Combination Therapy of Atorvastatin and Amlodipine Inhibits Sympathetic Nervous System Activation and Improves Cognitive Function in Hypertensive Rats

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**Background:** A previous study has demonstrated that orally administered atorvastatin reduces sympathetic nervous system (SNS) activation via an anti-oxidant in the rostral ventrolateral medulla (RVLM) of hypertensive rats, whereas amlodipine did not. Furthermore, several previous reports have suggested that atorvastatin or amlodipine improves cognitive dysfunction during hypertension. The aim of the present study was to determine whether a combination of atorvastatin and amlodipine causes sympathoinhibition via reduction of oxidative stress in the RVLM and improves cognitive dysfunction of hypertensive rats.

**Methods and Results:** Stroke-prone spontaneously hypertensive rats (SHRSPs), as a hypertensive model with sympathoeexcitation, were divided into 4 groups; a combination of atorvastatin and amlodipine-treated (COM), atorvastatin-treated (ATR), amlodipine-treated (AML), and vehicle-treated (VEH) SHRSPs. After treatment for 28 days, the mean blood pressure did not change in ATR rats, and was reduced to the similar levels in COM, AML, and HYD rats. However, SNS activation and oxidative stress in the RVLM were significantly lower only in COM than in ATR, AML, HYD, and VEH rats. Cognitive performance and manganese-superoxide dismutase activity in the hippocampus were significantly higher, and oxidative stress in the hippocampus was significantly lower in COM than in VEH, AML, and HYD rats to a greater extent than in ATR rats.

**Conclusions:** A combination of atorvastatin and amlodipine causes sympathoinhibition via an anti-oxidant in the RVLM and improves cognitive dysfunction via an anti-oxidant in the hippocampus in hypertensive rats, independent of the blood pressure-lowering effect. *(Circ J 2012; 76: 1934–1941)*

**Key Words:** Brain; Calcium channel blocker; Hypertension; Statin; Sympathetic nervous system

Hypertension and dyslipidemia can act multiplicatively or synergistically to increase the risk of a cardiovascular disease event. Many evidences have demonstrated advantages of combined vs. older sequential and individual approaches to the treatments for hypertension and dyslipidemia. Amlodipine is a long-acting dihydropyridine calcium channel blocker indicated for the treatment of hypertension, and previous clinical trials have suggested that amlodipine reduces cardiovascular events in different patient populations. Atorvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin) indicated for the treatment of dyslipidemia and the prevention of cardiovascular disease. The combination of atorvastatin and amlodipine emerged to be more effective than each single drug alone in reducing blood pressure and in improving the lipid profile.

In the development and progression in hypertension, sympathetic nervous system (SNS) activation is a main cause, and might play an additional pathophysiological role in the development of cardiovascular complications. SNS activation is mainly regulated by the brain, and in hypertension and heart failure models of rats, we have demonstrated that interventions to the brain have beneficial effects through sympathoinhibition. Especially 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. We have demonstrated that oxidative stress produced by an angiotensin II type 1 receptor and nicotinamide adenine dinucleotide phosphate [NAD (P) H] oxidase in the RVLM increases, and nitric oxide in the RVLM decreases the SNS activation, and that oxidative stress and nitric oxide in the RVLM have the potential to be the target of the treatments for hypertension.

Statins have been shown to reduce renal sympathetic nerve traffic, and the effect on nitric oxide and oxidative stress in the...
brain has been proposed as possible explanations. Our previous reports also suggested that orally administered atorvastatin or amlodipine inhibits the SNS activation via an antioxidant and/or upregulation of nitric oxide synthase in the RVLM and the brainstems of hypertensive rats. It has not been determined, however, whether a combination of atorvastatin and amlodipine has a sympathoinhibitory effect via the reduction of oxidative stress in the RVLM of hypertensive rats.

One of the important hypertensive organ damages is cognitive dysfunction. Both hypertension and dyslipidemia are the risk factors of cognitive dysfunction, and previous reports have suggested that atorvastatin and amlodipine improve cognitive dysfunction. Oxidative stress and/or antioxidant deficiency cause cognitive decline, and oxidative stress in the hippocampus impairs cognitive function. These previous studies indicate that each of atorvastatin or amlodipine might have a possible preventive effect on cognitive dysfunction via reduction of oxidative stress in the hippocampus. However, it has not been determined whether a combination of atorvastatin and amlodipine has a beneficial effect on cognitive dysfunction of hypertensive rats.

The aims of the present study were to investigate whether the combination of atorvastatin and amlodipine has the sympathoinhibitory effect and improvement of cognitive dysfunction in hypertensive rats. We divided stroke-prone spontaneously hypertensive rats (SHRSPs), as hypertensive and vascular dementia model rats with severe sympathetic hyperactivity, into 5 groups: single atorvastatin-treatment group (ATR), single amlodipine-treatment group (AML), a combination of atorvastatin and amlodipine-treatment group (COM), hydralazine-treatment group (HYD), and vehicle group (VEH). We determined the SNS activation by 24-h urinary norepinephrine excretion, and the oxidative stress in the RVLM and hippocampus by thiobarbituric acid-reactive substances (TBARS) methods. Cognitive function was assessed by the Morris water maze test, which has been widely used as a test of spatial memory and cognition.

Methods

Animals
This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male SHRSPs (12–14 weeks old), weighing 350–425 g were fed a standard feed (SLC Japan, Hamamatsu, Japan). They were housed individually in a temperature-controlled room (22–23°C) with a 12-h light-dark cycle (lights on at 07:00 h). We divided the SHRSPs into 5 groups: ATR, AML, COM, HYD, and VEH (n=5 for each).

Oral Administration of Amlodipine, Atorvastatin or Hydralazine

SHRSPs were treated for 4 weeks. ATR was administered atorvastatin (20 mg·kg⁻¹·day⁻¹, supplied from Pfizer Inc, New York, NY, USA). AML was administered amlodipine (5 mg·kg⁻¹·day⁻¹, supplied from Pfizer Inc, New York, NY, USA). COM was administered atorvastatin (20 mg·kg⁻¹·day⁻¹) plus amlodipine (5 mg·kg⁻¹·day⁻¹). HYD was administered hydralazine (5 mg·kg⁻¹·day⁻¹, Sigma Aldrich, St. Louis, MO, USA). VEH was administered 0.5% methylcellulose. All drugs were dissolved in 0.5% methylcellulose and administered by gastric gavage every day.

Measurement of Blood Pressure, Heart Rate and Urinary Norepinephrine Excretion

The mean blood pressure and heart rate were measured using the radio-telemetry system in rats in conscious state, every day as described previously. At 4 weeks, we calculated the urinary norepinephrine excretion for 24 h as an indicator of the SNS activation, as described previously.

Measurement of TBARS in the RVLM and Hippocampus

To obtain the RVLM and hippocampus tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with PBS (150 mmol/L NaCl, 3 mmol/L KCl, and 5 mmol/L phosphate; pH 7.4, 4°C). Prior to the removal of the brain, we made an incision on the dorsal surface of the head, and opened the bone of the head in a stereotaxic frame. RVLM was identified by the microinjected L-glutamate-induced significant and rapidly pressor response, as described previously, and we microinjected dye into the same site. We also microinjected dye into the hippocampus according to the rat brain atlas. After these procedures, the brains were removed quickly, and 1 mm thick slices of the dye-stained tissues from the RVLM or hippocampus were obtained by using the punch-out technique with a cryostat at −72 ± 1°C. The tissues were 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. Thiobarbituric acid (0.3%) was added to the homogenate, and the absorbance of the organic dye-stained tissues from the RVLM or hippocampus was measured at 532 nm. The amount of TBARS was determined by absorbance, as described previously.

NAD (P) H Oxidase Activity

NAD (P) H-dependent superoxide production in the RVLM and hippocampus was measured using a lucigenin luminescence assay, as described previously. A 10% (W/vol) RVLM or hippocampus tissue homogenate was homogenized in 50 mmol/L of phosphate buffer and a luminescence assay was performed with a luminescence reader (Berthold Technology). Quantification of NAD (P) H oxidase activity was expressed relative to that in VEH, which was assigned a value of 1.

Superoxide Dismutase Activity in the RVLM and Hippocampus

Cu/Zn-superoxide dismutase (SOD) and Mn-SOD activity in the RVLM and hippocampus were assayed by monitoring the inhibition of the rate of xanthine-mediated/xanthin oxidase-mediated reduction of cytochrome c (pH 7.4), as described previously. For discrimination between Cu/Zn-SOD and Mn-SOD activities, the assay was additionally performed after incubation in the presence of KCN, which selectively inhibits the Cu/Zn-SOD isoform. SOD activity was expressed relative to that in VEH, which was assigned a value of 1.

Analysis of Cognitive Function

Spatial leaning and memory function of the rats were investigated with the Morris water maze test in a circular pool filled with water at a temperature of 25.0 ± 1°C. In the hidden platform test, a transparent platform was submerged 1 cm below the water level. All the procedures of the Morris water maze were performed for 7 days. A pre-training session was carried out at day 0, in which animals were given 60 s free swimming without the platform. In the hidden-platform test for 4 days, the rats were given 2 trials (1 session) on day 1 and 4 trials (2 sessions) per day on days 2, 3, and 4. After mounting the plat-
form, the rats were allowed to remain there for 15 s. If a rat was unable to find the platform within 60 s, it was guided to the platform and allowed to rest on the platform for 15 s. Probe trials were performed on day 5. In the probe trial, the hidden platform was removed and the rats was released from the right quadrant and allowed to swim freely for 60 s. The time spent in the target quadrant, where the platform has been located during training, and the time spent in the other quadrants were measured. In the visible-platform test performed on day 6, the platform was elevated above the water surface and placed in a different position. The rats were given an inter-trial interval of 10 min for these trials.

**Statistical Analysis**
All values are expressed as mean ± SEM. Comparisons between any 2 mean values were performed using Bonferroni’s correction for multiple comparisons. ANOVA was used to compare all the parameters in all groups. Differences were considered to be statistically significant at a P value of <0.05.

**Results**

**Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion**
The mean blood pressure was reduced in AML, COM, and HYD rats to similar levels, and were significantly lower in AML, COM, and HYD rats than in VEH rats (Figure 1A). The mean blood pressure was not different between ATR and VEH rats (Figure 1A). The heart rate was significantly lower in COM than in ATR, HYD, and VEH rats (Figure 1B), and was not different between ATR, AML, HYD, and VEH rats. Urinary norepinephrine excretion was significantly lower in COM rats than in VEH rats, and was not different between ATR, AML, HYD, and VEH rats (Figure 1C).

**TBARS Levels and NAD(P)H Oxidase Activity in the RVLM and Hippocampus**
In the RVLM, TBARS levels (Figure 2A) and NAD(P)H oxidase activity (Figure 2B) were significantly lower only in COM rats than in VEH rats, and were not different between ATR, AML, HYD, and VEH rats.

In the hippocampus, TBARS levels (Figure 2C) were significantly lower in COM rats than in AML, HYD, and VEH rats to a greater extent than in ATR rats (Figure 2C). NAD(P)H oxidase activity was not different between ATR, AML, COM, HYD, and VEH rats (Figure 2D).

**SOD Activity in the RVLM and Hippocampus**
In the RVLM, Cu/Zn-SOD activity was not different between COM, ATR, AML, HYD, and VEH rats (Figure 3A). However, Mn-SOD activity was significantly higher only in COM rats than in VEH rats, and was not different between ATR, AML, HYD, and VEH rats (Figure 3B).

In the hippocampus, Cu/Zn-SOD activity was not different between COM, ATR, AML, HYD, and VEH rats (Figure 3C). Mn-SOD activity was significantly higher in COM rats than in AML, HYD, and VEH rats to a greater extent than in ATR rats (Figure 3D).

**Morris Water Maze Test**
In the hidden platform test, the escape latency was significantly lower in ATR rats than in AML, HYD, and VEH rats, and the performance was significantly improved in COM rats to a greater extent than in ATR rats (Figure 4A). In the probe
Figure 2.  (A) TBARS levels in the RVLM of each group (n=5 for each).  (B) NAD (P) H oxidase activity in the RVLM of each group (n=5 for each).  (C) TBARS levels in the hippocampus of each group (n=5 for each).  (D) NAD (P) H oxidase activity in the hippocampus of each group (n=5 for each).  TBARS, thiobarbituric acid-reactive substances; RVLM, rostral ventrolateral medulla; NAD (P) H, nicotinamide adenine dinucleotide phosphate; VEH, vehicle; ATR, atorvastatin; AML, amlodipine; COM, combination of atorvastatin and amlodipine; HYD, hydralazine.  *P<0.05 vs. VEH.

Figure 3.  (A) Cu/Zn-SOD activity in the RVLM of each group (n=5 for each).  (B) Mn-SOD activity in the RVLM of each group (n=5 for each).  (C) Cu/Zn-SOD activity in the hippocampus of each group (n=5 for each).  (D) Mn-SOD activity in the hippocampus of each group (n=5 for each).  Cu/Zn-SOD, Cu/Zn-superoxide dismutase; RVLM, rostral ventrolateral medulla; Mn-SOD, Mn-superoxide dismutase; VEH, vehicle; ATR, atorvastatin; AML, amlodipine; COM, combination of atorvastatin and amlodipine; HYD, hydralazine.  *P<0.05 vs. VEH, **P<0.05 in COM vs. ATR.
test, COM rats spent significantly more time in the target quadrant as compared with ATR rats (Figure 4B). In the visible platform test, there were no significant differences in the escape latency among all of the groups.

**Discussion**

In the present study, we have demonstrated 2 major findings. First, a combination of atorvastatin and amlodipine has a sympathoinhibitory effect via an antioxidant through the inhibition of NAD (P)H oxidase and activation of Mn-SOD in the RVLM of SHRSPs. Second, a combination of atorvastatin and amlodipine has a protective effect on the cognitive function via an antioxidant through the activation of Mn-SOD in the hippocampus of SHRSPs. These results suggest that the combination therapy of atorvastatin and amlodipine has a potential to be a novel treatment for hypertension with sympathoinhibition and protection against cognitive dysfunction.

In the present study, although atorvastatin or amlodipine alone does not cause sympathoinhibition and a reduction of oxidative stress in the RVLM, a combination of atorvastatin and amlodipine causes sympathoinhibition via reduction of oxidative stress through the inhibition of NAD (P)H oxidase and activation of Mn-SOD in the RVLM. Previous studies have demonstrated that atorvastatin inhibits NAD (P)H oxidase activity in various organs, and that statins activate Cu/Zn-SOD in the vasculature. We also have demonstrated that atorvastatin causes the sympathoinhibition via reduction of oxidative stress through the inhibition of NAD (P)H oxidase and activation of Mn-SOD in the RVLM. Previous studies have demonstrated that atorvastatin inhibits NAD (P)H oxidase and activation of Mn-SOD in the RVLM. We consider that the combination of atorvastatin and amlodipine has the potential to be a novel treatment for hypertension with sympathoinhibition and protection against cognitive dysfunction.

In the present study, although atorvastatin or amlodipine alone does not cause sympathoinhibition and a reduction of oxidative stress in the RVLM, a combination of atorvastatin and amlodipine causes sympathoinhibition via reduction of oxidative stress through the inhibition of NAD (P)H oxidase and activation of Mn-SOD in the RVLM. We consider that the combination of atorvastatin and amlodipine has the potential to be a new sympathoinhibitory therapy for hypertensive patients via reduction of oxidative stress in the RVLM.

A previous report suggests that atorvastatin improves the cognitive dysfunction of humans. Furthermore, there are many reports indicating the beneficial effects of calcium channel blockers on cognitive function. In SHRSP, amlodipine, lercanidipine, and azelnidipine have the protective effects on neuronal damage, and the effects are also not class-effects of calcium channel blockers. In the present study, atorvastatin alone improves the cognitive dysfunction of SHRSPs, whereas amlodipine alone does not have the benefit on cognitive function. Moreover, the combination of atorvastatin and amlodipine also reduces oxidative stress via up-regulation of Mn-SOD in the hippocampus. We consider that the combination of atorvastatin and amlodipine has the potential to be reasonable therapy with the protection for cognition in hypertensive patients.

The mechanisms in which the combination of atorvastatin and amlodipine has the beneficial effects on SNS activation and cognitive dysfunction in SHRSPs should be discussed. We have previously demonstrated that both atorvastatin and amlodipine have the sympathoinhibitory effect via reduction of oxidative stress in the RVLM of SHRSPs. The different mechanisms...
of atorvastatin and amlodipine could be related to the direct actions of the superoxide-related enzyme systems, because they belong to a different classes of drugs. Previous studies have demonstrated that atorvastatin directly reduces NAD (P) H oxidase activity by inhibition of Rac1 membrane translocation, and activates Mn-SOD indirectly due to the upregulation of nitric oxide synthase via a small G protein Rho isoform, and that amlodipine directly reduces NAD (P) H oxidase activity by inhibition of p22phox and activates Mn-SOD due to the upregulation of nitric oxide synthase phosphorylation. We consider that the results in combination of atorvastatin and amlodipine are due to the addition of these different mechanisms. The results in each mono-therapy group would not be “not” effective, but “not significant”, because each drug is administered in a low dose and, in part, the number of rats in each group was relatively low. Furthermore, previous studies have demonstrated that NAD (P) H oxidase is activated by an angiotensin II type 1 receptor and Mn-SOD is activated by nitric oxide. Although there is no evidence to indicate the direct interaction and common upper stream between NAD (P) H oxidase and Mn-SOD activity, NAD (P) H oxidase-produced superoxide decreased nitric oxide availability, which resulted in the inhibition of Mn-SOD indirectly. Interestingly, in the hippocampus, atorvastatin alone affected only Mn-SOD, not TBARS levels, NADPH oxidase activity, and CuZn-SOD. In the brain, superoxide is produced mainly by an angiotensin II type 1 receptor-NAD (P) H oxidase, and is reduced mainly by CuZn-SOD and Mn-SOD. However, Mn-SOD is not the only antioxidant factor in the brain, such as glutathione peroxidase and/or catalase. Although we cannot exclude the possibility that such superoxide-scavenging enzymes might affect TBARS levels in the hippocampus, we consider that the upregulation of Mn-SOD by atorvastatin alone is not sufficient to reduce oxidative stress in the hippocampus. We consider that the pathway in the production of oxidative stress via NAD (P) H oxidase and SOD might be different between the RVLM and hippocampus. Further studies are necessary to investigate the pharmacological effects of atorvastatin and amlodipine.

In the present study, we used only atorvastatin as a statin, and only amlodipine as a calcium channel blocker in each single dose. There are controversial reports about using other statins with regard to the effects on cognitive function in humans. Previous studies have demonstrated that the beneficial effects on cognitive function are observed with the use of nifedipine, but not with amlodipine. Although we previously demonstrated that amlodipine causes sympathoinhibition via reduction of oxidative stress through the inhibition of NAD (P) H oxidase and upregulation of Cu/Zn-SOD and Mn-SOD in the RVLM of SHRSPs, the combination of atorvastatin and amlodipine activated Mn-SOD in the RVLM and hippocampus, but not CuZn-SOD. The results obtained in the present study could not determine whether the beneficial effects in the present study are class-effects or were dose-dependent. Further pharmacological studies are necessary.

Clinical Implications

In the treatment for cardiovascular diseases, the combination of atorvastatin and amlodipine emerged to be even more effective than the single drugs alone in reducing blood pressure and in improving lipid profile. Moreover, it has been suggested that single-pill amlodipine/atorvastatin (Caduet) treatment improves cardiovascular events to a greater extent than co-administered antihypertensive and lipid-lowering therapy due to its high adherence. Atorvastatin or amlodipine is widely used in a world among statins or calcium channel blockers. The results obtained in the present study could have a novel clinical implication in the treatments for hypertension because the combination of atorvastatin and amlodipine has the potential to improve the SNS activation and cognitive dysfunction in patients with hypertension.

Study Limitations

There are some limitations in the present study. First, with regard to sympathoinhibition, we examined the oxidative stress via NAD (P) H oxidase and SOD only in the RVLM. As well as the RVLM, there are some important nuclear involved in cardiovascular control, such as nucleus tractus solitarii (NTS) or the hypothalamus. Among them, we focused on the RVLM because RVLM is the most important cite in the regulation of SNS activation. Second, we did not examine the renin-angiotensin system in the RVLM or hippocampus. In the RVLM, oxidative stress is mainly produced by an angiotensin II type 1 receptor, and many previous studies have demonstrated that the renin-angiotensin system in the brain regulates SNS activation, and that in the hippocampus is important for cognitive function. However, the aim of the present study was to determine the effects of the combination of atorvastatin and amlodipine. Third, we could not demonstrate that atorvastatin and/or amlodipine penetrates the blood-brain barrier to reach the RVLM and hippocampus. We must investigate the measurement of the dose of atorvastatin and amlodipine in the brain in a future study.

Conclusions

In the present study, we have demonstrated that a combination of atorvastatin and amlodipine has protective effects on the SNS activation and cognitive function via antioxidants in the RVLM and hippocampus of SHRSPs. These results suggest that the combination therapy of atorvastatin and amlodipine has a potential to be a novel treatment for hypertension with sympathoinhibition and protection against cognitive dysfunction.

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Conflict of Interest: None.

Disclosures


References


