Eplerenone Shows Renoprotective Effect by Reducing LOX-1–Mediated Adhesion Molecule, PKCε-MAPK-p90RSK, and Rho-Kinase Pathway

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Abstract—Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) may play an important role in atherosclerosis by inducing leukocyte adhesion molecules, such as intercellular and vascular cell adhesion molecule-1 (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1]). We hypothesized that eplerenone, a novel selective aldosterone blocker, produces inhibition of LOX-1–mediated adhesion molecules, suppresses mitogen-activated protein (MAP) kinase and its downstream effector 90 ribosomal S6 kinase (p90RSK) through the protein kinase Cε (PKCε) pathway, and improves endothelial function by inhibition of Rho-kinase in the renal cortex of Dahl salt-sensitive hypertensive (DS) and salt-resistant (DR) rats. Eplerenone (10, 30, and 100 mg/kg per day) was given from the age of 6 weeks to the left ventricular hypertrophy stage (11 weeks) for 5 weeks. At 11 weeks, expression levels of LOX-1, ICAM-1, VCAM-1, and Rho-kinase were higher in DS rats than in DR rats and were decreased by eplerenone. Similarly, upregulated phosphorylation of PKCε, MAP kinase, and p90RSK in DS rats was also inhibited by eplerenone. In contrast, downregulated endothelial nitric oxide synthase mRNA was increased by eplerenone to a similar degree as after treatment with Y-27632, a selective Rho-kinase inhibitor. Eplerenone administration resulted in significant improvement in glomerulosclerosis (eplerenone 10 mg, −61%; 30 mg, −78%; and 100 mg, −84% versus DS; \(P<0.01\), respectively) and urinary protein (10 mg, −78%; 30 mg, −87%; and 100 mg, −88% versus DS; \(P<0.01\), respectively). These results suggest that the renoprotective effects of eplerenone may be partly caused by inhibition of LOX-1–mediated adhesion molecules and PKCε–MAP kinase–p90RSK pathway, and improvement in endothelial function.

Key Words: aldosterone ■ hypertension ■ oxidative stress

Activation of the local renin-angiotensin-aldosterone system in the kidney and heart may play a critical role in the pathogenesis of hypertension and heart failure.1 The local renin-angiotensin-aldosterone system acts in a functionally independent paracrine/autocrine fashion and is thought to play an important role in the development of cardiac hypertrophy and remodeling.2 Therefore, blockade of the renin-angiotensin-aldosterone system locally, rather than in the circulatory system, might improve renal function. The pathophysiological role of aldosterone has received impor-
inhibition of Rho-kinase produced a reduction of macrophage infiltration and renal fibrosis in rats with renal failure. However, the underlying mechanisms of these important pathways associated with renoprotective effects of MR antagonists remain unknown. Accordingly, the purpose of this study was to evaluate whether eplerenone, a novel selective aldosterone blocker, contributed to the improvement of proteinuria and renal damage, and whether eplerenone showed renoprotective effects by reducing these critical pathways in the renal cortex of DS rats.

Methods
All procedures were in accordance with our institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal Models and Experimental Designs
Male inbred DS and Dahl salt-resistant (DR) rats (Eisai Co Ltd, Tokyo, Japan) were weaned and fed a diet containing 0.3% NaCl until age 6 weeks (DS-6w). Thereafter, they were fed a diet containing 8% NaCl until age 11 weeks. The systolic blood pressure (SBP) was measured by the tail-cuff method at the start of the 8% NaCl diet and at 1-week intervals thereafter. In DS rats fed an 8% NaCl diet after age 6 weeks, a stage of concentric left ventricular hypertrophy at 11 weeks is followed by a distinct stage of left ventricular failure, with chamber dilatation at 18 weeks. Twenty-nine 11-week-old DS hypertensive rats were randomly divided into 4 groups: rats treated with vehicle (DS-V; n=8), rats treated with eplerenone (Pfizer Inc) 10 mg/kg per day (DS-E10; n=7), rats treated with eplerenone 30 mg/kg per day (DS-E30; n=7), and rats treated with eplerenone 100 mg/kg per day (DS-E100; n=7). Eplerenone was administered in the chow containing 8% NaCl (Research Diets, Inc, New Brunswick, NJ) at a concentration of 0.11, 0.34, and 1.16 mg eplerenone per gram of chow resulting in approximate doses of 10, 30, and 100 mg/kg per day, respectively. This dose and route of administration were determined to result in optimal pharmacokinetic characteristics for effective in vivo inhibition of MR in the rat. Moreover, the osmotic minipump (model 2ML2; Alzet Corp) containing Y-27632, a selective Rho-kinase inhibitor, saline solution was implanted in 2 groups. Subpressor dose of Y-27632 in DS rats (3 mg/kg per day; DS-Y; n=6) and DR rats (3 mg/kg per day; DR-Y; n=6) was continuously infused for 5 weeks. Age-matched male DR rats served as a control group (DR; n=8).

Quantification of mRNA Using Reverse-Transcription Polymerase Chain Reaction
All procedures used for the mRNA extraction, cDNA synthesis, polymerase chain reaction (PCR), and quantification of PCR product were described in detail in our previous report. PCR was performed using synthetic oligonucleotide primers as previously reported. The numbers of PCR cycles for the 11 genes examined were as follows: LOX-1, 38; VCAM-1, 29; ICAM-1, 28; P-selectin, 29; endothelial nitric oxide synthase (eNOS), 30; Rho-kinase, 30; transforming growth factor (TGF)-β1, 32; connective tissue growth
were analyzed by standard methods. Creatinine clearance was calculated using standard formulas.

The 24-hour urine samples were collected from rats in metabolic cages at 5 weeks after eplerenone therapy for measuring protein and renal injury.

Renal Morphology and Renal Injury Score

After the right kidney sections were stained with hematoxylin and eosin, periodic acid-Schiff, and periodic acid-methenamine silver, glomerulosclerosis, arterio-arteriolar sclerosis, and tubulointerstitial damage were scored as described previously. Glomerulosclerosis was scored in 10 glomeruli in each section as G0 for a normal glomerulus; G1, mild sclerosis (<25%); G2, moderate segmental sclerosis (25% to 50%); G3, severe segmental sclerosis (50% to 75%); and G4, global sclerosis (Figure 1a; G0 through G4).

Arterio-arteriolar sclerosis was scored in 20 to 30 arteries and arterioles in each section as A0, normal; A1, thickening of media; A2, focal hyalinosis with thickening of media; A3, medial thickening with global hyalinosis; A4, fibronectin necrosis and/or cellular hyperplasia with narrowing of arteriolar lumen and/or thrombus formation (Figure 1a; A0 through A4). Tubulointerstitial damage was categorized in 25 areas per section randomly photographed as TI0, normal; TI1, periarterial fibrosis; TI2, peritubular fibrosis; TI3, hyaline casts with focal fibrosis; and TI4, focal fibrosis with cellular infiltration in a large area (Figure 1a; TI1 through TI4). The damage score was standardized in 5 rats in each group as (0×number of S0 + 1×number of S1 + 2×number of S2 + 3×number of S3 + 4×number of S4)/number of S0 + S1 + S2 + S3 + S4, where S represents glomerulosclerosis, arterio-arteriolar sclerosis, or tubulointerstitial damage.

Urine Collection

The 24-hour urine samples were collected from rats in metabolic cages at 5 weeks after eplerenone therapy for measuring protein and creatinine levels. Urinary protein, creatinine in serum, and urine were analyzed by standard methods. Creatinine clearance was calculated using standard formulas.

 Statistical Analysis

All values are expressed as mean±SEM. Mean values were compared among 5 to 7 groups by ANOVA and the Bonferroni post hoc test for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Results

Physiological Profiles Before and After 5 Weeks of Treatment of Eplerenone

Before the treatment was started, at 6 weeks in DS rats fed 0.3% NaCl diet (DS-6w), body weight (BW), SBP, kidney weight (KW), and heart rate were measured and are presented in Table 1. Moreover, at 11 weeks, BW, SBP, KW, kidney weight (KW), and heart rate in the 5 groups were measured and are presented in Table 1. BW was significantly lower in DS rats than in DR rats. Long-term eplerenone therapy in DS rats significantly increased BW. In contrast, DS rats had higher KW and KW/BW compared with DR rats. Chronic eplerenone therapy in DS rats significantly decreased KW and KW/BW. DS rats had markedly higher SBP using the tail-cuff method than DR rats. Long-term eplerenone therapy significantly decreased SBP in DS-E100, but not in DS-E10 and DS-E30. There were no significant differences in heart rate among the 5 groups.

Urineary Parameters Before and After 5 Weeks Treatment of Eplerenone

Urineary protein excretion was significantly increased in DS rats compared with DR rats. Chronic eplerenone therapy in DS rats significantly reduced urinary protein excretion. Creatinine clearance was significantly decreased in DS rats compared with DR rats. Long-term eplerenone therapy in DS rats significantly increased creatinine clearance (Table 1).

Renal Morphology and Renal Injury Score Before and After 5 Weeks of Treatment of Eplerenone

The types of photomicrographs of renal injury scores in glomerulosclerosis (G0 to G4), arterio-arteriolar sclerosis

<p>| TABLE 1. General Characteristics and Renal Function in DR and DS Rats and Eplerenone-Treated DS Rats |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>DR</th>
<th>DS-V</th>
<th>DS-E10</th>
<th>DS-E30</th>
<th>DS-E100</th>
<th>DS-6w</th>
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<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>BW, grams</td>
<td>355±8</td>
<td>316±11†</td>
<td>351±3‡</td>
<td>352±3‡</td>
<td>360±3‡</td>
<td>165±4†§¶</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>137±3</td>
<td>233±7⊠</td>
<td>236±5†</td>
<td>232±5‡</td>
<td>201±5†§¶</td>
<td>123±6¶ §</td>
</tr>
<tr>
<td>KW, mg</td>
<td>119±36</td>
<td>1653±74‡</td>
<td>1409±53‡</td>
<td>1368±47‡</td>
<td>1288±41‡</td>
<td>544±19§ ¶</td>
</tr>
<tr>
<td>KW/BW, mg/g</td>
<td>3.35±0.05</td>
<td>5.22±0.16��</td>
<td>4.01±0.19†</td>
<td>3.88±0.20‡</td>
<td>3.57±0.11‡</td>
<td>3.28±0.08§ ¶</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>463±11</td>
<td>470±13</td>
<td>452±10</td>
<td>457±12</td>
<td>466±11</td>
<td>447±14</td>
</tr>
<tr>
<td>U-pro, mg/day</td>
<td>13.5±1.6</td>
<td>124.8±14.1¶</td>
<td>27.5±3.3¶</td>
<td>16.1±0.9§¶</td>
<td>14.7±1.5¶</td>
<td>12.1±1.6¶</td>
</tr>
<tr>
<td>Ccr, mL/min</td>
<td>4.41±0.26</td>
<td>1.11±0.24†</td>
<td>3.61±0.31¶</td>
<td>3.86±0.24¶</td>
<td>3.99±0.26‡</td>
<td>4.65±0.27‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

BW indicates body weight; Ccr, creatinine clearance; HR, heart rate; KW, kidney weight; SBP, systolic blood pressure; U-pro, urinary protein.

DR, DS-V, and DS-E10 to E100 rats euthanized at 11 weeks and DS-6w rats euthanized at 6 weeks.

*P<0.05, †P<0.01 vs DR.
‡P<0.01 vs DS-V.
§P<0.01 vs DS-E10.
¶P<0.01 vs DS-E100.
‖P<0.01 vs DS-E30.
(A0 to A4), and tubulointerstitial damage (TI0 to TI4) are shown in Figure 1a. Before the treatment was started, at 6 weeks in DS rats fed 0.3% NaCl diet (DS-6w), typical histological photomicrographs of glomerulosclerosis (Figure 1b; P), arterio-arteriolar sclerosis (Figure 1b; Q), and tubulointerstitial damage (Figure 1b; R), and renal injury scores were measured and are shown in Table 2. Moreover, at 11 weeks, typical histological photomicrographs of glomerulosclerosis (Figure 1b; A to E), arterio-arteriolar sclerosis (Figure 1b; F to J), tubulointerstitial damage (Figure 1b; K to O), and renal injury scores were measured and are presented in Table 2. Glomerulosclerosis, arterio-arteriolar sclerosis, and tubulointerstitial damage were significantly increased in DS rats compared with DR rats. Chronic eplerenone therapy in DS rats significantly reduced these renal injury scores.

**LOX-1 and Adhesion Molecules Expression**

LOX-1, VCAM-1, ICAM-1, and P-selectin mRNA and protein levels were significantly increased in DS rats compared with DR rats. Long-term eplerenone therapy in DS rats significantly reduced these tissue LOX-1, VCAM-1, ICAM-1, and P-selectin mRNA and protein levels (Figure 2a through 2d).

**p65NF-κB, PKCε, p44/p42ERK, and p90RSK Phosphorylation**

The phosphorylation of p65NF-κB, PKCε, p44/p42ERK, and p90RSK were significantly higher in DS rats than in DR rats. Chronic eplerenone therapy in DS rats significantly decreased the phosphorylation of p65NF-κB, PKCε, p44/p42ERK, and p90RSK (Figure 4a through 4d).

**Gene Expression of TGF-β1, CTGF, Type I Collagen, and MCP-1**

Gene expression of TGF-β1, CTGF, type I collagen, and MCP-1 were significantly higher in DS-V than in DR. Long-term eplerenone treatment in DS rats reduced these increased tissue TGF-β1, CTGF, type I collagen, and MCP-1 mRNA levels (Figure I, available at http://www.hypertensionaha.org).

**Effect of Eplerenone on Rho-Kinase and eNOS**

To elucidate whether Rho-kinase pathway is involved in DS and DR rats, and whether this pathway is associated with MR, we evaluated the effect of eplerenone and selective Rho-kinase inhibitor, Y-27632, on Rho-kinase. In addition, to evaluate the mechanisms of the beneficial effect of inhibiting the MR and the Rho-kinase pathway, expression of eNOS mRNA was measured. There were no significant differences in the phosphorylation of p44/p42ERK, p90RSK, and p65NF-κB. Chronic eplerenone significantly decreased the phosphorylation of p44/p42ERK, p90RSK, and p65NF-κB (Figure 4a through 4d).
in RhoA protein, Rho-kinase mRNA, and myosin light chain phosphorylation between DR and DR-Y. However, the levels of eNOS mRNA were significantly increased in DR-Y compared with DR (Figure 5a through 5d). Moreover, the levels of RhoA protein were higher in DS-V than in DR, and were lower in DS-E10 to E100 and DS-Y than in DS-V. Expressions of Rho-kinase mRNA were greater in DS-V than in DR, and were smaller in DS-E10 to E100 and DS-Y than in DS-V. In addition, myosin light chain phosphorylation was higher in DS-V than in DR, and less in DS-E10 to E100 and DS-Y than in DS-V. Furthermore, the levels of eNOS mRNA were lower in DS-V than in DR, and were larger in DS-E10 to E100 and DS-Y than in DS-V (Figure 5c and 5d).

**Discussion**

Administration of a novel selective MR inhibitor, eplerenone, to DS rats reduced proteinuria, glomerulosclerosis, arterio-arteriolar sclerosis, and tubulointerstitial damage, and suppressed expression of the genes coding for growth factors, with all 3 doses of eplerenone, despite the absence of blood pressure reduction in the groups receiving the 2 lower doses. Moreover, eplerenone suppressed expression of the LOX-1–mediated adhesion molecule and PKCe–mitogen-activated protein kinase–p90RSK pathway and improved endothelial function by inhibiting the Rho-kinase pathway. These results suggest that these blood pressure–independent renoprotective mechanisms were related to reduction of NF-κB–mediated induction of adhesion molecules from LOX-1 pathway, suppression of intracellular signal transduction via activated mitogen-activated protein kinase, and its downstream effector p90RSK through PKCe pathway, and improvement of endothelial function by inhibition of the Rho-kinase pathway.

We have shown that there are major reductions in renal injury scores as well as expression of inflammatory molecules with all doses of eplerenone despite the absence of blood pressure reduction in the groups receiving the 2 lower doses. There are several recent publications that document the blood pressure–independent beneficial effects of MR blockade in the kidney in several animal models of hypertensive nephropathy. To evaluate the role...
of MR blockade in the progression of vascular injury in saline-drinking stroke-prone spontaneously hypertensive rats, Rocha et al18 implanted spironolactone pellets. The resulting MR blockade produced no difference in SBP, whereas it significantly decreased proteinuria and also increased survival. Therefore, these findings suggest that there is a clear role for aldosterone in the pathogenesis of renal and vascular damage, and aldosterone-related injury is independent of the effects on blood pressure.

LOX-1 facilitates the uptake of Ox-LDL and mediates several of the biological effects of Ox-LDL in endothelial cells.6 Nagase et al reported renal damage and glomerulosclerosis, as well as elevated renal expression of LOX-1 in the DS rat,19 and in the aortic endothelium of hypertensive rats such as spontaneously hypertensive rats, stroke-prone spontaneously hypertensive rats, and Ang II–infused models.20 In addition, LOX-1 is shown to induce expression of adhesion molecules such as VCAM-1 and ICAM-1 on the vascular endothelial cells. Recently, Rocha et al21 indicated that chronic administration of eplerenone to aldosterone/salt-induced hypertensive rats attenuated ICAM-1 expression. These findings suggest that NF-κB–mediated induction of adhesion molecules by expression of LOX-1 pathway is important for onset and advancement of hypertension with renal damage, and aldosterone-related injury is independent of the effects on blood pressure.

Isozymes of PKC differ in their involvement in many cellular functions, and isozyme-specific stimulation or inhibition of PKC may be important therapeutic targets. Recently, Mihailidou et al22 showed that aldosterone-induced upregulation of cotransporter activity and direct downregulation effect on pump activity are both mediated by PKCε, in that the effect of aldosterone can be mimicked by PKCε agonists and blocked by PKCε agonists. In addition, PKCε is essential for ERK activation in rabbit cardiomyocytes.10 Subsequent coupling of cytoplasmic signaling to gene expression occurs through ERK-mediated serine–threonine phosphorylation of their downstream targets, such as p90RSK, acting as transcription regulators.23 Therefore, these findings suggest that PKCε–ERK–p90RSK pathway may play a key role in the pathogenesis of hypertension, and that renoprotective effects of eplerenone may be partly caused by inhibition of this pathway.

The Rho-kinase acting at downstream Rho was found to play a critical role in the myosin regulation system, and the role of this regulation system in hypertension was elucidated by development of Y-27632.24 We have recently reported that the Rho-kinase was involved in a morbid state of hypertension in Ang II–induced hypertensive rat models, and that the cardiovascular remodeling was improved by treatment with Y-27632.25 Recently, we have showed that expression of eNOS was stimulated by administration of Y-27632 in the DS rat.26 Moreover, Hao et al26 showed that in the heart, aorta, and kidney, eNOS gene expression was upregulated in the eplerenone-treated 2-kidney, 1-clip renovascular hypertensive rats. These studies suggest the possibility that the Rho-kinase pathway may play a pivotal role in hypertension with renal damage, in which eplerenone stimulates eNOS production to improve the endothelial function suppressed by Rho-kinase, eventually contributing to renoprotection.

Figure 5. Effects of chronic eplerenone and Y-27632 treatment on RhoA protein and Rho-kinase mRNA (a, b), and myosin light chain phosphorylation and eNOS mRNA (c, d). Values are means±SEM. n=6 per group. *P<0.05, †P<0.01 vs DR, ‡P<0.05, §P<0.01 vs DS-V, ||P<0.05 vs DS-E10. DR, DR-Y, DS-V, DS-E10–100, and DS-Y rats euthanized at 11 weeks.
A study limitation was that we did not observe 24-hour blood pressure changes in this study because we used tail-cuff method. Also, we could not examine the effect of eplerenone on renal artery pressure. Because of these study limitations, we have to perform further investigations to elucidate whether the renoprotective effect of eplerenone that was shown in this study is completely independent from blood pressure control. Otherwise, if these blood pressure changes were significant during the observation period, it is sure that there existed a stage of high blood pressure. And we can also refer to some previous studies\(^{27,28}\) that showed that doses of eplerenone that we used in this study had a poor effect on decreasing blood pressure. This circumstantial evidence would support our hypothesis that eplerenone has blood pressure–independent renoprotective effects.

Acknowledgments

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