Mineralocorticoid Receptor Antagonism Attenuates Cardiac Hypertrophy and Failure in Low-Aldosterone Hypertensive Rats

Kohzo Nagata, Koji Obata, Jinglan Xu, Sahoko Ichihara, Akiko Noda, Hirotaka Kimata, Tomoko Kato, Hideo Izawa, Toyoaki Murohara, Mitsuhiro Yokota

Abstract—Chronic elevation of plasma aldosterone contributes to heart failure. Mineralocorticoid receptor (MR) antagonism is cardioprotective in such a setting, but whether such protection occurs in the presence of low-aldosterone concentrations remains unclear. We investigated whether MR blockade attenuates cardiac hypertrophy and failure in rats with salt-sensitive hypertension. Dahl salt-sensitive (DS) rats fed a high-salt diet from 7 weeks develop concentric left ventricular (LV) hypertrophy secondary to hypertension at 12 weeks followed by heart failure at 19 weeks (DS-CHF). DS rats on such a diet were treated with a non-antihypertensive dose of the selective MR antagonist eplerenone from 12 to 19 weeks. Renin activity and aldosterone concentration in plasma were decreased in DS-CHF rats compared with controls. LV hypertrophy and fibrosis, as well as macrophage infiltration around coronary vessels, were apparent in DS-CHF rats. The amounts of mRNAs for 11β-hydroxysteroid dehydrogenase type 1, MR, monocyte chemoattractant protein 1, and osteopontin were increased in these hearts. Treatment of DS-CHF rats with eplerenone inhibited these changes in gene expression, as well as coronary vascular inflammation and heart failure. Eplerenone attenuated both the decrease in the ratio of reduced to oxidized glutathione and the increase in NADPH oxidase activity apparent in DS-CHF rat hearts. MR blockade with eplerenone thus resulted in attenuation of LV hypertrophy and failure, without an antihypertensive effect, in rats with low-aldosterone hypertension. The beneficial cardiac effects of eplerenone are likely attributable, at least in part, to attenuation of myocardial oxidative stress and coronary vascular inflammation induced by glucocorticoid-activated MRs. (Hypertension. 2006;47:656-664.)

Key Words: hypertension, sodium-dependent □ hypertrophy □ fibrosis □ heart failure □ mineralocorticoids □ glucocorticoids □ oxidative stress

The advent of antihypertensive therapy has markedly reduced cardiovascular morbidity and mortality. The incidence of heart failure continues to increase, however, suggesting that a hypertension-independent mechanism is responsible for this trend. Abnormal activation of the renin–angiotensin–aldosterone system (RAAS) correlates directly with the incidence and extent of target organ damage. Indeed, a chronic increase in the plasma concentration of aldosterone has been shown to contribute to the pathophysiology of heart failure. The harmful effects of aldosterone on the heart include the induction of vascular inflammation and damage, myocardial fibrosis and hypertrophy, ventricular arrhythmia, and cardiac dysfunction. Clinical and experimental studies have shown that blockade of the RAAS with angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 (AT1) receptor blockers, or mineralocorticoid receptor (MR) antagonists provides substantial cardiovascular protection. Overactivity of the RAAS is, thus, thought to be a risk factor for cardiovascular disease. It has remained unclear, however, whether MR antagonism might ameliorate pathological changes in the heart also under conditions of reduced RAAS activity.

The MR was originally thought to be activated only by the mineralocorticoid aldosterone and to act primarily by promoting sodium retention and potassium excretion in the kidney, thereby increasing blood pressure. It is now known to be expressed in nonepithelial cells, such as cardiomyocytes, and to be activated by endogenous glucocorticoids, including cortisol in humans and corticosterone in rodents. Indeed, these 2 glucocorticoids manifest the same affinity for the MR as does aldosterone. Regulation of the access of endogenous glucocorticoids to the MR by 11β-hydroxysteroid dehydrogenase (11β-HSD) is, thus, potentially important in disease states associated with MR activation by these steroids.

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Whereas 11β-HSD type 1 (11β-HSD1) is widely expressed and acts predominantly as a reductase in vivo, converting inactive 11-dehydrocorticosterone into active corticosterone, 11β-HSD2 is expressed at high levels in classic aldosterone target tissues (kidney, colon, and salivary glands) and functions exclusively as an oxidase, mediating the inactivation of corticosterone.11 However, the possible contribution of endogenous glucocorticoids to MR activation by ligands other than aldosterone.10

The recent Randomized ALdactone Evaluation Study (RALES)12 and EPlerenone neuroHormonal Efficacy and SUrvival Study (EPHESUS)13 clinical trials have shown that the addition of MR antagonists to standard care substantially increases the survival and reduces the hospitalization rate of individuals with heart failure. In both trials, aldosterone levels of the patients were normal, and sodium status was unremarkable.13 These results, thus, underscore the importance of MR activation by ligands other than aldosterone. Both ACE inhibitors and AT1 receptor blockers have generally been found to be less effective in hypertensive patients with low renin and aldosterone levels (typically blacks and Japanese) than in such patients with high renin and aldosterone levels (typically whites).15 Given that it has remained unclear whether MR antagonists are cardioprotective in the setting of hypertension with low renin and aldosterone levels, we have now investigated whether selective MR blockade with eplerenone is able to attenuate the progression of cardiac remodeling and the development of heart failure in rats with salt-sensitive hypertension.

**Methods**

**Animals**

Male inbred Dahl salt-sensitive (DS) rats fed an 8% NaCl diet from 7 weeks of age were randomized at 12 weeks to receive the same chow either containing a non-antihypertensive dose of 30 or 100 mg of eplerenone (Pfizer) per kilogram of body mass per day (DS-CHF+EPL30 and DS-CHF+EPL100 rats, respectively, n=8) or not (DS-CHF rats, n=8) until 19 weeks. The doses of eplerenone were determined from our preliminary observations. DS rats maintained on the 0.3% NaCl diet after 7 weeks of age served as age-matched controls (n=6). Systolic blood pressure was measured weekly by the indirect tail-cuff method. At 19 weeks, all of the rats were killed with the use of a low lucigenin concentration (5 μmol/L) was used to minimize artifactual O2− production attributable to redox cycling. In brief, 1 mg of homogenate protein diluted in 1 mL of lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1% Triton X-100) was transferred to an assay tube, and NADPH and dark-adapted lucigenin were added to final concentrations of 100 and 5 μmol/L, respectively, immediately before measurement of chemiluminescence. All of the assays were performed in triplicate. The chemiluminescence signal was sampled every 12 minutes with a tube luminometer (20/20n; Turner Designs), and the respective background counts were subtracted from experimental values.

**Statistical Analysis**

Data are presented as mean±SEM. Differences among groups were assessed by 1-way factorial ANOVA followed by Fisher’s multiple comparison test. A P value of <0.05 was considered statistically significant.

**Results**

**Cardiac Remodeling and Function**

At 19 weeks of age, there was no significant difference in body weight among DS-CHF, DS-CHF+EPL30, DS-CHF+EPL100, and control rats (Table). Systolic blood pressure was significantly higher in DS-CHF rats than in control rats at 8 weeks and thereafter. There was no significant difference in systolic blood pressure among DS-CHF, DS-CHF+EPL30, and DS-CHF+EPL100 rats at any age (Table, data not shown). Heart rate was similar among all 4 of the groups (data not shown). The ratio of LV weight to tibial length, an index of LV hypertrophy, was 52% greater in DS-CHF rats than in control rats at 19 weeks of age, and the overload-induced increase in this parameter was attenuated by eplerenone (Table). The ratio of lung weight to tibial length, an index of pulmonary

**Histology and Immunohistochemistry**

The left ventricle was fixed with ice-cold 4% paraformaldehyde for 16 to 24 hours, embedded in paraffin, and processed for histology and immunohistochemistry as described.6,17 Sections were stained with a mouse monoclonal antibody to rat CD68 (clone ED1, Chemicon, Temecula, CA) to visualize macrophages or with rabbit polyclonal antibodies to mouse connective tissue growth factor (CTGF; Torrey Pines Biolabs, Houston, TX). For negative controls, primary antibodies were replaced with nonimmune immunoglobulin G. Details are available in the online supplement.

**RT-PCR Analysis**

Total RNA was extracted from LV tissue and subjected to quantitative RT-PCR analysis as described,6,17,18 with primers and TaqMan probes specific for rat cDNAs encoding atrial natriuretic peptide, brain natriuretic peptide (BNP), ACE, the AT1 receptor,6 11β-HSD1, 11β-HSD2, CYP11B1, CYP11B2, MR, CTGF, MCP-1, or osteopontin (available in the online supplement). TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase control reagents (Perkin-Elmer) were used for detection of glyceraldehyde-3-phosphate dehydrogenase mRNA as an internal standard. The PCR products of each target gene were subcloned by TA cloning (pGEM-T Easy, Promega) and verified by sequencing. Serial dilutions of cloned plasmid DNA were analyzed for each target gene to determine standard curves for quantitative analysis.

**Assay of Glutathione and Superoxide Production**

The amount of total glutathione (reduced [GSH] and oxidized [GSSG]) in LV tissue was determined by the glutathione reductase and 5,5′-dithiobis-(2-nitrobenzoic acid) recycling assay as described.19 The amount of GSSG was determined by Griffith’s method. Superoxide (O2−) production by total homogenates of LV tissue was measured with the use of a lucigenin-based enhanced chemiluminescence assay as described.19 A low lucigenin concentration (5 μmol/L) was used to minimize artifactual O2− production attributable to redox cycling. In brief, 1 mg of homogenate protein diluted in 1 mL of lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1% Triton X-100) was transferred to an assay tube, and NADPH and dark-adapted lucigenin were added to final concentrations of 100 and 5 μmol/L, respectively, immediately before measurement of chemiluminescence. All of the assays were performed in triplicate. The chemiluminescence signal was sampled every minute for 12 minutes with a tube luminometer (20/20n; Turner Designs), and the respective background counts were subtracted from experimental values.

**Echocardiographic and Hemodynamic Analyses**

At 19 weeks of age, rats were subjected to transthoracic echocardiography as described.6 The peak negative myocardial velocity gradient was derived from tissue Doppler imaging for evaluation of diastolic function.16 Left ventricular (LV) end-diastolic pressure was determined as described.17 Details are available in the online supplement.

**Plasma Analysis**

A blood sample was collected from the right carotid artery after hemodynamic measurements. Renin activity (Renin-Riabead, Abbott Japan) and the concentrations of aldosterone (SPAC-S Aldosterone kit, TFB) and corticosterone (Rat Corticosterone [125I]Biotrak Assay System, Amersham Biosciences) in plasma were measured with radioimmunoassay kits.
Renin Activity and Aldosterone and Corticosterone Concentrations in Plasma

Renin activity and aldosterone concentration in plasma were lower in DS-CHF rats than in control rats (Figure 3A and 3B). Treatment with eplerenone attenuated the decrease in renin activity but not that in aldosterone concentration. The plasma concentration of corticosterone did not differ significantly among the 4 groups of rats (Figure 3C).

Coronary Vascular Inflammation and Myocardial Oxidative Stress

Immunostaining of the LV myocardium with an antibody to the monocyte-macrophage marker CD68 revealed pronounced macrophage infiltration in the perivascular space of intramural coronary vessels in DS-CHF rats (Figure 4A). This infiltration was accompanied by an increase in the abundance of mRNAs for the proinflammatory cytokines monocyte chemoattractant protein 1 (MCP-1) and osteopontin (Figure 4B and 4C). These inflammatory responses were abrogated by treatment with eplerenone.

Hemodynamic overload resulted in an increase in the NADPH-dependent production of $O_2^-$ and a decrease in the GSH/GSSG ratio in the left ventricle of DS-CHF rats compared with controls (Figure 5), indicative of myocardial oxidative stress. These effects were attenuated by treatment with eplerenone.

Cardiac Expression of Steroid Metabolic Genes

The abundance of ACE and AT$_{1A}$ receptor mRNAs in the myocardium was increased in DS-CHF rats compared with control rats, indicative of the development of congestive heart failure; treatment with eplerenone also significantly reduced the load-induced increase in this parameter.

Echocardiography revealed that LV posterior wall thickness and LV end diastolic dimension (LVDd) were greater in DS-CHF rats than in control rats at 19 weeks of age (Table). Fractional shortening was decreased in DS-CHF rats relative to that in controls. Treatment with eplerenone attenuated the changes in these parameters. The peak negative myocardial velocity gradient, an index of LV early-diastolic function, was reduced in DS-CHF rats, and this decrease was attenuated by eplerenone. LV end-diastolic pressure was markedly increased in DS-CHF rats, and, again, this difference was reduced by eplerenone. These results thus indicate that long-term treatment with eplerenone inhibited the transition to heart failure in DS rats by suppressing LV remodeling and improving LV systolic and diastolic function without an antihypertensive effect.

Histological analysis revealed that hemodynamic overload had increased the cross-sectional area of cardiac myocytes in DS-CHF rats by 45% compared with age-matched controls (Figure 1A and 1B). The extent of load-induced cardiomyocyte hypertrophy was reduced by treatment with eplerenone. Hemodynamic overload resulted in upregulation of the expression of fetal-type cardiac genes, including those for atrial natriuretic peptide and BNP, in the heart of DS-CHF rats, and this effect was inhibited by eplerenone (Figure 1C and 1D). Marked perivascular and interstitial fibrosis was also detected in the left ventricle of DS-CHF rats as compared with age-matched controls (Figure 2A and 2B); this increase in cardiac fibrosis was also significantly reduced by eplerenone. Immunohistochemical analysis revealed the abundance of CTGF in the heart to be increased in DS-CHF rats compared with controls in a manner sensitive to treatment with eplerenone (Figure 2C). The abundance of CTGF mRNA was also increased in the heart of DS-CHF rats in a manner sensitive to treatment with eplerenone (Figure 2D).

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various experimental groups, and expression of the CYP11B2 gene was not detected (data not shown).

Discussion
We have shown that treatment with eplerenone attenuates cardiac remodeling and failure, without an antihypertensive effect, in DS rats fed a high-salt diet, a model of low-renin, low-aldosterone hypertension. In this model, glucocorticoid-mediated MR activation may contribute to the development of cardiac hypertrophy and fibrosis, as well as to coronary vascular inflammation. Increased oxidative stress appears to underlie activation of the glucocorticoid–MR complex in the cardiovascular system of these animals. The beneficial effects of eplerenone in this model are likely attributable, at least in part, to attenuation of the myocardial oxidative stress and coronary vascular inflammation induced by the glucocorticoid-activated MR.

The increase in MR gene expression apparent in the heart of DS-CHF rats may contribute to an increase in MR-mediated signaling. Transgenic mice that express the human MR were shown previously to develop mild dilated cardiomyopathy.20 Cardiac hypertrophy is an adaptive response to pathological conditions, such as systemic hypertension and myocardial infarction. High-affinity MRs are localized in cardiomyocytes and endothelial cells of the normal heart.21 Furthermore, both aldosterone and the MR antagonists spironolactone and eplerenone modulate LV hypertrophy.22,23 Aldosterone also directly promotes cardiomyocyte hypertrophy and induces the expression of fetal-type cardiac genes in vitro in a manner dependent on activation of the MR.24 In vivo, however, it is difficult to distinguish between a direct action on myocyte growth and an effect mediated by an increase in mechanical load. In the present study, we, therefore, used a nonantihypertensive dose of eplerenone to investigate whether antagonism of the MR attenuates cardiac hypertrophy and heart failure in DS rats in a manner independent of an antihypertensive effect. We found that, in these animals with low-aldosterone hypertension, selective blockade of the MR suppressed LV (and cardiomyocyte) hypertrophy and inhibited the upregulation of fetal-type cardiac genes normally apparent in the failing heart.

DS-CHF rats exhibited an increase in the extent of interstitial and perivascular fibrosis in the LV myocardium, consistent with previous observations with this animal model.6 Cardiac fibrosis...
is a pathological feature associated with hypertension, as well as with cardiac hypertrophy and failure. CTGF is a potent profibrotic factor that is implicated in fibroblast proliferation, cell adhesion, and the synthesis of extracellular matrix. In the present study, the amounts of CTGF mRNA and protein were increased in the heart of DS-CHF rats, and these effects were ameliorated by treatment with eplerenone. Upregulation of CTGF gene expression has been associated with heart failure, and aldosterone-induced MR activation was shown previously to increase CTGF gene expression in a mouse innermedullary collecting-duct cell line. CTGF expression is also rapidly increased in cardiac myocytes exposed to phenylephrine, endothelin-1, or prohypertrophic stimuli, consistent with the notion that upregulation of this growth factor contributes to cardiac hypertrophy and fibrosis. The eplerenone-induced inhibition of CTGF gene expression in the myocardium of DS-CHF rats in the present study may, thus, have contributed to the attenuation of cardiac remodeling and heart failure by this drug.

Corticosterone, the endogenous glucocorticoid of rodents, manifests the same affinity for the MR as does aldosterone and is present at concentrations 2 orders of magnitude greater than those of aldosterone. Corticosterone concentration of steroids available for binding to their cognate receptors depends on the free extracellular concentration and on the intracellular activities of 11β-HSD1 and 11β-HSD2. Excessive salt intake suppresses the systemic RAAS. Indeed, in the present study, both plasma renin activity and plasma aldosterone concentration were markedly reduced in DS-CHF rats, whereas the plasma corticosterone level did not differ between DS-CHF and control rats. We also found that myocardial expression of the 11β-HSD1 gene was greatly increased in DS-CHF rats, whereas that of the 11β-HSD2 gene was virtually undetectable in the heart of either DS-CHF or control rats, consistent with previous observations in the neonatal rat heart. The intracellular concentration of corticosterone available for binding to the MR is, thus, likely much higher than that of aldosterone in the heart of DS-CHF rats, suggesting that most MRs in the heart of these animals are occupied by corticosterone. Loss-of-function mutations or inhibition of 11β-HSD2 also result both in activation of the MR by glucocorticoids and in salt-sensitive (low-renin, low-aldosterone) hypertension. We speculate that, in DS-CHF rats, the increased expression of 11β-HSD1 in the virtual absence of 11β-HSD2 may contribute to local glucocorticoid excess in the heart. Our observation that the increase in 11β-HSD1 gene expression in the heart of DS-CHF rats was inhibited by eplerenone suggests that glucocorticoid-mediated MR activation might be responsible for upregulation of the expression of this gene, forming a positive

Figure 2. Fibrosis and the expression of CTGF in the left ventricle of control (n=6), DS-CHF (n=8), DS-CHF+EPL30 (n=8), and DS-CHF+EPL100 (n=8) rats. (A) Azan-Mallory staining of transverse sections. Scale bar, 100 μm. (B) Relative area of interstitial fibrosis determined from sections similar to those in A. (C) Immunohistochemical staining of CTGF. Scale bar, 100 μm. (D) Quantitation of CTGF mRNA. Data in B and D are mean±SEM; *P<0.05 vs control rats, †P<0.05 vs DS-CHF rats.
Two cytochrome P450 enzymes are responsible for catalyzing the terminal steps in corticosterone and aldosterone synthesis: 11β-hydroxylase (P45011β or CYP11B1) and aldosterone synthase (P450aldo or CYP11B2), respectively. Expression of the CYP11B1 gene was minimal and that of the CYP11B2 gene was not detected in the heart of DS-CHF or control rats. In contrast, myocardial expression of both the ACE and AT1A receptor genes was upregulated in DS-CHF rats in a manner sensitive to eplerenone. These data indicate that the local renin–angiotensin system is activated in the heart of DS-CHF rats without an accompanying increase in aldosterone synthesis.

Although glucocorticoids have been thought to act as antagonists of the MR in nonepithelial cells, such as cardiomyocytes and brain cells, glucocorticoid–MR complexes are activated as a result of the generation of reactive oxygen species. NADPH oxidase is implicated in O2− signaling in the vasculature, and aldosterone-induced MR activation results in activation of vascular NADPH oxidase. A phagocyte-type NADPH oxidase is also expressed in the heart, most prominently in cardiomyocytes, and is a major source of O2− during the development of pressure-overload LV hypertrophy. We found that NADPH-dependent generation of O2− was increased and that the GSH/GSSG ratio was decreased in the heart of DS-CHF rats, consistent with data showing that increased oxidative stress in the myocardium may contribute to the transition from cardiac hypertrophy to failure. Treatment with eplerenone inhibited these effects, suggesting that this drug suppressed the progression to heart failure by reducing oxidative stress.

Macrophage infiltration into the perivascular space of intramural coronary vessels was accompanied by upregulation of the expression of the genes for the proinflammatory cytokines MCP-1 and osteopontin in the heart of DS-CHF rats. The observation that these effects were abrogated by treatment with eplerenone suggests that coronary vascular inflammatory responses induced by MR activation contribute to the cardiovascular injury and fibrosis apparent in this model. MCP-1 plays an important role in the infiltration of inflammatory cells into cardiovascular tissue and has been associated with the progression and severity of heart failure in rats. Osteopontin is implicated in aldosterone- and salt-induced vascular pathology as a result of its effects on cell-mediated immunity and vascular remodeling. The eplerenone-induced inhibition of coronary vascular inflammation may, thus, have contributed to the attenuation of cardiovascular remodeling in DS-CHF rats and thereby led to the improvement of LV function.

It is possible that the increased expression of the 11β-HSD1 gene in the heart of DS-CHF rats may promote glucocorticoid receptor–mediated activation of the BNP gene in cardiomyocytes and that this effect might explain, at least in part, the difference in the apparent potency with which eplerenone inhibited the upregulation of BNP versus MCP-1 gene expression in these animals. However, eplerenone specifically blocks the MR, and it inhibited the upregulation of both BNP and MCP-1 gene expression, suggesting a role for the MR in the activation of both these genes.
Some limitations of this study are important to note. First, given that aldosterone (or MR agonists) exerts both epithelial and nonepithelial effects, it might be considered ideal to have a control group treated with a dose of amiloride equivalent to eplerenone at 100 mg/kg to distinguish nonepithelial versus epithelial effects of the latter drug in the present study. To our knowledge, however, previous animal studies of the effects of MR antagonists on the heart have not included a control group treated with amiloride. Indeed, it may be difficult to determine an appropriate dose of amiloride for use as a control for MR antagonists. Second, the indirect tail-cuff method we used to determine systolic blood pressure is not adequate for ruling out the possibility that small changes in blood pressure may have contributed, in part, to the attenuation of cardiac remodeling and failure caused by eplerenone. Finally, future studies are needed to additionally explore the pathophysiological role of the MR occupied fully by endogenous glucocorticoids in response to altered intracellular redox status in the cardiovascular system.

Perspectives
We have shown that selective MR blockade with eplerenone attenuates the development of heart failure, as well as inhibits the progression of cardiac hypertrophy and fibrosis and coronary vascular inflammation, in a manner independent of its antihypertensive effect, in rats with salt-sensitive hypertension. Our results suggest that glucocorticoid-mediated MR activation contributes to cardiac and coronary vascular injury in this model of low-renin, low-aldosterone hypertension. Increased oxidative stress appears to be a driver for activation...
of the glucocorticoid–MR complex in the cardiovascular system of these animals. The beneficial effects of eplerenone are likely attributable, at least in part, to attenuation of myocardial oxidative stress and coronary vascular inflammation induced by the glucocorticoid-activated MR. MR antagonism may, thus, be of wide therapeutic potential in various cardiovascular diseases, with its benefit not being limited to those characterized by aldosterone or salt excess.

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