Eplerenone Inhibits Atherosclerosis in Nonhuman Primates

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Abstract—Aldosterone may be involved in the pathogenesis of atherosclerosis. We investigated the effect of eplerenone, a selective mineralocorticoid receptor blocker, on atherosclerosis in monkeys fed a high-cholesterol diet. Monkeys fed a high-cholesterol diet for 9 months were divided into 3 groups: those treated with a low dose of eplerenone (30 mg/kg per day); those treated with a high dose of eplerenone (60 mg/kg per day); and the placebo-treated group. The normal group consisted of monkeys fed a normal diet. There were no significant differences in blood pressure and cholesterol levels between the placebo- and eplerenone-treated groups. On the other hand, monocyte chemoattractant protein-1 and malondialdehyde-modified LDL were significantly higher in the placebo-treated group than in the normal group, whereas they were suppressed in the eplerenone-treated groups. The ratio of intimal volume to total volume by intravascular ultrasound analysis imaging of the aortas was dose-dependently lower in the eplerenone-treated groups than in the placebo-treated group. Acetylcholine-induced vasorelaxation was significantly weaker in the placebo-treated group than in the normal group, but the vasorelaxation was strengthened in the eplerenone-treated groups. A significant upregulation of angiotensin-converting enzyme activity was observed in the placebo-treated group, but the activity was suppressed in the eplerenone-treated groups. In conclusion, eplerenone may strengthen the endothelium-dependent relaxation and suppress angiotensin-converting enzyme activity in the vasculature, thus preventing the development of atherosclerosis in nonhuman primates. (Hypertension. 2005;46:1135-1139.)

Key Words: aldosterone ■ angiotensin ■ atherosclerosis ■ endothelium ■ hypercholesterolemia

Aldosterone is a mineralocorticoid hormone that plays an important role in regulating electrolyte balance and blood pressure.1–2 Aldosterone also participates in endothelial dysfunction, vascular fibrosis, and inflammation in the vasculature, and is involved in the pathogenesis of hypertension.3–6 Hypertension and hyperlipidemia are risk factors for atherosclerosis, but reductions in blood pressure and lipid levels do not necessarily result in the prevention of atherosclerotic lesions in nonhuman primates.7–9 On the other hand, in the nonhuman primate atherosclerotic model, angiotensin-converting enzyme (ACE) inhibitor and angiotensin II type 1 receptor blocker (ARB) significantly reduce the progression of atherosclerosis without reducing blood pressure and plasma cholesterol levels.7–9 Such reports suggest that angiotensin II blockade may be useful for preventing the development of atherosclerosis.

In the Randomized Aldosterone Evaluation Study (RALES), a significant 30% survival advantage in chronic heart failure patients was observed with the use of a mineralocorticoid receptor blocker spironolactone in addition to standard therapy, including diuretics and ACE inhibitors.10 It has been shown that ACE inhibitors only transiently suppress aldosterone production, and the RALES results suggest that aldosterone plays a significant role in cardiovascular diseases. In a rabbit atherosclerotic model, eplerenone, a selective mineralocorticoid receptor blocker, prevented relaxation to the endothelial-dependent agonist acetylcholine.11 Moreover, eplerenone reduced the development of atherosclerosis and suppressed serum and macrophage oxidative stress in the mice atherosclerotic model.12 However, in nonhuman primates, it has not been determined whether a mineralocorticoid receptor blocker prevents the development of atherosclerosis.

In the present study, we evaluated the effect of eplerenone, a selective mineralocorticoid receptor blocker, on the development of atherosclerosis in monkeys fed a high-cholesterol diet.

Methods

Animals
All procedures were performed in careful conformance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Experimental protocols were approved by the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College).

Twenty-four male monkeys (Macaca fascicularis) were purchased from Keari Co. The body weight of these monkeys ranged from 3.0 to 4.8 kg. All monkeys were housed at room temperature (23°C to 26°C) with a 12-hour light/dark cycle and had free access to food and water.

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Experimental Protocol
The 24 monkeys were randomly divided into 4 groups. A normal diet and a high-cholesterol diet, which contained 4% cholesterol and 6% corn oil, were purchased from Oriental Yeast Co. Six monkeys were fed a normal diet for 9 months, and these monkeys were called the normal diet group. The remaining 18 monkeys were fed a high-cholesterol diet for 6 months and were divided into 3 groups as follows: a placebo-treated group; a group given eplerenone 30 mg/kg orally per day; and a group given eplerenone 60 mg/kg orally per day. Blood samples were taken to measure levels of cholesterol, ACE activity, renin activity, monocyte chemoattractant protein-1 (MCP-1), and malondialdehyde-modified LDL (MDA-LDL), which is one of the surrogate markers of oxidized LDL. Intravascular ultrasound (IVUS) examination was performed at 9 months in the placebo-treated and eplerenone-treated groups. The aortic arch was used for the ACE activity measurement, and the descending aorta was used for the analysis of the atherosclerotic area of the aortic surface. The left carotid artery was used for the histomorphometrical analysis, and the right carotid artery was used for the vascular response studies.

Levels of Cholesterol, Renin Activity, ACE Activity, and MCP-1
The levels of total cholesterol, LDL cholesterol, and HDL cholesterol were measured after 9 months using a lipid automatic analyzer (7250; Hitachi Co.). Plasma renin activity (PRA) was determined with a renin assay kit (TFB Co.). Plasma ACE activity was measured using a synthetic substrate, hippuryl-His-Leu (Peptide Institute Inc.), specifically designed for ACE.7,9 One unit of ACE activity was defined as the amount of enzyme that cleaved 1 μmol hippuric acid/min.

An ELISA kit was used to determine the serum concentration of MCP-1 and MDA-LDL (SRL Co.).

IVUS Analysis
A 3.2-F imaging catheter with a 30-MHz transducer (Boston Scientific Co.) was inserted through the left femoral artery into the aorta. Automatic pullback images were obtained starting 80 mm from the end of the arch of the descending aorta to the diaphragm. Catheter withdrawal was triggered using a standardized ECG source with 0.2-mm step intervals per cardiac cycle simulation, and the ratio of the intimal volume to the total volume was calculated from the automatic pullback images taken at 0.2-mm intervals (TomTec 3D software).

Histological Analysis
The areas of the thoracic aorta atherosclerotic lesions were measured as described previously.7,9 The thoracic aorta was fixed with buffered formalin. The fixed tissue was stained with oil red O to visualize the lipid deposits on the intimal surface. Using an image analyzer (VM-30; Olympus Co. Ltd.), the atherosclerotic area was calculated as the ratio of the oil red O–stained area to the total surface area. Three segments from the proximal site of carotid artery were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections. The sections were stained with hematoxylin-eosin. The ratio of the intimal area to the total artery area was measured using MacSCOPE Ver 2.2, a computerized morphometry system.

Vascular Response
The carotid arteries were removed to do the vasorelaxation experiments.5,9 Relaxation with acetylcholine administration was assessed by cumulatively adding the agent to the vessels that had been constricted previously to a steady-state tension using 1 μmol/L norepinephrine.9 Papaverine (3×10^{-4} mol/L) was used to induce complete relaxation of the vessels, and the relaxation was evaluated as the percentage of the maximal relaxation for papaverine.

Aortic ACE Activity
Aortic extracts for measurement of ACE activity were prepared as described previously.9 The ACE activity was measured using synthetic substrate hippuryl-His-Leu. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 μmol hippuric acid/min. Protein concentration was assayed with BCA (bicinchoninic acid) Protein Assay Reagents (Pierce), using BSA as a standard.

Statistical Analysis
All numerical data shown in the text are expressed as the mean±SEM. Significant differences among the mean values of multiple groups were evaluated by 1-way ANOVA followed by a post hoc analysis (Fisher’s test). P<0.05 was used as the threshold for statistically significant differences.

Results
Blood Pressure and Cholesterol Levels
All monkeys appeared to be healthy, and there were no differences in body weight, systolic blood pressure, and heart rate between the animals in the normal diet group and those in the high-cholesterol diet groups treated with placebo or eplerenone.

The monkeys that were fed the high-cholesterol diet had total cholesterol and LDL cholesterol levels that were significantly greater than the levels found in the monkeys fed a normal diet. In the high-cholesterol diet group, the level of HDL cholesterol was significantly decreased. Treatment with both doses of eplerenone did not affect cholesterol levels in the monkeys fed a high-cholesterol diet.

PRA, ACE Activity, MCP-1, and MDA-LDL Level
PRA, plasma ACE activity, and serum angiotensin II concentration were not significantly different among the groups (Figure 1). On the other hand, plasma aldosterone concentration was significantly increased in the eplerenone-treated groups, and the concentration in the group given the high dose of eplerenone was higher than in the group given the low dose of eplerenone (Figure 1).

At 9 months, the MCP-1 and MDA-LDL levels were higher in the placebo-treated group than in the normal diet group, and this level was decreased in the eplerenone-treated groups (Figure 2).

IVUS Analysis in Aorta
The ratio of intimal volume to total volume in the placebo-treated group was greater than in the normal diet group, whereas the ratio was significantly lower in a dose-dependent manner in the eplerenone-treated groups (Figure 3A).

Lipid Deposition on the Aortic Surface
Typical photographs of an oil red O–stained aortic surface from the normal diet, placebo-treated, and low and high doses of eplerenone-treated groups at 9 months are shown in Figure 3B. The oil red O–stained area was not observed in the normal diet group. In the placebo-treated group, the ratio of the atherosclerotic area to the total area on the aortic surface was 72±1%, whereas in the low and high doses of the eplerenone-treated groups, the ratios were significantly reduced to 59±3% and 53±5%, respectively (Figure 3C).
Ratio of Intimal Area to Total Area in Carotid Artery
The ratio of the intimal area to the total area in the carotid artery was significantly lower in both eplerenone-treated groups than in the placebo-treated group (Figure 4A).

Vascular Response
Acetylcholine (10 nmol/L to 1 μmol/L) caused an endothelium-dependent relaxation in a concentration-dependent manner in all groups (Figure 4B). The endothelium-dependent relaxation in the placebo-treated group was significantly decreased compared with that in the normal diet group, whereas the relaxation was strengthened in the eplerenone-treated groups.

ACE Activity
Figure 5 shows aortic ACE activity in the monkeys fed a normal diet and a high-cholesterol diet. The aortic ACE activities in the normal group and the placebo-treated group were 0.42±0.05 and 0.98±0.07 mU/mg protein, respectively, and the difference was significant (P<0.01). The level in the high-dose eplerenone-treated group was reduced to the level found in the normal diet group (Figure 5).
LDL and HDL cholesterol levels. However, eplerenone reduced the atherosclerosis without changing the circulating cholesterol levels. Therefore, the effect of eplerenone appears to be independent of reductions in blood pressure and circulating cholesterol levels.

On the other hand, the MDA-LDL level in plasma, like the LDL cholesterol level, was significantly higher in the placebo-treated group than in the normal group at 9 months, but the level was reduced by eplerenone treatment. MDA-LDL is recognized as a surrogate marker of oxidized LDL, and it has been suggested that circulating MDA-LDL levels could be a useful indicator for the identification of patients with coronary artery disease.13,14 Furthermore, a significant negative correlation is observed between the circulating MDA-LDL levels and brachial artery endothelial function in patients.15 In the present study, the endothelial-dependent relaxation was lower in the placebo-treated group than in the normal group, and the relaxation was improved by treatment with eplerenone. Therefore, although eplerenone did not reduce circulating LDL levels, it may prevent the oxidation of LDL, which may result in the improvement of endothelial function.

Serum MCP-1 levels were increased in all monkeys fed a high-cholesterol diet for 9 months, but this level was significantly decreased in the high-cholesterol diet monkeys treated with eplerenone. Oxidized LDL enhances expression of MCP-1, thus stimulating circulating monocytes to become adherent and to differentiate to macrophages.16,17 After treatments with aldosterone, mRNA expression of MCP-1 was increased in the rat heart, and macrophage and proliferating endothelial and vascular smooth muscle cells were observed in the perivascular space of intramural coronary arteries.18 Although the clinical trials RALES10 and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study Investigators (EPHESUS)19 have shown that mineralocorticoid receptor blockers added to standard of care substantially increase survival and hospitalization in patients with heart failure, the mechanism may be important in reducing vascular inflammation.20 The upregulation of MCP-1 is known to play an important role in the initial stages of the atherosclerotic inflammatory process.21 MCP-1 blockade resulted in the prevention of atherosclerosis in a mouse atherosclerotic model.22 Our model is of a very early stage of atherosclerosis, and the significant suppression of MCP-1 levels by eplerenone may contribute to the inhibition of lipid deposition onto the vasculature.

Endothelial dysfunction has been observed in atherosclerotic lesions found in patients and in atherosclerotic models.9,23,24 In the present study, the isolated carotid arteries of monkeys fed a high-cholesterol diet for 9 months, compared with monkeys fed a normal diet, showed a significantly reduced relaxation induced by acetylcholine, an endothelium-dependent vasodilator. However, acetylcholine-induced relaxation in monkeys fed a high-cholesterol diet was improved with eplerenone treatment. Endothelial dysfunction is associated with the progression of atherosclerosis.25 Significant intimal hyperplasia in the carotid artery was observed in the placebo-treated monkeys, but the hyperplasia was also reduced by eplerenone treatment. Therefore, the improvement of endothelial function mediated by eplerenone may be involved in the reduction of atherosclerosis.

Previously, we reported that ACE activity and angiotensin II concentration in the aorta were significantly increased in monkeys fed a high-cholesterol diet, although neither plasma ACE nor renin activities were changed.26 ACE mRNA expression was increased in human atherosclerotic regions, and expression was most prominent in macrophages.27 In the monkey atherosclerotic model, ACE activity, but not chymase activity, was increased in the aorta, and ARB and ACE inhibitor treatment equally reduced the atherosclerotic lesions.28 These findings suggest that ACE-dependent angiotensin II formation in the vasculature plays an important role in the development of atherosclerosis. In general, aldosterone

**Figure 4.** A, Ratio of intimal area to total area in the carotid arteries in monkeys fed a normal diet (N), a high-cholesterol diet with placebo (P), a high-cholesterol diet treated with the low dose of eplerenone (L-E), a high-cholesterol diet treated with the high dose of eplerenone (H-E). B, Acetylcholine-induced vasorelaxation in noradrenaline-precontracted carotid arteries in monkeys fed a normal diet (N), a high-cholesterol diet with placebo (P), a high-cholesterol diet treated with the low dose of eplerenone (L-E), a high-cholesterol diet treated with the low dose of eplerenone, or a high-cholesterol diet treated with the high dose of eplerenone (H-E). *P<0.05 and **P<0.01 (A) and ***P<0.01 vs the normal diet group (B).

**Figure 5.** ACE activities in aortas obtained from monkeys fed a normal diet, a high-cholesterol diet with placebo, a high-cholesterol diet treated with the low dose of eplerenone, a high-cholesterol diet treated with the low dose of eplerenone (30 mg/kg), or a high-cholesterol diet treated with the high dose of eplerenone (60 mg/kg). *P<0.01.
has been thought to be located downstream of angiotensin II in the renin-angiotensin-aldosterone system. However, recent articles have demonstrated that aldosterone could contribute to the induction of ACE expression. For example, Keidar et al. reported that aldosterone upregulated ACE expression in macrophages and resulted in the acceleration of the atherosclerotic process in mice. In the present study, we observed a significant induction of ACE activity in the placebo-treated group compared with the normal group and a reduction of ACE activity in the eplerenone-treated groups. Keider et al. also reported that oxidative stress in the peritoneal macrophages was reduced by eplerenone treatment in the apolipoprotein E-deficient mice. In mice with chronic pressure overload caused by ascending aortic constriction, the filtrated macrophages in cardiac tissues was reduced by treatment with eplerenone. Therefore, the reduction of macrophages by eplerenone may be related to feedback suppression of ACE activity, resulting in preventing atherosclerosis.

**Perspectives**

“Aldosterone escape” is a well-known phenomenon associated with the use of an ACE inhibitor or an ARB. Therefore, when eplerenone is combined with either an ACE inhibitor or an ARB, it may be possible to suppress aldosterone action occurring as a result of “aldosterone escape.” Furthermore, eplerenone significantly reduces the ACE activity in atherosclerotic areas. Eplerenone may act not only downstream of angiotensin II in the renin-angiotensin-aldosterone system but also upstream of the angiotensin II production system. Hence, the combination of eplerenone with either an ACE inhibitor or an ARB should be more useful than monotherapy with either an ACE inhibitor or an ARB.

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**References**

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