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Effects of Atorvastatin, Amlodipine, and Their Combination on Vascular Dysfunction in Insulin-Resistant Rats

Tomio Okamura[1,*], Masashi Tawa[1], Ayman Geddawy[1,#], Takashi Shimosato[1], Hirotaka Iwasaki[1], Haruo Shintaku[2], Yuichi Yoshida[3], Masahiro Masada[3], Kazuya Shinozaki[1], and Takeshi Imamura[1]

[1]*Department of Pharmacology, Shiga University of Medical Science, Otsu 520-2192, Japan*
[2]*Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan*
[3]*Laboratory of Biochemistry, Faculty of Horticulture, Chiba University, Matsudo 271-8510, Japan*

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Abstract. Deficiency of tetrahydrobiopterin (BH_4) in the vascular tissue contributes to endothelial dysfunction through reduced eNOS activity and increased superoxide anion (O_2^-) generation in the insulin-resistant state. We investigated the effects of atorvastatin, a 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor; amlodipine, a calcium antagonist; and their combination on blood pressure, arterial relaxation and contraction, and vascular oxidative stress in aortas of high fructose–fed rats. Oral administration of atorvastatin for 8 weeks did not significantly lower blood pressure, but normalized angiotensin II–induced vasoconstriction and endothelial function in the fructose-fed rats. Atorvastatin treatment of fructose-fed rats increased vascular BH_4 content, which was associated with an increase in endothelial NO synthase activity as well as a reduction in endothelial O_2^- production. On the other hand, administration of amlodipine did not affect the angiotensin II–induced vasoconstriction and endothelial function, but normalized the elevated blood pressure in the fructose-fed rats. The combined treatment did not show synergistic but additive beneficial effects. The present study suggests that combined therapy of HMG-CoA reductase inhibitors and calcium antagonists prevents functional vascular disorders in the insulin-resistant state, possibly resulting in the protection against or delay of development of hypertension, vascular dysfunction in diabetes, and thereafter atherosclerosis.

Keywords: 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitor, calcium antagonist, nitric oxide, tetrahydrobiopterin, insulin resistance

Introduction

The insulin-resistant state is commonly observed prior to essential hypertension (1), hyperlipidemia, and diabetes; and each lifestyle-related disease is an established risk factor for coronary artery disease (2) and atherosclerosis (3). To protect against or delay such vascular disorders, the insulin-resistant state is an appropriate target for the medical treatment. The insulin-resistant state is generally accompanied with elevation of blood pressure and plasma levels of insulin, cholesterol, and triglyceride. At the vasculatures, endothelial dysfunction, and increase in oxidative stress due to imbalance of nitric oxide (NO) and superoxide anion (O_2^-) generation have been found (4). We have reported that insulin resistance may be a pathogenic factor for endothelial dysfunction through impaired endothelial nitric oxide synthase (eNOS) activity caused by the enhanced formation of superoxide anion due to uncoupled eNOS (5), which is caused by relative deficiency of tetrahydrobiopterin (BH_4) in vascular endothelial cells. Increase in BH_4 at vascular tissues by oral supplementation of BH_4 (6), by a gene transfer of GTP cyclohydrolase (CH) 1 cDNA (7), or by treatment with statins (8) increased eNOS activity as well as decreased in endothelial O_2^- production, and restored the impaired endothelium-dependent arterial relaxation in insulin-resistant rats. However, these treatments to

*Corresponding author. okamura@belle.shiga-med.ac.jp
#Present address: Department of Pharmacology, Faculty of Medicine, Minia University, Egypt
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restore endothelial dysfunction failed to normalize blood pressure in the insulin-resistant rats (6 – 8). The remaining high blood pressure after these treatments may pull the trigger on the development of atherosclerosis in the future.

Calcium antagonists, effective anti-hypertensive agents, are known to induce vasodilatation by inhibition of L-type calcium channels located at vascular smooth muscle cell membranes, and some of them were reported to increase eNOS activity (9, 10). Therefore, combination therapy of statins and calcium antagonists may induce synergistic actions on vascular disorders in the lifestyle-related diseases.

Recently, combination therapy of atorvastatin and amlodipine has been extensively studied in cardiovascular disorders in vivo (11 – 15), and its effectiveness has been reported to have advantages compared with mono-therapy in patients with coronary heart disease (12) and hypertension (13 – 15), but the vascular mechanisms of their actions have not been well analyzed.

In the present study, we therefore compared the vascular effect of atorvastatin, amlodipine, and their combination in the rats fed high levels of fructose (fructose-fed rats), an acquired animal model of insulin-resistance, in order to elucidate the underlying mechanisms for the ameliorative effects of these treatments.

Materials and Methods

Materials

The following materials were purchased from the company shown in parentheses: acetylcholine (ACh; Daiichi-Sankyo, Tokyo), superoxide dismutase (SOD), L-phenylephrine and indomethacin (Sigma, St. Louis, MO, USA), sodium nitroprusside (SNP; Nacalai Tesque, Kyoto), calcium ionophore A23187 (Boehringer Ingelheim, Ingelheim, Germany), papaverine hydrochloride (Nichi-iko, Toyama), N^G-nitro-L-arginine (L-NA) and angiotensin II (Ang II) (Peptide Institute, Minoh), L-arginine (Kanto Chemical, Tokyo).

Experimental animals

The Animal Care and Use Committee at Shiga University of Medical Science approved the use of rat aortas in accordance with the experimental protocols of this study. Five-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka) were housed in an environmentally controlled room with a 12-h light/dark cycle and free access to a laboratory diet and water. The animals were divided into eight groups and fed ad libitum on one of the following diets for eight weeks: 1) standard chow (Control), 2) standard chow supplemented with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ amlodipine (AM), 3) standard chow supplemented with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ atorvastatin (AT), 4) standard chow supplemented with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ amlodipine and 10 $mg \cdot kg^{-1} \cdot d^{-1}$ atorvastatin (AM + AT), 5) a diet high in fructose (F), 6) a diet high in fructose with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ amlodipine (F + AM), 7) a diet high in fructose with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ atorvastatin (F + AT), 8) a diet high in fructose with 10 $mg \cdot kg^{-1} \cdot d^{-1}$ amlodipine and 10 $mg \cdot kg^{-1} \cdot d^{-1}$ atorvastatin (F + AM + AT). The normal chow (Oriental Yeast) consisted of 58% carbohydrate (no fructose), 12% fat, and 30% protein (N/N). The high-fructose diet (Oriental Yeast) consisted 67% carbohydrate (of which 98% was fructose), 13% fat, and 20% protein by energy percent. The high fructose–fed rats were used as an animal model for the common type of insulin resistance with endogenous hyperinsulinemia (16).

At the end of the 8-week feeding, food was not given for 12 h, and the animals were weighed and anesthetized intraperitoneally by injection of sodium pentobarbital (50 mg/kg). Venous blood was taken for measurement of plasma glucose, insulin, total cholesterol, and triglyceride. Aortas were removed for measurement of eNOS and NADPH oxidase activity, O_2^- production, biopterin content, GTPCH1 activity, and protein content.

Measurement of blood pressure

Blood pressure was measured the day before the experiment, and the rats were trained to the apparatus three times before measurement. Systolic blood pressure in the tail region was measured using an electrosphygmomanometer after the rats were pre-warmed for 15 min.

Isometric tension studies

The animals were administered an intraperitoneal injection of sufficient sodium pentobarbital for anesthesia and intravenous injection of sufficient heparin before they were killed. The thoracic aorta (0.6 – 0.8-cm outside diameter) was isolated and cut into strips with special care to preserve the endothelium. The specimens were suspended in organ chambers (10-ml capacity) and the resting tension was adjusted to 2.0 g, which is optimal for inducing the maximal contraction, as previously described (17). Isometric contractions and relaxations of aortic strips were recorded. Firstly, the contractile response to KCl (30 mM) was obtained and this was taken as 100% contraction. To prevent synthesis of prostaglandins, the following experiments were performed in the presence of 10 μM indomethacin. The strips were exposed to cumulative concentration of L-phenylephrine (10^{-9} to 10^{-6} M). Ang II (10^{-9} to 10^{-6} M) was added directly to the bathing media at a single concentration to avoid the development of tachyphylaxis. In another series of experiments, the strips were partially precontracted with L-phenylephrine ($1 - 3 \times 10^{-7}$ M).

After a plateau was attained, the strips were exposed to ACh (10^{-9} to 10^{-5} M), the calcium ionophore A23187 (10^{-9} to 10^{-7} M), or SNP (10^{-11} to 10^{-6} M) to construct dose–response curves. At the end of each experiment, 100 μM papaverine was added to induce maximal relaxation, which was taken as 100% for relaxation induced by agonists. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Endothelium removal was verified by abolition or marked suppression of the relaxations caused by 10^{-6} M ACh. The effect of SOD (200 U/ml) was also examined in some strips with endothelium.

Measurement of NO synthase activity in aortic endothelial cells

eNOS activity was measured by the conversion of L-(^{3}H)arginine to L-(^{3}H)citrulline as previously described (18).

Measurement of ex vivo aortic O_2^- production and NADPH oxidase activity

O_2^- production in aortic segments was measured using the lucigenin-enhanced chemiluminescence method as described previously (19). Lucigenin counts were expressed as counts per minute per milligram of dry weight of vessel. More than 80% of the chemiluminescence was inhibited by the pretreatment of arterial segments with 100 U/ml SOD.

To measure the vascular NADPH oxidase activity, the vessel was homogenized in 400 μl homogenizing buffer (50 mM phosphate buffer and 0.01 mM EDTA, pH 7.8), and the homogenate was centrifuged at 1,000 × *g* for 10 min. A 20-μl aliquot of the supernatant was then added to glass scintillation vials containing lucigenin (50 μM) in 2 ml homogenizing buffer and the chemiluminescence value was measured. NADPH (500 μM) was added to the incubation medium as a substrate for O_2^- production.

Measurements of pteridine derivatives levels and GTP-CH1 activity

Aortic tissues were homogenized in 25 mM triethanolamine-HCl Measurements of biopterin content were performed by high-performance liquid chromatography (HPLC) analysis as previously described (6, 20, 21). GTP-CH1 activity was assayed using the HPLC method by measurement of neopterin, which was released from dihydroneopterin triphosphate after oxidation and phosphatase treatment (22).

Protein assay

Protein content was determined by the method of Bradford (23) with bovine serum albumin as a standard.

Statistical analysis

The results are expressed as the mean ± S.E.M. Statistical analyses were made by Student's *t*-test for two groups and Tukey's method after one-way analysis of variance (ANOVA) for more than three groups. *P*-values less than 0.05 were considered to be significant.

Results

Metabolic characteristics and blood pressure of the rats

As shown in Table 1, all 8 groups gained weight to a similar extent over the study period without any significant difference. Blood glucose levels also did not differ among the groups. On the other hand, plasma insulin levels were significantly higher in the fructose-fed rats than those in the rats fed the standard chow. Fructose-fed rats also showed a significant elevation of plasma total cholesterol and triglyceride levels and blood pressure compared with normal diet–fed rats. These characteristics of fructose-fed rats used in the present study were almost the same as those in the fructose-fed insulin-resistant rats in the previous study (5). Treatment with amolodipine and/or atorvastatin did not affect the insulin levels among the rats fed either the normal or high-fructose chow. Amlodipine did not affect the lipid levels in either rats receiving the normal chow or rats receiving the high-fructose chow. Atorvastatin significantly lowered plasma cholesterol and triglyceride levels in the fructose-fed rats, but did not affect them in the rats fed the standard chow. These plasma lipid levels in the fructose-fed rats treated with both amlodipine and atorvastatin were significantly lower than those in fructose-fed rats treated with amlodipine alone.

Atorvastatin did not significantly affect blood pressure in either the rats fed the standard chow or those fed the high fructose chow. Amlodipine significantly lowered blood pressure in the fructose-fed rats, but did not affect that in the control rats. Blood pressure in the fructose-fed rats treated with both amlodipine and atorvastatin was significantly lower than that in fructose-fed rats treated with atorvastatin alone.

NO-dependent vascular relaxation in rat aorta

The addition of ACh at concentrations of 10^{-9} to 10^{-5} M produced a dose-dependent relaxation in aortic strips with endothelium, which was abolished by treatment with NO synthase inhibitors (data not shown) or removal of the endothelium (Fig. 1A). The ACh-induced relaxation was significantly reduced in the fructose-fed rats compared with that in control rats. The addition of SOD restored the reduced relaxation caused by ACh in the fructose-fed rats, which was not significantly different from that in the control rats (Fig. 1A).

Table 1. Metabolic characteristics and blood pressure in the rats

	Control	AM	AT	AM + AT
Body weight (g)	492 ± 9	489 ± 10	490 ± 7	484 ± 7
Glucose (mg/dl)	94.6 ± 2.3	95.3 ± 2.4	94.3 ± 3.0	94.3 ± 3.5
Insulin (pg/ml)	568 ± 28	549 ± 27	552 ± 35	498 ± 32
Total cholesterol (mg/dl)	51.5 ± 3.7	52.0 ± 4.5	49.8 ± 3.8	48.8 ± 5.1
Triglycerides (mg/dl)	63.4 ± 2.3	66.9 ± 4.9	64.3 ± 9.5	66.4 ± 6.5
Systolic blood pressure (mmHg)	120 ± 4	120 ± 5	122 ± 4	121 ± 3
	F	F + AM	F + AT	F + AM + AT
Body weight (g)	494 ± 6	488 ± 12	491 ± 11	502 ± 13
Glucose (mg/dl)	97.3 ± 2.9	93.7 ± 3.1	92.4 ± 2.8	91.4 ± 2.2
Insulin (pg/ml)	1601 ± 62*	1450 ± 48*	1542 ± 72*	1430 ± 60*
Total cholesterol (mg/dl)	82.3 ± 3.8*	79.3 ± 4.5*	58.6 ± 3.0$^{\#,\P}$	56.2 ± 2.5$^{\#,\P}$
Triglyceride (mg/dl)	169.4 ± 6.3*	171.7 ± 6.3*	91.4 ± 4.3$^{\#,\P}$	90.5 ± 5.1$^{\#,\P}$
Systolic blood pressure (mmHg)	142 ± 5*	119 ± 3$^{\#}$	129 ± 6	118 ± 4$^{\#}$

Rats were fed the normal or high-fructose diet with or without drug for 8 weeks. After 8 weeks, body weight measurement and blood sampling were performed. Blood pressure was measured on the day before the experiment. AM, amlodipine (10 mg·kg^{-1}·d^{-1}); AT, atorvastatin (10 mg·kg^{-1}·d^{-1}); F, High-fructose diet. n = 8 for each group, *$P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. F, $^{\P}P < 0.05$ vs. F + AM.

Treatment with amlodipine did not affect the ACh-induced relaxation in the fructose-fed rats, whereas treatment with atorvastatin significantly potentiated the endothelium-dependent relaxation. Combined treatment with amlodipine and atorvastatin also significantly potentiated the relaxation in the fructose-fed rats (Fig. 1B). Similar results were also obtained for the relaxation induced by A23187, a receptor-independent endothelium-derived relaxing factors–releasing substance (Fig. 1C). On the other hand, vasodilator responses to SNP were comparable among the 5 groups. Amlodipine treatment tended to potentiate the relaxation induced by SNP, but the difference was statistically insignificant (Fig. 1D).

Vascular reactivity in response to L-phenylephrine and Ang II

The contractile responses of aortic strips to L-phenylephrine and Ang II are shown in Fig. 2. The contraction induced by L-phenylephrine in the fructose-fed rats was similar to that in control rats. By contrast, contraction in response to Ang II was markedly increased in fructose-fed rats compared with that in control rats. Treatment with amlodipine did not affect the aortic contraction induced by Ang II or phenylephrine, whereas treatment with atorvastatin caused a lesser contractile response to Ang II, but not to L-phenylephrine, in fructose-fed rats. Combined treatment with amlodipine and atrovastatin also caused similar results in fructose-fed rats.

O_2^- production from aortic segments

As shown in Table 2, basal O_2^- production by endothelium-intact aortic segments from fructose-fed rats were twofold higher than that of control rats. Endothelial removal produced a slight reduction of O_2^- levels in vessels from control rats, while marked reduction of O_2^- production was found in endothelium-denuded vessels from fructose-fed rats. Thus, after removal of the endothelium, the O_2^- production rates between the two groups were no longer different. The O_2^- production was significantly increased by ACh in all groups and the increase was significantly greater in the fructose-fed rats than in the control rats. Treatment with amlodipine did not affect the O_2^- production in the fructose-fed rats, whereas treatment with atorvastatin significantly decreased both basal and ACh-stimulated O_2^- production in the fructose-fed rats. Combined treatment with amlodipine and atorvastatin also significantly reduced the O_2^- production in the fructose-fed rats. The increase of O_2^- production in the fructose-fed rats was abolished, resulting in basal-level production after incubation with L-NA.

Activity of eNOS and NADPH oxidase in the aorta

Fructose-fed rats (23.9 ± 2.5 pmol^{-1}·min^{-1}·mg protein^{-1}, n = 6) showed significant reduction of eNOS activity compared with control rats (64.4 ± 3.3 pmol^{-1}·min^{-1}·mg protein^{-1}, n = 6). Administration of atorvastatin to the fructose-fed rats significantly elevated the enzyme activity (49.7 ± 2.6 pmol^{-1}·min^{-1}·mg protein^{-1}), whereas that of amlodipine did not influence the enzyme activity (26.2 ± 3.5 pmol^{-1}·min^{-1}·mg protein^{-1}). Combined treatment with amlodipine and atorvastatin also significantly increased the enzyme activity (48.5 ± 4.3 pmol^{-1}·min^{-1}·mg protein^{-1}).

Consistent with increased O_2^- production under the ex

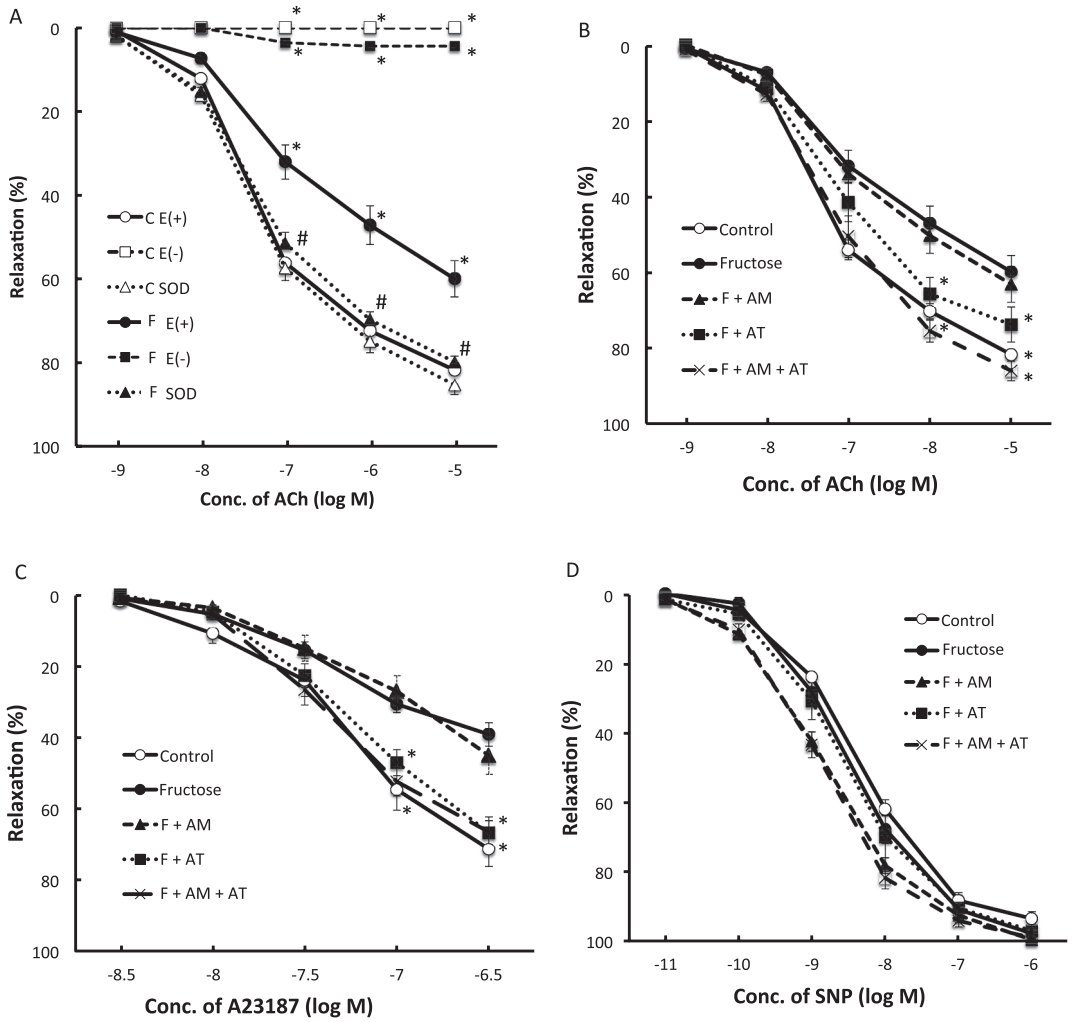

Fig. 1. Relaxant responses of rat aortic strips. Aortic segments were isolated and their functions were assessed in organ chamber experiments. Data are expressed as means ± S.E.M. (n = 8). A: Effect of acetylcholine (ACh) in the aorta with [E(+)] or without [E(−)] endothelium of control (C, normal diet) and fructose-fed (F) rats. The experiments were also performed in the presence of superoxide dismutase (SOD, 200 U/ml). B: Comparison of ACh-induced relaxations among the various groups. Control, normal diet–fed rat; Fructose (F), high fructose diet–fed rat; AM, amlodipine; AT, atorvastatin). C: Comparison of A23187-induced relaxations among the various groups. Control, normal diet–fed rat; Fructose (F), high fructose diet–fed rat; AM, amlodipine; AT, atorvastatin. D: Comparison of sodium nitroprusside (SNP)-induced relaxations among various groups. Control, normal diet–fed rat; Fructose (F), high fructose diet–fed rat; AM, amlodipine; AT, atorvastatin. A: $^{*}P < 0.05$ vs. C E(+), $^{\#}P < 0.05$ vs. F E(+); B – D: $^{*}P < 0.05$ vs. Fructose.

vivo condition, O_2^- production derived from NADPH oxidase (n = 5, per group) in aortic homogenates from the fructose-fed rats (21723 ± 2192 cpm/mg protein) was 1.6-fold higher ($P < 0.05$) than those from control rats (13282 ± 1226 cpm/mg protein). Treatment with amlodipine had no effect on the NADPH oxidase activity (19102 ± 1303 cpm/mg protein), while treatment with atorvastatin significantly reduced the enzyme activity (12977 ± 1385 cpm/mg protein, $P < 0.05$). Combined treatment with amlodipine and atorvastatin also reduced the enzyme activity significantly (13299 ± 1908 cpm/mg protein, $P < 0.05$).

Biopterin content and GTP cyclohydrolase I activity in the aorta

The BH_4 content of aorta in the fructose-fed rats was significantly lower than that in control rats (Fig. 3A). In contrast, fructose-fed rats showed 2.8-fold increase of 7,8-BH_2 + biopterin in the aorta compared with control rats (Fig. 3B). The ratio of BH_4 to 7,8-BH_2 + biopterin was significantly lower in the fructose-fed rats than that in control rats (Fig. 3C). The fructose-fed rats treated with amlodipine did not show significant changes in these values compared with the fructose-fed rats without the treatment. On the other hand, the atorvastatin-treated

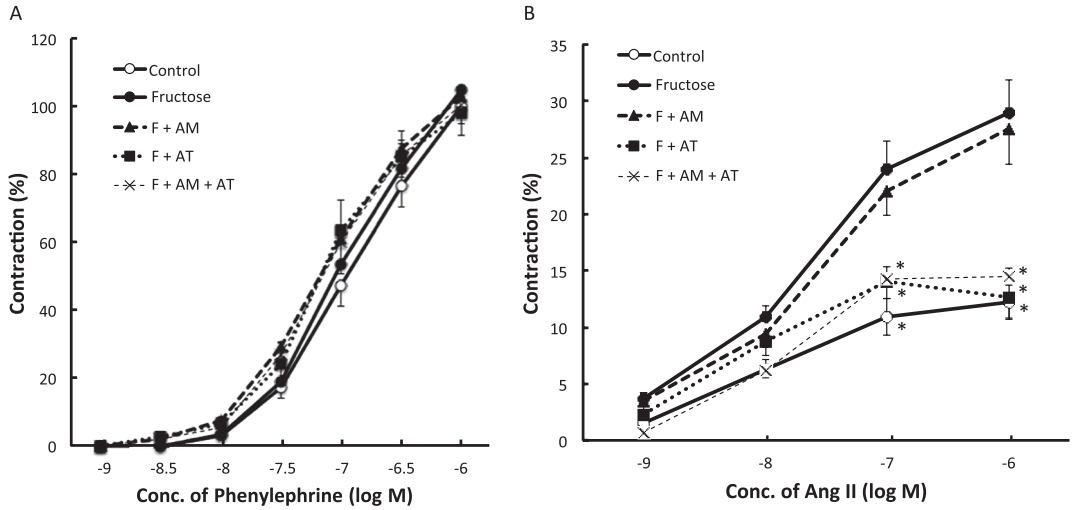

Fig. 2. Contractile responses of rat aortic strips. Aortic segments were isolated and their functions were assessed in organ chamber experiments. Data are expressed as means ± S.E.M. (n = 8). A: Comparison of phenylephrine-induced contractions among various groups. Control, normal diet–fed rat; Fructose (F), high fructose diet–fed rat; AM, amlodipine; AT, atorvastatin. B: Comparison of angiotensin II (Ang II)-induced contractions among various groups. Control, normal diet–fed rat; Fructose (F), high fructose diet–fed rat; AM, amlodipine; AT, atorvastatin. *$P < 0.05$ vs. Fructose.

Table 2. Effects of AM, AT, and AM + AT treatments on superoxide anion production in vascular segments

Endothelium	Incubation condition	Vascular superoxide production (cpm × 10^3 / mg dry tissue weight)				
		Control	Fructose (F)	F + AM	F + AT	F + AM + AT
–	Buffer alone	15.1 ± 1.0	22.2 ± 1.4*	24.1 ± 1.0	19.2 ± 1.1	22.5 ± 1.2
+	Buffer alone	23.5 ± 1.3	41.6 ± 1.8*	43.4 ± 2.7	29.8 ± 1.9$^{\#}$	30.7 ± 1.8$^{\#,\P}$
+	ACh	31.9 ± 2.8	123.1 ± 5.9*	131.4 ± 4.0	49.1 ± 2.4$^{\#}$	44.6 ± 1.3$^{\#,\P}$
+	ACh + L-NA	23.9 ± 1.3	46.1 ± 4.1*	39.4 ± 2.1	24.3 ± 1.2$^{\#}$	28.9 ± 2.4$^{\#,\P}$

Rats were fed the normal or high-fructose diet with or without drug for 8 weeks. After 8 weeks, the thoracic aorta of each rat was isolated, and O_2^- production in aortic segments was measured using the lucigenin-enhanced chemiluminescence method. AM, amlodipine (10 mg·kg^{-1}·d^{-1}); AT, atorvastatin (10 mg·kg^{-1}·d^{-1}); F, High-fructose diet. n = 8 for each group, *$P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. F, $^{\P}P < 0.05$ vs. F + AM.

fructose-fed rats demonstrated a significant elevation of BH_4 level as compared with control rats, whereas atorvastatin treatment significantly lowered the content of 7,8-BH_2 plus biopterin (Fig. 3: A, B). Combined treatment with amlodipine and atorvastatin also significantly increased the BH_4 content and decreased 7,8-BH_2 plus biopterin compared to those in the fructose-fed rats with or without treatment with amlodipine.

As shown in Fig. 3D, GTP-CH1 activity in the aortas of the fructose-fed rats was significantly lower than that of control rats. The atorvastatin-treated fructose-fed rats showed a significant elevation of the enzyme activity compared to fructose-fed rats, whereas the activity in the amlodipine-treated fructose-fed rats was not affected. Combined treatment with amlodipine and atorvastatin also showed a significant elevation of the enzyme activity compared to that in the fructose-fed rats with or without treatment with amlodipine.

Discussion

Oral administration of atorvastatin combined with amlodipine effectively prevented both endothelial dysfunction and elevation of blood pressure observed in the insulin-resistant rats. The administration of atorvastatin alone prevented the endothelial impairment, but did not significantly prevent the blood pressure elevation, whereas the administration of amlodipine alone prevented the elevation of blood pressure, but not the impairment of endothelial function in the insulin-resistant rats.

The mechanism underlying the prevention of endothelial dysfunction by atorvastatin is due in part to the inhibition of NADPH oxidase activity and uncoupled eNOS-dependent O_2^- production. Indeed, we found that atorvastatin treatment enhances eNOS activity (L-citrulline formation) in vivo. We think that enhancement

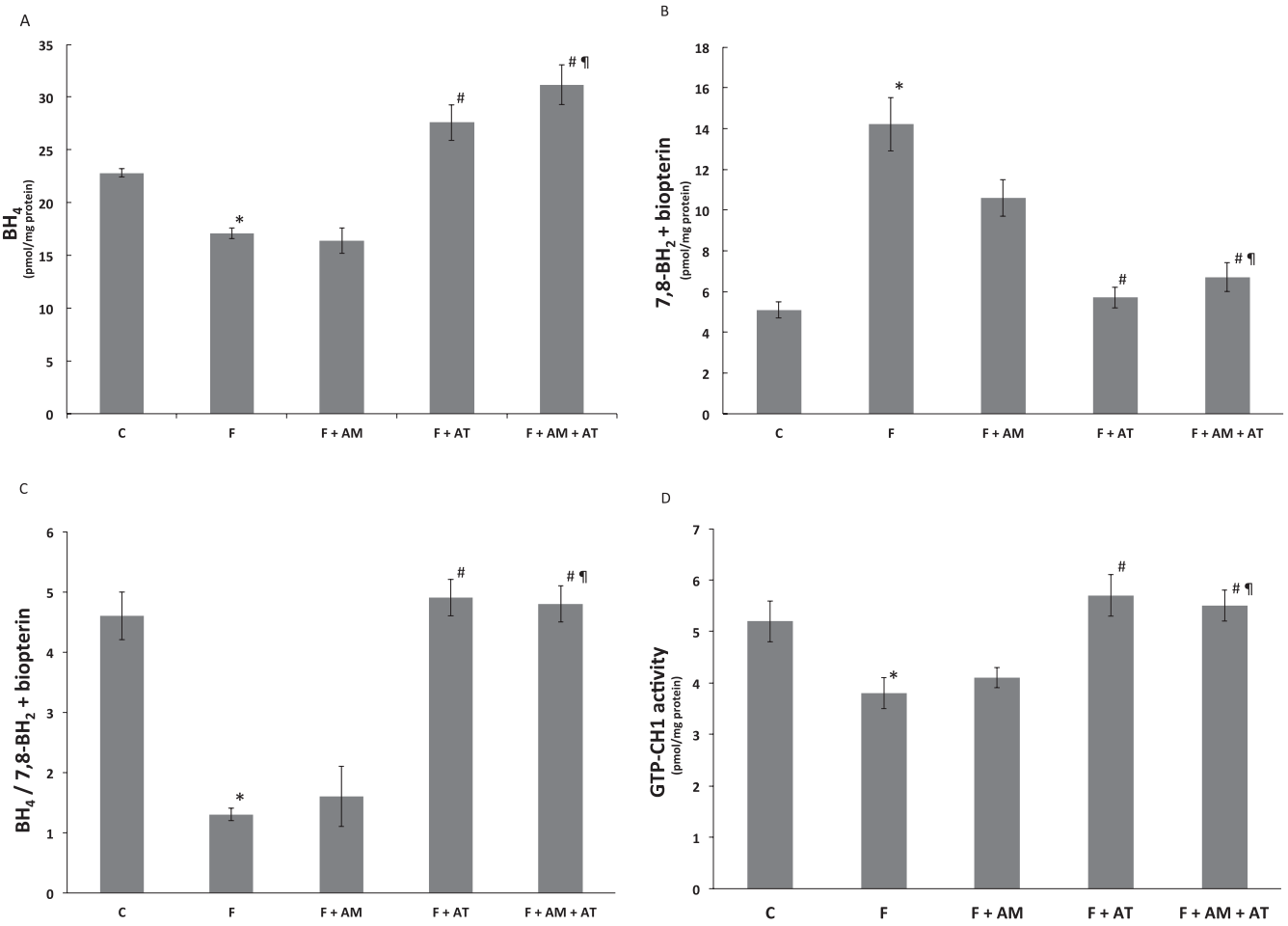

Fig. 3. Pteridine derivative levels and GTP cyclohydrolase I (GTP-CH1) activity in aortic tissue. Tissue levels of tetrahydrobiopterine (BH_4) (A) and 7,8-dihydrobiopterine (BH_2) + biopterin (B), the ratio of BH_4 to 7,8-BH_2 plus biopterin (C), and GTP-CH1 activity (D) were compared among the various groups. C, Control rats; F, Fructose-fed rats; AM, amlodipine; AT, atorvastatin. Data are expressed as means ± S.E.M. (n = 5). $^{*}P < 0.05$ vs. C, $^{\#}P < 0.05$ vs. F, $^{\P}P < 0.05$ vs. F + AM.

of eNOS activity by statin is one of the initial and fundamental steps for the observed beneficial effects of statin treatment in the insulin-resistant state. BH_4 was formerly suggested to be an important allosteric cofactor of NOS via stabilization of dimeric NOS, the active form of the enzyme, and thus may play an essential role in the control of NO and O_2^- production in vivo (24). BH_4 insufficiency leads to the uncoupled state of eNOS, resulting in decreased NO production and increased formation of oxygen radicals by NOS in vitro (25, 26). The excess amount of O_2^- reacts with NO and further limits the biological activity of eNOS (27). In vascular endothelial cells, BH_4 is synthesized from GTP via a de novo pathway. The rate-limiting enzyme in this pathway is GTP-CH1 (28). Augmented BH_4 biosynthesis in hyperglycemic human aortic endothelial cells by gene transfer of GTP-CH1 restored eNOS activity (29). Zheng et al. (30) performed ex vivo gene transfer of human GTP-CH1 to the arteries of deoxycorticosterone acetate (DOCA)-salt rats and demonstrated an improvement of endothelium-dependent relaxation accompanied by increased tissue BH_4 levels. We also have reported that long-term oral BH_4 supplementation (6) and intramuscular GTP-CH1 gene transfer improve endothelial dysfunction and oxidative stress in the aorta of insulin-resistant rats (7). Thus enhancement of GTP-CH1 activity by atorvastatin treatment in the vascular wall may normalize eNOS activity through augmentation of endothelial BH_4.

Rats fed a diet containing atorvastatin showed a significant increase of vascular BH_4 level as compared with the fructose-fed rats not given this statin, whereas atorvastatin treatment significantly lowered the content of 7,8-BH_2 plus biopterin. Evidence indicates that elevated O_2^- production causes an enhanced oxidation of BH_4 to 7,8-BH_2, which has been shown to inhibit the stimulatory

effects of BH_4 on eNOS (31). It has been reported that addition of 7,8-BH_2 in the presence of 10 μM BH_4 dose-dependently inhibits eNOS activity and stimulates eNOS-mediated O_2^- production (32). Direct effects of statin treatment on arterial redox state and nitric oxide bioavailability via BH_4-mediated eNOS coupling has also been reported in humans (33).

Partial improvement of plasma lipids and blood pressure in the fructose-fed rats treated with atorvastatin is probably associated with restored endothelial function. Atorvastatin treatment of fructose-fed rats normalized Ang II–induced vasoconstriction and endothelium-dependent relaxation induced by ACh and A23187, but did not influence the endothelium-independent relaxation by sodium nitroprusside. Our previous study demonstrated that HMG-CoA reductase inhibitors can specifically downregulate AT_1-receptor expression that was enhanced in insulin-resistant rats (8). The precise mechanism by which AT_1-receptor expression is upregulated in the insulin-resistant state is not clear. It appears that oxidative stress and/or a redox-sensitive transcription factor such as NFκB in the expression of AT_1 receptors may play a role, but this remains to be clarified. In addition, the atorvastatin treatment normalized vascular BH_4 content, and activities of GTP-CH1, NADPH oxidase, and eNOS in the fructose-fed rats. A previous study has demonstrated that manipulation of intracellular BH_4 levels by statin can stimulate an NO-mediated cGMP increase in cultured endothelial cells (34). Furthermore, decreased ACh-induced vasodilation persists in hypertensive patients after treatment with antihypertensive drugs designed to normalize blood pressure (35). These findings suggest that statin can modulate in vivo NO production by improving endothelial dysfunction, but may not be enough to normalize blood pressure.

In contrast, the elevation of blood pressure observed in the fructose-fed rats was effectively prevented by oral administration of amlodipine alone, but this treatment was unable to restore the endothelium-dependent vasodilation induced by ACh or A23187. Decreased vascular BH_4 levels and activities of both GTP-CH1 and eNOS and increased O_2^- generation and NADPH oxidase activity in the vascular wall observed in the fructose-fed rats were unaffected by treatment with amlodipine. Moreover, enhanced vasoconstriction by Ang II were also unaffected by amlodipine treatment. Therefore, the mechanism underlying the prevention of blood pressure elevation caused by amlodipine in the insulin-resistant rats is neither due to improvement of endothelial dysfunction nor down-regulation of AT_1 receptors, but due to inhibition of L-type calcium channels located in the vascular smooth muscle cells. Amlodipine has been reported to increase NO production by stimulation of eNOS mRNA expression (36) or by phosphorylation of eNOS (10). However, in the present study, effects of amlodipine on the endothelial function was not observed in our fructose-fed, insulin-resistant rats. Treatment with amlodipine, rather, tended to potentiate endothelium-independent relaxation by sodium nitroprusside. Calcium-dependent superoxide production in vascular smooth muscle cells may be involved in this phenomenon (37). Beneficial effects of amlodipine mono-therapy in the insulin-resistant state have been demonstrated in the inherited (38) and drug-induced animal models (39). Whether or not the discrepancy in the beneficial effect of amlodipine is due to the nature of animal models of insulin resistance remains to be clarified. Failure of amlodipine to improve endothelium-dependent vasodilation has been reported in a clinical study (40). In the present study, amlodipine treatment did not affect the radical balance between O_2^- and NO in the vascular wall. A similar phenomenon has been reported in hypertensive patients (41).

In conclusion, long-term treatment by atorvastatin together with amlodipine prevented both endothelial dysfunction and elevation of blood pressure observed in the insulin-resistant rats. The combined treatment did not cause potentiating or synergistic effects, but showed additive effects, since it did not interfere with the beneficial effect of each drug. The present study strongly suggests that combined therapy of HMG-CoA reductase inhibitors and calcium antagonists could prevent functional vascular disorders in the insulin-resistant state, possibly resulting in the protection against or delay development of hypertension, vascular dysfunction in diabetes mellitus, and thereafter atherosclerosis.

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Conflicts of Interest

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References

1 Ferrannini E, Buzzigoli, G, Bonadonna R, Giorico MA, Oleggini M, Graziadei I, et al. Insulin resistance in essential hypertension. N Engl J Med. 1987;317:350–357.
2 Zavaroni I, Bonora E, Pagliara M, Dall'Aglio E, Luchetti L, Buonanno G, et al. Risk factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. N Engl J Med. 1989;320:702–706.
3 Laakso M, Sarlund H, Salonen R, Suhonen M, Pyörälä K,

Salonen JT, et al. Asymptomatic atherosclerosis and insulin resistance. Arterioscler Thromb. 1991;11:1068–1076.
4 Wever RM, Lüscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation. 1998;97:108–112.
5 Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, et al. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O2- imbalance in insulin-resistant rat aorta. Diabetes. 1999;48:2437–2445.
6 Shinozaki K, Nishio Y, Okamura T, Yoshida Y, Maegawa H, Kojima H, et al. Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ Res. 2000;87:566–573.
7 Shinozaki K, Nishio Y, Yoshida Y, Koya D, Ayajiki K, Masada M, et al. Supplement of tetrahydrobiopterin by a gene transfer of GTP cyclohydrolase I cDNA improves vascular dysfunction in insulin-resistant rats. J Cardiovasc Pharmacol. 2005;46: 505–512.
8 Shinozaki K, Nishio Y, Ayajiki K, Yoshida Y, Masada M, Kashiwagi A, et al. Pitavastatin restores vascular dysfunction in insulin-resistant state by inhibiting NAD(P)H oxidase activity and uncoupled endothelial nitric oxide synthase-dependent superoxide production. J Cardiovasc Pharmacol. 2007;49: 122–130.
9 Kobayashi M, Kobayashi K, Hara K, Higashi T, Yanaka H, Yagi S, et al. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am J Hypertens. 1999;12:483–491.
10 Lenasi H, Kohistedt K, Fichtischerer B, Mulsch A, Busse R, Fleming I. Amlodipine activates the endothelial nitric oxide synthase by altering phosphorylation on Ser1177 and Thr495. Cardiovasc Res. 2003;59:844–853.
11 Curran MP. Amlodipine/Atorvastatin: a review of its use in the treatment of hypertension and dyslipidaemia and the prevention of cardiovascular disease. Drugs. 2010;70:191–213.
12 Dorval JF, Anderson T, Buithieu J, Chan S, Hutchison S, Huynh T, et al. Reaching recommended lipid and blood pressure targets with amlodipine/atorvastatin combination in patients with coronary heart disease. Am J Cardiol. 2005;95:249–253.
13 Koh KK, Quon MJ, Han SH, Lee Y, Park JB, Kim SJ, et al. Additive beneficial effects of atorvastatin combined with amlodipine in patients with mild-to-moderate hypertension. Int J Cardiol. 2011;146:319–325.
14 Derosa G, Maffioli P. Effects of amlodipine plus atorvastatin association in hypertensive hypercholesterolemic patients. Expert Rev Cardiovasc Ther. 2010;8:835–843.
15 Fogari R, Preti P, Zoppi A, Lazzari P, Corradi L, Fogari E, et al. Effects of amlodipine-atorvastatin combination on inflammation markers and insulin sensitivity in normocholesterolemic obese hypertensive patients. Eur J Clin Pharmacol. 2006;62:817–822.
16 Reaven GM. Insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension. Parallels between human disease and rodent models. Diabetes Care. 1991;14:195–202.
17 Okamura T, Miyazaki M, Inagami T, Toda N. Vascular renin-angiotensin system in two-kidney, one clip hypertensive rats. Hypertension. 1986;8:560–565.
18 Rees D, Ben-Ishay D, Moncada S. Nitric oxide and the regulation of blood pressure in the hypertension- prone and hypertension-resistant Sabra rat. Hypertension. 1996;28:367–371.
19 Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. J Biol Chem. 1998;273:2015–2023.
20 Masada M, Akino M, Sueoka T, Katoh S. Dyspropterin, an intermediate formed from dihydroneopterin triphosphate in the biosynthetic pathway of tetrahydrobiopterin. Biochim Biophys Acta. 1985;18;840:235–244.
21 Kaufman S. Metabolism of the phenylalanine hydroxylation cofactor. J Biol Chem. 1967;242:3934–3943.
22 Masada M, Matsumoto J, Akino M. Biosynthetic pathways of pteridines and their association with phenotypic expression in vitro in normal and neoplastic pigment cells from goldfish. Pigment Cell Res. 1990;3:61–70.
23 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254.
24 Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest. 1997;100:2153–2157.
25 Reif A, Fröhlich LG, Kotsonis P, Frey A, Bömmel HM, Wink DA, et al. Tetrahydrobiopterin inhibits monomerization and is consumed during catalysis in neuronal NO synthase. J Biol Chem. 1999;274:24921–24929.
26 Werner ER, Werner-Felmayer G, Wachter H, Mayer B. Biosynthesis of nitric oxide: dependence on pteridine metabolism. Rev Physiol Biochem Pharmacol. 1996;127:97–135.
27 Kontos HA, Kontos MC. Role of products of univalent reduction of oxygen in hypertensive vascular injury. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. New York: Raven Press; 1995. p. 685–696.
28 Kaufman S, Kapatos G, Rizzo WB, Schulman JD, Tamarkin L, Van Loon GR. Tetrahydropterin therapy for hyperphenylalaninemia caused by defective synthesis of tetrahydrobiopterin. Ann Neurol. 1983;14:308–315.
29 Cai S, Khoo J, Channon KM. Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. Cardiovasc Res. 2005;65:823–831.
30 Zheng JS, Yang XQ, Lookingland KJ, Fink GD, Hesslinger C, Kapatos G, et al. Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. Circulation. 2003;108:1238–1245.
31 Klatt P, Schmid M, Leopold E, Schmidt K, Werner ER, Mayer B. The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. J Biol Chem. 1994;269: 13861–13866.
32 Shinozaki K, Kashiwagi A, Masada M, Okamura T. Molecular mechanisms of impaired endothelial function associated with insulin resistance. Curr Drug Targets Cardiovasc Haematol Disord. 2004;4:1–11.
33 Antoniades C, Bakogiannis C, Leeson P, Guzik TJ, Zhang MH, Tousoulis D, et al. Rapid, direct effects of statin treatment on arterial redox state and nitric oxide bioavailability in human atherosclerosis via tetrahydrobiopterin-mediated endothelial nitric oxide synthase coupling. Circulation. 2011;124:335–345.
34 Hattori Y, Nakanishi N, Akimoto K, Yoshida M, Kasai K. HMG-CoA reductase inhibitor increases GTP cyclohydrolase I mRNA and tetrahydrobiopterin in vascular endothelial cells.

Arterioscler Thromb Vasc Biol. 2003;23:176–182.
35 Panza JA, Quyyumi AA, Callahan TS, Epstein SE. Effect of antihypertensive treatment on endothelium-dependent vascular relaxation in patients with essential hypertension. J Am Coll Cardiol. 1993;21:1145–1151.
36 Kobayashi N, Yanaka H, Tojo A, Kobayashi K, Matsuoka H. Effects of amlodipine on nitric oxide synthase mRNA expression and coronary microcirculation in prolonged nitric oxide blockade-induced hypertensive rats. J Cardiovasc Pharmacol. 1999;34:173–181.
37 Qian J, Chen F, Kovalenkov Y, Pandey D, Moseley MA, Foster MW, et al. Nitric oxide reduces NADPH oxidase 5 (Nox5) activity by reversible S-nitrosylation. Free Radic Biol Med. 2012;52:1806–1819.
38 Sueta D, Nakamura T, Dong YF, Kataoka K, Koibuchi N, Yamamoto E, et al. Amlodipine enhances amelioration of vascular insulin resistance, oxidative stress, and metabolic disorders by candesartan in metabolic syndrome rats. Am J Hypertens. 2012;25:704–710.
39 Srinivasan PS, Hakim ZS, Santani DD, Goyal RK. Effects of chronic treatment with amlodipine in streptozotocin-diabetic and spontaneously hypertensive rats. Pharmacol Res. 1997;35:423–428.
40 Naya M, Tsukamoto T, Morita K, Katoh C, Furumoto T, Fujii S, et al. Olmesartan, but not amlodipine, improves endothelium-dependent coronary dilation in hypertensive patients. J Am Coll Cardiol. 2007;50:1144–1149.
41 Ohtsuka S, Yamazaki A, Oyake Y, Yamaguchi I. Amlodipine improves vascular function in patients with moderate to severe hypertension. J Cardiovasc Pharmacol. 2003;42:296–303.