Cardioprotective Mechanisms of Eplerenone on Cardiac Performance and Remodeling in Failing Rat Hearts

Naohiko Kobayashi, Kohtaro Yoshida, Shigefumi Nakano, Tomoyuki Ohno, Takeaki Honda, Yusuke Tsubokou, Hiroaki Matsuoka

Abstract—Aldosterone may play a pivotal role in the pathophysiology of heart failure. To elucidate the beneficial cardioprotective mechanism of eplerenone, a novel selective aldosterone blocker, we hypothesized that eplerenone stimulates endothelial NO synthase (eNOS) through Akt and inhibits inducible NO synthase (iNOS) via nuclear factor κB (NF-κB) after the development of oxidative stress and activation of the lectin-like, oxidized, low-density lipoprotein receptor 1 (LOX-1) pathway in Dahl salt-sensitive rats with heart failure. Eplerenone (10, 30, and 100 mg/kg per day) was given from the age of the left ventricular hypertrophy stage (11 weeks) to the failing stage (18 weeks) for 7 weeks. The left ventricular end-systolic pressure-volume relationship was evaluated using a conductance catheter. Decreased percentage of fractional shortening by echocardiography and end-systolic pressure-volume relationship in failing rats was significantly ameliorated by eplerenone. Downregulated eNOS expression, eNOS and Akt phosphorylation, and NOS activity in failing rats were increased by eplerenone. Upregulated expression of the mineralocorticoid receptor aldosterone synthase (CYP11B2); NAD(P)H oxidase p22phox, p47phox, gp91phox, iNOS, and LOX-1; and activated p65 NF-κB, protein kinase CβII, c-Src, p44/p42 extracellular signal-regulated kinase, and p70S6 kinase phosphorylation were inhibited by eplerenone. Eplerenone administration resulted in significant improvement of cardiac function and remodeling and upregulation of sarcoplasmic reticulum Ca²⁺-ATPase expression. These findings suggest that eplerenone may have significant therapeutic potential for heart failure, and these cardioprotective mechanisms of eplerenone may be mediated in part by stimulating eNOS through Akt and inhibiting iNOS via NF-κB after activation of the oxidative stress-LOX-1 pathway and signal transduction pathway. (Hypertension. 2006;47:671-679.)

Key Words: aldosterone ■ oxidative stress ■ heart failure ■ nitric oxide synthase ■ signal transduction

Aldosterone is implicated in the development of myocardial interstitial fibrosis and may play a critical role in the pathophysiology of heart failure, because there is evidence of an increased expression of the aldosterone synthase gene in the failing human heart, suggesting a role for locally produced aldosterone in the heart. The pathophysiological role of aldosterone has received impressive support from 2 clinical studies, the Randomized ALdactone Evaluation Study (RALES) and the Eplerenone Post-acute myocardial infarction Heart failure Efficacy and Survival Study (EPHESUS). These studies showed that low doses of mineralocorticoid receptor (MR) antagonists led to a dramatic reduction of the mortality rate. However, little is known about the role of aldosterone in the development of cardiovascular remodeling and the progression to cardiac failure. The endothelium is an important modulator of vascular tone and function, in part by the synthesis and release of endothelial NO synthase (eNOS). Recent studies have shown that serine/threonine kinase Akt, a downstream effector of phosphatidylinositol 3-kinase, phosphorylates human eNOS at serine 1177 in response to varied stimuli, such as growth factors and shear stress. In addition, the production of reactive oxygen species (ROS) by NAD(P)H oxidase may be involved in vascular smooth muscle cell growth and the pathogenesis of hypertension, and we have shown recently that in deoxycorticosterone acetate–salt rats with left ventricular hypertrophy, NAD(P)H oxidase p22phox, p47phox, and gp91phox expression was upregulated in the left ventricle (LV), potentially contributing to endothelial dysfunction. Moreover, recent reports have demonstrated that oxidized low-density lipoprotein receptor 1 (LOX-1) expression in cardiac myocytes is induced by neurohormonal factors activated in heart failure. Nuclear factor κB (NF-κB) activation is widely acknowledged to be required for the expression of a number of inflammatory and stress-response genes and those resulting in inducible NO synthase (iNOS) production. In fact, we have reported recently that expression of eNOS and iNOS may play a key role in the cardiac performance and remodeling observed in heart failure. Additionally, aldosterone induces phosphorylation of signaling molecules, including protein kinase C (PKC), c-Src, and mitogen-activated protein (MAP) kinase. However, the underlying mechanisms of these important pathways associated with cardioprotective effects...
of MR antagonists in heart failure remain unknown. Accordingly, the purpose of the present study was to evaluate whether eplerenone, a novel selective aldosterone blocker, contributed to the improvement of cardiac dysfunction and remodeling and whether eplerenone showed cardioprotective effects by improving these critical pathways in the LV of failing Dahl salt-sensitive hypertensive (DS) rats. Specifically, we hypothesized that eplerenone stimulates eNOS through the Akt/protein kinase B (PKB) pathway, reduces iNOS via NF-κB after the development of the oxidative stress-LOX-1 pathway, and inhibits MAP kinase and its downstream effector p70S6 kinase through the PKCβII-c-Src pathway.

**Methods**

All of the 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 rats and Dahl salt-resistant (DR) rats (Eisai Co Ltd, Tokyo, Japan) were weaned and fed a diet containing 0.3% NaCl until 6 weeks of age. Thereafter, they were fed a diet containing 8% NaCl until 18 weeks of age. The systolic blood pressure (SBP) was measured by the tail-cuff method at the start of the 8% NaCl diet and until 18 weeks of age. 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. Transthoracic echocardiography evaluating the LV end-diastolic diameter (LVEDD) and fractional shortening (FS) were performed at 18 weeks, as described previously.6,14

**LV End-Systolic Pressure–Volume Relation**

We obtained the slope of the LV end-systolic pressure–volume relation (Ees) by a gradual inferior vena cava occlusion with the use of the conductance catheter technique, as reported previously.11,13

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DR-C</th>
<th>DSHF-V</th>
<th>DSHF-E10</th>
<th>DSHF-E30</th>
<th>DSHF-E100</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>BW, g</td>
<td>464±6</td>
<td>302±6*</td>
<td>395±11‡</td>
<td>397±7‡</td>
<td>406±7‡</td>
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<tr>
<td>SBP, mm Hg</td>
<td>132±3</td>
<td>245±5*</td>
<td>247±5*</td>
<td>243±5*</td>
<td>224±5*‡</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>7.3±0.1</td>
<td>9.4±0.3*</td>
<td>7.7±0.2</td>
<td>7.6±0.2</td>
<td>7.4±0.2</td>
</tr>
<tr>
<td>Ees, mm Hg/mL</td>
<td>2031±100</td>
<td>1143±97*</td>
<td>2567±310‡</td>
<td>2355±114‡</td>
<td>3406±146‡</td>
</tr>
<tr>
<td>Wall/aortic media ratio</td>
<td>0.12±0.01</td>
<td>0.39±0.03*</td>
<td>0.24±0.02‡</td>
<td>0.21±0.02‡</td>
<td>0.22±0.02‡</td>
</tr>
<tr>
<td>Perivascular fibrosis</td>
<td>0.24±0.03</td>
<td>0.80±0.07*</td>
<td>0.55±0.06‡</td>
<td>0.48±0.04‡</td>
<td>0.37±0.03‡</td>
</tr>
<tr>
<td>NADPH oxidase activity, %</td>
<td>100±5.8</td>
<td>247±13.2*</td>
<td>183±6.8*</td>
<td>171±5.3*</td>
<td>155±4.7*‡</td>
</tr>
<tr>
<td>Plasma aldosterone, ng/L</td>
<td>88.7±5.3</td>
<td>204.4±11.4*</td>
<td>97.8±6.8</td>
<td>94.9±7.2</td>
<td>83.8±6.1‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Abbreviations are defined in text. *P<0.01 vs DR-C; †P<0.05; ‡P<0.01 vs DSHF-V; §P<0.05; ¶P<0.01 vs DSHF-E10; ‡P<0.05 vs DSHF-E30.

**Histological Examination and Evaluation of Cardiovascular Remodeling**

Histological examination was performed as described in detail previously.6,14,15

**Plasma Aldosterone Level**

The plasma aldosterone level was measured by radioimmunoassay as reported previously.16

**Quantification of mRNA Using RT-PCR**

All of the procedures used for the mRNA extraction, cDNA synthesis, PCR, and quantification of PCR product were described in detail in our previous reports.6,17 PCR was done using synthetic oligonucleotide primers as reported previously.6,17 The numbers of PCR cycles for the 12 genes examined were as follows: eNOS, 30; iNOS, 31; NAD(P)H oxidase p22phox, 36; p47phox, 32; gp91phox, 33; LOX-1, 38; MR, 32; aldosterone synthase (CYP11B2), 31; sarcoplasmic reticulum Ca2+-ATPase (SERCA2), 32; transforming growth factor (TGF)-β1, 32; type I collagen, 27; and GAPDH, 22.
Figure 1. Micrographs show the effect of chronic eplerenone treatment on small coronary arteries with Masson’s trichrome stain (a to e) and detection of superoxide anion production (f to j) in the LV. a and f refer to DR-C; b and g, DSHF-V; c and h, DSHF-E10; d and i, DSHF-E30; and e and j, to DSHF-E100. Bar=100 µm (a to e) and 500 µm (f to j).
Statistical Analysis
All of the values are expressed as mean±SEM. Mean values were compared among the 5 groups by ANOVA and the Bonferroni post hoc test for multiple comparisons. P<0.05 was considered statistically significant.

Results
Physiological Profiles After 7 Weeks of Treatment of Eplerenone
Body weight (BW), SBP, LV weight (LVW)/BW, heart rate, and plasma aldosterone in the 5 groups are presented in the Table. 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 LVW/BW compared with DR rats. Chronic eplerenone therapy in DS rats significantly decreased LVW/BW. DS rats had markedly higher SBP using the tail-cuff method than DR rats. Long-term eplerenone therapy in DS rats significantly decreased SBP. Heart rate was significantly higher in DS rats than in DR rats. Chronic eplerenone therapy in DS rats significantly decreased heart rate.

Cardiac Function for LVEDD and %FS
LVEDD was significantly higher in DS rats than in DR rats. Long-term eplerenone therapy in DS rats significantly decreased LVEDD. In contrast, DS rats had lower %FS compared with DR rats. Long-term eplerenone therapy in DS rats significantly improved %FS. In addition, its effect was greater in DSHF-E100 than in DSHF-E10 and E30 (Table).

LV End-Systolic Elastance
Ees was significantly lower in DS rats than in DR rats. Chronic eplerenone therapy significantly prevented the fall in Ees seen in failing DS rats. Moreover, its effect was greater in DSHF-E100 than in DSHF-E10 and E30 (Table).

Cardiovascular Remodeling
The morphological appearance, wall:lumen ratio, and perivascular fibrosis of coronary artery in the 5 groups are shown in Figure 1a–1e and Table (n=20 vessels per group). Wall:lumen ratio and perivascular fibrosis were significantly increased in DS rats compared with DR rats. In contrast, chronic eplerenone therapy caused a significant reduction of these ratios. Moreover, its parameter was significantly decreased in DSHF-E100 compared with DSHF-E10 and E30.

Superoxide Anion Production and NADPH Oxidase Activity
To assess the involvement of oxidative stress in the failing DS rats, superoxide anion production and NADPH oxidase...
activity were evaluated. Superoxide anion production (n=5 per group, 2 to 3 sections for each LV) was increased in the failing LV of DS rats compared with DR rats. Chronic eplerenone therapy in DS rats significantly reduced superoxide anion production (Figure 1f–1j). Moreover, NADPH oxidase activity (n=5 per group) was significantly higher in failing DS rats than in DR rats. Long-term eplerenone treatment in DS rats significantly reduced the NADPH oxidase activity (Table). Moreover, its effect was greater in DSHF-E100 than in DSHF-E10 and E30.

Expression of eNOS, Phosphorylation of eNOS and Akt, and NOS Activity
The levels of eNOS mRNA and protein, phosphorylation of eNOS and Akt, and nitrite production were significantly decreased in failing DS rats compared with DR rats. Chronic eplerenone therapy in DS rats significantly increased expression of eNOS mRNA and protein, phosphorylation of eNOS and Akt, and nitrite production. Moreover, these indexes were significantly higher in DSHF-E100 than in DSHF-E10 and E30 (Figure 2A and 2B).

Expression of NADPH Oxidase Subunit, LOX-1, and iNOS
The levels of NADPH oxidase p22phox, p47phox, gp91phox, LOX-1, and iNOS mRNA and protein expression in the LV were significantly higher in failing DS rats than in DR rats.

Chronic eplerenone treatment in DS rats significantly suppressed the expression of p22phox, p47phox, gp91phox, LOX-1, and iNOS mRNA and protein (Figure 3A and 3B and Figure 4A and 4B).

Presence of MR and CYP11B2 mRNA and Plasma Aldosterone Levels in Failing DS Rats
Gene expression of MR and CYP11B2 mRNA was clearly increased in the LV of failing DS rats compared with DR rats (Figure 4A and 4B). In addition, plasma aldosterone levels were higher in DS rats than in DR rats (Table). These increases in failing DS rats were prevented by the administration of eplerenone. These results confirmed the presence of the MR and CYP11B2 mRNA and plasma aldosterone levels in failing DS rats.

Phosphorylation of p65NF-κB, PKCβII, c-Src, p44/p42ERK, and p70S6K
The phosphorylation of p65NF-κB, PKCβII, c-Src, p44/p42ERK, and p70S6K was significantly higher in failing DS rats than in DR rats. Chronic eplerenone therapy in DS rats significantly decreased the phosphorylation of p65NF-κB, PKCβII, c-Src, p44/p42ERK, and p70S6K (Figure 5A and 5B).

Expression of SERCA2, TGF-β1, and Type I Collagen
The levels of SERCA2 mRNA and protein expression were significantly decreased in failing DS rats compared with DR...
Long-term eplerenone treatment in DS rats significantly upregulated the expression of SERCA2 mRNA and protein. Moreover, its effect was greater in DSHF-E100 than in DSHF-E10 and E30. In contrast, the levels of TGF-β1 and type I collagen mRNA and protein expression were significantly higher in DSHF-V than in DR-C. Long-term eplerenone treatment in DS rats reduced the expression of TGF-β1 and type I collagen mRNA and protein (Figure I, available online at http://www.hypertensionaha.org).

Discussion

Figure 6 shows the schematic chart of signal transduction pathways in failing hearts of DS rats in this study. Administration of a novel selective MR inhibitor, eplerenone, to failing hearts of DS rats ameliorated cardiac dysfunction, cardiovascular remodeling, and SERCA2 expression 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. In addition, eplerenone stimulates eNOS expression and phosphorylation through Akt and suppresses expression of the iNOS via the NAD(P)H oxidase-LOX-1-NF-κB pathway and inhibits phosphorylation of p44/p42ERK-p70S6K associated with the PKCβII-c-Src pathway. These findings indicate that these blood pressure-independent cardioprotective mechanisms were related to improvement of endothelial function by the Akt/PKB pathway, reduction of NF-κB-mediated induction of iNOS from the oxidative stress-LOX-1 pathway, and suppression of intracellular signal transduction via activated MAP kinase and its downstream effector p70S6K through the PKCβII-c-Src pathway.

We have demonstrated that there are major improvements of cardiac dysfunction and cardiovascular remodeling, as well as endothelial function, 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 heart of several animal models. Recently, Kuster et al19 evaluated the role of eplerenone in mediating the transition from hypertrophy to failure in mice with chronic pressure overload caused by ascending aortic constriction. The beneficial effects of eplerenone occurred in the absence of a decrease in systemic blood pressure but were associated with a decrease in myocardial oxidative stress and inflammation, which may be involved in mediating the adverse effects of MR activation in pressure overload. Therefore, these results suggest that there is a clear role for aldosterone in the pathogenesis of cardiovascular remodeling and that eplerenone may have direct cardioprotective effects.

Recently, the molecular mechanism of Ca²⁺-independent eNOS activation was elucidated, in which Akt/PKB activation by shearing stress is involved, leading to phosphorylation of the N-terminal 1179 serine residue of eNOS. These findings suggest that eNOS stimulation may be suppressed by
impaired endothelial cells, which may be caused by decreased eNOS phosphorylation mediated by Akt. In a recent report on endothelial functions, Schafer et al. demonstrated that downregulated expression of eNOS protein in the aorta of failing rats with myocardial ischemia could be restored with eplerenone administration. Also, Fraccarollo et al. reported that reduced eNOS activity in the LV of rats with decreased cardiac functions and myocardial remodeling, which were caused by extensive myocardial infarction, was restored with eplerenone administration. Based on these findings together, it is considered that eplerenone may improve endothelial functions by enhancing both the expression of eNOS mRNA or protein and eNOS phosphorylation, and it is suggested that enhancement of Akt-mediated eNOS activation may be involved in the molecular mechanism of the pharmacological action of eplerenone. In addition, oxidative stress increased uptake of oxidized low-density lipoprotein via scavenger receptors. Sun et al. demonstrated that inhibition of NAD(P)H oxidase ameliorates the adverse myocardial effects of aldosterone, suggesting that NAD(P)H oxidase may be a source of ROS in response to MR activation. In addition, eplerenone treatment in hyperlipidemic rabbits reduced the superoxide generation and NAD(P)H oxidase activity of their aortas. Furthermore, oxidative stress is a stimulus for inflammation, such as cytokines and iNOS. ROS can activate ROS-sensitive transcription factors, such as NF-κB and activator protein-1, leading to the recruitment of inflammatory cells and the elaboration of iNOS. It has been reported that NF-κB is activated by various cytokines, including iNOS, suggesting important roles of the transcription factor not only in onset and/or maintaining of inflammation but also in the pathology of arteriosclerosis and organopathy. Recent reports on MR inhibitors demonstrated that NF-κB activation was suppressed by administration of an MR inhibitor spironolactone to rats treated with continuous infusion of aldosterone or to double transgenic rats harboring human renin and angiotensinogen genes (dTGR). These findings suggest the possibilities that activation of NF-κB–mediated induction of

Figure 5. Effects of chronic eplerenone treatment on p65NF-κB, PKCβII, c-Src, p44/p42ERK, and p70S6K phosphorylation. Values are mean±SEM. n=5 per group. *P<0.05, †P<0.01 vs DR-C; ‡P<0.05, §P<0.01 vs DSHF-V; ||P<0.05 vs DSHF-E30; * *P<0.05 vs DSHF-E30.
iNOS from the oxidative stress-LOX-1 pathway plays important roles in the pathology of heart failure and that eplerenone suppresses iNOS induction via suppressing NF-κB activation through oxidative stress-LOX-1, which may lead to improvements in cardiac function and remodeling.

Aldosterone induces phosphorylation of signaling molecules including PKC, c-Src, and MAP kinase. Activation of these pathways are known to be critically involved in vascular smooth muscle cell processes associated with remodeling, inflammation, and altered tone in hypertension. PKC is implicated in the nongenomic effects of aldosterone. In addition, Src also induces activation of MAP kinases associated with cell growth, apoptosis, and collagen deposition, as well as activation of other downstream proteins involved in cell adhesion processes.

Recently, Callera et al suggested that nongenomic signaling pathway by aldosterone occurred through c-Src-regulated activation of MAP kinase, and these responses were abrogated by eplerenone. These findings identify a signaling pathway for aldosterone, involving PKC-c-Src-regulated activation of ERK and their downstream target effector p70S6K, and these signaling pathways may be important mediators of the intracellular signal transduction pathways responsible for cell growth and differentiation.

A study limitation was that we did not observe 24-hour blood pressure changes in this study because we used the tail-cuff method. Because of this study limitation, we have to perform additional investigations to elucidate whether the cardioprotective effect 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 cardioprotective effects. Moreover, the other study limitation was that we assessed the activity of NADPH oxidase in the LV by the measurement of superoxide-enhanced lucigenin chemiluminescence. Many previous studies have investigated NADPH oxidase activity using lucigenin-enhanced chemiluminescence. However, lucigenin has been criticized recently for its ability to redox cycle and its propensity to measure cellular reductase activity independent from NADPH oxidase. In addition, this technique does not provide information concerning the location of superoxide production in situ. For this purpose, we used staining with dihydroethidium, which is specific for superoxide production.

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