Original Article

The Specific Mineralocorticoid Receptor Blocker Eplerenone Attenuates Left Ventricular Remodeling in Mice Lacking the Gene Encoding Guanylyl Cyclase-A

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Mineralocorticoid receptor (MR) blockers attenuate cardiac remodeling in experimental models of heart failure, myocardial infarction and pressure-overload, in which the renin-angiotensin-aldosterone system is activated. Mice lacking the gene encoding guanylyl cyclase-A (GC-A), a common receptor for atrial and brain natriuretic peptide (ANP and BNP, respectively), show marked cardiac hypertrophy and fibrosis, which are almost completely inhibited by both genetic and pharmacological blockade of type 1 angiotensin II receptors. However, the effect of eplerenone, a specific MR blocker, on cardiac remodeling in GC-A knockout (GC-A KO) mice remains unknown. Male 12-week-old GC-A KO mice were assigned to control, eplerenone and hydralazine groups (*n*=6–7/group). Treatment with eplerenone at a dose of 100 mg/kg body weight/d reduced heart weight/body weight ratios, interstitial fibrosis and blood pressure to levels similar to those seen in wild type mice, in association with reduced transcription of atrial natriuretic peptide, brain natriuretic peptide, transforming growth factor-β1, collagen I and collagen III. Although hydralazine (5 mg/kg body weight/d) exerted a similar effect on blood pressure, it did not inhibit the cardiac remodeling in GC-A KO mice. In conclusion, eplerenone attenuates cardiac remodeling in GC-A KO mice, most likely in a blood pressure–independent manner, which suggests that signaling downstream of MR is involved in the ventricular remodeling of GC-A KO mice. (*Hypertens Res* 2008; 31: 1251–1256)

***Key Words*: guanylyl cyclase A, mineralocorticoid receptor blocker, cardiac remodeling, aldosterone**

Introduction

The efficacy of mineralocorticoid receptor (MR) blockers in the treatment of heart failure and acute myocardial infarction was recently demonstrated in two large randomized clinical trials (*1*, *2*). To further confirm the usefulness of these drugs and to investigate their mechanism of action, the effects of spironolactone and eplerenone have been investigated in a number of experimental animal models of hypertension (*3*) and myocardial infarction (*4*), and pressure-overload model in which the renin-angiotensin-aldosterone and sympathetic

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nervous systems are initially over-activated to maintain cardiac pumping performance as an extra-cardiac compensatory mechanism (*5*). However, this over-activation eventually leads to cardiac remodeling and dysfunction (*6*, *7*). In these settings, MR blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers and β-adrenergic receptor blockers all appear to prevent cardiac remodeling and thus improve rates of mortality and morbidity (*3–5*).

Mice lacking the gene encoding guanylyl cyclase-A (GC-A), GC-A KO, a common receptor for atrial and brain natriuretic peptides (ANP and BNP, respectively), show salt-insensitive hypertension, left ventricular hypertrophy and interstitial fibrosis under unstressed conditions (*8–10*). An earlier report from our laboratory (*11*) revealed that cardiac remodeling in mice is almost completely blocked by either genetic or pharmacological blockade of the type 1 angiotensin II receptor (AT1), indicating that the cardiac manifestation of targeting GC-A is AT1-dependent and that the activity of the renin-angiotensin system is relatively upregulated in the heart. Considering the recent finding (*12*) that the gene encoding CYP11B2, an aldosterone synthase, is expressed in the heart, it remains to be clarified whether or not the aldosterone-MR pathway is involved in the ventricular remodeling in GC-A KO mice.

Here we report that eplerenone, a highly selective MR blocker, reduces the left ventricular hypertrophy and fibrosis normally seen in GC-A KO mice to levels similar to those seen in wild type (WT) mice, with a concomitant reduction in the gene expression of transforming growth factor-β (TGF-β) and collagen types I and III.

Methods

Animals and 4 Week Treatments with Eplerenone

All experimental procedures were performed in accordance with Nara Medical University standards for animal care. GC-A KO mice were the gift of David Garvers, University of Texas Southwestern Medical Center. The genetic background of the original GC-A KO and WT mice was C57BL/6. The GC-A KO and WT mice used in the present study were generated from heterozygous mice after crossing single GC-A KO and WT mice. Male 12-week-old GC-A KO mice were assigned to control, eplerenone and hydralazine groups (n=6–7/group). WT and untreated GC-A KO mice (control) were given a vehicle chow (D10001 donated by Pfizer Inc., New York, USA); mice in the eplerenone group were given the same chow containing eplerenone (1.25 g/kg D10001 chow donated by Pfizer Inc.), resulting in an approximate dose of 100 mg/kg body weight (BW)/d; and mice in the hydralazine group were given the vehicle chow (D10001), and hydralazine was supplied in the drinking water (50 mg/L) ad libitum, resulting in an approximate dose of 5 mg/kg BW/d. All animals were maintained on their respective diets for 4 weeks and examined at 16 weeks of age.

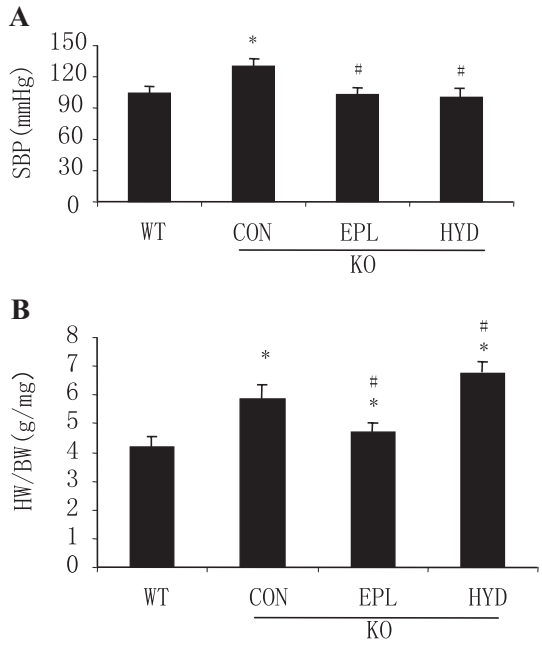

Fig. 1. *Systolic blood pressure (SBP) (A) and the heart weight to body weight (HW/BW) ratios (B) in WT mice and GC-A KO mice left untreated (CON) or treated with eplerenone (EPL) or hydralazine (HYD). Bars depict the means±SEM. *$p<0.05$ vs. WT, $^{\#}p<0.05$ vs. GC-A KO (CON).*

Measurement of Blood Pressure and Heart Rate

Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious mice using the tail-cuff method (Softron Co. Ltd., Tokyo, Japan).

Determination of Heart Weights/BW Ratios

Hearts were rapidly excised, rinsed in cold PBS and weighed. The ratios of the heart weights to the BW (HW/BW ratios) were then calculated and used as an index of cardiac hypertrophy.

Histological Analysis

For light microscopy, after hearts were cut in half transversely, the basal half was fixed in 10% formalin, embedded in paraffin and prepared for routine histology. To determine the degree of collagen fiber accumulation, we randomly selected 20 fields in 3 individual sections and calculated the ratio of the areas of Masson Trichrome staining of interstitial fibrosis to the total ventricular area using a KS400 image system (Zeiss, Jena, Germany).

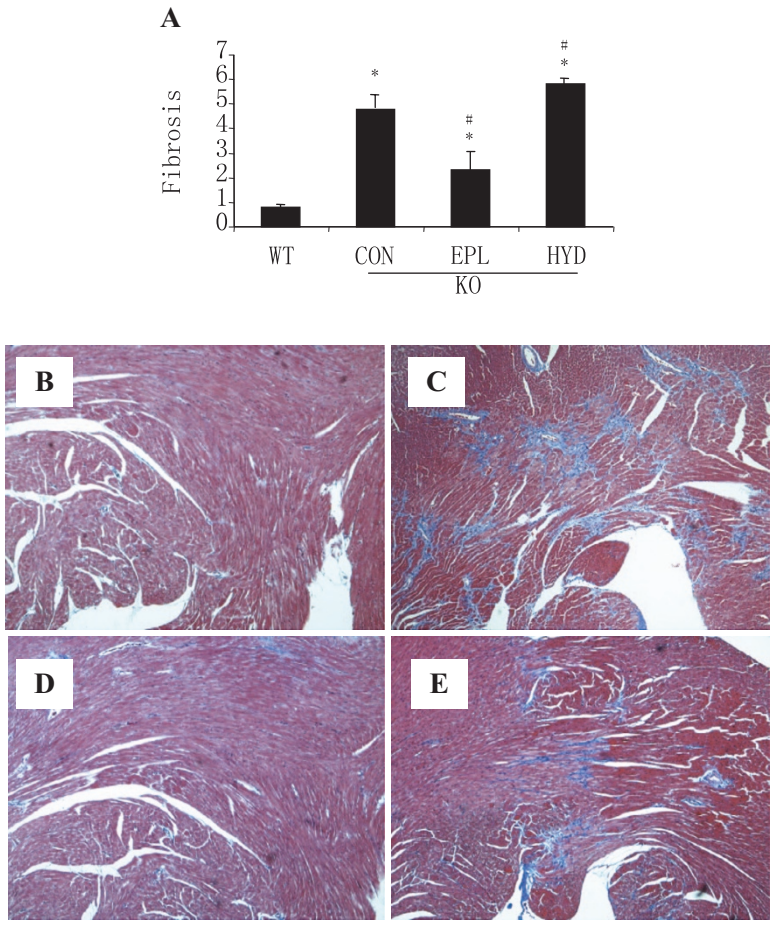

Fig. 2. *A: Evaluation of ventricular fibrosis in WT mice and GC-A KO mice left untreated (CON) or treated with eplerenone (EPL) or hydralazine (HYD). Bars depict the means±SEM. *$p<0.05$ vs. WT, $^{\#}p<0.05$ vs. GC-A KO (CON). B–E: Representative Masson Trichrome staining of hearts from WT (B), untreated GC-A KO (C), eplerenone-treated GC-A KO (D) and hydralazine-treated GC-A KO (E) mice.*

Analysis of mRNA

Excised hearts were cut in half transversely, after which the apical half was rapidly frozen and stored at −80°C until required for mRNA analysis. Total mRNA was prepared using TRIzol (Life Technologies Inc., Gaithersburg, USA). Expression of mRNAs encoding ANP, BNP, TGF-β1, collagen I and collagen III was evaluated using real time quantitative RT-PCR with the appropriate primers and probes in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, USA). To verify that equal amounts of mRNA were amplified, GAPDH mRNA was also amplified using the same method with a specific primer pair and probe (Applied Biosystems), and all mRNA levels were normalized to those of GARDH mRNA.

Statistical Analysis

All results are expressed as means±SEM. One-way ANOVA was used test to analyze differences among groups, after which post-hoc Tukey's tests were used to make comparisons between individual groups. Values of $p<0.05$ were considered significant.

Results

Effects of Eplerenone on SBP, HW/BW Ratios and Fibrosis in GC-A KO Mice

Consistent with earlier reports (*8–10*), we found that SBP was higher, HW/BW ratios were larger and left ventricular interstitial fibrosis was more severe in GC-A KO mice than in WT mice (Figs. 1 and 2). However, 4 weeks of treatment with eplerenone reduced SBP in GC-A KO mice by 23%, bringing it to the level seen in WT mice (Fig. 1A). Eplerenone also reduced HW/BW ratios in GC-A KO mice to the level seen in WT mice (Fig. 1B), and significantly reduced left ventricular fibrosis (Fig. 2). Although hydralazine lowered SBP to a sim-

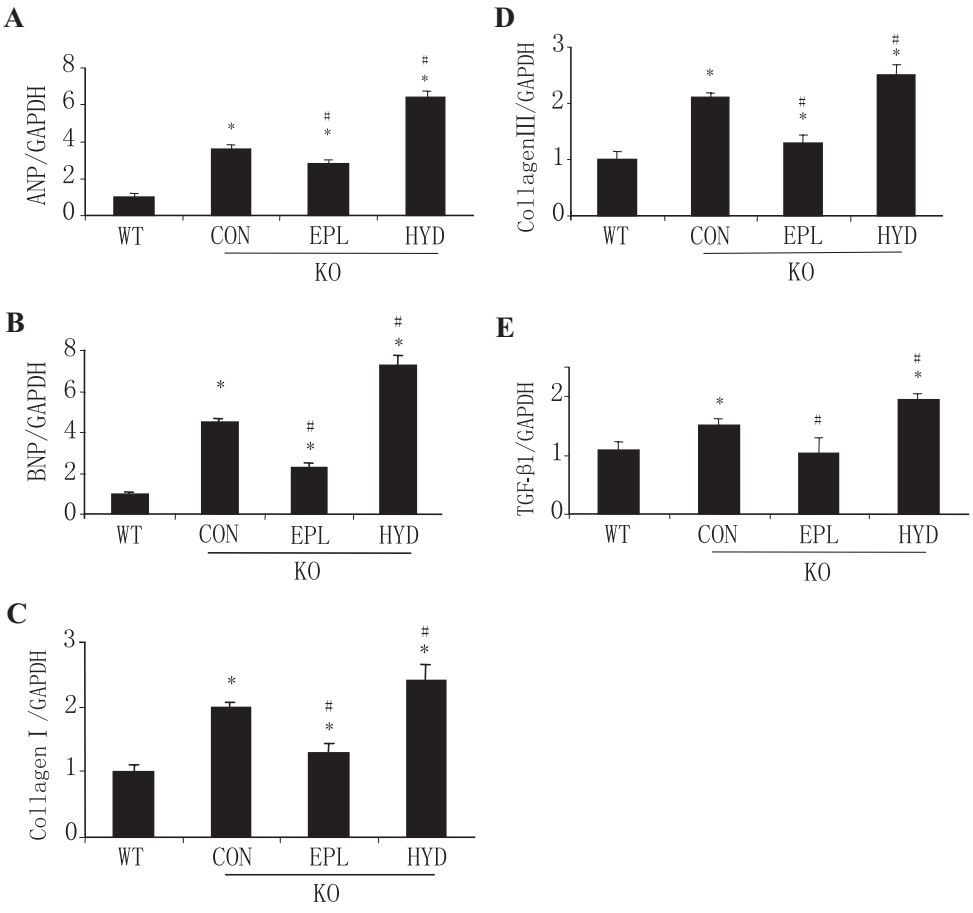

Fig. 3. *Relative levels of ANP (A), BNP (B), collagen I (C), collagen III (D) and TGF-β1 (E) mRNA expression measured using quantitative RT-PCR in WT mice and GC-A KO mice left untreated (CON) or treated with eplerenone (EPL) or hydralazine (HYD). Bars depict the means±SEM. *$p < 0.05$ vs. WT, $^{\#}p < 0.05$ vs. GC-A KO (CON).*

ilar degree, it did not reduce either HW/BW ratios or interstitial fibrosis in GC-A KO mice; on the contrary, it actually increased them somewhat (Figs. 1B and 2).

Effects of Eplerenone in Cardiac Gene Expression

As shown in Fig. 3A and B, levels of ANP and BNP mRNA were significantly higher in GC-A KO than WT mice, but were reduced by 4 weeks of treatment of eplerenone, which is consistent with the reduction in ventricular hypertrophy seen in GC-A KO mice treated with eplerenone. We also evaluated the expression profiles of collagen I, collagen III and TGF-β1 mRNA, which are widely accepted as molecular markers for ventricular fibrosis (Fig. 3C, D and E). Consistent with the fibrosis data (Fig. 2), ventricular levels of all three markers were significantly higher in GC-A KO than WT mice, and were reduced by eplerenone treatment. By contrast, hydralazine increased the expression of ANP, BNP, collagen I, collagen III and TGF-β1 mRNA in GC-A KO mice, which is consistent with its exacerbation of interstitial fibrosis and cardiac hypertrophy (Fig. 3).

Discussion

In the present study, we demonstrated that eplerenone attenuated cardiac hypertrophy and interstitial fibrosis with a concomitant reduction in the transcription of TGF-β and collagen types I and III in GC-A KO mice. Although eplerenone also reduced SBP by 23%, we suggest eplerenone attenuates cardiac remodeling in a pressure-independent manner for the following reasons: 1) hydralazine did not inhibit cardiac remodeling, despite normalization of SBP in GC-A KO mice; 2) we previously observed that genetic and pharmacological blockade of AT1 diminishes cardiac hypertrophy and fibrosis independently of SBP in GC-A KO mice (*11*); 3) Knowles *et al*. (*13*) showed pressure-independent enhancement of cardiac hypertrophy in GC-A KO mice; and 4) a sub-pressor dose of eplerenone reportedly prevents transition to myocardial failure in mice with pressure-overload (*5*). Taken together, these findings suggest that eplerenone exerts a direct effect on cardiac remodeling and that mineralocorticoid

receptor signaling is a key mediator of cardiac remodeling in GC-A KO mice.

In double KO mice in which both the GC-A and AT1 genes were targeted (*11*), HW/BW ratios and levels of fibrosis were similar to those in WT mice, clearly indicating that the cardiac remodeling is mainly AT1-dependent and AT1 signaling is relatively activated in GC-A KO mice. Moreover, ANP directly downregulates the CYP11B2 gene expression in cultured adrenal cortical cells and neonate cardiomyocytes (*14*, *15*). It is, therefore, possible that the level of CYP11B2 mRNA is elevated in ventricles of GC-A KO mice. However, we could not detect a substantial amount of CYP11B2 mRNA in the ventricular tissues of either GC-A KO or WT mice by a PCR technique (data not shown). Garnier *et al*. (*16*) reported that a more than 100-fold overexpression of CYP11B2 in cardiomyocytes did not induce either cardiac hypertrophy or fibrosis. Moreover, Lopez *et al*. (*8*) reported that the plasma aldosterone level in GC-A KO mice was similar to that in WT mice. Taken together, these findings indicate that MR is unlikely to be activated by either overproduced-aldosterone in the ventricle or circulating aldosterone in GC-A KO mice.

The mechanism for activation of MR signaling in the ventricles of GC-A KO mice is not clear at the present time. Recently, Nagata *et al*. (*17*) raised the possibility that MR signaling is activated by endogenous glucocorticoid in Dahl salt-sensitive rats, in which aldosterone levels are low. In contrast, other investigators have reported that glucocorticoids do not activate MR but act as antagonists in cardiomyocytes (*18*, *19*). Given the recent evidence that radical oxygen species (ROS) and cyclosporine A, an activator of the Calcineurin-Nuclear Factor of Activated T Cells (NFAT) pathway, activate MR signaling in the kidney (*20–22*), these pathways also should also be investigated to determine their possible roles in activating MR signaling in the ventricles of GC-A KO mice. Tokudome *et al*. (*23*) recently reported that the calcineurin-NFAT pathway is activated in GC-A KO mice.

Recently, Franco *et al*. (*24*) reported that eplerenone at a dose of 200 mg/kg BW/d did not prevent baseline left ventricular hypertrophy in ANP KO mice but did significantly prevent adverse cardiac remodeling induced by transverse aortic constriction-induced pressure overload in these mice. The discrepancy in the effect of eplerenone on basal left ventricular hypertrophy is probably explained by the preserved action of BNP in the ventricle. In addition, it should be noted that we used 100 mg/kg BW/d of eplerenone, which resulted in significant decline of BP in GC-A KO mice. However, earlier studies showed that eplerenone at a dose of 200 mg/kg BW/d had no cardiac or vascular effects in WT mice (*5*, *25*) and ANP KO mice (*24*). When we treated GC-A KO mice with 200 mg/kg BW/d eplerenone, SBP declined to below 60 mmHg in all mice and some mice died (data not shown). Further studies are necessary to investigate why GC-A KO mice are more sensitive to eplerenone.

TGF-β is a key mediator that induces fibrotic and inflammatory changes in various organs. For instance, aldosterone reportedly stimulates TGF-β1 expression in the kidney (*26*), and both angiotensin II and aldosterone may exert their injurious effects through TGF-β (*27*). In the present study, eplerenone markedly reduced the cardiac fibrosis seen in GC-A KO mice along with transcription of TGF-β1, collagen I and collagen III. This suggests that upregulation of TGF-β1 and collagen types I and III is due to AT1 signaling-mediated activation of MRs in the ventricles of GC-A KO mice.

Another interesting finding in the present study is that hydralazine treatment augmented the fibrosis and its related gene expression in GC-A KO mice despite the significant reduction of SBP. Hydralazine has been widely used as a control depressor agent in experiments in which the effects of depressor agents, such as angiotensin converting enzyme inhibitor, angiotensin receptor blocker, and α-adrenergic receptor blocker, on cardiac remodeling were investigated. Hydralazine causes direct relaxation of arterial smooth muscle, though its molecular mechanism is not known (*28*). Hydralazine-induced vasodilation is associated with direct or indirect stimulation of the sympathetic nervous system, and the renin angiotensin system, which counteract the hypotensive effect of hydralazine (*28*). In earlier works, the side effects of hydralazine were not found to induce deterioration of cardiac remodeling, but in GC-A KO mice, which are more susceptible to the action of angiotensin II (*11*) than WT mice, side effects might be apparent.

In conclusion, our findings indicate that eplerenone attenuates cardiac remodeling in GC-A KO mice, most likely in a blood pressure–independent manner, indicating that signaling downstream of MR is involved in the ventricular remodeling in GC-A KO mice.

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