Original Article

Amlodipine-Induced Reduction of Oxidative Stress in the Brain Is Associated with Sympatho-Inhibitory Effects in Stroke-Prone Spontaneously Hypertensive Rats

Yoshitaka HIROOKA[1),*], Yoshikuni KIMURA[1),*], Masatsugu NOZOE[1)], Yoji SAGARA[1)], Koji ITO[1)], and Kenji SUNAGAWA[1)]

Amlodipine is a dihydropyridine calcium channel blocker that is widely used for the treatment of hypertensive patients and has an antioxidant effect on vessels *in vitro*. The aim of the present study was to examine whether treatment with amlodipine reduced oxidative stress in the brains of stroke-prone spontaneously hypertensive rats (SHRSP). The animals received amlodipine, nicardipine or hydralazine for 30 days in their drinking water. Levels of thiobarbituric acid-reactive substances (TBARS) in the brain (cortex, cerebellum, hypothalamus, and brainstem) were measured before and after each treatment. Systolic blood pressure decreased to similar levels in the amlodipine-, nicardipine-, and hydralazine-treated groups. Urinary norepinephrine excretion was significantly reduced in SHRSP after treatment with amlodipine, but not with nicardipine or hydralazine. Levels of TBARS in the cortex, cerebellum, hypothalamus, and brainstem were significantly higher in SHRSP than in Wistar-Kyoto rats (WKY), and were reduced in amlodipine-treated, but not in nicardipine- or hydralazine-treated, SHRSP. Electron spin resonance spectroscopy revealed increased levels of reactive oxygen species in the brains of SHRSP, which were reduced by treatment with amlodipine. Intracisternal infusion of amlodipine also reduced systolic blood pressure, urinary norepinephrine excretion, and the levels of TBARS in the brain. These results suggested that oxidative stress in the brain was enhanced in SHRSP compared with WKY rats. In addition, antihypertensive treatment with amlodipine reduced oxidative stress in all areas of the brain examined and decreased blood pressure without a reflex increase in sympathetic nerve activity in SHRSP. (*Hypertens Res* 2006; 29: 49–56)

***Key Words*: blood pressure, heart rate, hypertension, oxidative stress, sympathetic nervous system**

Introduction

Amlodipine is a dihydropyridine calcium channel blocker that is widely used for the treatment of hypertension. Large clinical trials have confirmed its usefulness for preventing cardiovascular events by lowering blood pressure (*1*, *2*). Concern remains over the risk of cardiovascular events in patients

From the [1)]Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan.

*Each author contributed equally to this work.

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Address for Reprints: Yoshitaka Hirooka, M.D., Ph.D., Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. E-mail: hyoshi@cardiol.med.kyushu-u.ac.jp

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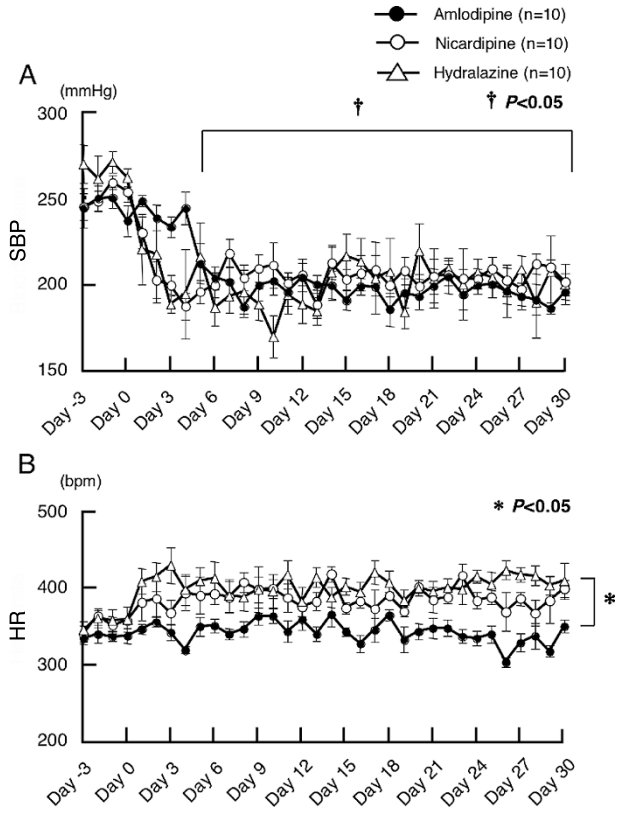

Fig. 1. *Time course of changes in systolic blood pressure (SBP) (A) and heart rate (HR) (B) induced by treatment with amlodipine, nicardipine and hydralazine.* †p < *0.05 compared with the baseline values.* *p < *0.05 for the difference between the two groups.*

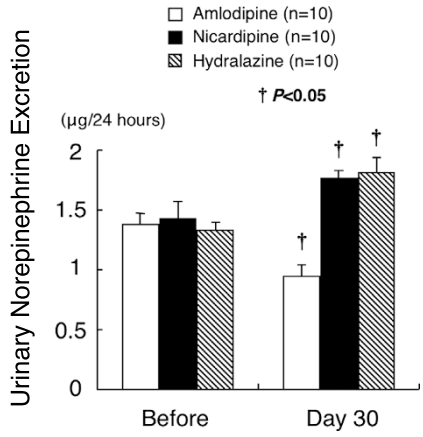

Fig. 2. *Urinary norepinephrine excretion for 24 h before and during the last day of (after) treatment with amlodipine, nicardipine and hydralazine.* †p < *0.05 compared with the values before treatment.*

with coronary artery disease, which is probably due to arterial baroreflex-mediated sympathoexcitation, particularly when short-acting calcium channel blockers are used (*3–5*). However, recent large clinical trials have indicated that this is not necessarily the case with long-acting dihydropyridine calcium channel blockers, such as amlodipine (*1*, *2*). In addition, amlodipine has been demonstrated to have anti-atherosclerotic and anti-inflammatory effects in animals (*6–9*) and humans (*10*). The mechanisms involved are complex, and include an increase in nitric oxide production (*11*) and a decrease in oxidative stress (*12–14*).

The reported effects of amlodipine on sympathetic nerve activity vary among human studies, although it appears to lower blood pressure (*15–17*). Receptor binding sites for calcium channel blockers have been identified in the brain (*18–20*). In conscious spontaneously hypertensive rats (SHR), intracerebroventricular administration of nifedipine or amlodipine decreases blood pressure, heart rate and renal sympathetic nerve activity (*21*, *22*). Furthermore, long-term i.v. infusion of nifedipine or amlodipine decreases these variables by inhibiting central sympathetic outflow (*21*, *22*).

Increased nitric oxide levels and decreased oxidative stress in the brain, particularly in the brainstem, inhibit sympathetic nerve activity, thereby reducing blood pressure in stroke-prone SHR (SHRSP) (*23*). Increased oxidative stress is also involved in the pathogenesis of hypertension and hypertensive vascular lesion formation (*24*). We demonstrated previously that oxidative stress in the brain is increased in SHRSP, which is related to the increased sympathetic outflow in this model (*23*). Amlodipine reduces oxidative stress in the vasculature of hypertensive animals (*25*) and humans (*26*, *27*). However, the antioxidant effect of amlodipine in the brain of hypertensive animals has not been reported previously. Therefore, the aim of the present study was to determine whether long-term oral treatment with amlodipine reduced oxidative stress in the brain of SHRSP, and to examine the associated changes in blood pressure, heart rate, and urinary norepinephrine excretion. For this purpose, we measured thiobarbituric acid-reactive substances (TBARS), which are the end products of lipid peroxidation and markers of oxidative stress (*23*). Electron spin resonance spectroscopy measurements (*23*) were also performed to analyze the production of reactive oxygen species.

Methods

General Preparation

This study was reviewed and approved by the Committee of Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, Japan. Male SHRSP/Izm (*14* weeks old; SLC Japan, Hamamatsu, Japan) were fed a standard diet with free access to drinking water. The animals received amlodipine in their drinking water at doses (3 or 10 mg/kg body weight/day) that were chosen based on previous studies (*12*, *28–30*). Control groups were fed a standard diet

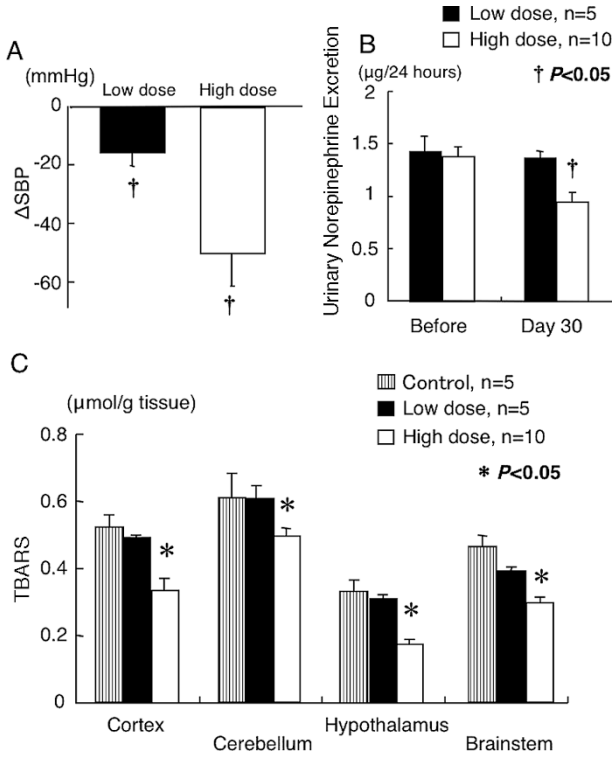

Fig. 3. *A: Amlodipine-induced changes in systolic blood pressure (ΔSBP) at doses of 3 or 10 mg/kg body weight/day. B: Urinary norepinephrine excretion for 24 h before and after amlodipine treatment. C: Levels of TBARS in the brain (cortex, cerebellum, hypothalamus and brainstem) in non-treated rats (control) and rats treated with amlodipine.* $^{\dagger}p < 0.05$ *compared with the baseline values.* $^{*}p < 0.05$ *for the difference between the two groups.*

and received nicardipine (10 mg/kg body weight/day) or hydralazine (20 mg/kg body weight/day) in their drinking water. The treatment commenced when the rats were 14 weeks of age and continued for 30 days. All drugs were dissolved in 45 ml of drinking water per day and, once this had been consumed, additional water was made available *ad libitum*.

Measurement of Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Systolic blood pressure and heart rate evaluated using the tail-cuff method were measured before and after treatment with amlodipine and the other drugs in SHRSP, as described previously (*31*). Urine was collected for 24 h in a metabolic cage. Urinary norepinephrine concentrations were measured before and after amlodipine, nicardipine or hydralazine treatment using high-performance liquid chromatography. Urinary norepinephrine excretion was calculated as a marker of sympathetic nerve activity (*23*, *31*).

Measurement of TBARS

Brain tissue was homogenized in 1.15% KCl (pH 7.4), and 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH and 0.3% TBA were added to the homogenate. The amounts of TBARS were determined by absorbance with a molecular extinction coefficient of 156,000 and expressed as μmol/g of wet weight tissue, as described previously (*23*, *32*).

Electron Spin Resonance Spectroscopy Measurements

Electron spin resonance spectroscopy measurements were performed at room temperature with an X-band (9.45-GHz) electron spin resonance spectrometer (JES-RE-1X; JEOL, Tokyo, Japan) at the following settings: microwave power of 10 mW, an external magnetic field range of 20 mT and a scan rate of 10 mT/min. The amounts of reactive oxygen species were quantified by monitoring the time-dependent decay of the amplitude of the electron spin resonance spectra elicited by the nitroxide radical 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (hydroxy-TEMPO) as a spin probe. The tissue was homogenized in 50 mmol/l phosphate-buffered saline (PBS) containing the following protease inhibitors: leupeptin (10 g/ml), phenylmethylsulfonyl fluoride (100 g/ml), dithiothreitol (1 mmol/l) and trypsin inhibitor (10 μg/ml). The homogenate was mixed rapidly with hydroxy-TEMPO (0.1 mmol/l) in PBS and drawn into glass tubes. The electron spin resonance spectra were recorded for up to 10 min at 10-s intervals, as described previously (*23*, *32*, *33*).

Continuous Intracisternal (i.c.) Infusion Experiments with Amlodipine

The SHRSP were randomly divided into two groups, which received either artificial cerebrospinal fluid vehicle ($n=5$) or amlodipine (dissolved in artificial cerebrospinal fluid; 0.1 mg/kg body weight/day; $n=6$) by continuous i.c. infusion (0.25 μl/h) for 2 weeks *via* an osmotic minipump (Alzet model 1002; DURECT Corp., Cupertino, USA), as described previously (*34*, *35*). The treatment commenced when the rats were 14 weeks of age and continued for 2 weeks. Systolic blood pressure, heart rate, urinary norepinephrine concentrations, and levels of TBARS were measured before and after the infusion.

Drugs

Amlodipine was provided from Pfizer Japan Inc. Other drugs were purchased from Sigma Chemical Co. (St. Louis, USA).

Statistical Analysis

All values are expressed as the mean±SEM). Two-way anal-

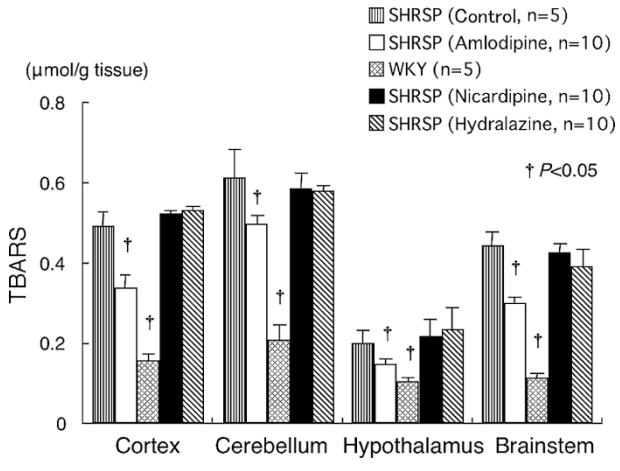

Fig. 4. *Levels of TBARS in the brain (cortex, cerebellum, hypothalamus and brainstem) in non-treated rats (control) and rats treated with amlodipine, nicardipine or hydralazine.* †p < *0.05 compared with the values for non-treated rats.*

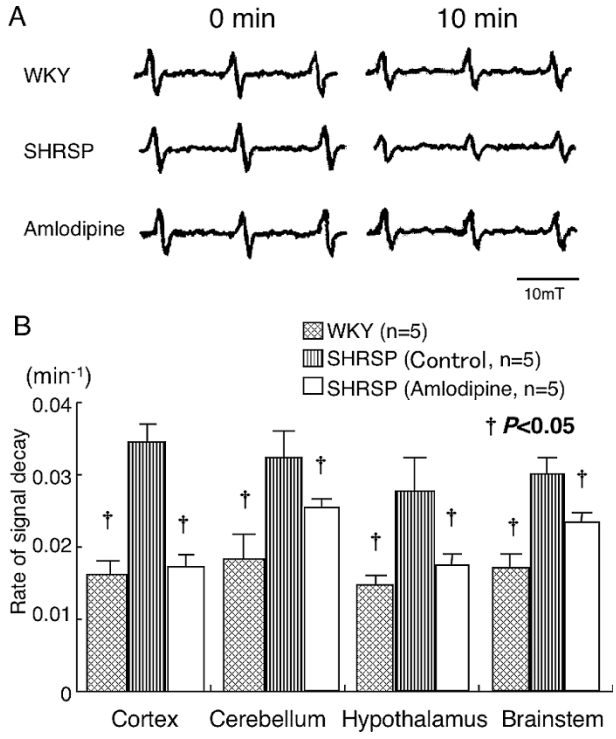

Fig. 5. *Electron spin resonance analysis of hydroxy-TEMPO in the tissues. A: Sequential sample of electron spin resonance spectra of hydroxy-TEMPO in brainstem tissues from SHRSP (middle spectra), SHRSP treated with amlodipine (lower spectra) and WKY rats (upper spectra). B: Summary data for the signal decay rate in the brain (cortex, cerebellum, hypothalamus and brainstem) in WKY rats, SHRSP and SHRSP treated with amlodipine.* †p < *0.05 compared with the values for non-treated SHRSP (control).*

ysis of variance (ANOVA) was used to compare the systolic blood pressure and heart rate between the amlodipine-treated and other groups. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. ANOVA was used to compare the amounts of TBARS and the electron spin resonance signal-decay rates in non-treated SHRSP and other rats in conjunction with a *post hoc* test using Scheffe's correction. A paired *t*-test was performed to compare the urinary norepinephrine excretion before and after treatment. Differences were considered to be statistically significant when p was less than 0.05.

Results

Effects of Amlodipine on Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Systolic blood pressure was reduced to similar levels in the high-dose amlodipine- and hydralazine-treated groups; the values for amlodipine, nicardipine and hydralazine were -40 ± 12, -45 ± 7 and -43 ± 8 mmHg, respectively ($n=10$ for each; Fig. 1A). By contrast, heart rate was not significantly affected by amlodipine treatment, but was increased by nicardipine and hydralazine treatment (Fig. 1B). Urinary norepinephrine excretion was significantly higher in SHRSP than in WKY rats, with values of 1.38 ± 0.10 and 0.76 ± 0.03 μg/day, respectively ($n=6$ for both; $p<0.05$). Furthermore, urinary norepinephrine excretion was decreased in SHRSP after amlodipine treatment, but was significantly increased after nicardipine or hydralazine treatment; the values were 1.37 ± 0.15 *vs*. 0.87 ± 0.10 μg/day, 1.45 ± 0.17 *vs*. 1.68 ± 0.06 μg/day and 1.33 ± 0.08 *vs*. 1.77 ± 0.14 μg/day for amlodipine, nicardipine, and hydralazine, respectively ($n=10$; $p<0.05$; Fig. 2). Treatment with a high dose of amlodipine decreased the systolic blood pressure to a greater extent than treatment with a low dose, with values of -40 ± 12 and -18 ± 7 mmHg, respectively ($p<0.05$; Fig. 3A). Urinary norepinephrine excretion was not significantly different before and after treatment with a low dose of amlodipine (1.44 ± 0.25 *vs*. 1.38 ± 0.15 μg/day; Fig. 3B).

Reactive Oxygen Species Generation in the Brain

Levels of TBARS in the cortex, cerebellum, hypothalamus and brainstem were significantly higher in SHRSP than in WKY rats ($p<0.05$; $n=5$ for both). Furthermore, levels of TBARS in each area of the brain examined were significantly reduced in the high-dose amlodipine-treated, but not in the nicardipine- or hydralazine-treated, SHRSP ($p<0.05$; $n=10$ for each; Fig. 4). The levels of TBARS in all areas of the brain examined were not significantly altered in the low-dose amlodipine-treated SHRSP (Fig. 3C). The intensity of electron spin resonance signals in each area of the brain decreased more rapidly in SHRSP than in WKY rats (Fig. 5A). The rates

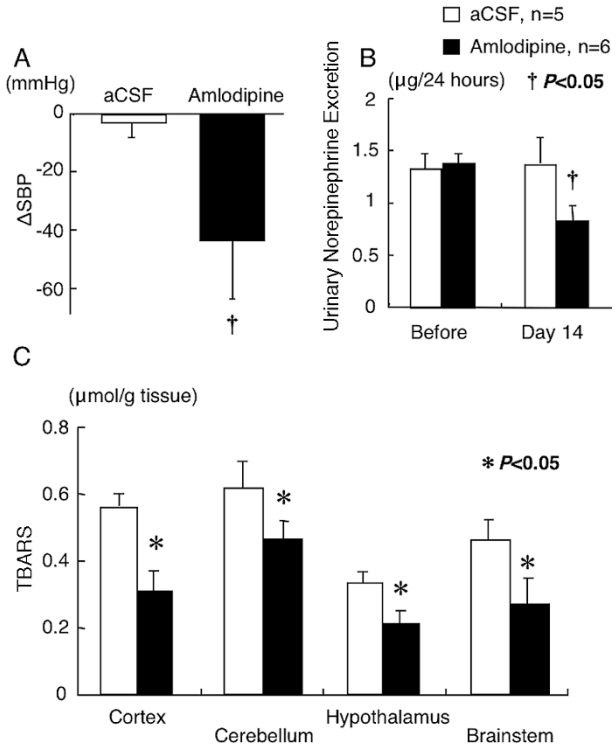

Fig. 6. *A: Changes in systolic blood pressure (ΔSBP) caused by continuous i.c. infusion with amlodipine or artificial cerebrospinal fluid (aCSF) for 2 weeks. B: Urinary norepinephrine excretion for 24 h at days 0 and 14. C: Levels of TBARS in the brain (cortex, cerebellum, hypothalamus and brainstem) in non-treated rats and rats treated with amlodipine at days 0 and 14.* $^{\dagger}p < 0.05$ *compared with the values before treatment.* $^{*}p < 0.05$ *for the difference between the two groups.*

of signal decay in the cortex, cerebellum, hypothalamus and brainstem, calculated from the slopes of the lines, were significantly higher in SHRSP than in WKY rats ($p<0.05$; $n=5$ for each; Fig. 5B). Furthermore, the rates of signal decay in these areas of the brain were significantly reduced in amlodipine-treated SHRSP ($p<0.05$; $n=5$ for each; Fig. 5B).

Effect of Continuous i.c. Infusion with Amlodipine

The changes in systolic blood pressure after the i.c. infusion of amlodipine for 2 weeks are shown in Fig. 6A. The changes in systolic blood pressure were significantly greater after treatment with amlodipine (−43±22 mmHg; $n=6$) than after treatment with artificial cerebrospinal fluid (−3±12 mmHg; $n=5$; $p<0.05$). Figure 6B shows that urinary norepinephrine excretion was significantly decreased in SHRSP after treatment with amlodipine (1.45±0.10 *vs*. 0.67±0.11; $n=6$; $p<0.05$), but was not significantly altered by treatment with artificial cerebrospinal fluid (1.42±0.08 *vs*. 1.48±0.20 μg/day; $n=5$). The levels of TBARS in all areas of the brain were significantly reduced in amlodipine- but not artificial cerebrospinal fluid-treated SHRSP ($n=6$ and 5, respectively; $p<0.05$; Fig. 6C).

Discussion

The major findings of the present study were that oral treatment with amlodipine did not induce reflex tachycardia and reduced sympathetic nerve activity. In addition, amlodipine decreased oxidative stress in the brains of SHRSP, as evaluated by the measurement of levels of TBARS. By contrast, treatment with hydralazine induced sympathoexcitation and reflex tachycardia, but did not alter levels of TBARS. Nicardipine, which is another calcium channel blocker, also induced sympathoexcitation and reflex tachycardia, but did not alter TBARS levels. The electron spin resonance spectroscopy results indicated increased reactive oxygen species production in SHRSP, which was attenuated after treatment with amlodipine. These findings suggest that long-term antihypertensive treatment with amlodipine does not cause reflex-induced sympathoexcitation and reduces the increased oxidative stress in the brains of SHRSP. In particular, the decreased oxidative stress levels in the brainstem and hypothalamus might be related to a decrease in sympathetic nerve activity.

A gradual decrease in blood pressure was observed over time in rats treated with amlodipine compared with those treated with hydralazine or nicardipine, due to differences in the pharmacokinetic profiles, plasma concentrations and lipophilicities of the drugs (*16*, *21–33*, *36*). Disrupted tight junctions caused by endothelial dysfunction are responsible for the increased permeability of tracers through the blood–brain barrier in chronic hypertension (*37*). L-type voltage-gated calcium channels in the central nervous system and dihydropyridines act on these receptors (*19*, *20*, *38–40*). Thus, it is possible that lipophilic dihydropyridines (such as nifedipine and amlodipine) are able to cross the blood–brain barrier in chronic hypertension (*21*, *22*) and reduce the generation of reactive oxygen species (*41–43*). However, this might not occur with all calcium channel blockers, as nicardipine did not reduce the generation of reactive oxygen species.

We believe that treatment with the lower dose of amlodipine in our study was not sufficient to reduce the oxidative stress in the brain. In addition, urinary norepinephrine excretion was not altered. By contrast, treatment with the higher dose of amlodipine induced a greater reduction in blood pressure, which was associated with a decrease in urinary norepinephrine excretion. Oxidative stress in the brain was also reduced. A greater reduction in blood pressure is thought to elicit a greater reflex increase in sympathetic nerve activity. Thus, these results suggest that treatment with amlodipine, at a dose that is sufficient to decrease blood pressure, reduces oxidative stress in the brain in association with sympathoinhibition.

Brain cell membranes contain a high concentration of polyunsaturated fatty acids (*44*). These are targets of free radicals, which cause chain reactions of lipid peroxidation (*45*). TBARS, which are the end products of lipid peroxidation and markers of oxidative stress, were increased in the brain of SHRSP (*23*). In the present study, we examined levels of TBARS in the cortex, cerebellum, hypothalamus and brainstem, and found that they were all increased in SHRSP compared with WKY rats. This was consistent with the results of our recent study, in which we compared levels of TBARS in the whole brain, the rostral ventrolateral medulla and the nucleus tractus solitarius of SHRSP and WKY rats (*46*). These areas are important for autonomic cardiovascular regulation (*47*, *48*). The electron spin resonance spectroscopy results further support the theory that there is increased generation of reactive oxygen species in the brain of SHRSP compared with WKY rats. Moreover, this increase was attenuated by amlodipine.

Although variable effects on the sympathetic nervous system have been reported in clinical studies in humans (*16–18*, *26*), lipophilic dihydropyridines (such as nifedipine and amlodipine) are believed to have sympatho-inhibitory and depressor effects through central nervous system mechanisms in SHR (*21*, *22*). During long-term i.v. infusion, nifedipine and amlodipine cross the blood–brain barrier and, thereafter, inhibit sympathetic nerve activity and reduce blood pressure (*21*, *22*). Furthermore, intracerebroventricular injection of these calcium channel blockers reduces blood pressure, heart rate, and renal sympathetic nerve activity (*21*, *22*). In addition, direct microinjection of calcium channel blockers into the nucleus tractus solitarius of the brainstem reduces blood pressure and heart rate *via* the inhibition of central sympathetic outflow (*49*). In the present study, amlodipine administered by i.c. infusion decreased blood pressure, urinary norepinephrine excretion and oxidative stress in the brain, further supporting the idea that it elicits these effects by acting on the central nervous system. There were no effects on blood pressure and heart rate when we intravenously administered the same concentration of amlodipine as used for the intracisternal infusion for 1 h (data not shown). Although the site of the sympatho-inhibitory actions of amlodipine in the central nervous system is not known, we consider the hypothalamus and brainstem to be likely candidates. In conjunction with the decrease in reactive oxygen species generation, an increase in endothelial nitric oxide synthase activity might be related to the decrease in oxidative stress and central sympathetic outflow in SHRSP (*31*, *50–52*). In fact, amlodipine enhances endothelial nitric oxide synthase activity (*53*), although we did not address this issue in the present study. Increased nitric oxide production in the brainstem also produces a decrease in central sympathetic outflow (*50–52*). Amlodipine may reduce reactive oxygen species by upregulating Cu/Zn superoxide dismutase in SHRSP (*54*).

Several previous studies have suggested that the generation of reactive oxygen species leads to hypertensive vascular-lesion formation (*55–60*). Therefore, therapies aimed at reducing the generation of reactive oxygen species might be useful for hypertensive patients. In particular, the brain is the organ that is most affected by hypertension (*55*). In the present study, we demonstrated that oral treatment with amlodipine reduced oxidative stress in the cortex and cerebellum, as well as the hypothalamus and brainstem; the effects on the latter might help reduce sympathetic nerve activity, thereby preventing cardiovascular events, whereas the effects on the former might help to protect brain function. Hypertension accelerates age-related organ damage, which is also associated with sympathetic dysregulation (*55*, *56*). In addition, dementia might be related to hypertension (*60*). Further studies will be required to examine how treatment with amlodipine leads to the reduction of reactive oxygen species. It is possible that long-term treatment for hypertension, as well as the reduction of oxidative stress in the brain, will result in a better quality of life for patients.

We cannot exclude the possibility that amlodipine might act on the peripheral sympathetic nervous system, thereby inhibiting the sympathetic nerve activity. In particular, amlodipine has been shown to block both N-type Ca^{2+} channels and L-type Ca^{2+} channels (*61*, *62*), although the extent of these actions has not been clarified *in vivo*. Nicardipine has also been reported to exhibit this blocking activity (*62*). However, we found different results between amlodipine and nicardipine. Furthermore, the present study does not provide direct evidence that an increase in oxidative stress in the brain inhibits sympathetic nerve activity, thereby reducing blood pressure. Thus, it remains unknown whether the decrease in reactive oxygen species in the brain is a cause or an effect of sympatho-inhibition or blood pressure reduction from the results of the present study. The reduction of blood pressure itself, however, did not decrease oxidative stress in the brain when we administered hydralazine or nicardipine. Finally, we used a high dose of amlodipine in the present study. Although this dose of amlodipine (10 mg/kg/day) has been used in other experimental studies (*12*, *28–30*), it did require us to adjust the level of blood pressure reduction among the treatments.

In conclusion, the results of the present study suggest that long-term treatment with amlodipine decreases the generation of reactive oxygen species in several areas of the brain, including the hypothalamus and brainstem. This mechanism might be associated with a reduction in sympathetic nerve activity in SHRSP.

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