorbance, as described previously (10,11,21,22).

Microinjection of Tempol or Angiotensin II into the RVLM

To inhibit the oxidative stress in the RVLM, we microinjected tempol (1 nmol) into the RVLM of SHRSP-EX, SHRSP-C, WKY-EX, and WKY-C that were anesthetized with sodium pentobarbital, as described previously (10,11,16). One hour after the microinjection of tempol, we determined the recovery of MAP and HR to the levels of baseline, and we microinjected angiotensin II (50 pmol) into the RVLM. A catheter was inserted into the femoral artery to record MAP. A tracheal cannula was connected to a ventilator, and the rats were artificially ventilated. The rats were placed in a stereotaxic frame. A glass micropipette was filled with tempol, angiotensin II, or L-glutamate and positioned at the injection site. Before the microinjection, the RVLM was identified by monitoring the MAP after injection of a small dose of L-glutamate. The identification of the RVLM was confirmed as described previously (10,11,16).

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. Differences were considered to be statistically significant at P value <.05.

RESULTS

MAP, HR, and Urinary Norepinephrine Excretion

Mean arterial pressure, HR, and urinary norepinephrine excretion were significantly higher in SHRSP-C than in WKY-C (Figures 1A, B and 2A). However, MAP, HR, and urinary norepinephrine excretion were significantly lower in SHRSP-EX than in SHRSP-C (Figures 1A, B, and 2A). Mean arterial pressure, HR, and urinary norepinephrine excretion did not differ between WKY-EX and WKY-C (Figures 1A, B and 2A). The exercise training-induced increase in HR just after the exercise training was significantly smaller at day 28 than at day 0 in SHRSP (increase in HR: 28 ± 4 bpm vs. 42 ± 5 bpm;

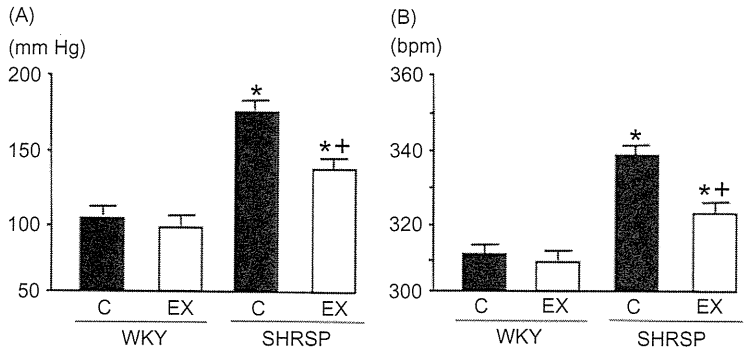

Figure 1. (**A**) Mean arterial pressure of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$ versus WKY-C and +$P < .05$ versus SHRSP-C in SHRSP-EX. (**B**) Heart rate of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$ versus WKY-C and +$P < .05$ versus SHRSP-C in SHRSP-EX.

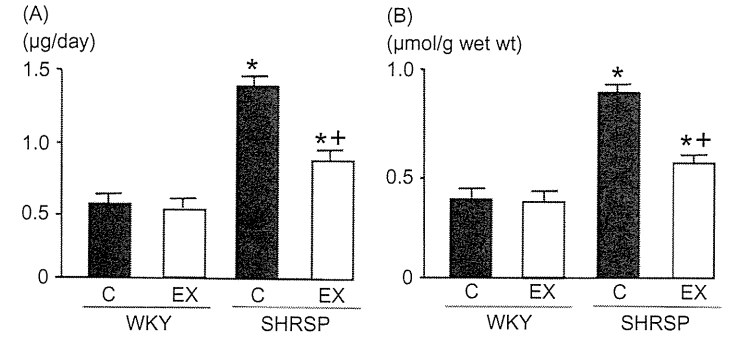

Figure 2. (**A**) Twenty-four-hour urinary norepinephrine excretions of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$ versus WKY-C and +$P < .05$ versus SHRSP-C in SHRSP-EX. (**B**) The levels of TBARS in the RVLM of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$ versus WKY-C, and +$P < .05$ versus SHRSP-C in SHRSP-EX.

increase in HR per basal HR: 9 ± 1% vs. 12 ± 1%; $n = 5$ for each, $P < .05$ for each). However, in WKY, the exercise training-induced increase in HR just after the exercise training did not differ between days 28 and 0 (increase in HR: 22 ± 3 bpm vs. 20 ± 3 bpm; increase in HR per basal HR: 7 ± 2 % vs. 6 ± 1 %; $n = 5$ for each; ns).

Conscious Baroreflex Sensitivity

Conscious baroreflex sensitivity measured by spontaneous sequence method was significantly lower in SHRSP-C than in WKY-C (10.7 ± 2.1 vs. 18.3 ± 3.0 ms/mm Hg; $n = 5$ for each, $P < .05$). However, the baroreflex sensitivity was significantly higher in SHRSP-EX than in SHRSP-C (16.0 ± 1.8 vs. 10.7 ± 2.1 ms/mm Hg; $n = 5$ for each, $P < .05$). The baroreflex sensitivity did not differ between WKY-EX and WKY-C (19.2 ± 2.0 vs. 18.3 ± 3.0 ms/mm Hg; $n = 5$ for each; ns).

TBARS Levels in the RVLM

The levels of TBARS in the RVLM were significantly higher in SHRSP-C than in WKY-C (Figure 2B) but were significantly lower in SHRSP-EX than in SHRSP-C (Figure 2B). The levels of TBARS in the RVLM did not differ between WKY-EX and WKY-C (Figure 2B)

Depressor Responses Caused by the Microinjection of Tempol into the RVLM

The depressor responses caused by the microinjection of tempol into the RVLM were significantly greater in SHRSP-C than in WKY-C and were significantly smaller in SHRSP-EX than in SHRSP-C (Figure 3A). Moreover, the degrees of the changes in MAP per basal MAP were also significantly smaller in SHRSP-EX than in SHRSP-C (−11 ± 1% vs. −15±2%; $n = 5$ for each, $P < .05$). The depressor responses caused by the microinjection of tempol into the RVLM did not differ between WKY-EX and WKY-C (Figure 3A).

Pressor Responses Caused by the Microinjection of Angiotensin II into the RVLM

The pressor responses caused by the microinjection of angiotensin II into the RVLM were significantly greater in SHRSP-C than in WKY and were significantly smaller in SHRSP-EX than in SHRSP-C (Figure 3B).

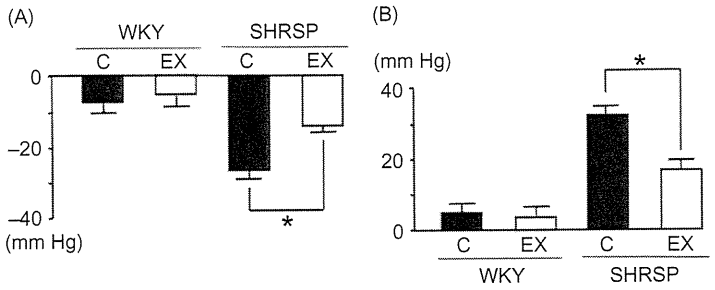

Figure 3. (**A**) Changes in mean arterial pressure due to the microinjection of tempol into the RVLM of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$. (**B**) Changes in mean arterial pressure due to the microinjection of angiotensin II into the RVLM of WKY-C, WKY-EX, SHRSP-EX, and SHRSP-C. Data are shown as mean ± SEM ($n = 5$ for each group). *$P < .05$.

Moreover, the degrees of the changes in MAP per basal MAP were also significantly smaller in SHRSP-EX than in SHRSP-C (13 ± 2% vs. 18 ± 2%; $n = 5$ for each, $P < .05$). The pressor responses caused by the microinjection of angiotensin II into the RVLM did not differ between WKY-EX and WKY-C (Figure 3B).

DISCUSSION

In this study, we have demonstrated three findings. First, exercise training caused the sympathoinhibition and improved the impaired baroreflex sensitivity in the SHRSP. Second, exercise training reduced the oxidative stress in the RVLM of SHRSP. Third, the depressor responses caused by the inhibition of oxidative stress in the RVLM locally and the pressor responses caused by the microinjection of angiotensin II into the RVLM were significantly smaller in SHRSP with exercise training than in control SHRSP. These results suggest that exercise training caused the sympathoinhibition and improved the impaired baroreflex sensitivity via antioxidant in the RVLM in part through the blocked AT_1R in the RVLM.

In the brain, oxidative stress due to the renin–angiotensin system mainly causes the sympathoexcitation (10–14,23,24). Especially, oxidative stress due to the AT_1R in the RVLM strongly causes the sympathoexcitation in SHRSP (10–14). To inhibit the activity of the SNS in hypertension, AT_1R-induced oxidative stress in the RVLM is considered to be a novel target. In fact, the infusion of small dose of AT_1R blocker into the brain of SHRSP causes the significant inhibition of the activity of the SNS (11). However, the oral administration of AT_1R blocker could not inhibit the AT_1R in the RVLM sufficiently. In this study, exercise training reduces the oxidative stress in the RVLM, which causes the sympathoinhibition in SHRSP. Although we did not determine the possibility that the reduction of oxidative stress blocks AT_1R in the RVLM, the mechanisms by which the exercise training reduces the oxidative stress in the RVLM might be due to the blocked AT_1R in the RVLM, because the depressor responses caused by the inhibition of oxidative stress in the RVLM locally and the pressor responses caused by the microinjection of angiotensin II into the RVLM were significantly smaller in SHRSP with exercise training than in control SHRSP. These results are compatible with the several previous studies in chronic heart failure and coronary artery disease (3,4,25–27). Furthermore, several previous studies suggest that the exercise training activates the antioxidant enzymes in various organs (28–33). In this study, we did not check the antioxidant enzymes in the RVLM. However, several previous studies indicate that the exercise training activates the antioxidant enzymes in the brain (30–33). Taken together, we consider that the exercise training causes the beneficial sympathoinhibition via the RVLM in part through the blocked AT_1R in the RVLM.

Baroreflex sensitivity is the important indicator of the long-term mortality of cardiovascular diseases (8). We have demonstrated that the increase in nitric oxide or the reduction of the oxidative stress in the RVLM improves the impaired baroreflex sensitivity of SHRSP (22). In this study, exercise training improves the impaired baroreflex sensitivity in SHRSP, and these results are compatible with previous studies (5,6,34). The mechanisms by which the exercise training improves the impaired baroreflex sensitivity in SHRSP are considered because of the reduction of oxidative stress through the blocked AT_1R in the RVLM.

The mechanisms by which the exercise training blocked AT_1R in the RVLM have not been fully determined in this study. 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 (4,6,25,26). One of the important activating factors of brain renin–angiotensin system is the inflammatory cascade (35), and exercise training is known to lower the inflammatory substances in the brain of rats (36). We consider that the exercise training-induced anti-inflammation might in part cause the inhibition of brain renin–angiotensin system. Further studies are necessary.

Interestingly, in this study, the effects of the exercise training were determined in SHRSP, not in WKY. These results are compatible with our previous studies (10,11). In our previous studies, the blocked AT_1R and the reduction of the oxidative stress in the RVLM inhibited the activity of the SNS only in SHRSP, not in WKY (10, 11). From these results, we consider that the exercise training affects the AT_1R in the RVLM. However, we could not deny that the longer exercise training or other protocols of the exercise training have the possibility to block AT_1R in the RVLM of WKY. To determine this question, we must further assess the strength and benefits of the exercise training and examine the relationship between the AT_1R–oxidative stress–the activity of the SNS axis and the benefits of the exercise training.

There are some limitations in this study. First, we examined the oxidative stress and the effects of AT_1R only in the RVLM. There are some important nuclei and areas involved in the cardiovascular control, such as nucleus tractus solitarii, hypothalamus, and so on. The reduction of oxidative stress due to exercise training may not be the unique phenomenon in the RVLM. From this reason, in this study, we examined only TBARS methods for the RVLM tissues obtained by the punch-out method and not the histological examination. However, in the regulation of activity of the SNS, RVLM is the most important site. Furthermore, in the RVLM, oxidative stress is the most powerful and important sympatho-exciting factor. In this study, we focused on the oxidative stress in the RVLM and performed the exercise training in only one protocol. Actually, at days 0 and 28, we checked the exercise-induced increase in HR just after the exercise training and determined that the increase in HR just after the exercise training was significantly smaller at day 28 than at day 0 in SHRSP and not in WKY. However, 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. We have to perform the further research.

CONCLUSIONS

Our results suggest that, in hypertensive rats with sympathoexcitation, exercise training causes the sympathoinhibition and improves the impaired baroreflex sensitivity via reduction of oxidative stress in the RVLM through the blocked AT_1R. We consider that exercise training should be included in the treatment for hypertension, because the sympathoinhibition and the improvement of the impaired baroreflex sensitivity have strong benefit on the long-term mortality in cardiovascular diseases.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the papers.

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ORIGINAL ARTICLE

Sympathoinhibition caused by orally administered telmisartan through inhibition of the AT_1 receptor in the rostral ventrolateral medulla of hypertensive rats

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In patients and animals with hypertension, sympathetic nervous system (SNS) activation is present. We have demonstrated that angiotensin II type 1 receptor (AT_1R)-induced oxidative stress in the rostral ventrolateral medulla (RVLM), a vasomotor center in the brainstem, causes SNS activation in hypertensive rats. The aim of the present study was to determine whether orally administered AT_1R blockers (ARBs) inhibit SNS activation through an anti-oxidant effect via inhibition of AT_1R in the RVLM of hypertensive rats and, if so, whether the benefits are class effects of ARBs. Stroke-prone spontaneously hypertensive rats (SHRSPs), a hypertensive model with sympathoexcitation, were divided into four groups: SHRSPs treated with telmisartan (TLM), candesartan (CAN), or hydralazine (HYD) and a vehicle group (VEH). Although systolic blood pressure was reduced in the TLM, CAN and HYD groups to the same level, heart rate, SNS activation and oxidative stress in the RVLM were significantly lower in the TLM group only. The pressor effect caused by the microinjection of angiotensin II into the RVLM and the depressor effect caused by the microinjection of tempol, a superoxide dismutase mimetic, into the RVLM were both significantly smaller in TLM, but not in CAN or HYD. These results suggest that orally administered TLM inhibits SNS activation through an anti-oxidant effect via inhibition of AT_1R in the RVLM of SHRSPs; these results are also independent of depressor effects and are not class effects of ARBs.
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Keywords: angiotensin II; brain; oxidative stress; sympathetic nervous system

INTRODUCTION

Sympathetic nervous system (SNS) activation is a main cause of the development and progression of hypertension.[1–4] SNS activation is mainly regulated by the brain,[5–7] and we have demonstrated in rat models with hypertension or heart failure that direct interventions to the brain have beneficial effects because of sympathoinhibition.[8–14] Particularly in the brain, SNS activation is mainly regulated by the rostral ventrolateral medulla (RVLM) in the brainstem, and the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone.[5,6] We have demonstrated that oxidative stress in the RVLM produced by the angiotensin II type 1 receptor (AT_1R) causes SNS activation.[11,14–17] Upregulation of the central AT_1R is important in the pathophysiology of hypertension.[6,7] Microinjection of AT_1R blockers (ARBs) into the RVLM or intracerebroventricular infusion of ARBs inhibits SNS activation in hypertensive rats.[15,18–20] However, AT_1R or oxidative stress in the RVLM have not been targets for the treatment of hypertensive patients because we do not have suitable oral agents to inhibit AT_1R or oxidative stress in the RVLM of hypertensive patients.

Interestingly, previous animal studies have suggested that peripherally administered ARBs inhibit the central actions of angiotensin II in the brain.[16,21–28] We demonstrated that orally administered ARBs reduced oxidative stress in the brains of hypertensive rats[16,27] and that orally administered telmisartan (TLM) inhibits SNS activation in hypertensive rats.[16] These results suggest that orally administered ARBs have the potential to inhibit SNS activation through reduction of oxidative stress via inhibition of AT_1R in the RVLM. In a previous clinical study, TLM, an ARB, is effective in reducing short-term ambulatory blood pressure variability and SNS activation in hypertensive patients with diabetic nephropathy.[29] However, in other clinical studies, ARBs do not have the same beneficial effects on the autonomic nervous system.[30,31] Moreover, it has not been determined whether the sympathoinhibition caused by orally administered ARBs is a class effect of ARBs.[32] Gohlke *et al.*[26] demonstrated that, following peripheral administration, TLM is able to penetrate the blood-brain barrier in a dose- and time-dependent manner to inhibit centrally mediated effects of angiotensin II and that

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the effects of ARBs might differ depending on the pharmacokinetics and properties of each drug. We hypothesized that orally administered TLM penetrates the blood-brain barrier to a greater extent than any other ARB.

In hypertensive patients, ARBs are preferable for hypertensive patients.[33] New mechanistic insight into antihypertensive treatment could be provided if systemic treatment with ARBs was shown to inhibit SNS activation through inhibition of AT_1R in the brain of hypertensive patients. The aim of the present study is to investigate whether orally administered TLM inhibits SNS activation through the reduction of oxidative stress via inhibition of AT_1R in the RVLM of hypertensive rats. If so, we also aim to determine whether the results are independent of its depressor effects and if these effects are class effects of ARBs. To this end, we divided stroke-prone spontaneously hypertensive rats (SHRSPs) with severe sympathetic hyperactivity, used as the hypertensive model into TLM-, CAN-, hydralazine (HYD)- or vehicle (VEH)-treatment groups. TLM and CAN are widely used ARBs, and both ARBs have powerful blood pressure-lowering effects.[32] We determined SNS activation by 24-h urinary norepinephrine excretion and determined the oxidative stress in the RVLM using the thiobarbituric acid-reactive substance (TBARS) method. We also determined the activity of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, which is a key AT_1R-activated component in the creation of oxidative stress moieties in the RVLM. Furthermore, we also performed microinjections of angiotensin II, superoxide dismutase mimetic (tempol) or NAD(P)H oxidase inhibitor (apocynin) into the RVLM of each group.

METHODS

Animals

This study was reviewed and approved by the committee on ethics for Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments by the Kyushu University. Male SHRSPs and age-matched Wistar–Kyoto (WKY) rats (12 to 14 weeks old) weighing 350 to 425 g were fed standard feed during this protocol (SLC Japan, Hamamatsu, Japan). They were housed individually in a temperature-controlled room (22 to 23 °C) with a 12-h/12-h light-dark cycle (lights on at 0700 hours). We divided SHRSPs and the WKY rats into four groups: a TLM-treatment group (TLM rats), a CAN-treatment group (CAN rats), a HYD-treatment group (HYD rats) and a VEH-treatment group (VEH rats).

Oral administration of TLM, CAN or HYD

SHRSPs and WKY rats were treated for 4 weeks. TLM rats received TLM ($2\,mg\,kg^{-1}$ per day, dissolved in 0.5% methylcellulose) and were given this oral gavage once daily (Sigma-Aldrich, St Louis, MO, USA). CAN rats received CAN ($2\,mg\,kg^{-1}$ per day, dissolved in 0.5% methylcellulose) and were given this oral gavage once daily (Sigma-Aldrich). HYD rats received HYD and were given this oral gavage once daily ($5\,mg\,kg^{-1}$ per day, dissolved in drinking water) (Sigma-Aldrich). VEH rats received 0.5% methylcellulose by oral gavage once daily.

Measurement of blood pressure, heart rate and SNS activation

Systolic blood pressure and heart rate were measured once weekly using the tail-cuff method (BP-98 A; Softron, Tokyo, Japan). At 4 weeks, we calculated urinary norepinephrine excretion for 24 h as an indicator of SNS activation as previously described.[9–11]

Microinjection of angiotensin II, tempol or apocynin into the RVLM

At the end of the study, we microinjected angiotensin II bilaterally into the RVLM of all rats ($n=5$ per group). To inhibit the local oxidative stress in the RVLM, we microinjected tempol (100 pmol) bilaterally into the RVLM of all rats ($n=5$ rats per group). To inhibit the NAD(P)H oxidase locally in the RVLM, we microinjected apocynin (1 nmol) bilaterally into the RVLM of all groups ($n=5$ per group). The doses of tempol or apocynin and the procedures of the microinjection are reported in our previous studies.[11,15]

Measurement of TBARS in the RVLM

To obtain RVLM tissue, the rats were deeply anesthetized with sodium pentobarbital ($100\,mg\,kg^{-1}$ IP) and transcardially perfused with PBS ($150\,mol\,l^{-1}$ NaCl, $3\,mmol\,l^{-1}$ KCl and $5\,nmol\,l^{-1}$ phosphate; pH 7.4, 4 °C). The brains were quickly removed, and 1-mm thick sections were obtained with a cryostat at -7 ± 1 °C. The RVLM was defined according to a rat brain atlas as described previously[9,11] and obtained using a punch-out technique. The RVLM tissues were homogenized in 1.15% KCl (pH 7.4), 0.4% sodium dodecyl sulfate and 7.5% acetic acid adjusted; the pH was adjusted to 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5 °C for 60 min followed by heating to 100 °C for 60 min. After cooling, the mixture was extracted with distilled water and *n*-butanolpyridine (15:1) and centrifuged at 1600 *g* for 10 min. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by absorbance, as described previously.[11,15]

Measurement of NAD(P)H oxidase activity

At the end of the study, NAD(P)H-dependent superoxide production in the RVLM was measured using a lucigenin luminescence assay as described previously.[14,15] Quantification of NAD(P)H oxidase activity was expressed relative to that in WKY rats treated with VEH; this level was assigned a value of 1.

Statistical analysis

All values are expressed as the means ± s.e.m. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. Analysis of variance 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

Blood pressure, heart rate and urinary norepinephrine excretion

Systolic blood pressure of SHRSPs was significantly lower in each TLM, CAN and HYD rats when compared with VEH rats after 4 weeks of treatment (Figure 1a) and there was no difference among TLM, CAN and HYD rats (Figure 1a). However, heart rate in SHRSPs was significantly lower in TLM rats compared with CAN and HYD rats (Figure 1b). In WKY rats, systolic blood pressure and heart rate were the same among the groups (Figures 1c and d). Urinary norepinephrine excretion was significantly lower in TLM rats versus CAN, HYD and VEH rats in SHRSPs. However, urinary norepinephrine excretion was the same in CAN and VEH rats (Figure 2a). In WKY rats, urinary norepinephrine excretion was the same among all groups (Figure 2b).

TBARS levels and NAD (P) H oxidase activity in the RVLM

In SHRSPs, TBARS levels (Figure 3a) and NAD(P)H oxidase activity (Figure 3b) in the RVLM rats were significantly lower in TLM rats versus CAN, HYD and VEH rats, but there was no difference between CAN and VEH rats. In WKY rats, TBARS levels (Figure 3c) and NAD(P)H oxidase activity (Figure 3d) in the RVLM were the same between all groups.

Effects of microinjection of angiotensin II into the RVLM

In SHRSPs, the pressor effects caused by the microinjection of angiotensin II into the RVLM were significantly smaller in TLM rats than in CAN, HYD or VEH rats (Figure 4a), and they were the same in CAN and VEH rats (Figure 4a). In WKY rats, the pressor effects

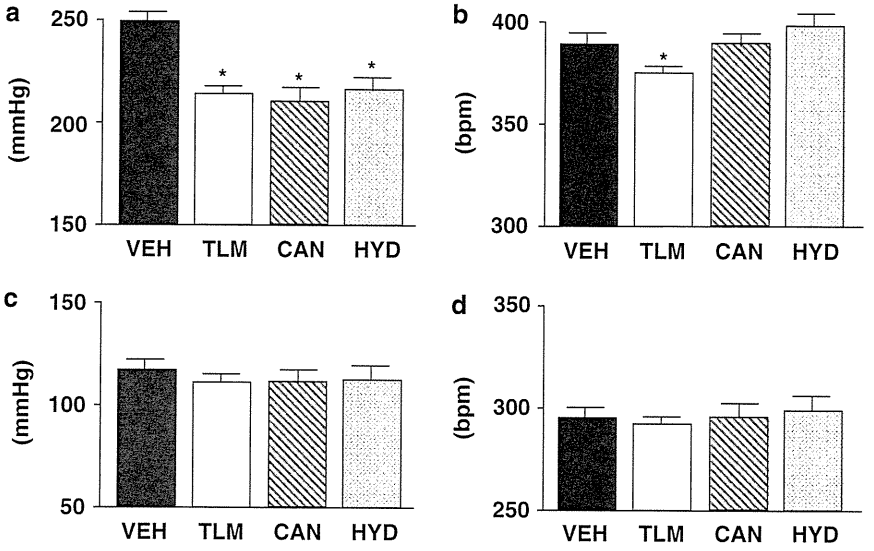

Figure 1 (**a**) Systolic blood pressure of each group of stroke-prone spontaneously hypertensive rats (SHRSPs) ($n=5$ each), (**b**) heart rate of each group ($n=5$ each) of SHRSPs, (**c**) systolic blood pressure of each group of Wistar–Kyoto (WKY) rats ($n=5$ each) and (**d**) heart rate of each group of WKY rats ($n=5$ each). *$P<0.05$ *vs.* VEH in each strain. CAN, candesartan; HYD, hydralazine; TLM, telmisartan; VEH, vehicle.

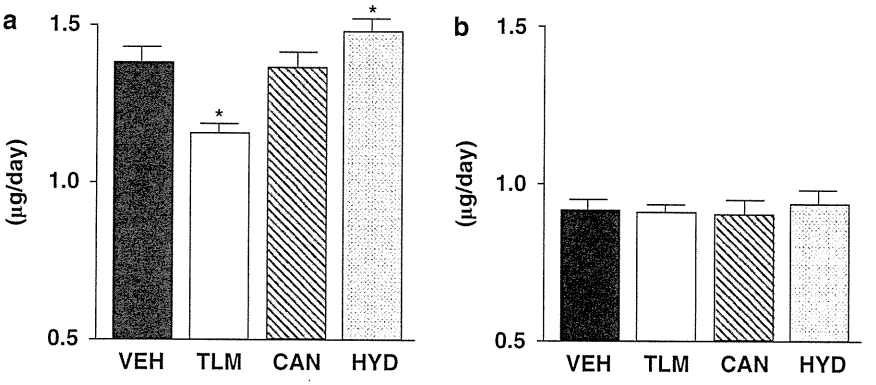

Figure 2. (**a**) 24-h urinary norepinephrine excretion of each group of stroke-prone spontaneously hypertensive rats (SHRSPs) ($n=5$ each) and (**b**) 24-h urinary norepinephrine excretion of each group ($n=5$ each) of Wistar–Kyoto (WKY) rats. *$P<0.05$ *vs.* VEH in each strain. CAN, candesartan; HYD, hydralazine; TLM, telmisartan; VEH, vehicle.

caused by the microinjection of angiotensin II into the RVLM were the same among groups (Figure 4b).

Effects of microinjection of tempol or apocynin into the RVLM

In SHRSPs, the depressor effects caused by the microinjection of tempol (Figure 5a) or apocynin (Figure 5b) into the RVLM were significantly smaller in TLM rats than in CAN, HYD or VEH rats, and there was no difference between CAN and VEH rats. In WKY rats, the depressor effects caused by the microinjection of tempol (Figure 5c) or apocynin (Figure 5d) into the RVLM were the same in all groups.

DISCUSSION

In the present study, we demonstrated two major findings. First, orally administered TLM inhibits SNS activation through the reduction of oxidative stress via inhibition of AT_1R in the RVLM of SHRSPs. Second, the sympathoinhibition caused by orally administered TLM in SHRSPs is independent of its depressor effect and is not a class effect of ARBs. These results suggest that orally administered TLM might have the potential to be a novel treatment for hypertension via sympathoinhibition.

SNS activation is determined mainly by neural activity in the RVLM in hypertensive patients.[5,6] We demonstrated that oxidative stress in the brain causes hypertension through sympathoexcitation.[11,15–17,34,35] We also demonstrated that AT_1R-inducced oxidative stress in the RVLM causes SNS activation in hypertensive rats.[11,14,15] Direct inhibition of the AT_1R in the RVLM inhibits SNS activation in hypertensive rats.[15,18–20] Peripherally administered ARBs also inhibit the central actions of angiotensin II in the brain.[21–28] Furthermore, we demonstrated that orally administered TLM inhibits SNS activation by reducing oxidative stress in the brains of hypertensive rats.[16] However, it has not been determined whether the sympathoinhibition caused by orally administered ARBs is a class effect of ARBs.[32] The effects of ARBs might differ depending on the pharmacokinetics and properties of each drug.[26] In the present study, orally administered TLM inhibits SNS activation and the angiotensin II-AT_1R-NAD(P)H oxidase-oxidative stress pathway in the RVLM of SHRSPs. However, orally administered CAN or HYD does not have

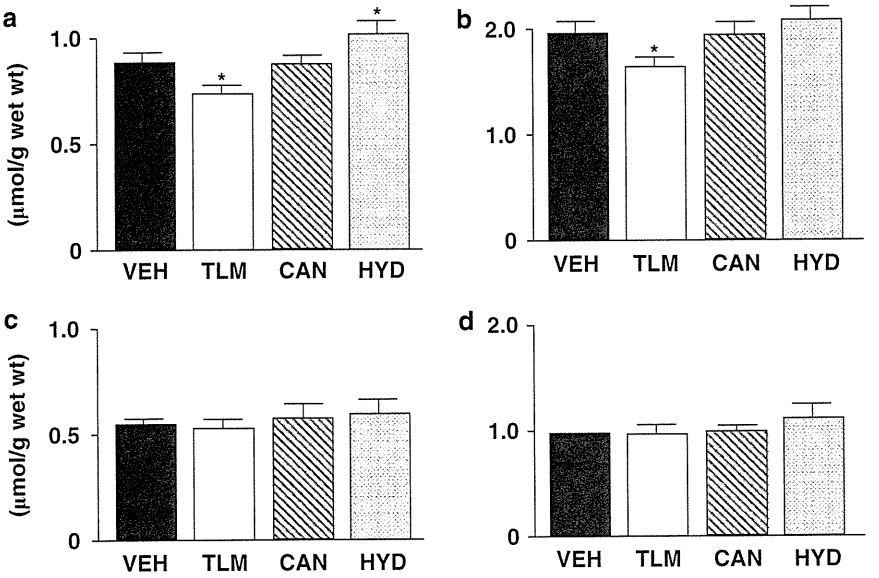

Figure 3 (**a**) Thiobarbituric acid-reactive substance (TBARS) levels in the rostral ventrolateral medulla (RVLM) of each group of stroke-prone spontaneously hypertensive rats (SHRSPs) ($n=5$ each), (**b**) nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase activity in the RVLM of each group ($n=5$ each) of SHRSPs, (**c**) TBARS levels in the RVLM of each group of Wistar–Kyoto (WKY) rats ($n=5$ each) and (**d**) NAD(P)H oxidase activity in the RVLM of each group of WKY rats ($n=5$ each). *$P<0.05$ *vs.* VEH in each strain. CAN, candesartan; HYD, hydralazine; TLM, telmisartan; VEH, vehicle.

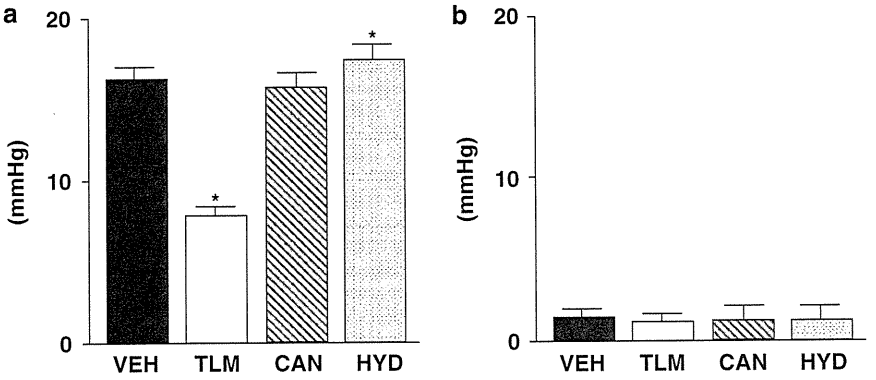

Figure 4 (**a**) Changes in mean arterial pressure caused by the microinjection of angiotensin II into the rostral ventrolateral medulla (RVLM) of each group of stroke-prone spontaneously hypertensive rats (SHRSPs) ($n=5$ each) and (**b**) changes in mean arterial pressure caused by the microinjection of angiotensin II into the RVLM of each group in Wistar–Kyoto (WKY) rats ($n=5$ each). *$P<0.05$ *vs.* VEH in each strain. CAN, candesartan; HYD, hydralazine; TLM, telmisartan; VEH, vehicle.

these results in SHRSPs despite having similar depressor effects. These results indicate that orally administered TLM might inhibit the AT_1R in the RVLM. These effects are independent of its depressor effects and are not class effects of ARBs.

The mechanisms by which sympathoinhibition, through the reduction of oxidative stress via inhibition of AT_1R in the RVLM, is different between orally administered TLM and CAN should be discussed. In the present study, oxidative stress and NAD(P)H oxidase activity in the RVLM are reduced in the TLM-treated SHRSPs but not in the CAN-treated SHRSPs. The pressor effect caused by the microinjection of angiotensin II into the RVLM and the depressor effect caused by the microinjection of tempol or apocynin into the RVLM are significantly smaller in TLM-treated than in CAN-treated SHRSPs. These results suggest that the pathway of AT_1R-NAD(P)H oxidase-oxidative stress in the RVLM is blocked by orally administered TLM but not by CAN. A previous study demonstrated that, following peripheral administration, TLM penetrates the blood-brain barrier in a dose- and time-dependent manner to inhibit centrally mediated effects of angiotensin II because of the high lipophilicity of TLM.[26] Previously, we also demonstrated that orally administered TLM inhibits SNS activation through the inhibition of AT_1R in the brain of hypertensive rats.[16] We posit that orally administered TLM ($2\,mg\,kg^{-1}$ per day) can penetrate the blood-brain barrier and reach the RVLM of SHRSPs, whereas CAN ($2\,mg\,kg^{-1}$ per day) cannot. Furthermore, previous studies have demonstrated differences between TLM and CAN.[36–39] Both TLM and CAN show clear efficacies.[36] However, the efficacy of CAN is linked to the presence of a carboxyl group at its imidazole-derived moiety, whereas TLM is efficacious despite the absence of a carboxyl group.[36] Moreover, in terms of inverse agonist activity, previous studies have demonstrated that CAN can stabilize AT_1R in an inactive state, therefore acting as an 'inverse agonist,' in the absence of angiotensin II, whereas TLM does not have

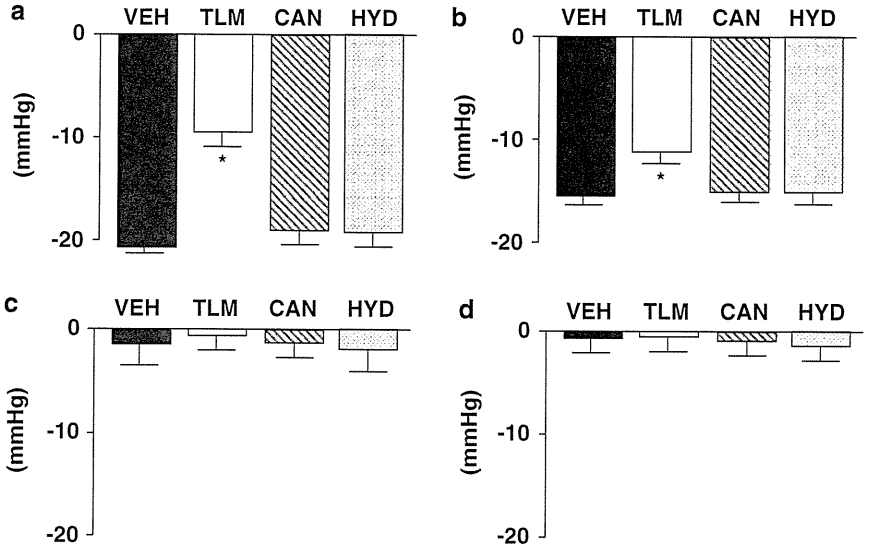

Figure 5 (**a**) Changes in mean arterial pressure caused by the microinjection of tempol into the rostral ventrolateral medulla (RVLM) of each group of stroke-prone spontaneously hypertensive rats (SHRSPs) ($n=5$ each), (**b**) changes in mean arterial pressure caused by the microinjection of apocynin into the RVLM of each group of SHRSPs ($n=5$ each), (**c**) changes in mean arterial pressure caused by the microinjection of tempol into the RVLM of each group in Wistar–Kyoto (WKY) rats ($n=5$ each) and (**d**) changes in mean arterial pressure caused by the microinjection of apocynin into the RVLM of each group in WKY rats ($n=5$ each). *$P<0.05$ *vs.* VEH in each strain. CAN, candesartan; HYD, hydralazine; TLM, telmisartan; VEH, vehicle.

such an effect.[37–39] In terms of the agonist activity of peroxisome proliferator-activated receptor (PPAR)-γ, a previous study suggested that orally administered rosiglitazone, a PPAR-γ agonist, promotes a central antihypertensive effect via upregulation of PPAR-γ and alleviation of oxidative stress in the RVLM of SHR.[40] Although both TLM and CAN function as partial agonists of PPAR-γ, only TLM achieves this effect at therapeutic doses.[41] Further studies are necessary to investigate the differences in central effects elicited by the various ARBs in terms of efficacy, inverse agonist activity and PPAR-γ agonist activity.

Several studies have suggested that orally administered CAN causes sympathoinhibition and attenuates the central effects of angiotensin II in the brain.[22,24,25,28] Direct inhibition of AT_1R in the RVLM or other areas of the brain inhibits SNS activation,[15,18–20] and superfusion with CAN decreases the electrophysiological activity of RVLM neurons examined using the patch-clamp technique.[42] In previous studies demonstrating sympathoinhibition caused by orally administered CAN in hypertensive rats, the doses of CAN were greater (4,[ref. 22] 5,[ref. 24] or 10,[ref. 25] mg kg^{-1} per day) than those in the present study (2 mg kg^{-1} per day). However, Sakata, *et al.*[28] demonstrated that 1 mg kg^{-1} per day of CAN for 2 weeks causes sympathoinhibition in SHR. Although blood pressure of SHRs in their study is lower than that of the SHRSPs in the present study, and the heart rate of the SHRs were similar to that of the WKY rats in their study, these previous studies suggested that the difference in sympathoinhibition between TLM and CAN in the present study may not be due to the dose of CAN. We hypothesize that orally administered CAN (2 mg kg^{-1} per day) was not sufficient to penetrate the blood-brain barrier of SHRSPs.

Interestingly, in the present study, sympathoinhibition through reduction of oxidative stress via inhibition of AT_1R in the RVLM was not obtained in WKY rats. These results are compatible with our previous studies.[11,15] In those studies, overexpression of superoxide dismutase in the RVLM or direct infusion of ARBs into the brain did not reduce oxidative stress and did not inhibit SNS activation in WKY rats.[11,15] From these results, two explanations are possible. First, orally administered TLM (2 mg kg^{-1} per day) could not penetrate the blood-brain barrier, which is not as damaged in WKY rats. In hypertensive rats, the blood-brain barrier is damaged;[43,44] thus, orally administered TLM can easily penetrate the blood-brain barrier of SHRSPs. This possibility would also support our results that orally administered ARB-induced sympathoinhibition through the reduction of oxidative stress via inhibition of the AT_1R in the RVLM is dependent on the penetration of the blood-brain barrier. Second, the role of AT_1R-induced oxidative stress on the regulation of SNS activation may differ between SHRSPs and WKY rats.

The increase in the number of hypertensive patients is a health problem because hypertension is considered to be a risk factor for cardiovascular diseases.[33] ARBs are widely used in hypertensive patients because of their powerful blood pressure-lowering effects and organ-protective effects.[33] However, one of the important treatment targets for hypertension is inadequate SNS activation, and it has not been determined whether ARBs have beneficial effects on SNS activation in hypertension. In the present study, we demonstrated that orally administered TLM, but not CAN, inhibits SNS activation through the reduction of oxidative stress via inhibition of AT_1R in the RVLM of SHRSPs. These results are compatible with a previous clinical study, which indicated that TLM (40 mg per day) causes sympathoinhibition to a greater extent than losartan (50 mg per day) in hypertensive patients with diabetic nephropathy.[29] We also previously demonstrated that orally administered atorvastatin, azelnidipine or amlodipine also causes sympathoinhibition through the reduction of oxidative stress in the RVLM of SHRSPs.[45–47] However, the results of the present study could not directly elucidate the clinical benefits of TLM in hypertensive patients because the dose of TLM in the present study is not a clinical dose, and there are no clinical trials demonstrating the same reduction in heart rate obtained in the present study. Furthermore, damage to the blood-brain barrier may be much more significant in SHRSPs than in hypertensive human patients. To determine whether the benefits of

TLM observed in the present animal study could be obtained in humans with hypertension, further clinical studies are necessary to examine brain oxidative stress, concentrations of ARBs in the brain and the permeability of the blood-brain barrier in hypertensive patients treated with clinical doses of ARBs.

There are several limitations to the present study. First, we examined the AT_1R-NAD(P)H oxidase-oxidative stress pathway only in the RVLM. In addition to the RVLM, some important foci are involved in cardiovascular regulation, such as the nucleus tractus solitarii and the hypothalamus.[6] AT_1R is rich in the specific brain nuclei that regulate SNS activation, such as the anteroventral third ventricle, paraventricular nucleus of the hypothalamus, nucleus tractus solitarii and RVLM.[48–50] Moreover, a high density of AT_1R is present in the brain regions involved in the regulation of SNS activation, such as the circumventricular organs outside of the blood-brain barrier, where peripherally administered ARBs are able to effect change without consideration of the blood-brain barrier, as well as inside of the blood-brain barrier.[50] The reduction of oxidative stress via inhibition of AT_1R caused by orally administered TLM may not be a phenomenon unique to the RVLM. However, in the regulation of the SNS activation, the RVLM is the most important site.[5,6] Furthermore, in the RVLM, oxidative stress is considered to be the most important sympathoexciting factor.[11,15–17] For these reasons, the RVLM is the focus of the present study. Second, we did not examine the dose-dependency of sympathoinhibition caused by orally administered TLM or CAN and the long-term effect of orally administered TLM or CAN in the present study. Our previous study and the present study suggest that the degrees of sympathoinhibition and the depressor effects caused by orally administered TLM are significantly smaller in the present study ($2\,mg\,kg^{-1}$ per day) than in our previous study (5 or $10\,mg\,kg^{-1}$per day).[16] Third, we could not directly demonstrate that TLM penetrates the blood-brain barrier to reach the RVLM; in future studies we will measure the concentration of TLM in the brain tissue.

In conclusion, in the present study, orally administered TLM inhibits SNS activation through the reduction of oxidative stress via inhibition of AT_1R in the RVLM of SHRSPs, and the effects are independent of its depressor effect and are not class effects of ARBs. These results suggest that orally administered TLM might have the potential to be a novel treatment for hypertension resulting from sympathoinhibition through the reduction of oxidative stress via inhibition of AT_1R in the RVLM.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Resveratrol attenuates angiotensin II-induced senescence of vascular smooth muscle cells

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ABSTRACT

Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol abundant in red wine, is known to extend the life span of diverse species. On the contrary, it was reported that angiotensin (Ang) II enhances senescence of vascular smooth muscle cells (VSMCs). We, therefore, examined whether resveratrol attenuates Ang II-induced senescence of VSMC. Senescence-associated β-galactosidase (SA β-gal) assay showed that Ang II induced senescence of VSMC. The Ang II-induced senescence was inhibited by losartan, an Ang II type 1 receptor (AT1R) antagonist but not by PD123319, Ang II type 2 receptor antagonist, indicating that AT1R is responsible for the induction of senescence. Resveratrol suppressed Ang II-induced senescence of VSMC in a dose-dependent manner. In addition, resveratrol suppressed Ang II-induced induction of p53 and its downstream target gene p21, both of which play an important role in the induction of senescence. Resveratrol suppressed senescence of VSMC possibly through inhibition of AT1R-dependent induction of p53/p21. Suppression of p53 induction may be involved in the longevity by resveratrol.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is one of the polyphenols contained in red wine and considered to be responsible for the French paradox, a phenomenon that the incidence of ischemic heart diseases is relatively low in France in spite of Western style diet that is rich in saturated fat [1]. Resveratrol has various beneficial effects on cardiovascular diseases [2], cerebral ischemic injuries [3] and cancer [4]. Previous studies have demonstrated that resveratrol extends the life span of diverse species through activation of silent information regulator 2 (Sir2) [5] even on a high-calorie diet [6]. Mammals have seven Sir2 homologs (sirtuins, SIRT1 to 7). It is generally believed that longevity is mainly promoted by SIRT1 and resveratrol affects various aspects of cell function through SIRT1 in mammalian cells. In addition to activation of SIRT1, resveratrol has multifaceted effects such as anti-oxidative, anti-inflammatory and anti-apoptotic effects.

Angiotensin (Ang) II is a major effecter molecule of the renin angiotensin system. The effect of Ang II is mediated by Ang II receptors. So far, 2 isoforms of Ang II receptor designated type 1 receptor (AT1R) [7] and type 2 receptor (AT2R) [8] have been identified. It is generally accepted that most of the traditional cardiovascular effects of Ang II such as vasoconstriction, regulation of fluid and electrolyte balance and drinking behavior are ascribed to AT1R. In addition, AT1R is involved in the progression of cardiovascular diseases including atherosclerosis, hypertension, cardiac hypertrophy and heart failure [9]. AT2R seems to antagonize most but not all of the effect of AT1R. A recent report showed that Ang II accelerates senescence of vascular smooth muscle cells (VSMC) through up-regulation of p53, a multifunctional transcription factor [10]. In contrast, fonsartan, an AT1R antagonist reportedly prolonged the lifespan of hypertensive rats [11]. It has been reported that cardiovascular risk factors such as hypertension, diabetes and aging are associated with shortening of telomere, which is considered to induce cellular senescence [12]. It is also suggested that VSMC senescence is involved in the progression of atherosclerosis.

Although these studies suggest that resveratrol and Ang II antagonize in terms of vascular senescence, the direct evidence whether resveratrol affects Ang II-accelerated VSMC senescence is lacking.

We examined whether resveratrol attenuates Ang II-induced acceleration of senescence in VSMCs.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL, Invitrogen Co. (Carlsbad, CA). Fetal bovine serum

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(FBS) was from JRH Biosciences Inc (Lenexa, KS). Resveratrol, bovine serum albumin (BSA) and PD123319 were purchased from Sigma Chemical Co (St. Louis, MO). Ang II was purchased from PEPTIDE Institute Inc. (Osaka, Japan). Anti-p53, anti-acetyl p53 (lysine 379) and anti-p21 antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). Other chemical reagents were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan) unless mentioned specifically. Losartan was a generous gift from MSD Co.

2.2. Cell culture

VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Cells between passages 8 and 12 were used. VSMCs were prepared in 10 cm plates, grown to sub-confluence, and then cultured in DMEM containing 0.1% BSA for 2 days. Ang II (100 nmol/L) with or without resveratrol were administered to VSMC for 1 day and 7 days by changing the medium everyday to new medium containing freshly prepared Ang II and resveratrol.

2.3. Preparation of nuclear extracts and Western blot analysis

Cells were scraped off, washed in ice-cold PBS followed by ice-cold hypotonic buffer (buffer A: 10 mmol/L HEPES, pH7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L PMSF, 0.5 mmol/L DTT), and then lysed for 10 minutes on ice in the buffer A containing 0.1% Nonident P-40. The lysates were centrifuged for 10 minutes at 10,000g. The pelleted nuclei were suspended in lysis buffer (20 mmol/L HEPES, pH7.9, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L PMSF, 0.5 mmol/L DTT), incubated for 15 minutes at 4 °C, and centrifuged for 10 minutes at 10,000g. The supernatant was used as nuclear extracts.

Nuclear extracts were electrophoresed in 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The blots were blocked with TBS-T (20 mmol/L Tris–HCl, pH 7.6, 137 mmol/L NaCl, 0.1% Tween 20) containing 10% non-fat dry milk at room temperature for 1 hour. p53 and p21 were detected by ECL chemiluminescence (Amersham Biosciences) according to the manufacturer's instructions. The specific band was scanned with an imaging analyzer and H3 histone was used as a loading control.

2.4. Senescence-associated β-galactosidase (SA β-gal) assay

Cells were washed with PBS (pH 7.2–7.4) and fixed with 0.5% glutaraldehyde in PBS for 5 minutes at room temperature. Then, cells were washed twice with PBS and incubated at 37 °C (no CO_2) with fresh senescence-associated (SA)-β-galactosidase staining solution [1 mg/mL 5-bromo-4-chrolo-3-indolyl β-D-galactopyranoside (X-Gal), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 40 mmol/L sodium phosphate (12.0% Na_2HPO_4 and 88.0% NaH_2PO_4 pH 6.0), 150 mmol/L NaCl, and 2 mmol/L $MgCl_2$] for 16 hours. SA β-gal-positive cells were detected and counted by light microscopy. Cells were permealized with 0.2% Triton X-100 in PBS and DNA was visualized by staining with DAPI (1 μg/mL). DAPI positive cells were

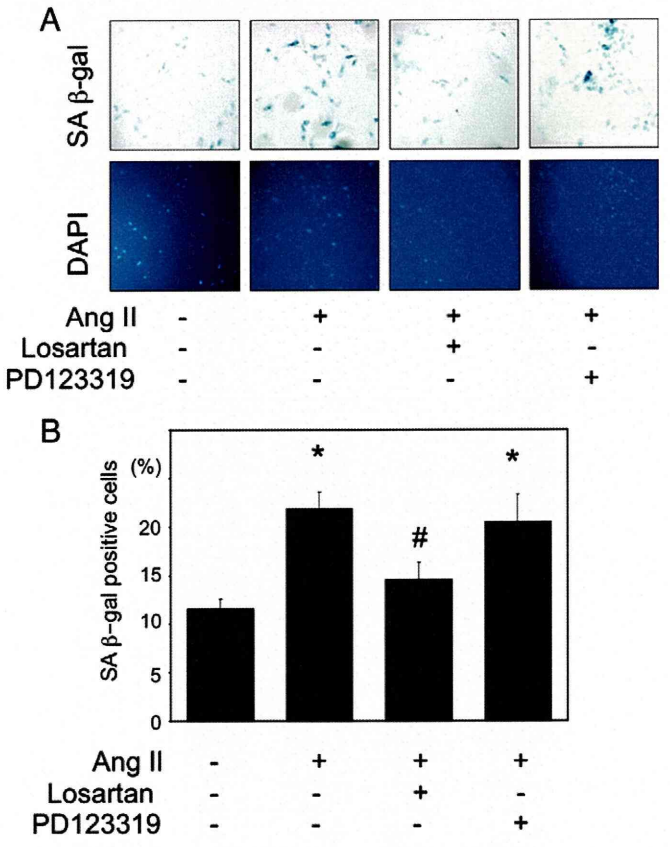

Fig. 1. Ang II induced senescence of VSMC through AT1R.VSMCs were cultured with Ang II (100 nM) for 7 days in the absence or presence of losartan (10 μM), an AT1R antagonist, or PD123319 (10 μM), an AT2R antagonist. Then the cells were subjected to SA β-gal assay. The number of SA β-gal positive cells and DAPI (nucleus) in 5 randomly selected high power field was counted. The bar graph indicates the ratio of the number of SA β-gal positive cells to the number of DAPI. $N=4$, $^{*}P<0.05$ vs. Ang II (−), $^{\#}P<0.05$ vs. Ang II (+).

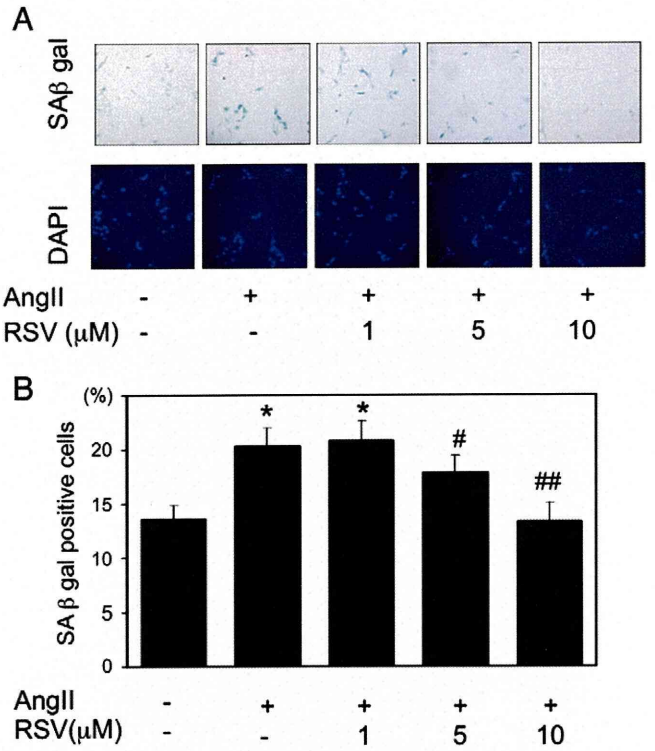

Fig. 2. Resveratrol suppressed Ang II-induced senescence of VSMCs. VSMCs were cultured with Ang II (100 nM) for 7 days in the absence or presence of varying concentration of resveratrol. The number of SA β-gal positive cells and DAPI (nucleus) in 5 randomly selected high power field was counted. The bar graph indicates the ratio of the number of SA β-gal positive cells to the number of DAPI. $N=4$, $^{*}P<0.05$ vs. Ang II (−), $^{\#}P<0.05$ vs. Ang II (+).

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detected under fluorescent microscope. An independent investigator blind to the treatment of the samples counted the number of SA β-gal- and DAPI-positive cells in five randomly chosen high power field in each sample.

2.5. Measurement of oxidative stress

5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) was loaded to VSMC and the cells were stimulated with AngII (1 μM) in the absence or presence of resveratrol for 1 day and 7 days. Fluorescent intensity at 488 nm was measured.

2.6. Statistical analysis

Statistical analysis was performed with performed 1-way ANOVA and Fisher's test, if appropriate. Data are shown as mean ± SEM. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Ang II induced VSMC senescence through AT1R

After 7 days of culture with Ang II (100 nmol/L), SA β-gal positive cells were significantly increased (Fig. 1). The increase in the number of SA

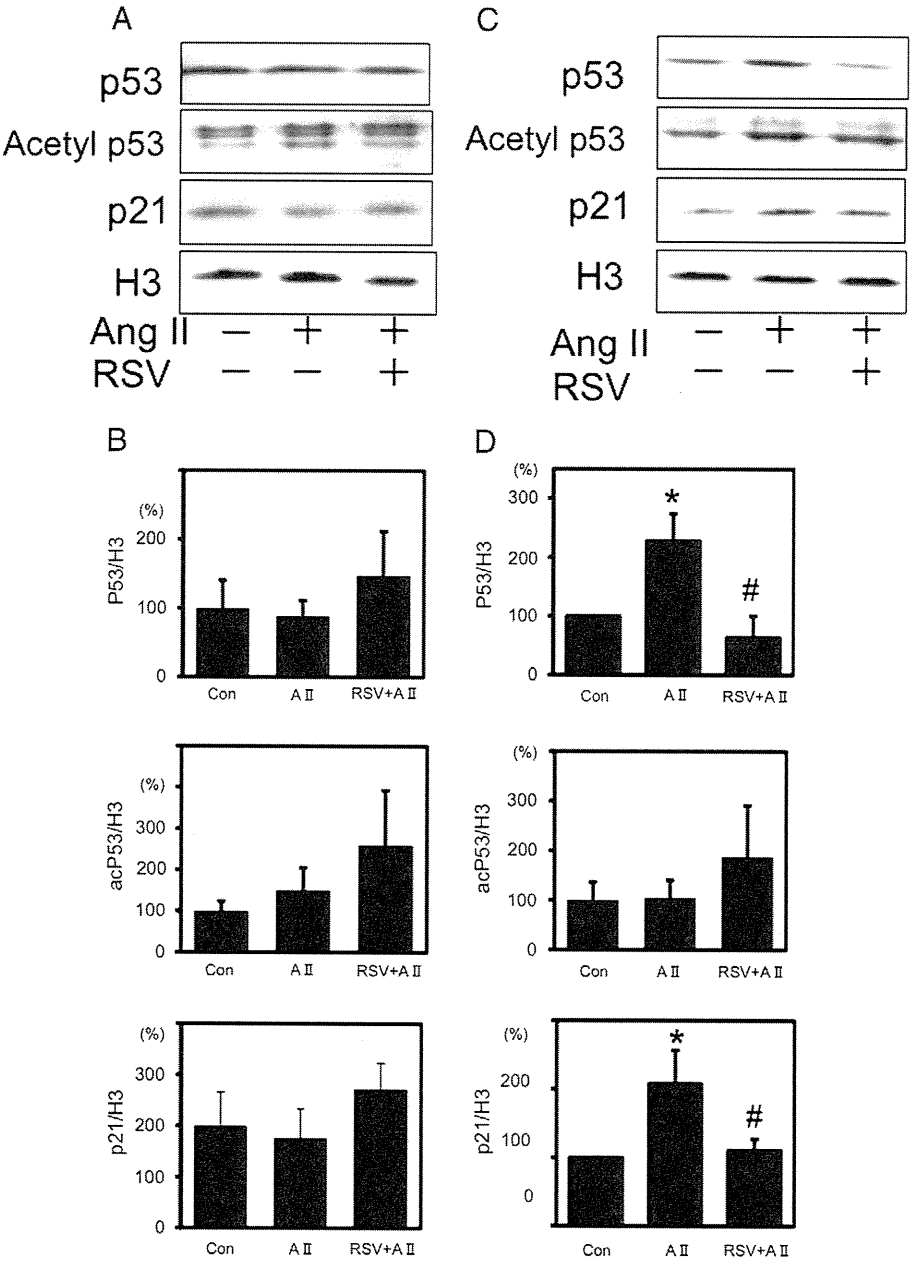

Fig. 3. Resveratrol suppressed Ang II-induced expression of p53 and p21 at day 7 but not at day 1.(A) VSMCs were cultured with Ang II (100 nM) for 1 day in the absence or presence of resveratrol (10 μM). Western blot analysis of nuclear protein for p53, acetyl p53, p21 and histone H3 protein was performed. (B) Bar graphs indicate the ratio of p53, acetyl p53 or p21 to histone H3 protein level.(C) VSMCs were cultured with Ang II (100 nM) for 7 days in the absence or presence of resveratrol (10 μM). Western blot analysis of nuclear protein for p53, acetyl p53, p21 and histone H3 protein was performed. (D) Bar graphs indicate the ratio of acetyl p53, p53 or p21 to histone H3 protein level.Control culture (Ang II (−), resveratrol (−)) was set as 100%. $N=3$, $^{*}P<0.05$ vs. Ang II (−), $^{\#}P<0.05$ vs. Ang II (+).

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β-gal-positive cells was almost completely blocked by co-incubation with losartan, an AT1R antagonist, but not by co-incubation with PD123319, an AT2R antagonist. These data suggest that AT1R mediates Ang II-induced senescence of VSMC.

3.2. Resveratrol inhibited Ang II-induced VSMC senescence

Resveratrol at various concentrations was added to the culture medium of VSMC concomitantly with Ang II. Resveratrol dose-dependently reduced the number of SA β-gal positive cells induced by Ang II (Fig. 2).

3.3. Resveratrol inhibited Ang II-induced p53 and p21 expression

It was reported that p53 mediates Ang II-induced senescence of VSMC. [10] We therefore, examined the effect of resveratrol on p53 expression and its target gene p21.

AngII stimulation for 24 hours did not affect the expression of p53, or p21 and acetylation level of p53, and resveratrol did not show any effect either (Fig. 3A and B). However, Ang II increased expression of p53 and p21 in VSMC after 7 days of stimulation (Fig. 3C and D). Co-incubation with resveratrol suppressed Ang II-induced p53 and p21 expression, indicating that down-regulation of p53 may be responsible for attenuation of VSMC senescence by resveratrol. However, we could not detect the changes of acetylation level of p53 by resveratrol at days 1 and 7.

4. Discussion

In the present study, we showed that resveratrol suppressed Ang II-induced senescence of VSMC concomitantly with down-regulation of p53. Although experimental evidence of causal relationship between p53 and senescence is not presented in the present study, it is likely that down-regulation of p53 is responsible for inhibition of senescence because a previous report showed that p53 inhibition attenuates vascular senescence [13].

Although we showed that resveratrol suppressed p53 expression in VSMC, several studies have reported opposite results. Mnjoyan and Fujise showed that resveratrol reversed serum-induced down-regulation of p53 and thereby inhibited proliferation of VSMC [14], and a recent study showed that resveratrol induced nuclear translocation and expression of p53 and activated p53 target genes such as Gadd45 and Cdkn1a in VSMC [15]. These data suggest that resveratrol rather activates p53 pathway. The reason for this apparent discrepancy between previous studies and our study is not immediately clear and further study is needed.

It is reported that Ang II accelerates atherogenesis in apolipoprotein E knockout (ApoEKO) mice, which was correlated with SA β-gal positive area and p53 expression in the aorta [13]. It is also shown that Ang II increases senescent VSMC through Ras activation in association with an up-regulation of inflammatory cytokine expression [10]. A recent study showed that AngII-induced oxidative DNA damage plays an important role in VSMC senescence [16]. In contrast, resveratrol is reported to suppress atherogenesis in ApoEKO mice [17]. Therefore, our data that resveratrol suppressed Ang II-induced senescence and p53 expression of VSMC seem to agree with these previous in vivo studies. In addition, suppression of Ang II-induced senescence and p53 expression may contribute to anti-atherogenic effect of resveratrol. Because oxidative stress plays a critical role in atherogenesis and vascular senescence, the anti-oxidative effect of resveratrol may be involved in the inhibition of VSMC senescence. However, we could not detect the effect of resveratrol on ROS production at days 1 and 7 of stimulation (data not shown). Therefore, further study is needed to clarify the detailed mechanism for the effect of resveratrol.

We have previously reported that resveratrol reduced expression of AT1R in VSMC [18]. Therefore, we cannot exclude the possibility that AT1R down-regulation may contribute to the resveratrol inhibition of Ang II-induced VSMC senescence. However, resveratrol at 10 μM did not affect AT1R expression and higher concentration of resveratrol (50 μM~) was necessary to sufficiently suppress AT1R expression in VSMC, suggesting that down-regulation of AT1R may not explain the attenuation of Ang II-induced senescence or p53 down-regulation by resveratrol.

Acetylation is believed to be crucial for the functional activity of p53 and indispensable for transcriptional activity [19]. However, we could not detect any changes of acetylation level of p53 by resveratrol. This may be due to that activation of SIRT1 by resveratrol occurs relatively higher concentration (50–100 μM) of resveratrol [5] as required for the downregulation of AT1R.

A recent study showed that genetic ablation of AT1R in mice prolonged the life span [20]. Old AT1R-deficient mice showed less cardiac hypertrophy and fibrosis. Vascular structure was relatively preserved in AT1R-deficient mice compared with wild type mice. Oxidative stress level was decreased in the heart, aorta and kidney of the AT1R-deficinet mice. Resveratrol is also known to extend life span of many species [21]. Therefore, suppression of Ang II-induced senescence by resveratrol may contribute to the longevity by resveratrol at least in part.

The upstream signaling of resveratrol that inhibits Ang II-induced VSMC senescence and p53 expression is not clear at this point. However, a previous report showed that N-acetylcysteine, a potent antioxidant inhibited Ang II-induced VSMC senescence [13]. Because resveratrol also has an antioxidant effect [22], it is possible that reduction of oxidative stress is responsible for the p53 down-regulation by resveratrol.

In summary, we showed in the present study that resveratrol suppressed Ang II-induced VSMC senescence, which may contribute to suppression of atherogenesis and extension of life span by resveratrol.

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Combination Therapy of Olmesartan and Azelnidipine Inhibits Sympathetic Activity Associated with Reducing Oxidative Stress in the Brain of Hypertensive Rats

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Abstract

It has been demonstrated that the antihypertensive drugs with the antioxidant action on the brainstem inhibit the sympathetic activity and consequently decrease blood pressure and heart rate (HR) in hypertensive rats. Combination drugs of the angiotensin receptor blocker and calcium channel blocker, such as olmesartan (OLM)/azelnidipine (AZ) and candesartan (CAN)/amlodipine (AM), are widely used for treating hypertension in Japan. In this study, it was investigated whether there are differences in the antioxidant effect in the brain and the sympathoinhibitory effect between OLM/AZ and CAN/AM combination therapies in stroke-prone spontaneously hypertensive rats (SHRSP). OLM/AZ (10/8 mg kg^{-1} day^{-1}), CAN/AM (4/2.5 mg kg^{-1} day^{-1}), or vehicle was orally administered for 30 days to SHRSP. OLM/AZ and CAN/AM markedly decreased systolic blood pressure to the same extent. OLM/AZ decreased HR to a greater extent than CAN/AM. Urinary norepinephrine excretion as a marker of sympathetic activity was unchanged in the CAN/AM group, but reduced in the OLM/AZ group. Oxidative stress in the whole brain assessed using the in vivo electron spin resonance method was similarly decreased in both OLM/AZ and CAN/AM groups. Importantly, thiobarbituric acid reactive substance levels in the brainstem were significantly lower in the OLM/AZ group, but not in the CAN/AM group, than in the vehicle group. These results suggest that combination therapy of either OLM/AZ or CAN/AM does not induce reflex-mediated sympathetic activation despite the marked blood pressure reduction, which is associated with an antioxidant effect in the brain regions affecting the sympathetic activity. Furthermore, the antioxidant effect in the brainstem and the sympathoinhibitory effect of OLM/AZ combination may be greater than those of CAN/AM combination treatment.

Keywords: hypertension, angiotensin receptor blocker, calcium channel blocker, sympathetic nervous system, brain, oxidative stress

INTRODUCTION

Hypertension is an important risk factor for cardiovascular and cerebrovascular diseases; however, in many countries, blood pressure (BP) is not adequately controlled in more than 50% of patients (1,2). Combination therapy using an angiotensin receptor blocker (ARB)/calcium channel blocker (CCB) is one of the recommended therapies for achieving the targeted level of BP in various types of hypertensive patients (1,2). In addition, the use of fixed-combination drug is advantageous for improving adherence (2). However, there is concern about the powerful BP-lowering therapy, in which excessive and rapid reduction of BP causes reflex-mediated activation of the sympathetic nervous system (3). Recent studies emphasize that activation of the sympathetic nervous system is involved in the various aspects of pathophysiology of hypertension in the clinical setting (4). Therefore, the use of drugs not causing sympathoactivation despite the large BP reduction needs to be considered.

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Olmesartan (OLM)/azelnidipine (AZ) and candesartan (CAN)/amlodipine (AM) combinations are often used as fixed-dose ARB/CCB combination therapies in Japan and are reported to have the powerful antihypertensive action (5,6). In general, ARBs have been shown to decrease sympathetic activity, although not equal in each ARB (7–9). In contrast, a long-acting CCB, AM is suggested to be neutral or elicit a slight increase in sympathetic activity probably due to reflex-mediated activation (10,11). Another long-acting CCB, AZ is suggested to have a sympathoinhibitory effect (12,13). In addition, accumulating evidence indicates that oxidative stress has an important role in hypertension (14). In particular, oxidative stress in the brain contributes to the activation of the sympathetic nervous system (8,9,15–18). It has been demonstrated that the reduction of oxidative stress in the brain decreases BP and heart rate (HR) through sympathoinhibition in hypertensive rats (7–9,17,19). However, it remains unknown whether OLM/AZ and CAN/AM combination therapies still have the antioxidant effect in the brain and the sympathoinhibitory effect without eliciting reflex sympathoexcitation due to the powerful antihypertensive action. Therefore, in this study, it was investigated whether there are differences in the antioxidant effect in the brain and the subsequent sympathoinhibitory effect between combination therapies using OLM/AZ and CAN/AM.

METHODS

Animals and General Procedures

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical and Pharmaceutical Sciences, and performed in accordance with the Guidelines for Animal Experiments at Kyushu University. Male stroke-prone spontaneously hypertensive rats (SHRSP) (11 weeks old; SLC Japan, Hamamatsu, Japan) were housed in a room with controlled lighting, temperature, and humidity. After a 1-week acclimatization period, SHRSP were assigned to one of four groups: (i) treatment with a combination of olmesartan medoxomil (OLM)/AZ (OLM/AZ group, OLM: 10 mg kg^{-1} day^{-1}; AZ: 8 mg kg^{-1} day^{-1}); (ii) treatment with a combination of candesartan cilexetil (CAN)/amlodipine besylate (AM) (CAN/AM group, CAN: 4 mg kg^{-1} day^{-1}; AM: 2.5 mg kg^{-1} day^{-1}); (iii) treatment with vehicle (untreated group, vehicle: 0.5% of methyl cellulose); and (iv) no treatment (before treatment group). All drugs were dissolved in 0.5% of methyl cellulose and administered once a day by oral gavage, and treatment continued for 30 days. OLM, AZ, and CAN were supplied by Daiichi-Sankyo Company, Ltd., Tokyo, Japan, and AM was supplied by LKT Laboratories, Inc., Tokyo, Japan.

Measurements of BP, HR, and Urinary Norepinephrine Excretion

Systolic blood pressure (SBP) and HR were measured using the tail-cuff method for each group (BP-98A; Softron, Tokyo, Japan). Urine was collected from each rat for 24 hours in a metabolic cage. Urinary norepinephrine concentration was measured using high-performance liquid chromatography to calculate urinary norepinephrine (UNE) excretion as a marker of sympathetic activity (7,12,17,19,20).

In Vivo Electron Spin Resonance Spectroscopy

Oxidative stress in the brain was evaluated using in vivo electron spin resonance (ESR) spectroscopy as previously described (7). [3-(Methoxycarbonyl)-2,2,5,5-tetramethylpyrrolizinooxy]radical (MC-PROXYL), a nitroxyl radical species, was used as the blood–brain barrier permeable spin probe because of its lipophilicity (21). MC-PROXYL undergoes an in vivo biological reaction to nonradical derivatives such as hydroxylamine (22). Additionally, increased reactive oxygen species (ROS) precipitate in the reaction nonenzymatically (23). This provides a basic mechanism for the in vivo ESR method; increased ROS production increases the in vivo ESR signal decay rate in comparison with an appropriate control value.

An anesthetized rat (sodium pentobarbital, 50 mg kg^{-1}, intraperitoneally) was placed on a handmade rat carrier. MC-PROXYL solution (150 mmol L^{-1} in saline, 0.33 mmol kg^{-1} body weight) was injected into the tail vein of the rat, and the carrier was slid into the resonator cavity such that the head of the rat, the area between interorbital and interaural lines, was located in the ESR detecting area (coaxial discoid space; 6 cm in diameter, 1.5 cm in depth). Measurement was started immediately using 300 MHz ESR spectroscopy (JEOL, Tokyo, Japan) under the following conditions: radiowave frequency, 300 MHz; radiowave power, 2.5 mW; field modulation width, 0.1 mT; field modulation frequency, 100 kHz; scan rate, 0.083 mT $second^{-1}$; scan width, 1.0 mT; time constant, 0.1 second; and accumulation number, 5.

Signal intensity, defined as the height of the center line of a spectrum, was obtained using an averaged spectrum processed using five consecutively accumulated spectra within a 60-second interval. Measurement data were collected for 5 minutes. Natural logarithmic values of the data were plotted as a function of time, and the signal decay rate was determined using the negative slope of the linear regression of the plots, which showed good linearity.

Measurement of Thiobarbituric Acid Reactive Substances

To obtain brain tissues, rats were deeply anesthetized using sodium pentobarbital (100 mg kg^{-1} intraperitoneally) and perfused transcardially with