Addition of Eplerenone to an Angiotensin-Converting Enzyme Inhibitor Effectively Improves Nitric Oxide Bioavailability

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Abstract—Angiotensin II and aldosterone both promote endothelial dysfunction and atherosclerosis. We investigated the effect of a combination of eplerenone, a selective aldosterone antagonist, and enalapril, an angiotensin-converting enzyme inhibitor, on NO bioavailability and spontaneous atherosclerotic changes. Twenty-four myocardial infarction–prone Watanabe heritable hyperlipidemic rabbits were treated with vehicle (control), eplerenone (50 mg/kg per day), enalapril (3 mg/kg per day), or eplerenone plus enalapril for 8 weeks (n=6 in each group). After treatment, acetylcholine-induced NO production was measured as a surrogate for endothelium-protective function, and vascular peroxynitrite (a product of superoxide and NO) was measured to assess dysfunctional endothelial NO synthase activity. Plaque area was quantified by histology. Intra-aortic infusion of acetylcholine produced an increase in plasma NO concentration that was significantly higher with all of the drug treatments compared with the control. Eplerenone and enalapril, in combination, increased acetylcholine-induced NO by 7.9 nM, which was significantly higher than with either eplerenone or enalapril alone. Vascular peroxynitrite was significantly higher in the control group (1.3 pmol/mg of protein) and significantly lower with combination treatment (0.4 pmol/mg of protein) compared with the enalapril or eplerenone group. The highest tetrahydrobiopterin levels were observed after cotreatment with eplerenone and enalapril. Histology of the thoracic aorta showed a significantly decreased plaque area with combination therapy compared with monotherapy. Combined treatment with a selective aldosterone antagonist and an angiotensin-converting enzyme inhibitor has additive protective effects on endothelial function and on atherosclerotic changes via decreased nitrosative stress. (Hypertension. 2008;51:734-741.)

Key Words: NO ■ endothelial function ■ aldosterone ■ nitrosative stress

The renin-angiotensin-aldosterone system is upregulated in the vasculature of atherosclerotic vessels. Angiotensin II generated locally by modulation of nicotinamide adenine dinucleotide phosphate (NADPH)–dependent oxidases may result in inactivation of NO, and we have shown that chronic treatment with angiotensin II impairs endothelial function by reducing the basal and acetylcholine (ACh)-induced plasma NO concentration (measured by a catheter-type NO sensor) in anesthetized New Zealand white rabbits.1 Blockade of the renin-angiotensin-aldosterone system with angiotensin-converting enzyme (ACE) inhibitors has beneficial effects in high-risk patients, but cardiovascular events are not always appropriately controlled.2 This may be partly because of the effects of aldosterone, the second component of the renin-angiotensin-aldosterone system, and there is increasing evidence to suggest that ACE inhibitors only suppress the production of aldosterone transiently.3,4 Aldosterone also increases NADPH oxidase activity and generation of reactive oxygen species, and mineralocorticoid receptor (MR) blockade improves endothelial function in angiotensin II–infused rats5 and in a high-lipid–diet rabbit model.6 Endothelial dysfunction is characterized by reduced endothelium-dependent relaxation, suggesting reduced availability because of less NO production or enhanced NO inactivation. However, increased production of vasoconstictor factors could also be responsible for reduced endothelium-dependent relaxation, and Blanco-Rivero et al7 have demonstrated that arachidonic acid–derived vasoconstictor prostanoids are involved in endothelial dysfunction produced by aldosterone treatment in normotensive and hypersensitive rats. Therefore, the extent to which aldosterone plays a role in improving NO bioavailability and atherosclerotic change requires further investigation.

In the present study, we hypothesized that aldosterone directly induces impairment of NO bioavailability and con-
tributes to the pathogenesis of atherosclerosis. To test this hypothesis, we examined the effect of eplerenone, a selective aldosterone receptor antagonist, on NO bioavailability (measured with our sensor)\(^1\)\(^-\)\(^8\)\(^,\)\(^9\) and atherosclerotic lesion formation and vascular peroxynitrite level in myocardial-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits. We also examined the effect of combination therapy of eplerenone and an ACE inhibitor, enalapril, on NO bioavailability and plaque formation.

### Materials and Methods

#### Catheter-Type NO Sensor

The integrated architecture and performance of the catheter-type NO sensor have been described previously.\(^1\)^\(^-\)\(^3\)^\(^,\)\(^8\)\(^,\)\(^9\) In brief, the NO sensor (amino-700 XL, Innovative Instruments, 700-μm diameter at the detection tip) measures the oxidative current of NO using an NO monitor (model mNO-T, Innovative Instruments). The baseline (0 level) was set arbitrarily using the amperometric method and calibrated with NO-saturated pure water. The change in current from baseline is expressed as the “change in NO concentration (nM)”.

#### Animal Preparation

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol was approved by the institutional animal care and use committee of Wakayama Medical University. Twenty-four myocardial infarction–prone WHHLMI rabbits (3 months old) were assigned randomly to 1 of 4 groups. The respective groups received oral administration of vehicle and 0.5% carboxymethylcellulose sodium (control group), 50 mg/kg per day of eplerenone, 3 mg/kg per day of enalapril, and 50 mg/kg per day of eplerenone plus 3 mg/kg per day of enalapril daily for 8 weeks. Doses of enalapril and eplerenone were chosen based on previous reports: Hoshida et al\(^10\) showed that chronic treatment with enalapril (3 mg/kg per day) significantly reduces ACE activity in rabbits, and Mihailidou et al\(^1\)\(^1\)\(^-\)\(^3\) reported that eplerenone (50 mg/kg per day) significantly inhibits aldosterone-induced changes in systolic blood pressure and the Na\(^+\)K\(^+\) pump current in New Zealand white rabbits administered aldosterone (50 μg/kg of body weight) via an implanted osmotic minipump.

Rabbits were anesthetized IM with xylazine (10 mg/kg) and ketamine (50 mg/kg) and IV with pentobarbital sodium (10 mg/kg), followed by heparin (1000 U) for anticoagulation. A catheter for ACh or N\(^6\)-methyl-L-arginine (L-NMMA, NO synthase inhibitor) infusion was located in the aortic arch from the external carotid artery, and the NO sensor was inserted through the left femoral artery and located in the abdominal aorta. Aortic blood pressure was monitored simultaneously through a stiff cannula with a strain gauge pressure transducer (Nihon Kohden). Plasma cholesterol and aldosterone levels were measured at 4 and 8 weeks after treatments using the cholesterol oxidase method (Cholesterol E-test, WAKO Chemical Industries, Ltd) and an ELISA kit (Northwest Life Science Specialties, LLC) according to the manufacturer’s protocol.\(^12\)

#### Vessel Preparation

The aorta was placed in chilled, modified Krebs/HEPES buffer (buffer A composition [mmol/L]: NaCl, 99.01; KCl, 4.69; CaCl\(_2\), 1.87; MgSO\(_4\), 1.20; KH\(_2\)PO\(_4\), 1.03; NaHCO\(_3\), 25.8; Na-HEPES, 20.1; and glucose, 11.1 [pH 7.4]), cleaned of excessive adventitial tissue, and cut into 5-mm ring segments.

#### Estimation of Aortic O\(_2^-\) Production

O\(_2^-\) anion production was measured in aortic segments using lucigenin (5 μmol/L) chemiluminescence as described previously.\(^13\) Briefly, after preparation, the vessels were placed in a modified Krebs/HEPES buffer and equilibrated for 30 minutes at 37°C. Scintillation vials containing 2 mL Krebs/HEPES buffer with 5 μmol/L of lucigenin were placed into a scintillation counter operating in the out-of-coincidence mode. After dark adaptation, background counts were recorded, and a vascular segment was added to the vial. Scintillation counts were then recorded every 2 minutes for 15 minutes, and the respective background counts were subtracted. The vessels were then dried in an oven at 90°C for 24 hours for the determination of dry weight.

#### Estimation of Reduced Nicotinamide-Adenine Dinucleotide/NADPH Oxidase Activity in Vessel Homogenates

Aortic segments were placed in chilled buffer A as described above. A 10% vessel homogenate was prepared in 50 mmol/L of phosphate buffer by homogenizing aortic segments in a glass-to-glass motorized homogenizer. The homogenizing buffer (buffer B) was a 50-mmol/L phosphate buffer containing 0.01 mmol/L of EDTA. The homogenate was subjected to low speed centrifugation (1000g) for 10 minutes to remove unbroken cells and debris. Aliquots of the supernatant were then added to scintillation vials containing lucigenin (5 μmol/L) in 2 mL of buffer B. Chemiluminescence over the ensuing 5 minutes in response to the addition of either reduced nicotinamide-adenine dinucleotide (NADH) or NADPH (both 100 μmol/L) was recorded by a luminescence counter (LB9505, Berthold Technologies). Values were standardized to the amount of protein present, which was measured using a commercially available kit (DC Protein Assay, BioRad Laboratories).

#### Western Blotting

Aorta samples were homogenized in ice-cold radioimmunoprecipitation assay buffer (150 mmol/L of NaCl, 50 mmol/L of Tris-Cl, 5 mmol/L of EDTA, 1% v/v of Nonidet P-40, 0.5% wt/v of deoxycholate, 10 mmol/L of phenylmethylsulfonyl fluoride, 2 μg/mL of aprotinin, and 2 μg/mL of leupeptin). Aortic extracts (30 μg protein per lane) were mixed with sample loading buffer and separated on a 12% SDS polyacrylamide gel. Proteins were electro-transferred to polyvinylidene fluoride membranes (Immun-Blot 0.2 μm, Bio-Rad), and bands were detected with a chemiluminescence assay (ECL Plus, Amersham), using primary antibodies for endothelial NO synthase (eNOS; Signal Transduction Laboratories, Cell Signaling Technology) and phosphorylated (Ser\(^1177\)) eNOS (Cell Signaling Technology). The profile of each band was plotted using National Institutes of Health Image, and the densitometric band intensity was determined.

#### Measurement of Aortic Luminal Surface Area

Aortas (n=6 per group) were opened longitudinally and stained with Oil red O solution (Certistain, Merck). The percentage of Oil red O–positive area in relation of the total vessel area was quantified using Adobe Photoshop and National Institutes of Health Image software.

oxidation/nitration, nitrotyrosine can be used as a surrogate index of in vivo–uncoupled NO synthase–dependent damage. Vascular nitrotyrosine in proteins was measured using the NWLSS nitrotyrosine ELISA kit (Northwest Life Science Specialties, LLC) according to the manufacturer’s protocol.\(^12\)
Histological Examination of Atherosclerosis
For the histological examination, a small portion of the thoracic aorta was pressure fixed in 10% neutral-buffered formalin for 48 hours. After fixation and standard paraffin embedding, serial cross-sections were processed for general histological staining with hematoxylin-eosin. The method for determining the intimal area:medial area ratio of the thoracic aorta, which is used as a measure of atherosclerotic burden, has been described previously.14 These slides were scanned microscopically (Keyence, Inc) to enable the use of computerized image analysis with Adobe Photoshop and National Institutes of Health Image analysis software. Six sections from each rabbit aorta were used to determine the intimal area:medial area ratio. Some vessels may have deformed during the procedure, but we assumed that vessel deformation did not influence the calculation of the intimal area:medial area ratio.

Macrophages in the lesion were stained immunohistochemically with a Vectorstain Universal ABC kit (Vector Laboratories, catalog No. PK-6200) using an antimacrophage antibody (RAM-11, Dako Japan Co, Ltd) as the primary antibody and a biotin-labeled anti-mouse IgG antibody (MBL Co, Ltd) as the secondary antibody.

Measurement of Tetrahydrobiopterin in Aortic Segments
Measurement of tetrahydrobiopterin (BH4) was performed by high-performance liquid chromatography after iodine oxidation in acidic or alkaline conditions, as described previously.15 Briefly, aortic segments from rabbits treated with vehicle (control), eplerenone, enalapril, and eplerenone and enalapril were harvested, snap-frozen in liquid nitrogen or dry ice, and stored at −80°C. The frozen segments were divided into 2 fractions of known weight, 1 of which was suspended in HCl (0.25 mL, 0.1N), and the other was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 80°C. The frozen segments were divided into 2 fractions of known weight, 1 of which was suspended in HCl (0.25 mL, 0.1 N), and the other was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 80°C. The frozen segments were divided into 2 fractions of known weight, 1 of which was suspended in HCl (0.25 mL, 0.1 N), and the other was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 80°C. The frozen segments were divided into 2 fractions of known weight, 1 of which was suspended in HCl (0.25 mL, 0.1 N), and the other was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 80°C. The frozen segments were divided into 2 fractions of known weight, 1 of which was suspended in HCl (0.25 mL, 0.1 N), and the other was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 80°C.

Statistical Analysis
All of the data are expressed as means±SEMs based on 6 independent experiments. Differences between groups were analyzed by ANOVA followed by Scheffe test and were considered to be significant when the P value was <0.05.

Table. Mean Blood Pressure and Plasma Cholesterol and Aldosterone Levels in WHHLMI Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mm Hg</th>
<th>Cholesterol, mg/dL</th>
<th>Aldosterone, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.5±1.1</td>
<td>610±18</td>
<td>286±13</td>
</tr>
<tr>
<td>Eplerenone</td>
<td>66.5±1.2</td>
<td>608±12</td>
<td>762±14</td>
</tr>
<tr>
<td>Enalapril</td>
<td>67.1±1.5</td>
<td>611±14</td>
<td>145±12</td>
</tr>
<tr>
<td>Eplerenone plus enalapril</td>
<td>66.8±1.6</td>
<td>580±14</td>
<td>665±12</td>
</tr>
</tbody>
</table>

WHHLMI rabbits were treated with vehicle (control), eplerenone (50 mg/kg per day), enalapril (3 mg/kg per day), and eplerenone plus enalapril for 8 weeks. Data are the means±SEMs, n=6 for each group. MAP indicates mean aortic pressure.

Results
Mean Blood Pressure and Plasma Cholesterol and Aldosterone Levels
We first examined the effects of eplerenone, enalapril, or a combination of the 2 drugs on mean blood pressure and plasma cholesterol and aldosterone concentrations. As shown in the Table, neither mean blood pressure nor plasma cholesterol levels differed significantly among the groups. In contrast, treatment with eplerenone and eplerenone plus enalapril alone, aldosterone decreased significantly during the first 4 weeks and was similar to that in control animals after 8 weeks, suggesting aldosterone breakthrough during treatment with the ACE inhibitor.16

Calibration of Sensors
The mean peak response with NO concentration was 331±14 pA/nM among the 7 sensors in the present study, and this value was comparable to those obtained with the original sensor.17

ACh-Induced Increase in NO Synthesis After Treatment for 8 Weeks
Endothelial function was monitored by ACh-induced NO synthesis (Figure 1A). Intra-aortic infusion of ACh (4 μg/kg per minute for 5 minutes) produced an increase in plasma NO concentration, as shown by the peak response (Figure 1B), and integrated response over the entire period (Figure 1C), which were both significantly greater with each drug treatment compared with the control (P<0.01). The increase in the plasma NO concentration was greater with eplerenone plus enalapril compared with eplerenone or enalapril alone (Figure 1B and 1C).

Basal NO Synthesis After Treatment for 8 Weeks
The effect of treatment for 8 weeks on local basal NO concentration was evaluated based on the decrease in NO concentration in the presence of 5 mg/kg of l-NMMA (Figure 1D). All of the drug treatments affected the basal NO concentration significantly compared with the control (P<0.01). The decrease in the basal peak change in NO concentration by l-NMMA infusion was significantly higher with eplerenone plus enalapril in combination (−4.2±0.2
nM) compared with eplerenone (1.7±0.01 nM) or enalapril (3.1±0.01 nM; P<0.01; Figure 1E). Similar results were obtained for the change in basal integrated plasma NO concentration induced by L-NMMA (Figure 1F).

**Change in Vascular Nitrotyrosine**
Vascular nitrotyrosine was measured as a surrogate index of vascular peroxynitrite and was significantly lower in drug-treated animals than in controls (Figure 2). The eplerenone plus enalapril combination produced a significantly lower level of vascular nitrotyrosine (0.41±0.03 pmol/mg of protein) compared with eplerenone (0.88±0.06 pmol/mg of protein) or enalapril (0.65±0.03 pmol/mg of protein) alone (P<0.01).

**Vascular Reactive Oxygen Species**
O$_2^-$ anion formation was significantly lower in drug-treated animals than in controls (Figure 3A). The eplerenone plus enalapril combination produced a significantly lower level of O$_2^-$ production than either eplerenone or enalapril alone. The oxidase activities in response to NADH and NADPH in the various animal groups are shown in Figure 3B and 3C, respectively. The activities of the NADH- and NADPH-dependent oxidases were both significantly lower in drug-treated animals compared with controls, and the combination treatment produced a significantly lower level of oxidase activity than either eplerenone or enalapril alone.

**Vascular eNOS and eNOS Phosphorylation**
Vascular eNOS was significantly higher in animals treated with enalapril alone or eplerenone plus enalapril compared with control animals or those receiving eplerenone alone.
Vascular eNOS phosphorylation at Ser1177 was significantly higher in treatment with eplerenone or eplerenone plus enalapril compared with control or enalapril alone (Figure 4B).

Atherosclerotic Plaque Formation

Atherosclerotic plaque formation was observed in typical histological sections of thoracic aorta in WHHLMI rabbits. En face Oil red O staining revealed that the plaque area was significantly smaller in animals treated with eplerenone or enalapril alone than in controls and was decreased further by eplerenone plus enalapril in combination (Figure 5A and 5b). Atherosclerotic changes were also quantified by calculating the ratio of the intimal area:medial area in the section, as shown in Figure 5C. The ratio was smaller in animals treated with eplerenone or enalapril alone than in controls and was decreased further by eplerenone plus enalapril (Figure 5D). Immunostaining of the sections with the monoclonal antibody RAM-11, a macrophage marker, revealed that the plaque composition was almost exclusively monocytes/macrophages, regardless of the type of treatment (Figure 5C).

Vascular BH4 Levels

BH4 is of fundamental importance for normal endothelial NO synthase, and all of the drug treatments significantly increased BH4 (Figure 6). The BH4 level in the thoracic aorta was significantly higher with eplerenone plus enalapril (0.56±0.02 ng/mg of tissue) compared with eplerenone...
Discussion

In the present study, we demonstrated for the first time that the administration of enalapril or eplerenone increased the ACh-induced and basal plasma NO concentrations using a catheter-type NO sensor in WHHLMI rabbits. Moreover, combined treatment with eplerenone and enalapril increased the ACh-induced and basal plasma NO concentrations to a significantly higher level than either eplerenone or enalapril alone. In addition, we found that the combination of eplerenone and enalapril was more effective than either drug alone in improving atherosclerotic lesion formation.

There is increasing evidence that ACE inhibitors, such as enalapril, only suppress the production of aldosterone transiently, which is consistent with our finding that the addition of eplerenone, a selective aldosterone antagonist, to enalapril improved NO bioavailability. With enalapril alone, the plasma aldosterone concentration decreased significantly during the first 4 weeks of treatment and reached levels similar to those in control animals after 8 weeks. A recent study showed that short-term MR blockade in patients with congestive heart failure who were already taking an ACE inhibitor resulted in a marked improvement in forearm resistance vessel function, suggesting that MR blockade improves endothelial function independently of and in addition to ACE inhibition.18

There are several potential mechanisms to account for the improvement in NO bioavailability with eplerenone. First, eplerenone may modulate the influence of aldosterone on \( \text{O}_2^- \)-generating oxidases. Such an effect would be consistent with previous observations that blockade of the MR results in reduced free radical injury under conditions of excess aldosterone.19 In the present study, eplerenone or enalapril treatment alone significantly inhibited production of both \( \text{O}_2^- \) and peroxynitrite in WHHLMI rabbits, and combined treatment significantly reduced the concentrations of these anions compared with monotherapy.20 Furthermore, both \( \text{O}_2^- \) and peroxynitrite can oxidize BH4, a critical eNOS cofactor, and lead to eNOS “uncoupling.”21 Uncoupled eNOS produces \( \text{O}_2^- \) rather than NO, and BH4 appears to have a particularly important role in regulating NO and \( \text{O}_2^- \) production by eNOS. In the present study, we showed that both eplerenone and enalapril increased vascular BH4 levels in WHHLMI...
derived O$_2^-$ production and promotion of atherogenesis.\textsuperscript{25} Our histological study demonstrated that both eplerenone and enalapril reduced the plaque area and that combined treatment with eplerenone and enalapril dramatically reduced the plaque area with marked suppression of the production of vascular peroxynitrite. Therefore, our present results suggest the possibility that aldosterone might contribute hypercholesterolemia-induced atherogenesis.

There are several limitations to the study. First, we were unable to clarify the mechanism by which treatment with eplerenone increases plasma aldosterone levels. Saruta et al\textsuperscript{26} demonstrated in a clinical study that eplerenone increases in active plasma renin and also aldosterone levels dose dependently in patients with hypertension. This suggests that the inhibitory effect of eplerenone on aldosterone action disturbs the feedback loop through which aldosterone normally inhibits renin release so that eplerenone actually stimulates the release of renin from the kidney and activates the renin-angiotensin-aldosterone system, leading to an increase in plasma aldosterone levels.

A second limitation is that rapid nongenomic aldosterone effects have been proposed to be of importance in human essential hypertension, in addition to the classic genomic aldosterone effects.\textsuperscript{27} Aldosterone has been reported to induce vasodilation by stimulating NO release through rapid nongenomic effects,\textsuperscript{28,29} but these data are still controversial. For example, Nagata et al\textsuperscript{30} have shown that aldosterone effects may be mediated via a genomic mechanism, because reactive oxygen species could not be detected within 2 hours of aldosterone exposure. In the present study, we examined the chronic effect of eplerenone on NO bioavailability, and the extent to which nongenomic action could contribute to the results is unclear.

Third, the binding affinity of eplerenone to the glucocorticoid receptor is similar to that with the MR. However, the plasma concentration of glucocorticoids is $>1000$ times higher than that of aldosterone, and the dose of eplerenone that blocks aldosterone binding to MR is unlikely to be sufficient to block glucocorticoid binding to the glucocorticoid receptor. However, we cannot completely exclude the possibility of interactions between eplerenone and the glucocorticoid receptor. Finally, the extent to which eNOS uncoupling may contribute to NO bioavailability is uncertain. To provide clear evidence for eNOS uncoupling, it will be necessary to analyze the O$_2^-$ anion production attributable to uncoupled eNOS by quantifying reduction of O$_2^-$ anion formation in the presence of a NOS inhibitor.\textsuperscript{31} However, within these limitations, we concluded that our results provide the first evidence that combined treatment with eplerenone and enalapril has beneficial effects on NO bioavailability and vascular remodeling.

**Perspectives**

The concept of aldosterone escape is an important one to consider when blocking the renin-angiotensin-aldosterone system with ACE inhibitors. The present results provide an experimental rationale for combination therapy with an ACE inhibitor and an aldosterone antagonist for treatment of hypertension and related cardiovascular diseases. Future stud-
ies in a clinical setting are needed to ascertain whether the combined treatment improves NO bioavailability more effectively than monotherapy and results in plaque regression and/or stabilization, as assessed with intravascular ultrasound and optical coherence tomography.

Disclosures

None.

References

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