Calcium Channel Blocker Azelnidipine Enhances Vascular Protective Effects of AT1 Receptor Blocker Olmesartan

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Abstract—The present studies were undertaken to investigate the potential effect of a calcium channel blocker (CCB) to enhance the inhibitory effect of an angiotensin (Ang) II type 1 (AT1) receptor blocker (ARB) on vascular injury and the cellular mechanism of the effect of CCB on vascular remodeling. In polyethylene cuff-induced vascular injury of the mouse femoral artery, proliferation of vascular smooth muscle cells (VSMCs) and neointimal formation associated with activation of extracellular signal-regulated kinase (ERK), and tyrosine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, inflammatory response assessed by monocyte chemoattractant protein-1 and tumor necrosis factor-α expression, as well as oxidative stress such as expression of NADH/NADPH oxidase p22phox subunit and superoxide production, were less in AT1 receptor null mice. Administration of nonhypotensive doses of a CCB, azelnidipine (0.5 or 1 mg/kg per day) attenuated these parameters in wild-type and AT1 receptor null mice. Coadministration of lower doses of an ARB, olmesartan (0.5 mg/kg per day), and azelnidipine (0.1 mg/kg per day), which did not affect vascular remodeling, significantly inhibited these parameters in wild-type mice. Moreover, the effective dose of azelnidipine (1 mg/kg per day) exaggerated the inhibitory action of olmesartan at effective doses of 1 or 3 mg/kg per day on VSMC proliferation in the injured arteries. These results suggest that azelnidipine could inhibit vascular injury at least partly independent of the inhibition of AT1 receptor activation and that azelnidipine could exaggerate the vascular protective effects of olmesartan, suggesting clinical possibility that the combination of CCB and ARB could be more effective in the treatment of vascular diseases. (Hypertension. 2004;43:263-269.)

Key Words: angiotensin ■ calcium channel ■ oxidative stress ■ vascular remodeling ■ inflammation

Evidence indicates that angiotensin (Ang) II is more than a hormone with hemodynamic and renal actions. It is also a local, biologically active mediator that has direct effects on endothelial and vascular smooth muscle cells (VSMCs) and plays a key role in the initiation and amplification of pathobiological events that lead to vascular disease.1 Ang II is a major mediator of oxidative stress and reduced nitric oxide (NO) activity by activating NADH/NADPH oxidase, which results in the production of the superoxide anion.2 Other mechanisms whereby Ang II may promote vascular remodeling and the formation of vascular lesions are the modulation of VSMC migration and decreased VSMC apoptosis,3,4 suggesting that Ang II is a pleiotropic local mediator of vascular remodeling and lesion formation. These major cardiovascular actions of Ang II have been reported to be mediated by the type 1 Ang II (AT1) receptor, and AT1 receptor blockers (ARBs) have been widely used as antihypertensive drugs with the expectation of a vascular protective effect.5

Calcium channel blockers (CCBs) have been shown to retard atherogenesis in animal models and to prevent the development of early lesions in human coronary arteries. They are used in the treatment of angina pectoris and hypertension. However, the mechanism by which CCB reverse endothelial dysfunction and prevent vascular damage is largely unknown. One of the mechanisms proposed to explain the beneficial effect of CCBs on endothelial function is antioxidant activity.6,7 Recently, Fukuo et al reported that the CCB nifedipine indirectly upregulates endothelial superoxide dismutase expression by stimulating vascular endothelial growth factor production by adjacent VSMC.8 It has been reported that DNA synthesis induced by Ang II and growth factors such as platelet-derived growth factor (PDGF) in VSMCs is significantly blunted by CCBs, and that CCBs inhibit neointimal formation in the injured artery.9-11 Accumulating evidence has suggested that the intracellular signaling mechanisms, by which AT1 receptors exert hypertrophic and/or hyperplastic effects on their targets such as VSMC, are closely associated with receptor and non-receptor tyrosine kinases, and that some AT1 receptor-mediated signaling requires Ca2+-sensitive tyrosine kinases.12 These results suggest the possibility that combination therapy of ARB and CCB could more effectively prevent vascular damage than monotherapy.

Arterial neointimal thickening is an important process in the development of atherosclerosis, bypass graft failure, and...
restenosis after angioplasty. We have developed a mouse model of vascular disease induced by polyethylene cuff placement around the femoral artery, in which the AT1 receptor is upregulated, followed by VSMC proliferation, vascular inflammation, and neointimal thickening. In the present study, we used this mouse model of cuff-induced vascular injury and explored the following possibilities: (1) dihydropyridine CCB, azelnidipine, can prevent vascular remodeling, especially when focusing on growth-promoting signals and oxidative stress. Moreover, we investigated that azelnidipine can exaggerate the inhibitory effect of an ARB, olmesartan, on vascular remodeling.

Methods

Animals and Operation
Adult male AT1a receptor knockout mice (AT1aKO) (donated by Tanabe Seiyaku Co., Ltd., Osaka, Japan) and wild-type (WT) mice (based on C57BL/6d strain; Clea Japan Inc., Osaka, Japan) aged 10 to 12 weeks were used in this study. The Animal Studies Committee of Ehime University approved the experimental protocol. The surgical procedure for cuff-induced vascular injury in the femoral artery was performed according to the method previously described.13-16

Administration of CCB and ARB
Azelnidipine (CS-905; donated by Sankyo Pharmaceutical Co., Tokyo, Japan) was administered orally after cuff placement for 7 or 14 days. Olmesartan (RNH-6270; donated by Sankyo Pharmaceutical Co., Tokyo, Japan) was administered using an osmotic minipump (model 1002; Alza Corp.) implanted intraperitoneally at the same time as cuff-placement, as previously described.13 Blood pressure was measured by the indirect tail-cuff method with blood pressure monitor (MK-1030; Muromachi Kikai Co. Ltd, Tokyo, Japan).

Morphometric Analysis, Measurement of DNA Synthesis, and Immunohistochemical Study
Morphometric analysis, measurement of DNA synthesis determined by the incorporation of bromodeoxyuridine (BrdU), and immunohistochemical study were performed according to the methods previously described.13-16

Detection of Inflammatory Factors, NADH/NADPH Oxidase, and Signaling Molecules by Western Blot
Total proteins were prepared from pooled arteries (6 to 8 arteries for each group), and Western blot was performed using specific antibodies as previously described.13-16 Anti-p22phox antibodies were purchased from Santa Cruz Biotechnology Inc. Densitometric analysis was performed using an image scanner (EPSON GT-8000) and NIH imaging software.

Immunofluorescent Study
Paraffin-embedded sections were incubated with anti-p22phox antibody, washed, and incubated with biotin-labeled secondary antibodies, then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axiostar microscope equipped with a computer-based imaging system.18

Superoxide Detection
Frozen, enzymatically intact, 10-μm-thick sections of sham-operated and injured femoral arteries were incubated at the same time with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light.18 DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For ethidium detection, a 543-nm argon laser combined with a 500- to 550-nm band-pass filter was used.

Statistical Analysis
Values are expressed as mean±SEM in the text and figure. Data were analyzed by 2-way ANOVA, followed by post-hoc analysis. A value of P<0.05 was considered to be statistically significant.

Results

Effect of Azelnidipine on Neointimal Formation and VSMC Proliferation After Cuff Placement in WT and AT1aKO Mice
To examine the possibility of whether CCB could exert a vascular protective effect, in part independently of antagoniz-
ing AT1 receptor stimulation, we used AT1aKO mice and examined the effect of azelnidipine on vascular injury. As previously reported,14 BrdU labeling index in VSMCs in the media and neointima measured 7 days after cuff placement was significantly lower in AT1aKO mice, and neointimal formation 14 days after operation was also significantly less than that in WT mice (Figure 1). These results support the notion that AT1 receptor stimulation plays a role in vascular remodeling via enhancing VSMC proliferation. Azelnidipine at a dose of 0.5 or 1 mg/kg per day, which did not influence blood pressure and heart rate, significantly inhibited DNA synthesis in VSMC and neointimal formation in WT and AT1aKO mice (Table, Figure 1).

**Signaling Mechanism of Inhibitory Effect of Azelnidipine on Neointimal Formation**

To examine the signaling mechanism, we focused on extracellular signal-regulated kinase (ERK) activity, because the ERK pathway, which is activated by the AT1 receptor and growth factors, is critical for cell proliferation.19 We also examined the tyrosine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3 in the injured artery, because STAT seem to be involved in AT1 receptor-mediated c-fos expression in VSMCs.20 Consistent with previous reports,15,16 cuff-induced vascular injury increased phosphorylation of ERK, STAT1, and STAT3 in the injured artery 7 days after cuff placement without changes in total protein levels of these proteins, whereas these increases were smaller in AT1aKO mice (Figure 2). Azelnidipine at a dose of 1 mg/kg per day significantly inhibited activation of ERK and STAT in the injured artery in WT and AT1aKO mice.

**Effect of Azelnidipine on Inflammatory Response and Oxidative Stress Induced by Cuff Placement**

Western blot using pooled artery samples showed that the expression of monocyte chemotactic protein (MCP)-1 and tumor necrosis factor (TNF)-α was increased 7 days after cuff placement in WT and AT1aKO mice; however, this increase was less in AT1aKO mice (Figure 1a, available at http://www.hypertensionaha.org). Azelnidipine (1 mg/kg per day)
inhibited the expression of MCP-1 and TNF-α not only in WT mice but also in AT1aKO mice (Figure Ia). Consistent with these results, immunohistochemical staining showed similar results (Figure Ib and Ic, available online at http://www.hypertensionaha.org). Next, we examined the effect of azelnidipine on expression of p22phox, a membrane-associated NADH/NADPH oxidase subunit, and superoxide anion production in the injured artery. Expression of p22phox and superoxide anion production was increased after cuff placement in WT and AT1aKO mice; however, these increases were less in AT1aKO mice (Figure 3). Azelnidipine inhibited the expression of p22phox and superoxide anion production in both WT and AT1aKO mice (Figure 3).

**Effects of Olmesartan and Azelnidipine on Vascular Remodeling**

Olmesartan at 1 or 3 mg/kg per day significantly decreased neointimal formation and decreased BrdU-positive VSMC in the media and neointima in WT mice (Figure 4) without a change in blood pressure (Table), as previously reported.15 Zhu et al reported that cultured aortic VSMCs prepared from AT1aKO mice display functional AT1β receptors, and activation of these receptors leads to calcium entry and metabolism. To examine the role of AT1β receptor in this cuff-induced vascular injury model, we examined the effect of effective dose of olmesartan (3 mg/kg per day) on DNA synthesis in VSMC in AT1aKO mice and demonstrated that olmesartan did not significantly inhibit BrdU incorporation in VSMCs (Figure 4). Moreover, we demonstrated that the expression of AT1β receptor was very low in femoral artery and did not change after cuff placement.21 These results suggest that the involvement of AT1β receptor is less possible in this cuff-induced vascular injury model in femoral artery. Administration of olmesartan (3 mg/kg per day) significantly decreased the activation of ERK, STAT1, and STAT3 without any change in their total protein levels (Figure II, available online at http://www.hypertensionaha.org.) and inhibited the expression of MCP-1, TNF-α, and p22phox (Figure 3). Because it has been shown that NADH/NADPH oxidases are a major enzymatic source of superoxide generation in vascular cells,22 the upregulation of NADH/NADPH oxidases might cause a burst of superoxides, thereby contributing to the pathophysiology of cuff-induced vascular injury. We demonstrated that proliferation of VSMC, neointimal formation, BrdU index, activation of ERK, STAT1, and STAT3, expression of p22phox and superoxide anion production were attenuated in AT1α KO mice (Figure 3 and I). Administration of a nonhypotensive dose of an ARB, olmesartan, significantly attenuated these vascular responses, supporting the notion that Ang II stimulation via the AT1 receptor exerts pleiotropic effects on vascular remodeling in WT mice (Figure 5 and 6).

**Discussion**

In a mouse model of vascular injury, we have documented that cuff placement around the femoral artery induces neointimal thickening with an increase in VSMC proliferation and inflammation, as well as upregulation of components of the renin–angiotensin system such as the AT1 receptor in injured arteries.14 In the present study, we further demonstrated that superoxide anion production and expression of p22phox were increased in injured arteries induced by cuff placement (Figure 3). Because it has been shown that NADH/NADPH oxidases are a major enzymatic source of superoxide generation in vascular cells,22 the upregulation of NADH/NADPH oxidases might cause a burst of superoxides, thereby contributing to the pathophysiology of cuff-induced vascular injury. We demonstrated that proliferation of VSMC, neointimal formation, inflammation assessed by MCP-1 and TNF-α expression, expression of p22phox, and superoxide anion production were attenuated in AT1αKO mice (Figure 3 and I). Administration of a nonhypotensive dose of an ARB, olmesartan, significantly attenuated these vascular responses, supporting the notion that Ang II stimulation via the AT1 receptor exerts pleiotropic effects on vascular remodeling in WT mice (Figure 5 and 6).
CCB are also widely used for the treatment of hypertension with the expectation of their potential vascular protective effect. It seems to be possible that combination therapy of an ARB with a CCB could exert more beneficial effects on vascular remodeling than monotherapy, although the effects of CCB on vascular diseases are not well established. To explore this possibility and examine the possible cellular and signaling mechanisms involved in the potential vasoprotective effect of CCB and ARB, we used a cuff-induced vascular injury model, in which Ang II plays a role in the pathogenesis of vascular remodeling as mentioned above. We demonstrated that azelnidipine even at a nonhypotensive dose effectively inhibited DNA synthesis of VSMC and neointimal formation in WT and AT1 aKO mice (Figure 1). These results suggest that azelnidipine could inhibit the VSMC proliferation in vascular injury and, further, that this effect of azelnidipine is at least partly independent of the inhibition of AT1 receptor activation. Moreover, we demonstrated that olmesartan or azelnidipine alone at lower doses did not affect neointimal formation; however, coadministration of both olmesartan and azelnidipine at these doses (0.5 and 0.1 mg/kg per day, respectively) significantly inhibited VSMC proliferation and neointimal formation (Figure 4).

It has been reported that AT1 receptor stimulation increases intracellular Ca\(^{2+}\) via an influx of extracellular Ca\(^{2+}\) by opening cell membrane calcium channels or release from the intracellular Ca\(^{2+}\) pool, which leads to activation of Ca\(^{2+}\)-sensitive kinases, such as Pyk2 and Src and transactivation of epidermal growth factor (EGF) receptor, and consequent activation of downstream mitogen-activated protein (MAP) kinases such as ERK, thereby contributing to VSMC growth.\(^{12}\) Seewald et al\(^ {23}\) reported that the Ang II-induced stimulation of ERK is a Ca\(^{2+}\)-dependent process mainly through the influx of Ca\(^{2+}\) from outside of the VSMC and is important for Ang II-induced DNA synthesis in VSMCs. It has also been reported that nifedipine decreased DNA synthesis and total cellular protein content as well as the levels of phosphorylated ERK and Pyk-2 and even the phosphorylation of Pyk-2 in cultured VSMC, and that nifedipine suppressed VSMC proliferation in balloon-injured rat thoracic aorta.\(^ {11}\) Consistent with these results, we demonstrated that administration of a nonhypotensive dose of azelnidipine effectively inhibited activation of ERK in the cuff-induced injured artery (Figure 2). It is established that sequentially activated protein kinases such as ERK mediate further transmission of growth signals to the nucleus through expression of the immediate early growth response gene c-fos, which is regulated by the net interaction of different transcription factors. STAT are known to be activated by many different extracellular signal-
ing proteins including cytokines, growth factors, and Ang II via the AT₁ receptor. We observed that azelnidipine decreased the phosphorylation of STAT1 and STAT3 in WT and AT₁aKO mice (Figure 2). Moreover, we observed that coadministration of a lower dose of azelnidipine and olmesartan attenuated the activation of ERK and STAT, although azelnidipine or olmesartan alone at these doses (0.5 or 0.1 mg/kg per day, respectively) did not affect activation of ERK and STAT (Figure II). These results suggest that azelnidipine-mediated inactivation of ERK and STAT may contribute to the exaggeration of the inhibitory effect of olmesartan on vascular injury.

Oxidative stress induces the expression of redox-sensitive genes for chemoattractant proteins, such as MCP-1, and leukocyte adhesion molecules and intracellular adhesion molecules. The superoxide anion may function as a signaling molecule, mediating increased activity of NF-κB that coordinates the upregulation of these proinflammatory genes. Ang II activates membrane oxidase NADH/NADPH oxidase, which results in production of the superoxide anion, and this proinflammatory action of Ang II on the vessel wall interacts synergistically with those of other cardiovascular risk factors, such as dyslipidemia and diabetes. We examined the possibility that CCB could exert an antioxidative stress effect in the injured artery, and observed that a nonhypotensive dose of azelnidipine inhibited the expression of p22phox, superoxide anion production, and MCP-1 and TNF-α expression in WT and AT₁aKO mice (Figure 3 and I). We also observed that coadministration of a lower non-effective dose of azelnidipine and olmesartan (0.5 and 0.1 mg/kg per day, respectively) inhibited oxidative stress in the injured artery. These results suggest that azelnidipine could inhibit AT₁ receptor-mediated as well as AT₁ receptor-independent oxidative stress in vascular injury, at least partly because of inhibition of the expression of p22phox, thereby decreasing superoxide anion production. Once produced, reactive oxygen species can activate several receptor and nonreceptor protein tyrosine kinases possibly via inhibition of a protein tyrosine phosphatase. These tyrosine kinases include the JAK and Src families, Pyk2/CAK, and the receptors for EGF and PDGF. Therefore, it is conceivable that azelnidipine-mediated inhibition of oxidative stress could contribute to the attenuation of ERK and STAT activation in the injured artery, thereby decreasing VSMC proliferation.

Taken together, our results provide the evidence of a synergistic vascular protective effect of CCBs and ARBs, and further extend the clinical notion that the combination of CCBs and ARBs could be more effective in the treatment of vascular diseases and hypertension than monotherapy.

**Perspectives**

We demonstrated that azelnidipine could exaggerate the vascular protective effects of olmesartan, suggesting that the combination
of CCB azelodipine and ARB olmesartan could be more effective in the treatment of vascular diseases. In addition, a low dosage of combined drugs may be able to reduce the side effects in clinical applications. Moreover, we reported that combination of ARB, valsartan with fluvastatin, or estrogen exaggerate their vascular protective effect in cuff-induced vascular injury model.\textsuperscript{15,16} In contrast, evidences demonstrated that the combination of an ACE inhibitor, perindopril, and an ARB, candesartan cilexetil, may be more effective in the treatment of cardiac and vascular diseases in stroke-prone spontaneously hypertensive rats than the combination of a CCB, amlopidine, with an ACE inhibitor or an ARB or monotherapy with each agent\textsuperscript{27} and that despite a similar reduction in blood pressure, the addition of the CCB, amlopidine, to the ARB, valsartan, failed to provide similar renoprotection to that observed with an equihyptensive regimen of ARB as monotherapy in diabetic nephropathy.\textsuperscript{28} In addition, Lassila et al\textsuperscript{29} reported that CCB, amlodipine, despite a similar reduction in blood pressure, the addition of the signaling mechanism of CCB will provide further insights into discrepancies would be because of different animal models and different dosages. More detailed analysis of the cellular and signaling mechanism of CCB will provide further insights into the pathogenesis of hypertension and atherosclerosis and may initiate new rational and therapeutic concepts.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, the Japan Research Foundation for Clinical Pharmacology, the Tokyo Biochemical Research Foundation, and a grant from the Smoking Research Foundation.

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*Hypertension*. 2004;43:263-269; originally published online January 5, 2004; doi: 10.1161/01.HYP.0000113627.08110.6f

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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