Original Article

Different Effects of Amlodipine and Enalapril on the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase–Extracellular Signal-Regulated Kinase Pathway for Induction of Vascular Smooth Muscle Cell Differentiation *In Vivo*

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Although recent clinical trials have shown that amlodipine exerts antiatherogenic effects, the mechanism of these effects remains unknown. This study was designed to examine which signal transduction pathway might be important for the antiatherogenic property of amlodipine, as assessed by aortic smooth muscle cell (SMC) phenotypes in hypertension *in vivo*. Stroke-prone spontaneously hypertensive rats (SHRSP) were randomly treated with a vehicle, amlodipine, or enalapril while Wistar-Kyoto rats (WKY) used as controls were treated with only the vehicle. Both drugs were equally effective at reducing systolic blood pressure, and inhibiting the progression of aortic remodeling and fibrosis in comparison to those of vehicle-treated SHRSP. In the aortas of vehicle-treated SHRSP, the level of contractile-type smooth muscle (SM) myosin heavy chain (MHC) SM2 was significantly lower, whereas the level of synthetic-type MHC NMHC-B/SMemb was significantly higher compared with those in the WKY aortas. Compared to the vehicle-treated SHRSP group, both drugs significantly and equally shifted the aortic SMC phenotype in SHRSP toward the differentiated state by reducing NMHC-B/SMemb and increasing SM2. The levels of MKK6, p38 MAPK, MEK1 and p-42/44 ERK were significantly higher in the vehicle-treated SHRSP than in the WKY. Both drugs significantly reduced these values in the SHRSP aorta. Furthermore, the levels of MEK1 and p-42/44 ERK were significantly lower in the amlodipine- than in the enalapril-treated SHRSP group, whereas enalapril was more effective than amlodipine at increasing p-Akt and endothelial NO synthase in SHRSP aortas, which were significantly lower in the vehicle SHRSP group than in the WKY group. Thus, the MEK-ERK pathway might be one of the crucial determinants of the aortic SMC phenotype activated by amlodipine treatment of hypertension *in vivo*. (*Hypertens Res* 2006; 29: 179–186)

***Key Words*: hypertension, calcium antagonist, signal transduction, smooth muscle cell**

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This study was supported in part by grants from Eisai Co., Ltd., Sumitomo Pharmaceuticals, and the Takeda Science Foundation.

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Received August 5, 2005; Accepted in revised form December 26, 2005.

Introduction

Vascular smooth muscle cells (SMCs) exist in a diverse range of phenotypes, and the phenotypic modulation of vascular SMCs is a hallmark of vascular dysfunction in hypertension (*1*, *2*). Under physiologic conditions, vascular SMCs exhibit the differentiated/contractile phenotype, whereas, under pathologic conditions, vascular SMCs display a proliferative/dedifferentiated phenotype and are involved in the progression of atherosclerosis (*1*). SMCs in the media express 2 isoforms of the smooth muscle (SM) myosin heavy chain (MHC; SM1 and SM2) and 2 types of nonmuscle MHC (NMHC) isoforms (NMHC-B/SMemb and NMHC-A) (*1*, *2*). We recently reported that angiotensin II receptor type 1–mediated NAD(P)H oxidase–generated reactive oxygen species, endothelial NO synthase (eNOS) and phosphoinositide 3-kinase (PI3-K)/protein kinase B (PKB) (Akt), as well as GATA-6, a zinc finger transcription factor that regulates SM-MHC isoforms in vascular SMCs, might be crucial determinants for the vascular SMC phenotype in hypertension *in vivo* (*3*, *4*). It has also been reported that the forced expression of active forms of mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 and MAP kinase (MAPK) kinase (MKK) 6, which are the upstream kinases of ERK and p38 MAPK, respectively, induced de-differentiation of SMCs, indicating that the SMC phenotype would be determined by the balance between the strengths of the Akt pathway and the ERK and p38 MAPK pathways (*5*).

The calcium antagonists have been widely used in the treatment of patients with hypertension (*6*–*8*), in whom they inhibit cardiovascular events as effectively as angiotensin-converting enzyme (ACE) inhibitors (*7*–*9*). In addition, recent clinical studies have revealed that the long-acting L-type dihydropyridine calcium antagonist amlodipine may have antiatherogenic properties independent of its effects on vasodilatation (*10*–*12*). We have also shown that amlodipine may have antiatherosclerotic antioxidative action beyond blood-pressure lowering in stroke-prone spontaneously hypertensive rat (SHRSP) hearts (*13*). However, the precise mechanisms regulating vascular SMC phenotypic modulation and the critical signal transductions affecting the vascular SMC phenotype in hypertension by amlodipine remain unclear *in vivo*. The purpose of this study was to examine which signal transduction pathway(s) might be crucial to the antiatherogenic property of amlodipine, and regulate the aortic SMC phenotype in SHRSP *in vivo*.

Methods

Experimental Protocol

The Ethics Committee for Animal Experimentation at the Yamaguchi University School of Medicine approved the experimental protocol. The long-acting L-type dihydropyridine calcium antagonist amlodipine and the ACE inhibitor enalapril were provided by Pfizer Pharmaceuticals Inc. (Tokyo, Japan) and Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Twelve-week-old male Wistar-Kyoto rats (WKY group; $n=20$) and SHRSP ($n=60$) were obtained from Charles River Japan (Yokohama, Japan). The WKY group was treated with a vehicle, and the SHRSP were randomly treated with a vehicle (SHRSP group), amlodipine (5 mg/kg per day, amlodipine group), or enalapril (10 mg/kg per day, enalapril group). The doses used in the experiments were determined according to Umemoto *et al*. (*13*). Without anesthetizing the rats, systolic blood pressure (SBP) and heart rate were obtained by tail-cuff plethysmography. After the 6-week treatment period, the rats were weighed, euthanized with a sodium pentobarbital overdose and perfusion-fixed for 5 min at a pressure of 90 mmHg with heparinized saline followed by Bouin's solution; then, paraffin slices (4-μm thick) of the thoracic aortas were stained with Sirius red as previously reported (*3*). Care was taken not to damage either the endothelium or the medial layer. The other rats were also euthanized by an intraperitoneal sodium pentobarbital overdose, and then the rat thoracic aortas were immediately rinsed with phosphate-buffered saline to remove adventitial fat and connective tissue by blunt dissection, frozen in liquid nitrogen, and stored at −80°C until used for immunoblots (*3*). In some experiments, the endothelium was denuded as previously reported (*3*).

Histological Analysis

The total cell number in the aortic tunica media, the wall thickness/lumen ratio (the medial thickness to the internal diameter), and the percentage of the total collagen volume fraction determined by Sirius red staining were measured from 5 randomly selected fields in 1 cross-section of the aorta in a blind fashion using a computer-assisted image analysis system as previously reported (*3*), and the mean value of each aorta was used for statistical analysis.

Immunoblotting

The following were applied for immunoblotting using the enhanced chemiluminescence (ECL™) system (Amersham Biosciences, Piscataway, USA) as previously reported (*3*): mouse monoclonal antibody against human α-SM actin (DakoCytomation, Kyoto, Japan), mouse monoclonal antibodies against rabbit contractile-type SM-MHC isoform SM2 and NMHC-B/SMemb (Yamasa, Choshi, Japan), and human eNOS (Transduction Laboratories, Lexington, USA), goat polyclonal antibodies against human Akt and MKK6, rabbit polyclonal antibody against mouse p38 MAPK and rat MEK1, mouse monoclonal antibodies against human phospho-42/44 ERK (p-42/44 ERK) and phospho-p38 MAPK (p-p38 MAPK) (Santa Cruz Biotech, Santa Cruz, USA), and rabbit polyclonal antibodies against rat 42/44 ERK and mouse

Table 1. Heart Rate, Body Weight, Wall-to-Lumen Ratio, Total Cell Number, and Collagen Volume Fraction in 18-Week-Old Rats

Parameter	WKY	SHRSP	Amlodipine	Enalapril
Heart rate (bpm)	306±13	330±13	309±14	288±9
Body weight (g)	409±16	315±9*	315±5*	320±5*
Wall-to-lumen ratio	0.05±0.004	0.086±0.002*	0.069±0.003†,§	0.067±0.004†,‡
Total cell number	2,046±181	2,474±126	2,326±106	2,054±89
Collagen volume fraction (%)	2.8±0.2	5.0±0.3*	3.9±0.2†,§	3.9±0.3†,§

Values are means±SEM. SHRSP were treated with vehicle, amlodipine (5 mg/kg per day), or enalapril (10 mg/kg per day) for 6 weeks. *$p<0.01$, †$p<0.05$ *vs*. the WKY group; ‡$p<0.01$, §$p<0.05$ *vs*. the vehicle SHRSP group. Experiments: $n=5$–6. WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats.

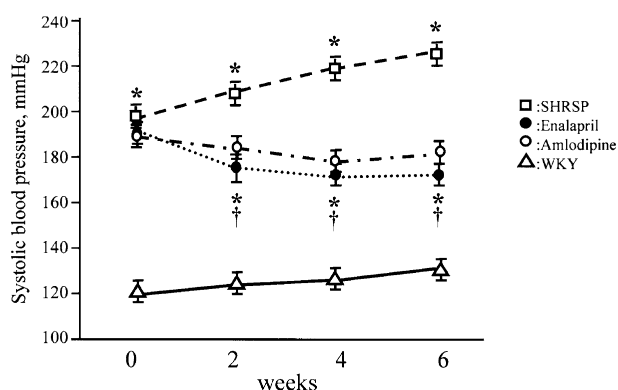

Fig. 1. *Graphs showing systolic blood pressure (SBP) in the vehicle SHRSP, amlodipine, and enalapril groups. Bars indicate SEM.* *p< *0.01* vs. *the WKY group,* †p< *0.01* vs. *the vehicle SHRSP group. Experiments,* n = *5–7.*

phospho-Akt (p-Akt) (Cell Signaling Technology, Beverly, USA). The ratios of each protein (SM2, NMHC-B/SMemb, eNOS, MKK6, and MEK1) to α-SM actin (as an internal standard), the ratio of p-Akt to total Akt, the ratio of p-p38 MAPK to total p38 MAPK, and the ratio of p-42/44 ERK to total 42/44 ERK were obtained by calculating the percentage of each protein expression *vs*. the vehicle WKY group, respectively.

Statistical Analysis

All values are expressed as the means±SEM. The experimental groups were compared by ANOVA followed by Scheffe's multiple comparisons. Values of $p<0.05$ were considered statistically significant.

Results

The heart rate was unaltered among the 4 groups (Table 1). Throughout the experiments, SBP in the vehicle SHRSP group was significantly higher than that in the WKY group. The two drugs significantly and equally reduced SBP compared with that in the vehicle SHRSP group. However, both the amlodipine and enalapril groups showed significantly higher SBP than the WKY group (Fig. 1). Body weight was greater in the WKY group than in the 3 SHRSP groups, but there was no difference in the body weights among the vehicle and the 2 drug-treated SHRSP groups (Table 1).

The wall-to-lumen ratio and collagen volume fraction were increased in the vehicle SHRSP group compared with the WKY group. The 2 drug-treated groups had significantly lower values than the vehicle SHRSP group. There were no significant differences in these values between the 2 drug-treated SHRSP groups, but the values were still significantly higher than in the WKY group. There was little difference in the total cell number in the aortic media among the 4 groups (Table 1).

Figure 2 shows that SM2 expression in the aorta was significantly decreased in the vehicle SHRSP group compared with the WKY group. Compared with the vehicle SHRSP group, SM2 expression in the aorta was significantly higher in both the amlodipine and enalapril groups, with no significant difference seen between the 2 drug-treated groups. However, the level of SM2 expression in both drug-treated groups was still significantly lower than that in the WKY group. In contrast, NMHC-B/SMemb expression in the aorta was significantly higher in the vehicle SHRSP group compared with the WKY group. Compared with the vehicle SHRSP group, NMHC-B/SMemb expression in the aorta was significantly lower in both the amlodipine and enalapril groups, with no significant difference seen between the 2 drug-treated groups. The level of NMHC-B/SMemb expression in both the drug-treated groups was, however, still significantly greater than that in the WKY group.

We further examined which signal transduction regulates the rat aortic SMC phenotype *in vivo*. The expressions of p-p38 MAPK and p-42/44 ERK in the aortas were significantly higher in the vehicle SHRSP group than in the WKY group (Fig. 3). The 2 drug-treated groups had significantly lower aortic expressions of both p-p38 MAPK and p-42/44 ERK than the vehicle SHRSP group. Both drugs reduced p-p38 MAPK in SHRSP aortas, with no significant difference seen between the 2 drug-treated groups, whereas amlodipine was

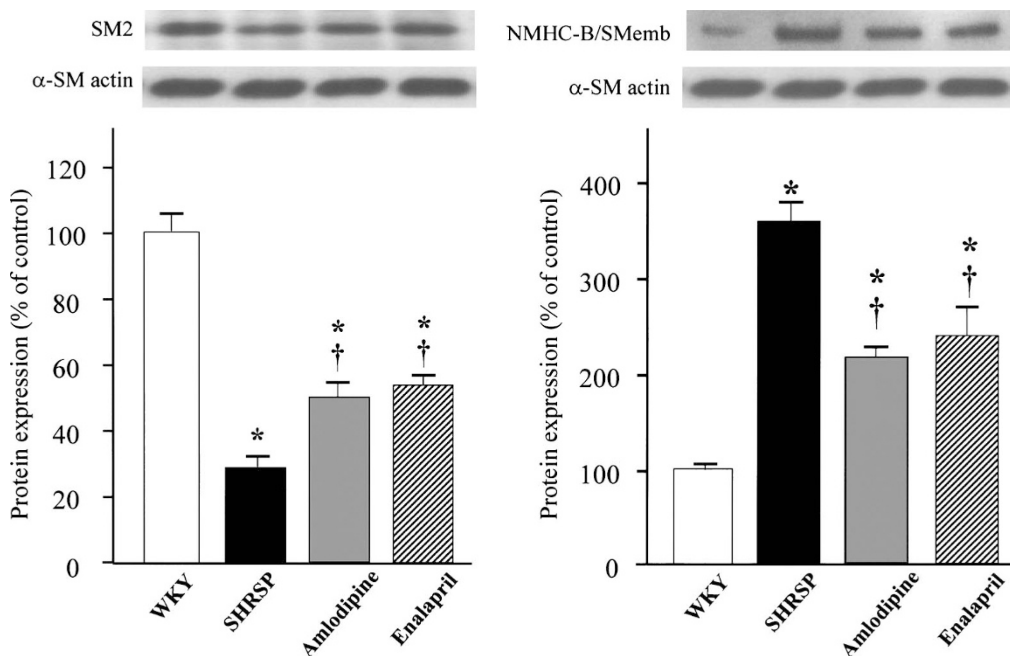

Fig. 2. *Quantitative analysis of SM2 and NMHC-B/SMemb expressions in the rat aortas. Bars indicate SEM.* *$p < 0.01$ vs. *the WKY group;* †$p < 0.01$ vs. *the vehicle SHRSP group. Experiments:* $n = 6$.

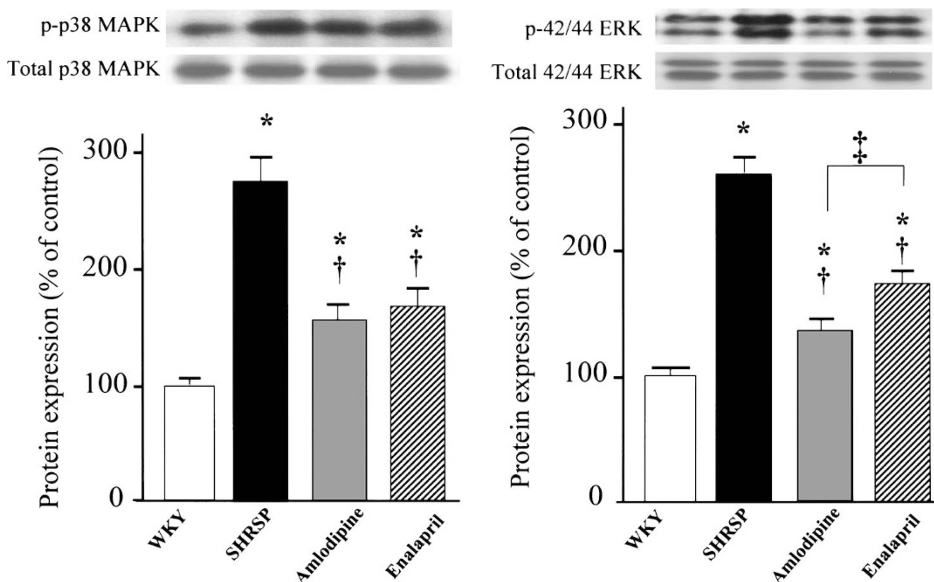

Fig. 3. *Quantitative analysis of p-p38 MAPK and p-42/44 ERK expression in the rat aortas. Bars indicate SEM.* *$p < 0.05$ vs. *the WKY group;* †$p < 0.01$ vs. *the vehicle SHRSP group;* ‡$p < 0.05$ vs. *the amlodipine group. Experiments:* $n = 6$.

more effective than enalapril at decreasing p-42/44 ERK expression in SHRSP aortas. The level of p-p38 MAPK and p-42/44 ERK expressions in both the drug-treated groups was, however, still significantly greater than that in the WKY group.

We also examined the aortic expressions of MKK6 and MEK1, the upstream kinases of MAPK and ERK, respectively, which were significantly higher in the vehicle SHRSP group than in the WKY group (Fig. 4). The two drugs significantly reduced these values in SHRSP aortas. Both drugs reduced MKK6 in SHRSP aortas, with no significant difference seen between the 2 drug-treated groups, whereas amlodipine was more effective than enalapril at decreasing MEK1 expression in SHRSP aortas to the same level as in the WKY

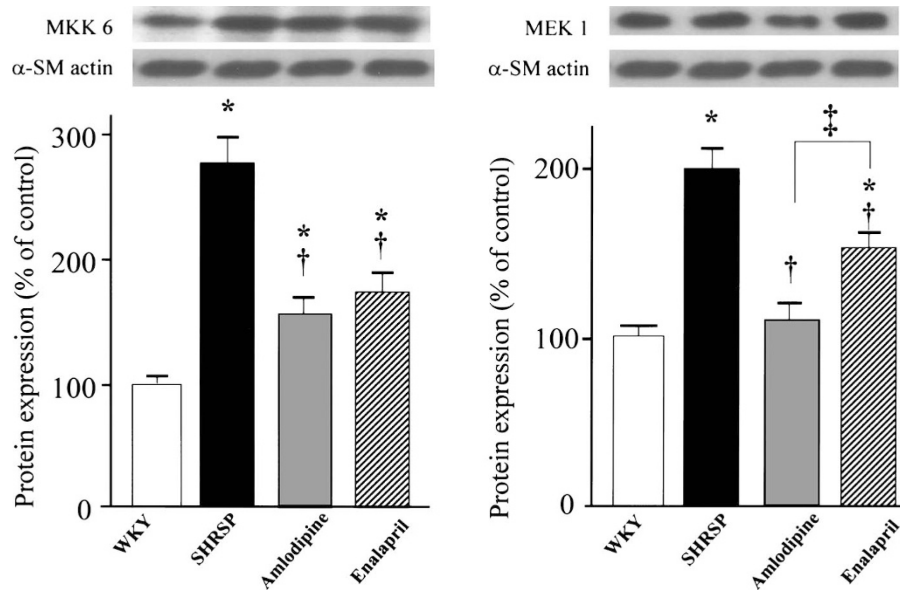

Fig. 4. *Quantitative analysis of MKK6 and MEK1 expression in the rat aortas. Bars indicate SEM.* *$p<0.05$ vs. *the WKY group;* †$p<0.01$ vs. *the vehicle SHRSP group;* ‡$p<0.05$ vs. *the amlodipine group. Experiments:* $n=6$.

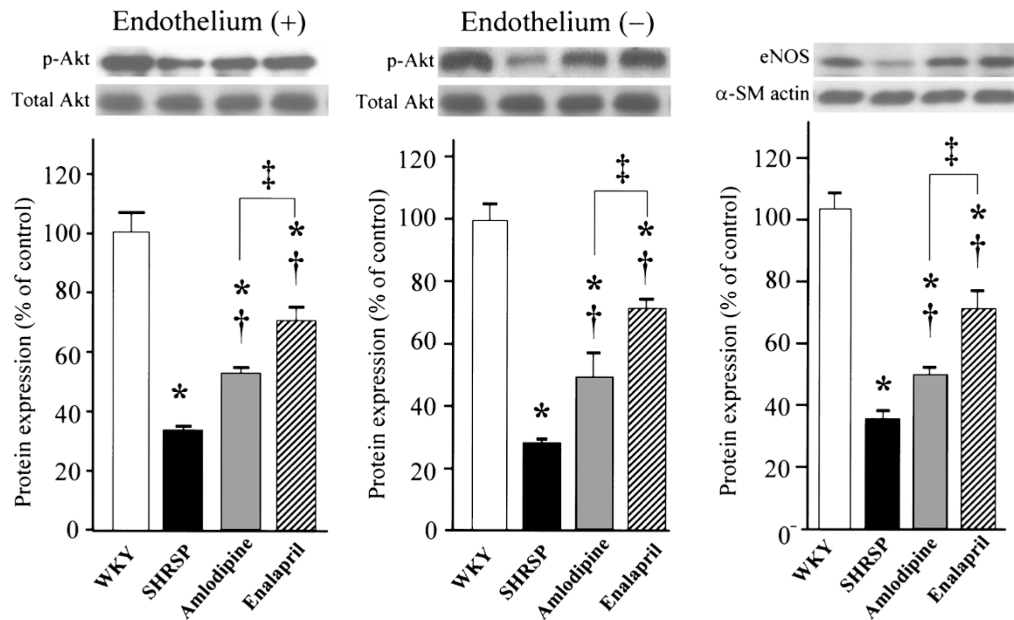

Fig. 5. *Quantitative analysis of p-Akt expression with and without endothelium, and eNOS expression in the rat aortas. Bars indicate SEM.* *$p<0.01$ vs. *the WKY group;* †$p<0.05$ vs. *the vehicle SHRSP group;* ‡$p<0.01$ vs. *the amlodipine group. Experiments:* $n=3$–6.

group. The level of MKK6 and MEK1 expressions in the enalapril group was still significantly greater than that in the WKY group.

Figure 5 shows the results of p-Akt expression in the rat aorta with and without endothelium, and demonstrates that p-Akt was significantly decreased in the vehicle SHRSP group compared with the WKY group. The 2 drug-treated groups had significantly higher p-Akt expression than the vehicle SHRSP group, both with and without endothelium. Enalapril was more effective than amlodipine at increasing p-Akt expression in SHRSP aortas both with and without endothelium.

To clarify whether endothelial dysfunction affects the regulation of the vascular SMC phenotype, we examined the

level of eNOS expression in the rat aortas, and found that the level of eNOS expression was significantly decreased in the vehicle SHRSP group compared with the WKY group (Fig. 5). The two drugs significantly restored eNOS expressions in SHRSP aortas, but the levels of eNOS expression in the 2 drug-treated groups were still lower than that in the WKY group. Enalapril was more effective than amlodipine at restoring eNOS expression in SHRSP aortas.

Discussion

In this study, we demonstrated that within 6 weeks and at the doses used, both amlodipine and enalapril significantly and equally reduced blood pressure, and shifted vascular SMCs in the SHRSP aorta toward the differentiated (contractile) type by reducing NMHC-B/SMemb and increasing SM2, and inducing equipotent inhibition of the MKK6 and p38 MAPK pathways. The major differences in the effects of amlodipine and enalapril on signaling pathways for aortic SMC differentiation in SHRSP observed in our experiments were as follows: amlodipine induced a significantly greater reduction in MEK1 and p-42/44 ERK compared with enalapril, whereas enalapril was more effective than amlodipine at increasing p-Akt and eNOS in SHRSP aortas.

Meta-analyses of the recent clinical trials in hypertension suggest that blood pressure control is important, and that all antihypertensive drugs, including calcium antagonists and ACE inhibitors, have similar long-term efficacy and safety (*6–8*). In addition, it has been demonstrated that amlodipine improved left ventricular hypertrophy by controlling 24-h blood pressure (*14*), and reduced the rate of atherosclerosis in the carotid arteries as assessed by B-mode ultrasonography with fewer cases of cardiac events compared to the placebo in patients with coronary artery disease (*11*). Furthermore, in a study that measured the progression of atherosclerosis using intravascular ultrasound, administration of amlodipine but not enalapril reduced adverse cardiovascular events and retarded atherosclerosis progression in patients with coronary artery disease and normal blood pressure (*12*). These results suggest that amlodipine, as a vascular protective agent, may effectively improve outcomes in high-risk patients with coronary artery disease (*15*). However, the precise mechanism of the antiatherogenic property of amlodipine in hypertension has remained obscure.

Since the main function of vascular SMCs is Ca^{2+}-dependent contraction controlled by actin-myosin linked dual regulation (*16*, *17*), dihydropyridine calcium antagonists play an important role in the treatment of hypertension by lowering blood pressure through a mechanism of blocking L-type calcium channels in vascular SMCs, which express a large number of voltage-dependent calcium channels (*18*). In addition, several potential antiatherosclerotic mechanisms of pleiotropic action independent of L-type calcium channel modulation have been reported for amlodipine (*10–12*, *15*), including inhibition of lipid peroxide formation, increase in NO production, modification of SMC atherosclerotic membrane defects, a proteoglycan-mediated mechanism by vascular SMCs and low-density lipoprotein–proteoglycan interaction, and SMC phenotypic change, proliferation, and migration through potent inhibition of Ca^{2+} movements involved in cell-cycle initiation/progression (*19*, *20*). These cellular actions by amlodipine would inhibit vascular remodeling (*10*, *15*). We recently reported that both enalapril and amlodipine might have additional benefits for the reduction of oxidative stress, vascular remodeling, and cardiac fibrosis in SHRSP hearts beyond blood-pressure lowering, and that amlodipine inhibited vascular remodeling of intramyocardial arteries in SHRSP by efficiently modifying Cu/Zn superoxide dismutase, more so than did enalapril (*13*). Amlodipine may also have antioxidant properties in addition to antihypertensive activity to inhibit neuronal cell death (*21*), which would be useful to prevent cerebrovascular accidents in hypertensive patients. It has also been reported that amlodipine exerts its potent growth inhibitory effects by inhibiting the expression of early growth–response genes (*10–12*, *22–24*), and by interfering with multiple branches of mitogenic signaling pathways (*19*, *25–27*). Furthermore, Stepien *et al*. also reported that amlodipine specifically alters Ca^{2+} mobilization by interacting with the sarcoplasmic reticulum and inhibiting voltage-dependent Ca^{2+} influx and the resulting ERK activation (*22*).

The phenotypic modulation of vascular SMCs is a hallmark of vascular dysfunction in hypertension (*1*, *2*). However, the mechanisms regulating vascular SMC phenotypic modulation and the critical signal transductions affecting the vascular SMC phenotype remain controversial (*3*, *28–31*). Under culture conditions that maintained cultured vascular SMCs in the differentiated type, which is similar to the *in vivo* phenotype, Hayashi *et al*. recently demonstrated that maintenance of a differentiated phenotype of SMCs depends on the Akt pathway, whereas the coordinate activation of the ERK and p38 MAPK pathways induces SMC de-differentiation (*5*), indicating that the SMC phenotype should be determined by the balance between the strengths of the Akt pathway and the ERK and p38 MAPK pathways. We also reported that the ACE inhibitor cilazapril might inhibit NAD(P)H oxidase activity and shifted vascular SMCs in the SHRSP aorta toward the differentiated (contractile) type, and that the Akt pathway, but not the p38 MAPK or ERK pathways, may play a key role in determining SMC differentiation in the SHRSP aorta *in vivo* (*3*). Our results for enalapril in the present experiments are consistent with those of our previous reports (*3*), suggesting that enalapril may induce differentiation (contractility) of SMCs in SHRSP aortas, and that the balance and cross-regulation between the Akt- and MEK-signaling cascades determine the temporal pattern of ERK phosphorylation and may thereby guide the phenotypic modulation of vascular SMCs (*32*). Furthermore, we showed that both amlodipine and enalapril increased Akt in SHRSP aortas with or without an endothelium, suggesting that the Akt present not

only in the endothelium but also in the media may participate in the regulation of SMC phenotypic change in SHRSP aortas. Since the blood pressure–lowering actions of amlodipine and enalapril were equal, and no significant differences were observed in the reduction of the expressions of p38 MAPK and ERK between the 2 drug-treated groups, the activations of the p38 MAPK and ERK cascades in SHRSP aortas might be mainly dependent on blood pressure rather than on the drugs used.

Hypertension is a central pathogenic factor of endothelial dysfunction that is caused, in part, by the impairment of endothelial NO production by eNOS (*3*, *33*). Dihydropyridine calcium antagonists also modulate endothelial functions by "pleiotropic effects" such as enhancement of the bioavailability of endothelial NO, which plays a pivotal role in the regulation of vasorelaxation, leukocyte adhesion and platelet aggregation; impaired NO release is associated with the genesis and progression of atherosclerotic diseases and an increase in endothelial NO formation (*10*), which may contribute to the antiatherogenic effects of amlodipine (*34*). ACE inhibitors also attenuate endothelial dysfunction and enhance NO release from the vascular endothelium, which may contribute to the reduction of vascular structural alterations (*35*). Furthermore, On *et al*. demonstrated that treatment with an ACE inhibitor or a calcium antagonist resulted in improvement by a mechanism that is probably related to antioxidant activity (*33*). Our findings in the endothelium are consistent with those of our previous reports (*3*, *13*), suggesting that both types of drugs may restore the level of NO by increasing eNOS to induce differentiation of vascular SMCs.

Furthermore, within 6 weeks and at the doses used, enalapril may have restored eNOS by inducing a greater change than amlodipine in SHRSP aortas in our experimental model. Although the MEK-ERK pathway present in the endothelium may be involved in the effects of amlodipine on SMC phenotypic change in SHRSP aortas, based upon our findings the MEK-ERK pathway present in vascular SMCs may be more important for the phenotypic change of vascular SMCs compared with that present in the endothelium: amlodipine significantly reduced the MEK1 and p-42/44 ERK expression, while there was a significant difference in their expression in SHRSP aortas between the amlodipine and enalapril groups, even though the eNOS expression could not be restored to the same level as that of the WKY group by the administration of either drug. It has also been reported that inhibition of MEK1/2 does not alter blood pressure despite improved endothelial function and reduced arterial reactivity to Ang II (*36*), which may support our conclusions.

There are several limitations present in this study. We cannot exclude the possibility that signal transductions other than the MEK-ERK pathway may have been involved in the SMC phenotypic change in the aorta induced by the calcium antagonist amlodipine, since it has been reported that a variety of factors—such as hemodynamic and mechanical forces, and biochemical, cytokine and extracellular matrix stimulation—could lead to changes in SMC phenotype (*37*, *38*). In our study, blood pressures in SHRSP treated for 6 weeks with amlodipine at the doses used were still significantly higher compared with those in WKY, and p-42/44 ERK expression in the SHRSP aorta was significantly higher than that in the WKY while MEK1, the upstream kinase of ERK, decreased to the same level as in the WKY. It is likely that the 6-week treatment of SHRSP with amlodipine was not long enough to decrease aortic p-42/44 ERK expression to the level seen in the WKY in our experiments.

In summary, our study demonstrated that enalapril and amlodipine inhibited SMC de-differentiation through the inhibition of the MEK1-ERK pathway as well as the MKK6-p38 MAPK pathway, and through the upregulation of the eNOS and Akt pathways in SHRSP aortas. Furthermore, amlodipine was more effective than enalapril at reducing the MEK-ERK pathway and increasing SM2 expression in SHRSP aortas at the doses used, suggesting that the MEK-ERK pathways might be one of the crucial determinants for the phenotypic change of vascular SMCs, which in turn might be responsible for the antiatherogenic property of amlodipine in hypertension *in vivo*. Further studies will be required to understand the detailed signaling pathways that regulate the vascular SMC phenotype and the functional linkage between such signaling pathways *in vivo*.

Acknowledgements

We would like to thank Rie Ishihara and Kazuko Iwamoto for their excellent technical assistance.

References

1. Owens GK: Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 1995; **75**: 487–517.
2. Fujii K, Umemoto S, Fujii A, *et al*: Angiotensin II type 1 receptor antagonist downregulates nonmuscle myosin heavy chains in spontaneously hypertensive rat aorta. *Hypertension* 1999; **33**: 975–980.
3. Kawahara S, Umemoto S, Tanaka M, *et al*: Up-regulation of Akt and eNOS induces vascular smooth muscle cell differentiation in hypertension *in vivo*. *J Cardiovasc Pharmacol* 2005; **45**: 367–374.
4. Kubo M, Umemoto S, Fujii K, *et al*: Effects of angiotensin II type 1 receptor antagonist on smooth muscle cell phenotype in intramyocardial arteries from spontaneously hypertensive rats. *Hypertens Res* 2004; **27**: 685–693.
5. Hayashi K, Takahashi M, Kimura K, *et al*: Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogen-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. *J Cell Biol* 1999; **145**: 727–740.
6. Opie LH, Schall R: Evidence-based evaluation of calcium channel blockers for hypertension: equality of mortality and cardiovascular risk relative to conventional therapy. *J Am Coll Cardiol* 2002; **39**: 315–322.
7. Staessen JA, Wang JG, Thijs L: Cardiovascular protection and blood pressure reduction: a meta-analysis. *Lancet* 2001;

358: 1305–1315.
8. Staessen JA, Li Y, Thijs L, *et al*: Blood pressure reduction and cardiovascular prevention: an update including the 2003–2004 secondary prevention trials. *Hypertens Res* 2005; **28**: 385–407.
9. ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group: Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker *vs* diuretic: the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *JAMA* 2002; **288**: 2981–2997.
10. Mason RP, Marche P, Hintze TH: Novel vascular biology of third-generation L-type calcium channel antagonists: ancillary actions of amlodipine. *Arterioscler Thromb Vasc Biol* 2003; **23**: 2155–2163.
11. Pitt B, Byington RP, Furberg CD, *et al*, for the PREVENT Investigators: Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. *Circulation* 2000; **102**: 1503–1510.
12. Nissen SE, Tuzcu EM, Libby P, *et al*: Effect of antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. *JAMA* 2004; **292**: 2217–2225.
13. Umemoto S, Tanaka M, Kawahara S, *et al*: Calcium antagonist reduces oxidative stress by upregulating Cu/Zn superoxide dismutase in stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2004; **27**: 877–885.
14. Ishimitsu T, Kobayashi T, Honda T, *et al*: Protective effects of an angiotensin II receptor blocker and a long-acting calcium channel blocker against cardiovascular organ injuries in hypertensive patients. *Hypertens Res* 2005; **28**: 351–359.
15. Mason RP: Mechanisms of plaque stabilization for the dihydropyridine calcium channel blocker amlodipine: review of the evidence. *Atherosclerosis* 2002; **165**: 191–199.
16. Umemoto S, Sellers JR: Characterization of *in vitro* motility assays using smooth muscle and cytoplasmic myosins. *J Biol Chem* 1990; **265**: 14864–14869.
17. Sobue K, Hayashi K, Nishida W: Molecular mechanism of phenotypic modulation of smooth muscle cells. *Horm Res* 1998; **50** (Suppl 2): 15–24.
18. Gollasch M, Haase H, Ried C, *et al*: L-type calcium channel expression depends on the differentiated state of vascular smooth muscle cells. *FASEB J* 1998; **12**: 593–601.
19. Stepien O, Zhang Y, Zhu D, *et al*: Dual mechanism of action of amlodipine in human vascular smooth muscle cells. *J Hypertens* 2002; **20**: 95–102.
20. Berridge MJ: Unlocking the secrets of cell signaling. *Annu Rev Physiol* 2005; **67**: 1–21.
21. Yamagata K, Ichinose S, Tagami M: Amlodipine and carvedilol prevent cytotoxicity in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2004; **27**: 271–282.
22. Stepien O, Gogusev J, Zhu DL, *et al*: Amlodipine inhibition of serum-, thrombin-, or fibroblast growth factor–induced vascular smooth-muscle cell proliferation. *J Cardiovasc Pharmacol* 1998; **31**: 786–793.
23. Ko Y, Totzke G, Graack GH, *et al*: Action of dihydropyridine calcium antagonists on early growth response gene expression and cell growth in vascular smooth muscle cells. *J Hypertens* 1993; **11**: 1171–1178.
24. Mancini GB: Antiatherosclerotic effects of calcium channel blockers. *Prog Cardiovasc Dis* 2002; **45**: 1–20.
25. Zhang YZ, Gao PJ, Wang XY, *et al*: The inhibitory mechanisms of amlodipine in human vascular smooth muscle cell proliferation. *Hypertens Res* 2000; **23**: 403–406.
26. Lai YM, Fukuda N, Su JZ, *et al*: Novel mechanisms of the antiproliferative effects of amlodipine in vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertens Res* 2002; **25**: 109–115.
27. Jackson CL, Bush RC, Bowyer DE: Mechanism of antiatherogenic action of calcium antagonists. *Atherosclerosis* 1989; **80**: 17–26.
28. Laplante MA, Wu R, El Midaoui A, *et al*: NAD(P)H oxidase activation by angiotensin II is dependent on p42/44 ERK-MAPK pathway activation in rat's vascular smooth muscle cells. *J Hypertens* 2003; **21**: 927–936.
29. Touyz RM, El Mabrouk M, He G, *et al*: Mitogen-activated protein/extracellular signal–regulated kinase inhibition attenuates angiotensin II–mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells. *Circ Res* 1999; **84**: 505–515.
30. Su B, Mitra S, Gregg H, *et al*: Redox regulation of vascular smooth muscle cell differentiation. *Circ Res* 2001; **89**: 39–46.
31. Ushio-Fukai M, Alexander RW, Akers M, *et al*: Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 1999; **274**: 22699–22704.
32. Reusch HP, Zimmermann S, Schaefer M, *et al*: Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J Biol Chem* 2001; **276**: 33630–33637.
33. On YK, Kim CH, Oh BH, *et al*: Effects of angiotensin converting enzyme inhibitor and calcium antagonist on endothelial function in patients with essential hypertension. *Hypertens Res* 2002; **25**: 365–371.
34. Berkels R, Taubert D, Rosenkranz A, *et al*: Vascular protective effects of dihydropyridine calcium antagonists. Involvement of endothelial nitric oxide. *Pharmacology* 2003; **69**: 171–176.
35. Gohlke P, Stoll M, Lamberty V, *et al*: Cardiac and vascular effects of chronic angiotensin converting enzyme inhibition at subantihypertensive doses. *J Hypertens Suppl* 1992; **10**: S141–S144.
36. Touyz RM, Deschepper C, Park JB, *et al*: Inhibition of mitogen-activated protein/extracellular signal–regulated kinase improves endothelial function and attenuates Ang II–induced contractility of mesenteric resistance arteries from spontaneously hypertensive rats. *J Hypertens* 2002; **20**: 1127–1134.
37. Stegemann JP, Hong H, Nerem RM: Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol* 2005; **98**: 2321–2327.
38. Raines EW, Ferri N: Thematic review series: the immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. *J Lipid Res* 2005; **46**: 1081–1092.