Guanylyl Cyclase-A Inhibits Angiotensin II Type 1A Receptor–Mediated Cardiac Remodeling, an Endogenous Protective Mechanism in the Heart

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Background—Guanylyl cyclase (GC)-A, a natriuretic peptide receptor, lowers blood pressure and inhibits the growth of cardiac myocytes and fibroblasts. Angiotensin II (Ang II) type 1A (AT 1A ), an Ang II receptor, regulates cardiovascular homeostasis oppositely. Disruption of GC-A induces cardiac hypertrophy and fibrosis, suggesting that GC-A protects the heart from abnormal remodeling. We investigated whether GC-A interacts with AT 1A signaling in the heart by target deletion and pharmacological blockade or stimulation of AT 1A in mice.

Methods and Results—We generated double-knockout (KO) mice for GC-A and AT 1A by crossing GC-A–KO mice and AT 1A–KO mice and blocked AT 1 with a selective antagonist, CS-866. The cardiac hypertrophy and fibrosis of GC-A–KO mice were greatly improved by deletion or pharmacological blockade of AT 1A. Overexpression of mRNAs encoding atrial natriuretic peptide, brain natriuretic peptide, collagens I and III, transforming growth factors \( \beta_1 \) and \( \beta_3 \), were also strongly inhibited. Furthermore, stimulation of AT 1A by exogenous Ang II at a subpressor dose significantly exacerbated cardiac hypertrophy and dramatically augmented interstitial fibrosis in GC-A–KO mice but not in wild-type animals.

Conclusions—These results suggest that cardiac hypertrophy and fibrosis of GC-A–deficient mice are partially ascribed to an augmented cardiac AT 1A signaling and that GC-A inhibits AT 1A signaling-mediated excessive remodeling. (Circulation. 2002;106:1722-1728.)

Key Words: natriuretic peptides  angiotensin  receptors  heart diseases  remodeling

Normal cardiac structure is maintained by a sophisticated set of mechanical and cellular “checks and balances.” A disturbance of these checks and balances induces a remodeling process, the distinguishing features of which are phenotypic modulation of various cell types; cellular hypertrophy and hyperplasia; and production, deposition, and degradation of extracellular matrix.1 Many studies have demonstrated important roles of the local renin-angiotensin (Ang) II system (RAS) in cardiac remodeling under various basal or pathological conditions.2,3 Ang II was implicated in the development of cardiomyocyte hypertrophy and cardiac fibrosis in humans and in animal models.4–6 Most of the Ang II functions in the cardiovascular system are mediated through the Ang II type 1 (AT 1 ) receptor.7 Treatments with ACE inhibitors or AT 1 blockers effectively lower blood pressure (BP)8,9 and prevent or ameliorate myocardial hypertrophy.10

Atrial natriuretic peptide (ANP) is a cardiac hormone that acts through guanylate cyclase A (GC-A) to lower BP and dilate blood vessels in vivo11,12 and to inhibit the growth of cardiac myocytes and fibroblasts in vitro.13,14 Brain natriuretic peptide (BNP) also activates GC-A and has effects similar to those of ANP. As we15 and others16 have previously reported, mice lacking GC-A exhibit hypertension, cardiac hypertrophy, and interstitial fibrosis, suggesting the roles of GC-A in controlling BP and protecting the heart from abnormal remodeling. The mechanisms, however, especially for the cardiac protection of GC-A, are still unknown.

In the present study, we sought to determine whether the RAS plays a role in the cardiac pathology of GC-A–knockout (KO) mice and whether there is any functional antagonism between the RAS and GC-A pathways. To investigate this hypothesis, we adopted 2 kinds of blocking strategies, genetic and pharmacological, because of the advantages of each. Targeting disruption of the GC-A gene can lead to complete blockade throughout life, but pharmacological blockade cannot. In contrast, pharmacological blockade can be applied after the development of cardiac abnormalities. We also examined cardiac responsiveness to exogenous Ang II and...
showed that GC-A protects the heart by inhibiting cardiac AT$_{1A}$-mediated hypertrophy and interstitial fibrosis.

Methods

Animals and Treatments

All experimental procedures were performed according to Kyoto University standards for animal care. GC-A–KO and AT$_{1A}$-KO mice were generated by methods described previously. The genetic background of the original GC-A–KO and AT$_{1A}$-KO mice was C57BL/6. Wild-type (WT), AT$_{1A}$-KO, GC–A–KO, and double-KO mice used in the present study were generated from heterozygous mice after crossing of single GC–A–KO mice and AT$_{1A}$–KO mice. All comparisons were made among littermates. Male mice 16 weeks old were examined. Pharmacological treatments were begun when the mice were 12 weeks old. CS-866, an AT$_1$ antagonist (gift from Sankyo Co Ltd; 10 mg · kg$^{-1}$ · d$^{-1}$, suspended in 0.5% carboxymethyl cellulose sodium salt solution) was given orally by gavage once a day for 4 weeks. A suppressor dose of Ang II (0.1 mg · kg$^{-1}$ · d$^{-1}$, dissolved in 0.01 mol/L acetic acid) was infused subcutaneously for 2 weeks with an osmotic minipump (model 2002, Alza Corp). 6-Hydroxydopamine (6-OHDA, 100 mg/kg, dissolved in saline; Sigma) was injected intraperitoneally twice over 5 to 6 days for 4 weeks. Control mice were given only vehicle.

Measurement of BP and Heart Rate

Systolic BP (SBP) and heart rate (HR) were measured in conscious mice by the tail-cuff method (Softtron Co Ltd).

Plasma Ang II Determination

Blood samples were rapidly withdrawn from mice under ether anesthesia and immediately centrifuged to isolate the plasma, which was then stored at $-20^\circ$C until determination of Ang II concentrations with a radioimmunoassay.

Determination of Heart and Left Ventricular Weights and Interstitial Fibrosis

Hearts were dissected out, the weights of the whole heart and left ventricle were measured, and the ratios of those weights to the body weight (HW/BW and LVW/BW) were calculated and used as an index of ventricular hypertrophy. The left ventricles were then fixed in 10% formalin 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 stained interstitial fibrosis to the total left ventricular area using KS400 image system (Zeiss).

Analysis of mRNA

Total mRNA was prepared from the left ventricle by use of TRIzol (Life Technologies Inc). Expression of mRNAs encoding ANP, BNP, AT$_{1A}$, ACE, angiotensinogen, transforming growth factor (TGF)-β, TGF-β$_2$, collagen I, and collagen III was measured by reverse transcription–polymerase chain reaction (PCR) with the appropriate primers in a ABI PRISM 7700 Sequence Detector (Applied Biosystems). To verify that equal amounts of mRNA were subjected to reverse transcription–polymerase chain reaction, GAPDH mRNA was also amplified by use of the same method with specific primers and probe (Applied Biosystems).

Statistical Analysis

All results are expressed as mean±SEM. Data were analyzed by 1-factor ANOVA. If a statistically significant effect was found, the Newman-Keuls test was performed to isolate the difference between the groups. Values of $P<0.05$ were considered statistically significant.

Results

Effects of AT$_{1A}$ Deletion on HR, SBP, HW/BW, LVW/BW, and Interstitial Fibrosis

There was no significant difference in HR among genotypes (WT, 631.6±22.3; AT$_{1A}$–KO, 592.4±14.0; GC–A–KO, 602.2±19.6; and double-KO, 584.1±12.5 bpm). GC–A–KO induced significant increases in SBP, HW/BW, and LVW/BW. Genetic deletion of AT$_{1A}$ similarly decreased SBP in WT (by 26.2%) and GC–A–KO (by 20.6%) background (Figure 1a). In contrast, it showed stronger inhibitions on HW/BW and LVW/BW in GC–A–KO (by 28.4% and 35.4%, respectively) than in WT (by 9.3% and 12.9%, respectively) background (Figure 1, b and c). Left ventricular interstitial fibrosis was 7-fold higher in GC–A–KO than in WT mice, whereas fibrosis in AT$_{1A}$–KO mice was similar to that in WT mice (Figure 1, d and e), although fibrosis in double-KO mice was still higher than in WT but was 58.9% lower than in GC–A–KO mice. Thus, AT$_{1A}$–KO attenuated the hypertension, cardiac hypertrophy, and fibrosis otherwise seen in GC–A–KO mice. Interestingly, the effects of AT$_{1A}$ deletion on cardiac remodeling were more marked in GC–A–KO mice than in WT mice, whereas those on SBP were comparable among genotypes.

Ventricular Expression of mRNAs Encoding Angiotensinogen, ACE, AT$_{1A}$, ANP, BNP, Collagen I, Collagen III, TGF-β$_{1}$, and TGF-β$_{2}$

We observed no significant difference between plasma Ang II levels in WT and GC–A–KO mice (92.4±8.9 and 90.9±16.2 pg/mL, respectively). Furthermore, we found that expression levels of ventricular angiotensinogen, ACE, and AT$_{1A}$ mRNA were comparable between WT and GC–A–KO mice (Figure 2, a through c). Therefore, it appears that GC-A does not affect plasma levels of Ang II or its receptor expression in the heart. In contrast, AT$_{1A}$–KO significantly decreased angiotensinogen mRNA in both WT and GC–A–KO animals (Figure 2a).

ANP gene expression is increased by a variety of hypertrophic stimuli, and both the expression and secretion of BNP are elevated in patients with cardiac hypertrophy. When we analyzed ANP and BNP mRNA levels in the left ventricles of GC–A–KO and WT mice, we found their expression to be significantly elevated in the former (Figure 3, a and b), which is consistent with the cardiac hypertrophy seen in those animals. AT$_{1A}$–KO was associated with significantly lower ANP and BNP expression in both GC-A and WT mice (Figure 3, a and b).

Because TGF-β$_{1}$ is reportedly involved in Ang II–induced synthesis of collagen,$^{21,22}$ we also analyzed the left ventricular expression of collagen I, collagen III, TGF-β$_{1}$, and TGF-β$_{2}$ mRNAs. Transcription of all 4 was higher in GC–A–KO than in WT mice; consistent with the observed reduction in fibrosis, their expression in double-KO mice was decreased to the same levels as those in AT$_{1A}$–KO mice, suggesting that the overexpression of these genes is almost completely AT$_{1A}$-dependent (Figure 3, c through f).

Effects of AT$_{1}$ Blockade on SBP, HW/BW, LVW/BW, and Interstitial Fibrosis

We also examined the effects of chronic administration of CS-866 on SBP, HW/BW, LVW/BW, and fibrosis and found
that the effects of CS-866 were similar to those of AT₁a-KO (Figure 4, a through e). Furthermore, as with genetic deletion, pharmacological blockade of AT₁ also appeared to affect myocardial hypertrophy and fibrosis more markedly in GC-A−KO than in WT mice.

To clarify whether the aforementioned effects of AT₁ blockade reflected prevention or amelioration of the abnormalities seen in GC-A−KO mice, we compared SBP, HW/BW, LVW/BW, and fibrosis in 12- and 16-week-old WT and GC-A−KO mice receiving no other treatment. All 4 parameters were elevated in GC-A−KO mice at both times, and there was no significant difference between them at either time (Figure 5, a through d). Thus, the AT₁ blocker ameliorated the hypertension, myocardial hypertrophy, and fibrosis that were already established in GC-A−KO mice.

**SBP and Cardiac Responsiveness to Exogenous Ang II**

Considering the lack of difference in mRNA levels of angiotensinogen, ACE, and AT₁A between WT and GC-
A–KO mice, it is likely that the phenotypic changes in GC-A–KO mice were a result of hyperresponsiveness to Ang II. To rule out confounding interference caused by changes in BP, a suppressor dose of Ang II was chosen. Ang II injection had no effect on SBP, HW/BW, LVW/BW, or fibrosis in WT mice (Figure 6, a through d). Likewise, infusion of Ang II at this dosage did not affect SBP in GC-A–KO mice (Figure 6a). By contrast, the infusion greatly increased HW/BW, LVW/BW, and fibrosis in GC-A–KO mice (Figure 6, b through d), revealing an increased cardiac responsiveness to Ang II in those animals.

**Effects of Decreased BP on HW/BW and Cardiac Fibrosis**

To further clarify the influences of BP decline itself on cardiac hypertrophy and fibrosis in GC-A mice, we adapted 6-OHDA for chemical sympathectomy to lower BP, 18 6-OHDA significantly decreased SBP in the same manner in WT and GC-A–KO mice (WT control, 115.9±2.3; WT 6-OHDA, 101.8±1.9; GC-A–KO control, 140.3±3.0; and GC-A–KO 6-OHDA, 123.0±6.3 mm Hg). Although this decline in GC-A–KO mice was comparable to that induced by AT1A-KO and CS-866, it did not induce any reduction of HW/BW and cardiac fibrosis in either genotype (data not shown), supporting the concept that cardiac remodeling in GC-A–KO mice is independent of BP.

**Comparison of Effects of AT1A-KO, AT1 Blockade, and Ang II Infusion on SBP, HW/BW, and Cardiac Fibrosis**

AT1A-KO and AT1 blockade significantly lowered SBP in WT and GC-A–KO mice, indicating the important role of AT1A in maintenance of BP in both WT and GC-A–KO mice. However, the responsiveness of SBP to AT1A deletion or AT1 blocker was similar in both types (Figure 7, a through c), suggesting that specific elevation of BP in GC-A–KO mice is independent of AT1A signaling. Conversely, the HW/BW and cardiac fibrosis in GC-A–KO mice were more sensitive to the treatments than those in WT mice, suggesting the dependency of cardiac remodeling on AT1A signaling (Figure 7, a through c).

**Discussion**

In the present study, we demonstrate that GC-A disruption–induced cardiac hypertrophy and interstitial fibrosis are markedly inhibited by AT1A-KO or pharmacological blockade of AT1, implicating the involvement of AT1A in the cardiac remodeling. Furthermore, in the absence of GC-A, exogenous Ang II greatly exacerbates cardiac remodeling, suggesting that GC-A suppresses cardiac responsiveness to Ang II. As reported previously, mice deficient for GC-A display hypertension and cardiac abnormalities, 15,16 suggesting the important roles of GC-A in controlling BP and protecting the heart from excessive remodeling. However, it remains to be clarified what systems promoting cardiac remodeling were inhibited by GC-A. Ang II is a potent growth stimulator in cardiomyocytes and in fibroblasts21,22 in vitro, and an important role of local Ang II signaling is suggested in establishment of cardiac remodeling.23 AT1A is crucially involved in the hypertensive and hypertrophic effects of Ang II. To examine the role of Ang II signaling in the cardiovascular phenotypes of GC-A–deficient mice, we crossed them with an AT1A-deficient strain, yielding double-KO mice, which exhibited lower BP and HW/BW and reduced fibrosis compared with GC-A–deficient mice (Figure 1). Although the effects of AT1A-KO on SBP were comparable in WT and GC-A–KO mice, the reductions in HW/BW and fibrosis induced by AT1A-KO or AT1 blockade were more marked in GC-A–KO mice (Figure 7, a and b). Therefore, we speculated that cardiac AT1A signaling is enhanced in the absence of GC-A.

Previous studies provide circumstantial evidence of counteregulation by the natriuretic peptide system and RAS.24 Cardiomyocytes are the main source of ANP and BNP, 11,15 GC-A, their receptor, is also expressed in cardiomyocytes25 and possibly in cardiac fibroblasts.26 Likewise, components of the RAS have been demonstrated in the heart.27 In fact, we detected transcription of angiotensinogen, ACE, and AT1A in the cardiac ventricles, as shown in Figure 2. We initially speculated that cardiac Ang II was upregulated in GC-A–KO mice. That proved not to be the case, however; nor did we detect a difference in AT1A expression, which suggests that it
Ang II induces postreceptor signaling that differs in GC-A–KO and WT mice. Therefore, we next examined the responsiveness to Ang II in GC-A–KO mice. To rule out the effect of BP change, we chose Ang II at a subpressor dose. In WT mice, the infusion increased neither hypertrophy nor fibrosis. However, to our surprise, it augmented cardiac hypertrophy and fibrosis of GC-A–KO mice (Figure 6, b through d). The heart of GC-A–KO mice is much more responsive to Ang II infusion than that of WT mice (Figure 6c), suggesting that GC-A inhibits Ang II signaling at a postreceptor level, thereby suppressing cardiac remodeling independently of BP. The fact that decline of BP by another kind of depressor agent, 6-OHDA, did not influence cardiac hypertrophy and fibrosis in GC-A supports the hypothesis that cardiac remodeling in GC-A–KO mice is independent of BP.

Knowles et al.28 reported that an increase in cardiac afterload caused by transverse aortic constriction resulted in a 55% increase in LVW/BW of GC-A–KO mice, but only an 11% increase in WT mice. GC-A–KO mice thus appear to be more susceptible to the hypertrophic effects of increasing an external load. This is consistent with our current findings in that the increased myocyte stretch caused by the increased afterload would be expected to activate the cardiac RAS, which together with other growth factors would in turn mediate stretch-induced cardiac hypertrophy.29 In contrast to our results with CS-866, however, those investigators failed to observe significant antihypertrophic actions of an AT₁ blocker, losartan. Although the reason for the discrepancy is currently unclear, the fact that AT₁A–KO yielded similar inhibitions of cardiac remodeling further strengthens our conclusions.

The intracellular mechanism for counteraction between GC-A and AT₁A signaling is not clear at present. However, several lines of evidence suggest that ANP and intracellular cGMP elevation inhibit proliferation of nonmyocardial cells through inhibition of extracellular signal-regulated kinases (ERKs) in vitro.30,31 In cultured renal tubular cells, ANP also prevented the Ang II–mediated hypertrophy through a decrease in the phosphorylation peak of ERK.32 We therefore examined basal phosphorylation of cardiac ERK1/2 in GC-A–KO and WT, which showed no apparent difference between genotypes (data not shown). Knowles et al.28 also demonstrated that both the basal activities of mitogen-activated protein kinases and their induction by pressure overload were not different between GC-A–KO and WT mice and speculated that the relative activation of mitogen-activated protein kinases does not appear to be a controlling factor in the hypertrophic response in GC-A–KO mice caused by pressure overload.28 Further examination is necessary to determine the molecular effectors responsible for the cross-talk between the GC-A and AT₁A pathways in cardiac remodeling in vivo.
TGF-β1 is a potent stimulator of extracellular matrix protein (ECMP) synthesis (e.g., collagen and fibronectin). Ang II appears to stimulate the synthesis of ECMPs in cardiac fibroblasts, vascular smooth muscle cells, and renal mesangial cells through induction of TGF-β1 expression. In the present study, AT1A deletion or AT1 blockade markedly attenuated the cardiac fibrosis seen in GC-A KO mice (Figure 1, d and e, and Figure 4, d and e), along with the increased transcription of TGF-β1, TGF-β3, collagen I, and collagen III (Figure 3, c through e). These results further support the hypothesis that AT1A is involved in the fibrosis of GC-A-KO mice.

Our findings suggest that the GC-A–ligand system is an endogenous mechanism that protects the heart from AT1A-mediated remodeling. Recently, Nakayama et al described a functional mutation in the 5'-flanking region of human GC-A gene that is associated with essential hypertension and cardiac hypertrophy. GC-A gene expression is most likely diminished in these patients because of the mutation, predisposing them to cardiac hypertrophy and fibrosis like that seen in GC-A–KO mice. The activation of cardiac GC-A by natriuretic peptides or related drugs might therefore represent a new approach with which to improve treatment of cardiac structural abnormalities. The present results also suggest that blockade of AT1 or Ang II signaling using an AT1 blocker or ACE inhibitor would be the treatment of choice to prevent the cardiac hypertrophy and fibrosis in patients with lower expression level of GC-A.

In conclusion, our results provide direct evidence that GC-A inhibits cardiac remodeling partially via antagonizing AT1A signaling. The findings offer new insight into endogenous mechanisms for protection of the heart.
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