Amlodipine Treatment Reduces Stroke Size in Apolipoprotein E–Deficient Mice

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Background: This study investigated the effects of amlodipine, an L-type calcium channel blocker, on stroke size after focal brain ischemia in apolipoprotein E–deficient (ApoE KO) mice.

Methods: Mice were subjected to middle cerebral artery (MCA) occlusion after being given a high-cholesterol (HCD) or normal diet for 10 weeks with or without amlodipine at a nonhypotensive dose of 3 mg/kg/day. Ischemic brain area was measured by 2,3,5-triphenyltetrazolium chloride staining. Cerebral blood flow was analyzed by laser-Doppler flowmetry. Superoxide anion production in the brain was detected by dihydroethidium staining.

Results: The ApoE KO mice given HCD for 10 weeks showed a larger ischemic lesion size than mice with a normal diet. Amlodipine treatment in parallel with HCD feeding reduced the ischemic lesion size in ApoE KO mice. Interestingly, amlodipine treatment for only the last 2 weeks was also effective in reducing the ischemic lesion size in HCD-fed ApoE KO mice. The neurologic deficit after MCA occlusion was also improved by amlodipine treatment for either 10 weeks or 2 weeks. The decrease in surface cerebral blood flow after MCA occlusion was significantly attenuated in the peripheral region of the MCA territory in amlodipine-treated mice. Amlodipine treatment in HCD-fed ApoE KO mice also reduced superoxide production in the ischemic area of the brain.

Conclusions: These results suggest that amlodipine treatment reduces stroke size and neurologic deficit after focal brain ischemia, possibly through an increase in cerebral blood flow and inhibition of superoxide production. Am J Hypertens 2006;19:1144–1149 © 2006 American Journal of Hypertension, Ltd.

Key Words: Stroke, calcium channel blocker, oxidative stress, cerebral blood flow.

Stroke is one of the leading causes of morbidity and mortality in the industrialized world. However, treatment for stroke after its onset is limited, and management of hypertension is the most effective way to prevent stroke. A recent clinical study, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT), showed that an L-type calcium channel blocker (CCB), amlodipine, had an inhibitory effect on the primary onset of stroke, with similar blood pressure (BP) lowering compared with the diuretic agent chlortalidone. We speculate that CCB could have some unique effects beyond their BP-lowering effect. CCB have an inhibitory effect on carotid atherosclerosis, as reported in the Verapamil in Hypertension and Atherosclerosis Study (VHAS) and in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT). Moreover, a platelet-inhibitory effect acting on a different site from aspirin has been reported, resulting in antithrombotic qualities that could help prevent stroke. However, the inhibitory mechanisms of CCB in atherosclerosis-based brain damage have not been fully elucidated experimentally using cerebral ischemia models.

Apolipoprotein E–deficient (ApoE KO) mice have been widely used as a model of hyperlipidemia, and spontaneously develop atherosclerosis even with a standard chow diet. However, lesion formation is accelerated after treatment with a high-cholesterol diet (HCD). In addition, ApoE KO mice have increased susceptibility to focal cerebral ischemia and also show a worse deficit in locomotor activity after middle cerebral artery (MCA) occlusion, indicating a larger ischemic area partly caused by atherosclerosis-based cerebrovascular injury. Therefore,
we examined the effects of amlodipine on focal brain ischemia after MCA occlusion and explored its potential inhibitory mechanisms, using ApoE KO mice fed HCD.

**Methods**

**Animals**

Adult male ApoE KO mice based on the C57BL/6J strain were obtained from Jackson Laboratory (Bar Harbor, ME), and C57BL/6J mice were purchased from Nihon Clea (Tokyo, Japan). The mice were housed in a room where lighting was controlled (12 h on, 12 h off) and room temperature was kept at 25°C. They were given a standard diet (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

**Treatment With HCD and Amlodipine**

Six-week-old ApoE KO mice and C57BL/6J mice were fed either a standard diet or an HCD consisting of 1.25% cholesterol and 10% coconut oil for 10 weeks. Amlodipine (Pfizer Inc., New York, NY) was administered orally at a dose of 3 mg/kg/day from the day 1 of the HCD for 10 weeks, or for 2 weeks after 8 weeks of HCD. Blood pressure was measured at several points during the experiments by the indirect tail-cuff method with a BP monitor (MK-1030, Muromachi Kikai Co. Ltd., Tokyo, Japan).

**Middle Cerebral Artery Occlusion**

Focal cerebral ischemia was induced by occlusion of the middle cerebral artery (MCA) using an intraluminal filament technique according to a method previously described. Briefly, mice were anesthetized with 6 mg/kg ketamine and 120 mg/kg xylazine in saline administered intraperitoneally. After making a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicone resin (Xantopren; Bayer Dental, Osaka, Japan) was introduced through a small incision in the common carotid artery and advanced to a position 9 mm distal to the carotid bifurcation, for occlusion of the MCA. In sham-operated mice, the carotid arteries were exposed but no suture was inserted. Brain samples were obtained 24 h after MCA occlusion, and their coronal sections with 1-mm thickness were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) as previously described. The ischemic area was regarded as the white region. Ischemic ratio was calculated as the percentage of total brain area.

**Neurologic Score**

Neurologic deficit was evaluated 24 h after MCA occlusion using the neurologic scoring developed by Huang et al. Neurologic score was defined as follows: 0 = no neurologic deficit; 1 = failure to extend left forelimb; 2 = circling to the contralateral side; 3 = falling to the contralateral side at rest; and 4 = no spontaneous motor activity.

**Measurement of Cerebral Blood Flow**

Cerebral blood flow was determined in the territory of the MCA by laser-Doppler flowmetry using a flexible fiber-optic extension to the master probe (Omegaflo FLO-C1, Omegawave, Tokyo, Japan). The tip of the probe was fixed with a stereotactic frame to the intact skull over the territory supplied by the proximal part of the MCA (core; 2 mm caudal to bregma and 6 mm lateral to midline) and the peripheral part of the MCA (periphery; 2 mm caudal to bregma and 3 mm lateral to midline) using a tissue adhesive (Aron Alpha; Toa, Tokyo, Japan). Laser Doppler flow measurements do not quantify cerebral blood flow per gram of tissue, but their use at precisely defined anatomic landmarks serves as a means of comparing cerebral blood flow in the same animal serially over time.

Changes in cerebral blood flow after MCA occlusion were expressed as a percentage of the baseline value obtained by laser-Doppler flowmetry.

**Detection of Superoxide Anion in Brain Sections**

Histologic detection of superoxide anion in the boundary zone of the infarcted cortex was carried out as previously described. In brief, frozen, enzymatically intact, 10-μm-thick sections were prepared from murine brains 24 h after MCA occlusion and incubated immediately with dihydroethidium (DHE; 10 μmol/L) in phosphate-buffered saline for 30 min at 37°C in a humidified chamber protected from light. The substance DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For detection of ethidium, samples were examined with an Axioskop microscope (Axioskop 2 Plus with AxioCam, Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. The intensity of the fluorescence was analyzed and quantified using computer-imaging software (Densitograph, ATTO Corp., Tokyo, Japan).

**Statistical Analysis**

Values are expressed as mean ± SEM. The data were analyzed by two-way analysis of variance. If a statistically significant effect was found, post-hoc analysis was performed to detect the difference between the groups. A value of P < .05 was considered to be statistically significant.

**Results**

**Effect of Amlodipine in Decreasing Ischemic Lesion Size and Attenuating Neurologic Deficit After MCA Occlusion in ApoE KO Mice**

Mice were subjected to MCA occlusion, and the brain was removed 24 h after treatment and stained with TTC. In wild-type C57BL/6 mice, MCA occlusion induced focal
ischemia of approximately 25% of the total area in coronal sections of the brain as previously described. As shown in Fig. 1A, ApoE KO mice with a normal diet exhibited a slightly but not significantly larger ischemic lesion size after MCA occlusion compared with wild-type mice. On the other hand, HCD for 10 weeks in ApoE KO mice significantly increased the ischemic lesion size, to approximately 35% of the total area in coronal sections of the brain (Fig. 1A). Amlodipine treatment for 10 weeks attenuated the HCD-induced increase in ischemic lesion size. A histogram of the ischemic ratio in each section of brain showed a reduction of approximately 30% to 40% reduction in the mid-cerebral cortex and a 60% reduction in the occipital region. Amlodipine treatment also reduced stroke volume in nonatherogenic mice; however, the percent improvement in stroke volume was smaller than that in atherogenic mice (20% reduction in nonatherogenic mice and 41.0% to 42.3% reduction in atherogenic mice) (Figs. 1B and 1C). Interestingly, administration of amlodipine just 8 weeks after the start of HCD, for the last 2 weeks, was also effective in attenuating the ischemic lesion size (Figs. 1B and 1C).

Moreover, neurologic score at 24 h after MCA occlusion was lower in mice treated with amlodipine not only for 10 weeks but also for 2 weeks than in untreated mice (Fig. 2). In these experiments, HCD-fed ApoE KO mice with or without amlodipine treatment showed no difference in BP (Table 1).

**Effect of Amlodipine in Increasing Cerebral Blood Flow After MCA Occlusion**

Cerebral surface blood flow was measured in the core region and peripheral region of the MCA territory by

![FIG. 1.](image1.png)  
**FIG. 1.** Triphenyltetrazolium chloride staining of brain sections from C57BL/6 and apolipoprotein E–deficient (ApoE KO) mice with or without amlodipine (Aml) treatment, 24 h after middle cerebral artery (MCA) occlusion. Representative figures (A) are shown. Brain sections were taken 24 h after MCA occlusion. Coronal sections (0 to 6) were stained with 2,3,5-triphenyltetrazolium chloride (TTC) as described in Methods. Some mice were administered amlodipine (3 mg/kg/day) orally for 10 weeks or 2 weeks before MCA occlusion. The ischemic area (B) and ischemic volume (C) were determined morphometrically by TTC staining and expressed as a percentage of total area in ApoE KO mice. Brain sections are numbered from frontal (section 0) to caudal (section 6). Each group, n = 5 to 8. HCD = high cholesterol diet (1.25% cholesterol), given for 10 weeks; ND = normal diet. *P < .05 v C57BL6 without amlodipine treatment. †P < .05 v ND-fed ApoE KO mice. §P < .05 v HCD-fed ApoE KO mice without amlodipine treatment. Values are mean ± SEM.

**FIG. 2.** Neurologic score of HCD-fed ApoE KO mice after MCA occlusion. The MCA occlusion was performed, and neurologic scoring was used to evaluate the neurologic deficit 24 h after operation, as described in Methods. Some mice were orally treated with amlodipine (3 mg/kg/day) for 10 weeks or 2 weeks before MCA occlusion. Each group, n = 5 to 6. *P < .05 v ND. †P < .05 v HCD without amlodipine treatment. Values are mean ± SEM. Abbreviations as in Fig. 1.

**Table 1.** Blood pressure and plasma cholesterol level in apolipoprotein E–deficient (ApoE KO) mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mm Hg)</th>
<th>Cholesterol (mg/dL)</th>
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<tr>
<td>ND</td>
<td>99.4 ± 1.0</td>
<td>630.8 ± 23.0</td>
</tr>
<tr>
<td>HCD 10W</td>
<td>100.5 ± 2.0</td>
<td>1164.2 ± 55.7*</td>
</tr>
<tr>
<td>Aml 2W</td>
<td>99.5 ± 1.5</td>
<td>1194.6 ± 73.6*</td>
</tr>
<tr>
<td>HCD 10W + Aml 10W</td>
<td>99.3 ± 1.3</td>
<td>1193.6 ± 65.9*</td>
</tr>
</tbody>
</table>

Aml = amlodipine (3 mg/kg/day); BP = blood pressure; HCD = high-cholesterol diet (1.25% cholesterol); ND = normal diet.

Data are mean ± SD. Each group, n = 7.

* P < .05 v ND.
laser-Doppler flowmetry. Cerebral blood flow decreased just after MCA occlusion to about 10% of the basal level in the core region, and to about 60% in the periphery in HCD-fed ApoE KO mice (Fig. 3). This reduction of cerebral blood flow continued for at least 24 h after MCA occlusion. The decrease in cerebral blood flow in the core was not significantly different between mice with or without amlodipine treatment. However, the decrease in cerebral blood flow in the peripheral region was significantly attenuated in mice treated with amlodipine.

**Effect of Amlodipine in Decreasing Oxidative Stress After MCA Occlusion**

To assess the involvement of oxidative stress in the exaggeration of focal brain ischemia, superoxide anion production in the area described in Fig. 4A was evaluated by DHE staining. Superoxide anion production was increased on the occluded side, but not on the nonoccluded side, as shown in Fig. 4B. No increase in superoxide anion production was observed in the brain of nontreated or sham-operated mice. Administration of amlodipine decreased superoxide production in the ischemic area (Figs. 4B and 4C).

**Discussion**

In the present study, we demonstrated that amlodipine treatment attenuated the area of focal ischemia after MCA occlusion in HCD-fed ApoE KO mice, at least partly because of an increase of cerebral blood flow in the peripheral territory of the MCA and a reduction in oxidative stress in the ischemic region.

Dihydropyridine Ca\(^{2+}\) entry blockers have been reported to reduce focal ischemic damage in rats and mice by enhancing marginal blood flow to the ischemic penumbra.\(^{15}\) We demonstrated the benefit of a CCB, amlodipine, in atherosclerosis-based focal brain damage using ApoE KO mice. In ApoE KO mice, treatment with HCD induces hyperlipidemia and the spontaneous development of atherosclerosis. Plasma cholesterol level was significantly increased by HCD treatment, but was not influenced by amlodipine treatment (Table 1). Amlodipine is reported to reduce significantly the intracellular cholesterol level in smooth muscle cells of human atherosclerotic plaques\(^{16}\) and to prevent cholesterol from accumulating in normal cells from patients with coronary atherosclerosis.\(^{17}\) We did not assess cholesterol accumulation in the cerebral vessels in these mice; however, our previous study demonstrated that even 2 weeks of treatment with amlodipine significantly reduced atherosclerotic lesions in the aortic arch.\(^{18}\) Amlodipine treatment could improve the cell milieu presumably via a reduction of oxidative stress or improvement of endothelial function.\(^{19}\) It has been reported that ApoE KO mice with HCD exhibit endothelial dysfunction via impairment of endothelial nitric oxide (NO)–dependent vasorelaxation.\(^{20}\) Increased superoxide production and NADPH oxidase are also observed in ApoE KO mice compared with wild-type animals, indicating the potential importance of oxidative stress in the pathology of atherogenesis in ApoE KO mice. Experimental evidence suggests that calcium channel blockers can improve endothelial dysfunction by restoring NO availability through a mechanism probably related to their antioxidant effect, and therefore could protect endothelial cells from free-radical injury. Amlodipine is a low-clearance, long-acting, vaso-selective dihydropyridine calcium blocker.\(^{21}\) In our study, amlodipine significantly attenuated the increase of superoxide production in the ischemic brain region of ApoE KO mice, as shown in Fig. 4. These results indicate that amlodipine could be effective to prevent atherosclerosis through a reduction of peroxidation and preservation of superoxide dismutase (SOD). Moreover, amlodipine treatment for 2 days before MCA occlusion also showed a tendency to bring about a partial reduction in stroke size, but this was a weaker effect than that of long-term treatment with amlodipine (data not shown). Therefore, a vaso-active property of amlodipine may be involved in its protective effect on the brain.

Amlodipine is also therapeutically effective for lowering BP, as shown in numerous clinical trials. However, in the Comparison of Amlodipine versus Enalapril to Limit Occurrence of Thrombosis (CAMELOT) study, treatment with amlodipine in patients with coronary artery disease and normal BP resulted in reduced adverse cardiovascular events and slowed the progression of atherosclerosis measured by intravascular ultrasound.\(^{22}\) Moreover, amlodipine is reported to have a more potent neuroprotective effect than neutral calcium channel blockers such as nifedipine and nimodipine in rat cerebellar granule cells,\(^{23}\) and also has anti-inflammatory effects mediated not only by inhibition of mono-
cytokine chemoattractant protein (MCP)-1 but also by a decrease in chemokine (C-C motif) receptor 2 in circulating monocytes. Such amlodipine-specific experimental results support our finding of the prevention of stroke expansion by amlodipine.

Moreover, it is reported that clinically used amlodipine is a racemic mixture comprising 50% of the stereoisomer of amlodipine (R(+)H and S(−)H enantiomer). The amlodipine used in this study was also a racemic mixture with the same composition as amlodipine used in clinical settings. Hintze et al. demonstrated that R(+) enantiomer, which lacks calcium channel–blocking activity, can stimulate the release of NO. The substance NO has been demonstrated to have antistroke activity in rodent models, indicating that the protective effect of amlodipine on stroke expansion could be mediated at least partly by the R(+) enantiomer, although we have not examined the direct effect of the stereoisomer of amlodipine in this study.

Recently we reported the possibility that a combination of a CCB and angiotensin receptor blocker might be a useful and effective therapy for the treatment of cardiovascular disease. Our recent report that an L-type calcium blocker, azelnidipine, significantly reduced the atherosclerotic lesion area and oxidative stress in cultured vascular smooth muscle cells when co-administered with lower doses of an angiotensin II (Ang II) type 1 receptor blocker, olmesartan, also supports our hypothesis that improvement of the atherogenic milieu by amlodipine is effective in preventing stroke expansion. Furthermore, we also showed a possible reduction of the ischemic lesion size by administration of an Ang II type 1 receptor blocker via Ang II type 2 receptor stimulation, indicating that combination treatment with amlodipine and an angiotensin

![FIG. 4. Change in superoxide anion production after MCA occlusion in ApoE KO mice. Regions of interest (ROI) (A) are shown. "Ischemic" area is in the ischemic cortex close to the penumbra. "Nonischemic" area is in the contralateral nonischemic cortex. Representative photomicrographs (B) show reproducible dihydroethidium (DHE) staining of brain cortex sections from noninfarcted and infarcted area 24 h after MCA occlusion. Superoxide anion production was analyzed as the intensity of staining with DHE (10 μmol/L) in fresh-frozen sections of brain. Mice were orally treated with or without amlodipine (3 mg/kg/day) for 10 weeks before MCA occlusion. Intensity of fluorescence (C) was determined using computer imaging software as described in Methods. Each group, n = 6 to 7. *P < .05 vs noninfarct area. §P < .05 vs infarct area in HCD mice without amlodipine treatment. Values are mean ± SEM. Abbreviations as in Fig. 1.]
receptor blocker could be therapeutically more effective than each monotherapy and therefore strongly indicated for patients who have more risk factors for atherosclerosis.

Taken together, our findings suggest that amlodipine treatment in patients who have more risk factors for atherosclerosis-based cerebral ischemia (such as higher atherosclerosis scores of the carotid artery or a high serum cholesterol levels) is effective in preventing the potential complications of stroke and possibly in decreasing the incidence of stroke, independent of its BP-lowering effect.

References