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 AT₁a 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. Ang II is a pleiotropic local mediator of vascular remodeling and the formation of vascular lesions are the modulation of VSMC migration and decreased VSMC apoptosis, 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.

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. 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. 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. 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. 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
injury, independently of the AT1 receptor; and (2) possible
dihydropyridine CCB, azelnidipine, can prevent vascular
vascular injury and explored the following possibilities: (1)
present study, we used this mouse model of cuff-induced
remodeling, especially when focusing on growth-promoting
signaling mechanism of CCB-mediated prevents vascular
inflammation, and neointimal thickening.13

Figure 1. Effects of azelnidipine on DNA synthesis and morpho-
metric changes after cuff placement. Samples were prepared
from cuffed femoral arteries of WT and AT1aKO mice as
described in Methods. A, BrdU uptake in VSMC in media and
neointima was assayed 7 days after operation. B, Morphometric
analysis of media and neointima was performed 14 days after
operation, n=8 to 10 for each group. †P<0.05. ‡P<0.01 versus
without azelnidipine. †‡P<0.05 versus WT without azelnidipine.

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.13–16 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
injury, independently of the AT1 receptor; and (2) possible
signaling mechanism of CCB-mediated prevents 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)17 (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 immunohis-
tochemical 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 anti-
bodies 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 Axioskop 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 etidium, which binds to DNA in the
nucleus and fluoresces red. For etidium 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 1a). Consistent with these results, immunohistochemical staining showed similar results (Figure 1b and 1c, 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 VSMCs 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 AT1b receptors, and activation of these receptors leads to calcium entry and metabolism. To examine the role of AT1b 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 AT1b receptor was very low in femoral artery and did not change after cuff placement.21 These results suggest that the involvement of AT1b 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 5 and Figure 6). Next, we examined the possibility of whether azelnidipine together with olmesartan could exert a synergistic effect to prevent vascular injury. Olmesartan or azelnidipine alone at lower doses (0.5 or 0.1 mg/kg per day, respectively) did not affect neointimal formation, BrdU index, activation of ERK, STAT1, and STAT3, expression of p22phox and superoxide anion production. However, coadministration of olmesartan and azelnidipine at these doses significantly decreased these parameters (Figure 4 to 6 and II). We also observed that a lower dose of azelnidipine (0.1 mg/kg per day) significantly exaggerated the inhibitory effects of olmesartan at effective doses (1 or 3 mg/kg per day) on BrdU index (Figure 4). Moreover, we observed that effective dose of azelnidipine (1 mg/kg per day) exaggerated the inhibitory effect of olmesartan at effective doses of 1 or 3 mg/kg per day on VSMC proliferation in the injured arteries (Figure 4).

**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, and p22phox (Figure 5 and Figure 6) decreased in AT1aKO mice (Figure 3). Because it has been shown that sodium nitroprusside (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).
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 AT1aKO 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 Ca2+ via an influx of extracellular Ca2+ by opening cell membrane calcium channels or release from the intracellular Ca2+ pool, which leads to activation of Ca2+-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 al23 reported that the Ang II-induced stimulation of ERK is a Ca2+-dependent process mainly through the influx of Ca2+ 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 p22^{phox}, superoxide anion production, and MCP-1 and TNF-α expression in WT and AT1aKO mice (Figure 3 and 1). 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 p22^{phox}, 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 azelnidipine 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, amlodipine, 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, amlodipine, to the ARB, valsartan, failed to provide similar renoprotection to that observed with an equihypotensive CCB, amlodipine, to the ARB, valsartan, failed to provide despite a similar reduction in blood pressure, the addition of the hypertensive rats compared with valsartan. These apparent 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.

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Figure 1

(a) Western blot analysis showing MCP-1, TNF-α, and α-SM actin expression in WT and AT1aKO mice. The bar graphs indicate a significant increase in MCP-1 expression in AT1aKO compared to WT (p<0.05). TNF-α expression is also increased in AT1aKO, but the significance is not reported. α-SM actin expression remains unchanged.

(b) Immunohistochemical staining for AT1aKO and WT. The images show a comparison of tissue sections with and without Azel treatment.

(c) Graphs showing the effect of Cuff and Azel on MCP-1 and TNF-α expression. MCP-1 expression is significantly increased in AT1aKO compared to WT (p<0.01) with Azel treatment, while TNF-α expression shows a trend towards increase in AT1aKO with Cuff treatment.

WT

AT1aKO

Azel (-)

Azel (+)

Azel (-)

Azel (+)

MCP-1 (fold increase)

TNF-α (fold increase)