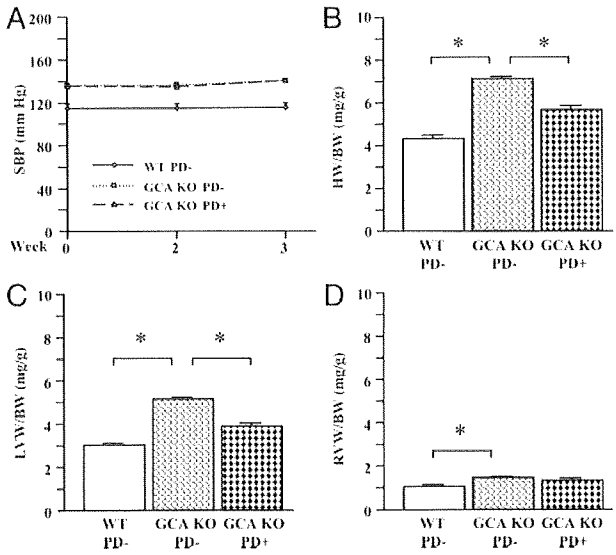

FIG. 5. Pharmacological blockade of AT2 ameliorated cardiac hypertrophy but did not affect hypertension in GCA-deficient mice. The AT2 antagonist PD123319 (30 mg/kg) was injected (ip) daily for 4 wk. The corresponding control animals were treated with saline alone. A–D, SBP (A), HW/BW (B), LVW/BW (C), and RVW/BW (D) in saline-treated (PD−) or PD123319-treated (PD+) mice. Values are means ± SEM (n = 5 each group). *, $P < 0.05$.

of cardiac weights to body weight, cardiomyocyte cross-sectional area, and collagen accumulation and overexpression of hypertrophic genes ANP, BNP, and collagens I and III in GCA-deficient hearts were all suppressed by AT2 deletion. Similarly, the cardiac hypertrophy in GCA-deficient mice was also attenuated by pharmacological blockade with PD123319. These results clearly indicate that AT2-dependent pro-hypertrophic signaling is dependent on GCA deficiency. Thus, similar to the situation with AT1a (6), AT2-mediated pro-hypertrophic signaling in the heart is inhibited by GCA.

Many studies have demonstrated a functional link between Ang II and TGF-β1 in the heart, and both are potent inducers of cardiac hypertrophy. Ang II has been shown to induce the expression of TGF-β1 in cardiac myocytes and fibroblasts (26). The absence of the TGF-β1 gene prevented the development of cardiac hypertrophy in response to subpressor doses of Ang II (27). We have demonstrated previously that genetic or pharmacological blockade of AT1 suppressed cardiac TGF-β1 overexpression and attenuated cardiac hypertrophy in GCA-deficient mice (6, 7). It has also been demonstrated that pharmacological blockade of AT2 is able to attenuate Ang II-stimulated TGF-β1 secretion in valvular interstitial cells (28). In the present study, overexpression of TGF-β1 in GCA deficiency was modulated by deletion of AT2, which also diminished the extent of cardiac hypertrophy. Thus, the present findings suggest that cardiac TGF-β1 participates in GCA-elicited inhibition of AT2-mediated pro-hypertrophic signaling in the heart.

Genetic deletion and pharmacological blockade of AT1 both reversed cardiac hypertrophy in GCA KO mice, thus implicating AT1 in growth promotion (6). Ablation of AT2 in the present study also partially attenuated cardiac hypertrophy in GCA KO mice, but somewhat surprisingly, the AT1 antagonist olmesartan did not produce further decreases in the HW/BW or LVW/BW ratios despite exerting beneficial effects on SBP. Importantly, however, it has been reported that blockade of AT2 abolished the anti-hypertrophic effect of AT1 antagonists in hearts of aged rats (29) and that combined AT1/AT2 blockade did not influence Ang II infusion-dependent cardiac hypertrophy in Sprague Dawley rats (30). The implication of these studies in intact animals, that AT2 is essential for the anti-hypertrophic effects of AT1 antagonists, is supported by the present findings in gene-targeted animals.

The present study further distinguishes the impact of pharmacological AT1 antagonism on SBP from effects on cardiac remodeling. Thus, olmesartan decreased SBP in intact, GCA-null, AT2-null, and GCA/AT2-double null mice, whereas cardiac hypertrophy was reversed by olmesartan only in GCA KO mice. Ang II signaling leading to altered gene transcriptional activation and function may involve multiple intracellular pathways. The AT1 is coupled to heterotrimeric G proteins and may activate phospholipases to increase calmodulin kinase activity and calcium release, which effects vasoconstriction (31). Balanced against this is the activation of ANP and BNP transcription and cross talk with the AT2, which modulate hypertensive actions (31). On the other hand, cardiomyocyte proliferation and hypertrophy are also stimulated by Ang II acting via AT1 signaling through the epidermal growth factor receptor coupled to MAPKs (32, 33). Thus, the present observations that cardiac hypertrophy in GCA deficiency is independent of blood pressure reg-

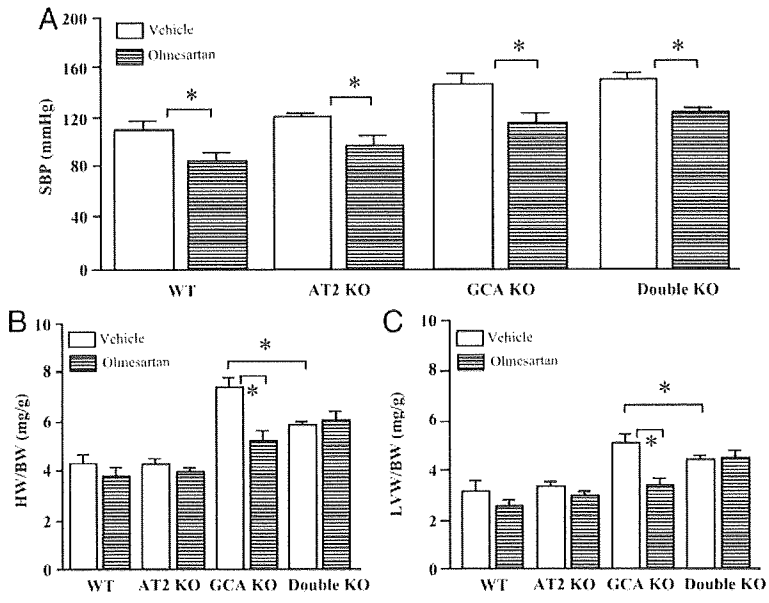

FIG. 6. The AT1 antagonist olmesartan lowered SBP but did not affect cardiac hypertrophic phenotype in double KO mice. A–C, Effect of olmesartan (10 mg/kg orally for 4 wk) on SBP (A), HW/BW (B), and LVW/BW (C) in hearts of mice carrying each of the genotypes under investigation. Values are means ± SEM (n = 7–9). *, $P < 0.05$.

ulation is consistent with the differential activation of alternate signaling cascades by AT1 and AT2.

Recently, several lines of evidence have suggested that GCA activity is relatively decreased in certain populations of patients with cardiovascular diseases. A functional polymorphism in the 5′-flanking region of the human GCA gene that is associated with essential hypertension and cardiac hypertrophy has been described (34). This variant most likely diminishes GCA gene expression in these patients, predisposing them to hypertension and cardiac hypertrophy such as that observed in GCA-deficient mice. Another polymorphism in the human GCA gene 5′-flank modulates left ventricular mass in essential hypertension (35). We also reported that a further polymorphism in the GCA 5′-flanking region, which decreases the transactivation of the GCA promoter in vascular smooth muscle cells, is associated with essential hypertension (36). Furthermore, it is emerging that desensitization of GCA signaling occurs in patients with severe heart failure (37, 38). These lines of evidence suggest that functional deterioration of GCA signaling may contribute to the progression of certain cardiovascular diseases. At this time, it is unclear whether an AT2-dependent mechanism could be operative in these patients. The present findings that cardiac hypertrophy in GCA-null mice is attenuated by blocking AT2 may provide important information for further detailed mechanistic research and eventual application of AT2 antagonists in patients with hypertension and cardiac hypertrophy caused by decreased GCA activity.

Taken together, the present findings demonstrate that GCA inhibits AT2-mediated cardiac growth-promoting signaling pathways and provides new insights into endogenous protective mechanisms against cardiac remodeling.

Acknowledgments

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Disclosure Summary: The authors have nothing to declare.

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Endogenous cardiac natriuretic peptides protect the heart in a mouse model of dilated cardiomyopathy and sudden death

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Yasuno S, Usami S, Kuwahara K, Nakanishi M, Arai Y, Kinoshita H, Nakagawa Y, Fujiwara M, Murakami M, Ueshima K, Harada M, Nakao K. Endogenous cardiac natriuretic peptides protect the heart in a mouse model of dilated cardiomyopathy and sudden death. *Am J Physiol Heart Circ Physiol* 296: H1804–H1810, 2009. First published April 3, 2009; doi:10.1152/ajpheart.01033.2008.—Ventricular myocytes are known to show increased expression of the cardiac hormones atrial and brain natriuretic peptide (ANP and BNP, respectively) in response to pathological stress on the heart, but their function during the progression of nonischemic dilated cardiomyopathy remains unclear. In this study, we crossed a mouse model of dilated cardiomyopathy and sudden death, which we generated by cardioselectively overexpressing a dominant-negative form of the transcriptional repressor neuron-restrictive silencer factor (dnNRSF Tg mice), with mice lacking guanylyl cyclase-A (GC-A), a common receptor for ANP and BNP, to assess the effects of endogenously expressed natriuretic peptides during progression of the cardiomyopathy seen in dnNRSF Tg mice. We found that dnNRSF Tg;GC-A$^{-/-}$ mice were born normally, but then most died within 4 wk. The survival rates among dnNRSF Tg;GC-A$^{+/-}$ and dnNRSF Tg mice were comparable, but dnNRSF Tg;GC-A$^{+/-}$ mice showed greater systolic dysfunction and a more severe cardiomyopathic phenotype than dnNRSF Tg mice. Collectively, our findings suggest that endogenous ANP/BNP protects the heart against the death and progression of pathological remodeling in a mouse model of dilated cardiomyopathy and sudden death.

neuron-restrictive silencer factor; guanylyl cyclase-A; cardiomyopathy; sudden death

HEART FAILURE IS A LEADING cause of mortality and morbidity in the Western world (17). In United States, for example, ~550,000 new cases are diagnosed each year (12). Despite recent progress in both medical and surgical management, heart failure remains an extremely lethal condition associated with a very poor quality of life and a 5-year survival rate of only ~50% (12, 34). Therefore, a better understanding of the molecular mechanisms underlying the progression of heart failure would be highly desirable, since it could serve as the basis for developing novel therapeutic approaches to treating the ailment.

Heart failure is accompanied by dysregulation of myocardial expression of a set of cardiac genes. One of the best-characterized genetic alterations seen in failing ventricles is reactivation of fetal cardiac genes, including those encoding atrial natriuretic peptide (ANP), skeletal α-actin, and β-myosin heavy chain, which are active within the fetal ventricles but quiescent in normal postnatal ventricles (3, 26). Such transcriptional alterations have been shown to correlate with deterioration of cardiac function and, conversely, improvement of cardiac function in response to medical and/or nonmedical interventions is accompanied by normalization of these genetic alterations (1, 2, 15, 17). Thus reprogramming cardiac gene expression appears to modify the pathological process during the progression of heart failure.

ANP is a cardiac hormone usually synthesized in the atrium and released in response to wall stretch. Upon its release, ANP acts at multiple sites to exert diuretic, natriuretic, and vasorelaxant effects (21). These biological properties are shared by brain natriuretic peptide (BNP), which, despite its name, is primarily secreted from the ventricles (18, 23, 31). Moreover, recent evidence indicates that ANP and BNP also act as paracrine factors, exerting antihypertrophic and antifibrotic effects in the heart (5, 9, 14, 24, 29). They exert both their hormonal and paracrine effects through activation of their common receptor, guanylyl cyclase-A receptor (GC-A), also known as natriuretic peptide receptor-A, which is expressed in a variety of tissues, including kidneys, blood vessels, adrenal glands, and heart (22), and is coupled to an increase in the intracellular concentration of cGMP (10). Ventricular expression of both ANP and BNP is upregulated in several pathological conditions of the heart, and their plasma concentrations are markedly elevated in patients with congestive heart failure (CHF). In fact, measurements of plasma ANP and BNP levels are used clinically to assist in the diagnosis of CHF, to assess prognosis, and to determine therapeutic strategy (16, 27, 30, 33). In addition, ANP and BNP are already being used to treat patients with acute heart failure (4, 32).

We recently found that, following myocardial infarction, mice lacking GC-A showed a higher incidence of acute heart failure, more severe left ventricular (LV) remodeling, and greater impairment of LV systolic function than mice expressing GC-A (20). This suggests that endogenous ANP/BNP may protect heart after myocardial infarction, but the role of intrinsic ANP/BNP signaling during the development of nonischemic dilated cardiomyopathy remains unclear. To address that question, in this study, we crossed a transgenic (Tg) mouse cardioselectively overexpressing a dominant-negative form of the transcriptional repressor neuron-restrictive silencer factor (dnNRSF Tg mice), which is a mouse model of dilated cardiomyopathy and sudden death, with mice lacking GC-A (GC-A$^{-/-}$; see Ref. 11). Almost all of dnNRSF Tg;GC-A$^{-/-}$

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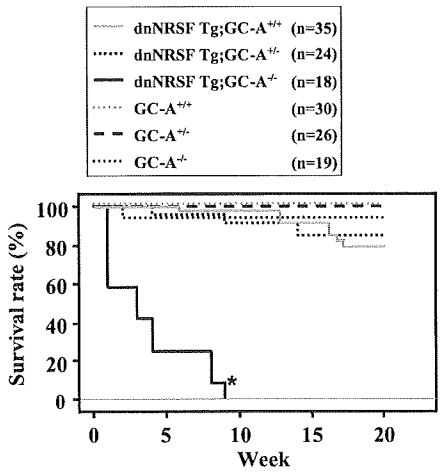

Fig. 1. Kaplan-Meier analysis of survival after birth among guanylyl cyclase-A (GC-A)-sufficient (GC-A$^{+/+}$) mice, GC-A heterozygous knockout (GC-A$^{+/-}$) mice, mice lacking GC-A (GC-A$^{-/-}$), mice overexpressing a dominant-negative form of the transcriptional repressor neuron-restrictive silencer factor (dnNRSF Tg);GC-A$^{+/+}$, dnNRSF Tg;GC-A$^{+/-}$, and dnNRSF Tg;GC-A$^{-/-}$ mice. dnNRSF Tg line 474 was used in this study. *$P < 0.05$ vs. dnNRSF Tg;GC-A$^{+/-}$. *n*, No. of mice.

mice died by 4 wk after birth. The survival rates among dnNRSF Tg;GC-A$^{+/-}$ and dnNRSF Tg mice were comparable, but dnNRSF Tg;GC-A$^{+/-}$ mice showed greater systolic dysfunction and a more severe cardiomyopathic phenotype than dnNRSF Tg mice. These findings suggest that endogenous ANP/BNP protects the heart against the sudden death and the progression of pathological remodeling in the mouse model of dilated cardiomyopathy.

MATERIAL AND METHODS

Animals. The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee in the Kyoto University Graduate School of Medicine. GC-A knockout (KO) mice generated as described previously were kindly provided by D. L. Garbers (The University of Texas Southwestern Medical Center) (14). Using methods described previously (11), we established two dnNRSF Tg lines (471 and 474) having different survival rates. In the present study, we used dnNRSF Tg line 474, whose survival rate was ~80% at 20 wk of age. The GC-A KO (GC-A$^{-/-}$), GC-A heterozygous KO (GC-A$^{+/-}$), dnNRSF Tg;GC-A$^{-/-}$, and dnNRSF Tg;GC-A$^{+/-}$ mice used to examine effects on survival were generated by crossing male GC-A$^{-/-}$ mice and female dnNRSF Tg;GC-A$^{+/-}$ mice. The wild-type (WT), GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF Tg;GC-A$^{+/-}$ mice used in other experiments were generated by crossing male GC-A$^{+/-}$ mice and female dnNRSF Tg;GC-A$^{+/-}$ mice. The genetic background of the original GC-A KO and dnNRSF Tg mice was C57BL/6.

Echocardiographic and hemodynamic analyses. After anesthetizing mice by intraperitoneal injection of a 2.5% wt/vol solution (8 μl/g) of tribromoethanol/amylene hydrate (Avertin), echocardiography was carried out using a Toshiba Power Vision 8000 echocardiographic system equipped with a 12-MHz imaging transducer as described previously (11). For hemodynamic analyses, mice were intubated and anesthetized with 0.5–1.5% isoflurane. A 2-French Millar Micro-Tip catheter (Millar Instruments) was then inserted in the right carotid artery and advanced in the left ventricle to record LV systolic and diastolic pressures, as well as the maximum and minimum rates of LV pressure development (dP/d*t*) (7).

Histological examination. Hearts were fixed in 10% formalin and prepared for histological analysis as described previously (13).

Quantitative RT-PCR analysis. Using 1- or 50-ng samples of total RNA prepared from ventricles, levels of mRNA encoding mouse ANP and BNP; skeletal α-actin; sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) 2; hyperpolarization-activated cyclic nucleotide-gated potassium channel (HCN) 2 and HCN4, which encode channels that carry the hyperpolarization-activated current; calcium channel, voltage-dependent, T-type, α1H-subunit (CACNA1H), which encodes the α_1H T-type Ca^{2+} channel; GC-A; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were then determined by quantitative real-time PCR following the manufacturer's protocol (Applied Biosystems, Zaventam, Belgium) as described previously (20). The real-time PCR primers and probes for ANP, BNP, skeletal α-actin, SERCA2, HCN2, HCN4, CACNA1H, GC-A, and GAPDH were all purchased from Applied Biosystems.

Statistical analysis. Data are presented as means ± SE. ANOVA was used to make multiple group comparisons. If ANOVA showed a significant difference ($P < 0.05$), a post hoc Fisher least-significant difference test was used to identify which group differences accounted for the significant *P* value. Survival rate was analyzed using the

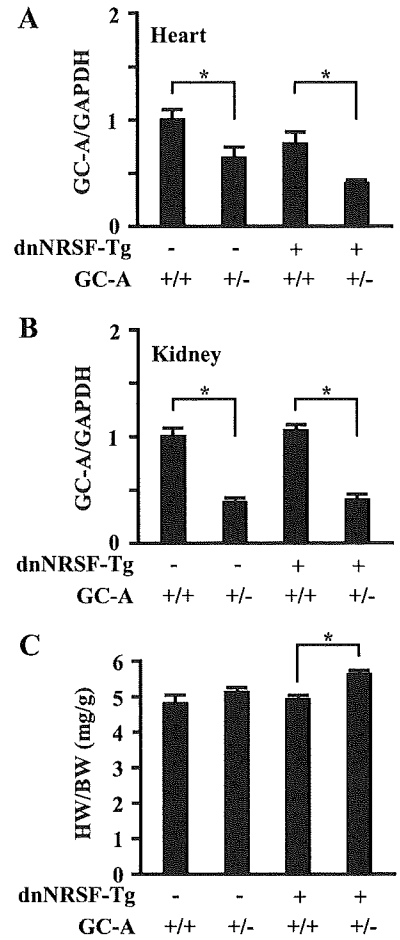

Fig. 2. Expression of GC-A mRNA and heart weight (HW)-to-body weight (BW) ratios (mg/g) in wild-type, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-A$^{+/-}$ mice. *A* and *B*: expression of GC-A mRNA of the heart (*A*) and the kidney (*B*) in wild-type, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-A$^{+/-}$ mice. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *$P < 0.05$. *C*: HW-to-BW ratios (mg/g) in wild-type, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-A$^{+/-}$ mice. Note that the ratio is significantly increased in dnNRSF Tg;GC-A$^{+/-}$ mice. *$P < 0.05$.

Kaplan-Meier method with the log-rank test. Values of $P < 0.05$ were considered significant.

RESULTS

Loss of GC-A is perinatally lethal in dnNRSF Tg mice. To determine the effects of the increased cardiac expression of ANP/BNP during the development and progression of dilated cardiomyopathy leading to sudden death, we generated dnNRSF Tg mice having a GC-A-null background by crossing dnNRSF Tg (line 474) with GC-A$^{-/-}$ mice (14). The resultant dnNRSF Tg;GC-A$^{-/-}$ mice were born at a rate similar to GC-A$^{-/-}$ mice. Moreover, the heart weight-to-body weight ratios on *postnatal day 2* did not differ significantly between the two genotypes (data not shown). Both dnNRSF Tg mice and dnNRSF Tg mice with a heterozygous GC-A background (dnNRSF Tg;GC-A$^{+/-}$ mice) grew normally until about 3 wk of age and started to die at ~4 wk of age (Fig. 1) (11). By contrast, ~80% of dnNRSF Tg;GC-A$^{-/-}$ mice died by 4 wk of age (by the time they were weaned), suggesting that GC-A is crucial for the survival of mice with dilated cardiomyopathy and lethal arrhythmia.

Diminished GC-A expression leads to deterioration of cardiac function in dnNRSF Tg mice. The perinatal lethality of the dnNRSF TG;GC-A$^{-/-}$ genotype made it difficult to assess cardiac function in these mice. We therefore used dnNRSF Tg;GC-A$^{+/-}$ mice, which expressed ~50% less GC-A mRNA than dnNRSF Tg mice, to evaluate the functional contribution of GC-A to the dnNRSF Tg heart (Fig. 2, *A* and *B*) (25). We initially compared the heart weight-to-body weight ratios in 8-wk-old WT, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF Tg;GC-A$^{+/-}$ mice. At that age, the cardiac structure and function of dnNRSF Tg mice were not yet disturbed (11). As shown in Fig. 2*C*, the heart weight-to-body weight ratios did not significantly differ among WT, dnNRSF Tg, and GC-A$^{+/-}$ mice but was significantly higher in dnNRSF Tg;GC-A$^{+/-}$ mice than in the other three groups. Moreover, subsequent echocardiography revealed dnNRSF Tg;GC-A$^{+/-}$ mice to have enlarged LV systolic and diastolic dimensions, increased LV mass, and reduced systolic function compared with dnNRSF Tg mice (Table 1). The hemodynamic parameters obtained through intracardiac catheterization showed significantly reduced LV systolic pressure and impaired dP/d*t* (Table 1). Thus impairment of GC-A signaling appears to degrade cardiac function in dnNRSF Tg mice.

Reducing GC-A promotes cardiac pathology in dnNRSF Tg mice. Histological analysis revealed additional effects of diminished GC-A expression on the structure of the dnNRSF Tg heart. The left ventricles were dilated to a greater extent in 8-wk-old dnNRSF Tg;GC-A$^{+/-}$ mice than in dnNRSF Tg or GC-A$^{+/-}$ mice (Fig. 3*A*). In addition, microscopic examination showed fibrosis to be more extensive in dnNRSF Tg;GC-A$^{+/-}$ mice than dnNRSF Tg mice, suggesting that endogenous ANP/BNP acts via GC-A to attenuate cardiac fibrosis in dnNRSF Tg hearts (Fig. 3*B*).

Finally, we assessed the mRNA expression of ANP, BNP, skeletal α-actin, and SERCA2, four marker genes used to evaluate cardiac pathology, and the mRNA expression of HCN2, HCN4, and CACNA1H, which we previously reported to be upregulated in dnNRSF Tg hearts (11). In that earlier study, we also observed that cardiac expression of ANP, BNP, and skeletal α-actin mRNA is upregulated in 8-wk-old dnNRSF Tg mice but that expression of SERCA2 mRNA is similar in 8-wk-old WT and dnNRSF Tg mice (11). In the present study, we found that the levels of ANP mRNA in dnNRSF Tg;GC-A$^{+/-}$ mice were even higher than in dnNRSF Tg hearts, whereas the levels of BNP mRNA were similar in dnNRSF Tg;GC-A$^{+/-}$ and dnNRSF Tg hearts (Fig. 4). Moreover, levels of skeletal α-actin mRNA were higher, whereas those of SERCA2 mRNA were significantly lower in dnNRSF Tg;GC-A$^{+/-}$ hearts than dnNRSF Tg hearts (Fig. 4). Taken together, these findings are consistent with the notion that reducing GC-A expression promotes pathological remodeling of dnNRSF Tg hearts. The cardiac expression of HCN2, HCN4, and CACNA1H mRNA did not significantly differ in dnNRSF Tg;GC-A$^{+/+}$ and dnNRSF Tg;GC-A$^{+/-}$ mice (Fig. 5).

DISCUSSION

Although it is well recognized that ventricular expression of both ANP and BNP is upregulated in hearts affected by dilated

Table 1. *Echocardiographic and hemodynamic analysis of 8-wk-old mice*

	GCA$^{+/+}$	GCA$^{+/-}$	dnNRSF Tg;GCA$^{+/+}$	dnNRSF Tg;GCA$^{+/-}$
Echocardiographic data	$n = 6$	$n = 4$	$n = 6$	$n = 4$
HR, beats/min	367.0±4.0	378.3±14.3	330.5±5.5	416.0±44.2
LVDd, mm	4.02±0.11	4.43±0.21	4.00±0.29	4.90±0.19*†
LVDs, mm	2.76±0.14	2.90±0.19	2.87±0.20	4.26±0.15*†‡
IVST, mm	0.66±0.04	0.63±0.03	0.65±0.03	0.58±0.05
PWT, mm	0.67±0.04	0.68±0.03	0.69±0.03	0.58±0.03
FS, %	32.8±1.9	34.0±4.5	27.9±1.5	12.8±1.5*†‡
EF, %	69.7±2.6	70.0±5.2	62.7±2.2	33.5±5.4*†‡
LVM, mg	90.2±8.1	104.2±1.8	89.3±3.0	116.6±11.7*†
Hemodynamic data	$n = 4$	$n = 5$	$n = 5$	$n = 5$
dP/dt_{max}, mmHg/s	4,865±201	5,005±283	4,757±325	3,747±202*†‡
dP/dt_{min}, mmHg/s	−4,935±218	−5,150±579	−4,756±237	−3,465±308*†‡
HR, min^{-1}	498±26.9	457±8.58	533±29.4	566±33.5‡
LVSP, mmHg	98.9±4.5	102.7±5.5	95.1±3.6	83.5±3.9*†
LVEDP, mmHg	2.25±0.56	3.15±0.38	2.36±0.69	2.73±0.85

Values are means ± SE; *n*, no. of mice. GC-A$^{+/+}$, guanylyl cyclase-A (GC-A)-sufficient; GC-A$^{+/-}$, GC-A heterozygous knockout mice; dnNRSF Tg, mice overexpressing a dominant-negative form of the transcriptional repressor neuron-restrictive silencer factor; HR, heart rate; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVST, interventricular septal thickness; PWT, posterior wall thickness; FS, fractional shortening; EF, ejection fraction; LVM, left ventricular mass; dP/d*t*, first derivative of pressure; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. $P < 0.05$ vs. control wild-type mice (*), vs. dnNRSF Tg mice (†), and vs. GCA$^{+/-}$ mice (‡).

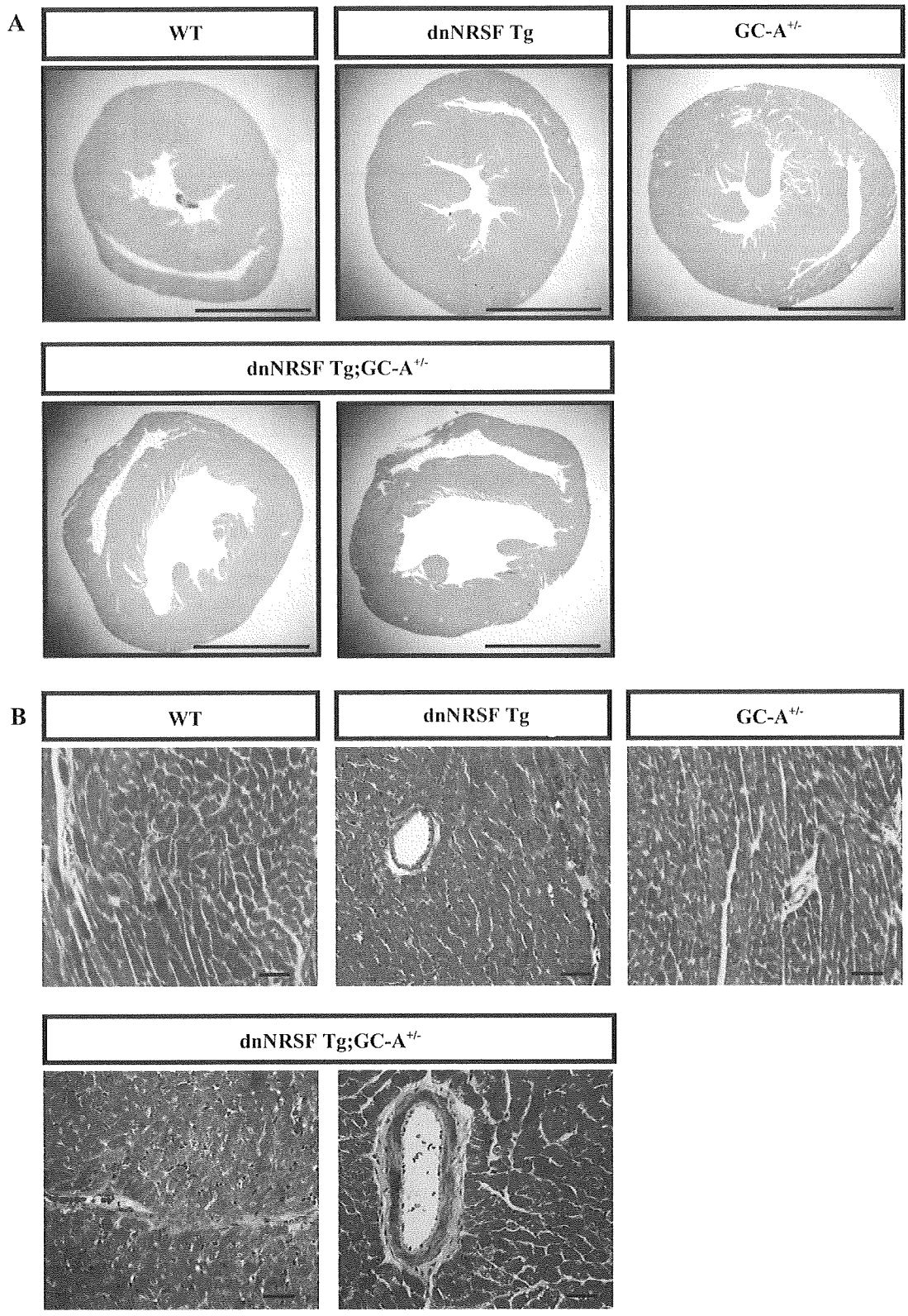

Fig. 3. Histological analysis. *A*: hematoxylin and eosin staining low-magnification photomicrographs showing the histology of wild-type, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-A$^{+/-}$ ventricles at 8 wk of age. Scale bars are 2.5 mm. *B*: Masson's trichrome staining showing fibrosis in sections from the left ventricles of wild-type, GC-A$^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-A$^{+/-}$ mice at 8 wk of age. Scale bars are 40 μm.

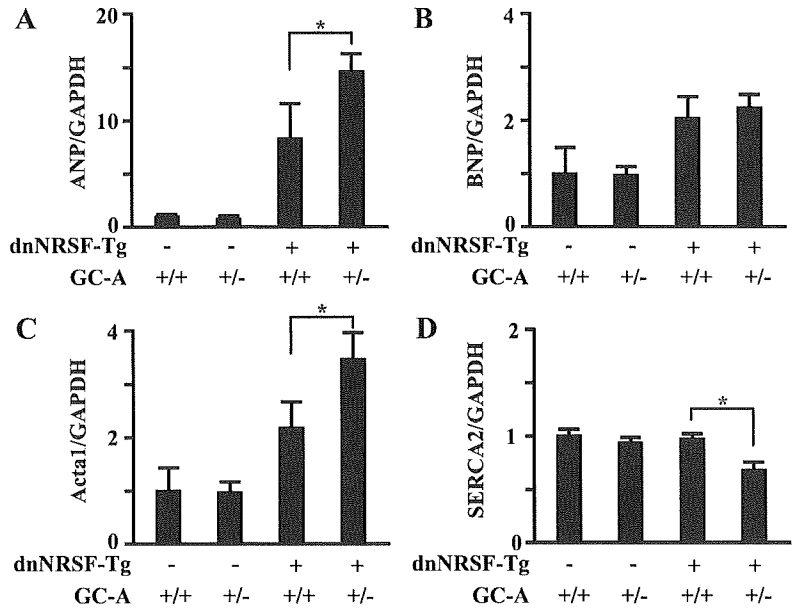

Fig. 4. Expression of atrial natriuretic peptide (ANP; *A*), brain natriuretic peptide (BNP; *B*), skeletal α-actin (*C*), and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2; *D*) mRNA in wild-type, GC-$A^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-$A^{+/-}$ mice. Samples (50 ng) of total RNA prepared from the ventricles of mice with the indicated genotypes were subjected to quantitative real-time PCR. Relative levels of ANP, BNP, skeletal α-actin, and SERCA2 mRNA, normalized to those of GAPDH mRNA, are shown. The levels in wild-type mice were assigned a value of 1. *$P < 0.05$.

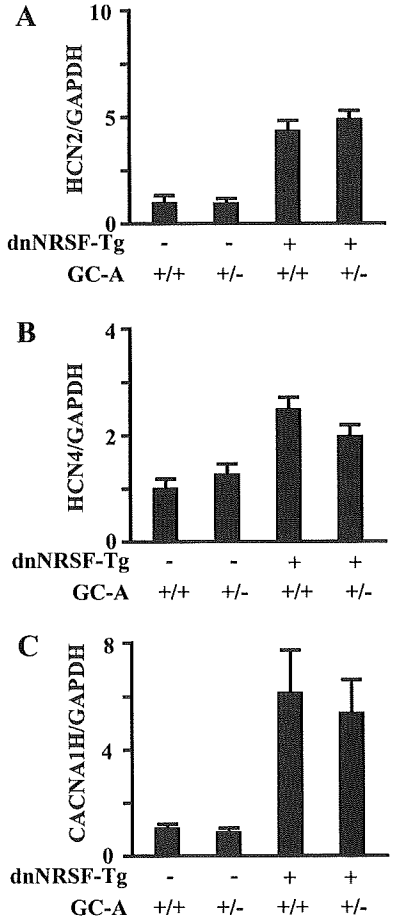

Fig. 5. Expression of HCN2 (*A*), HCN4 (*B*), and CACNA1H (*C*) mRNA in wild-type, GC-$A^{+/-}$, dnNRSF Tg, and dnNRSF-Tg;GC-$A^{+/-}$ mice. Relative levels of HCN2, HCN4, and CACNA1H mRNA, normalized to those of GAPDH mRNA, are shown. The levels in wild-type mice were assigned a value of 1.

cardiomyopathy (24), the effects of endogenous ANP/BNP during the development and progression of the ailment were not known. In the present study, we crossed dnNRSF Tg mice, a mouse model of dilated cardiomyopathy leading to sudden death, with GC-$A^{-/-}$ mice, which lack the receptor for ANP and BNP. Almost all dnNRSF Tg;GC-$A^{-/-}$ mice died within 4 wk after birth, whereas dnNRSF Tg;GC-$A^{+/+}$ mice lived at least 6 wk; dnNRSF Tg;GC-$A^{+/-}$ mice showed cardiomyopathic phenotypes that were more severe than dnNRSF Tg;GC-$A^{+/+}$. The results indicate that an insufficiency of GC-A accelerates the progression from latent to more evident cardiomyopathy in dnNRSF Tg mice, suggesting endogenous ANP/BNP exerts a protective effect against the progression of pathological cardiac remodeling and sudden death.

In healthy hearts, ANP is primarily secreted from the atrium, whereas BNP is primarily secreted from the ventricle, although small amounts of BNP are secreted from the atrium (18, 21, 23, 31). Ventricular expression of both ANP and BNP is upregulated under such pathological conditions as cardiac hypertrophy and heart failure, which makes plasma ANP/BNP levels a good prognostic indicator of clinical severity in a variety of cardiac diseases (21). Moreover, because improvement of cardiac function in response to medical and/or nonmedical therapy is accompanied by reductions in plasma ANP/BNP levels, they can serve as objective indicators with which to monitor the efficacy of therapy (30). As hormones, ANP and BNP exert diuretic, natriuretic, and vasorelaxant effects and counteract the effects of the renin-angiotensin-aldosterone and sympathetic nervous systems (21, 24, 25). In addition, they also act as paracrine factors, exerting antihypertrophic and antifibrotic effects in the heart (5, 9, 29). For these reasons, ANP and BNP are already being used clinically in patients with acute heart failure (4, 32). The roles played by endogenous ANP/BNP in the pathophysiology of heart failure had nonetheless remained unresolved. However, we recently showed that endogenous ANP/BNP is protective against acute heart failure and cardiac remodeling following experimental myocardial infarction in

mice (20). In the present study, moreover, we have shown that endogenous ANP/BNP also protects the heart against the progression of cardiac dysfunction in a mouse model of nonischemic dilated cardiomyopathy. Together, these two findings demonstrate that endogenous ANP/BNP protect against pathological ventricular remodeling, regardless of the etiology of the cardiomyopathy.

We previously showed that dnNRSF Tg;GC-A$^{+/+}$ mice grow normally until around 6 wk of age but then progress into cardiac dysfunction and die as a result of lethal arrhythmias some time later (11). The perinatal lethality of the dnNRSF Tg;GC-A$^{-/-}$ genotype would seem to indicate that endogenous ANP/BNP is able to protect dnNRSF Tg mice from sudden cardiac death, perhaps by exerting an antiarrhythmic effect. That said, we were unable to confirm that dnNRSF Tg;GC-A$^{-/-}$ mice die from lethal ventricular arrhythmias because of the technical difficulty of continuously collecting electrocardiography from perinatal mice. Blood pressures are elevated in GC-A$^{-/-}$ mice (14), which raises the possibility that increased blood pressure accelerates the progression of cardiac dysfunction in dnNRSF Tg;GC-A$^{-/-}$ mice. There is also a possibility that an as yet unidentified fundamental alteration caused by the GC-A-null background may have affected the phenotype. On the other hand, the idea that ANP/BNP exerts an antiarrhythmic effect is consistent with findings of an earlier report showing that older (12 mo of age) GC-A$^{-/-}$ mice have an increased susceptibility to ventricular arrhythmias (8). It also suggests the potential usefulness of ANP/BNP in the treatment of cardiomyopathies with a high susceptibility to arrhythmias. Indeed, ANP reportedly exerts a protective effect against arrhythmias induced by ischemia-reperfusion in dogs (28) and against those induced by proarrhythmic drugs in rabbits (6). The mechanism by which ANP/BNP might prevent arrhythmias remains unknown, although the recent report that sildenefil, a specific phosphodiesterase type 5 inhibitor, reduces the severity of arrhythmias during ischemia in dogs (19) suggests the effect is mediated by increasing cGMP levels via activation of GC-A. All of these data are suggestive of the therapeutic potential of ANP/BNP for the prevention of malignant arrhythmias in patients with heart failure or myocardial ischemia.

In conclusion, we have demonstrated that endogenous cardiac natriuretic peptides are able to markedly slow adverse cardiac remodeling during the progression of nonischemic cardiomyopathy toward sudden cardiac death. ANP and BNP are already being used to treat patients with acute heart failure. It is our hope that these findings begin to form the basis for novel and improved approaches to the treatment of patients with chronic heart failure and a high susceptibility to sudden cardiac death.

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Natriuretic Peptide Signaling *via* Guanylyl Cyclase (GC)-A: An Endogenous Protective Mechanism of the Heart

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Abstract: Atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones, secretions of which are markedly upregulated during cardiac failure, making their plasma levels clinically useful diagnostic markers. ANP and BNP exert potent diuretic, natriuretic and vasorelaxant effects, which are mediated *via* their common receptor, guanylyl cyclase (GC)-A (also called natriuretic peptide receptor (NPR)-A). Mice deficient for GC-A are mildly hypertensive and show marked cardiac hypertrophy and fibrosis that is disproportionately severe, given their modestly higher blood pressure. Indeed, the cardiac hypertrophy seen in these mice is enhanced in a blood pressure-independent manner and is suppressed by cardiomyocyte-specific overexpression of GC-A. These results suggest that the actions of a local cardiac ANP/BNP-GC-A system are essential for maintenance of normal cardiac architecture. In addition, GC-A was shown to exert its cardioprotective effects by inhibiting angiotensin II-induced hypertrophic signaling, and recent evidence suggests that regulator of G protein signaling (RGS) subtype 4 is involved in the GC-A-mediated inhibition of Gαq-coupled hypertrophic signal transduction. Furthermore, several different groups have reported that functional mutations in the promoter region of the human GC-A gene are associated with essential hypertension and ventricular hypertrophy. These findings suggest that endogenous GC-A protects the heart from pathological hypertrophic stimuli, and that humans who express only low levels of GC-A are genetically predisposed to cardiac remodeling and hypertension.

INTRODUCTION

Normal cardiac structure is maintained by a sophisticated set of mechanical and cellular "checks and balances", disturbance of which leads to a process called remodeling [1]. Although this process is initially adaptive, the beneficial effects are transient, and chronic cardiac remodeling leads to pathological molecular, cellular and interstitial changes that hinder cardiac function and ultimately lead to heart failure [2, 3]. Among the genes upregulated in cardiac remodeling, there are two that encode peptide hormones, atrial natriuretic peptide (ANP) [4, 5] and brain natriuretic peptide (BNP) [6]. ANP and BNP are synthesized, processed and secreted exclusively by the heart [7,8]. In response to the over-activation of various neurohumoral and mechanical stimuli that occur during heart failure, both the expression and secretion of ANP and BNP are dramatically upregulated, making their plasma levels clinically useful markers for the diagnosis and assessment of cardiac failure [9, 10]. Both natriuretic peptides exert potent diuretic, natriuretic and vasorelaxant effects through activation of their common receptor, guanylyl cyclase (GC)-A [also called natriuretic peptide receptor (NPR)-A] (Fig. (**1**)). GC-A is a prototype of plasma membrane-bound GCs, which serve as receptors that produce cyclic GMP (cGMP) in response to ligand binding [11]. C-type natriuretic peptide (CNP), the third member of the natriuretic peptide family, exerts its biological actions through another GC-coupled receptor called GC-B. In addition, the third receptor called clearance receptor mediates the metabolism of the natriuretic peptides. Because GC-A signaling stimulated by ANP and BNP results in a decrease in cardiac pre- and after-load, their mobilization during cardiac failure is thought to be one of the compensatory mechanisms activated in response to heart damage. In addition to the hemodynamic effects of their actions as circulating hormones, recent evidences suggest that ANP and BNP also act as autocrine/paracrine hormones. In the present review, we discuss the cardioprotective functions of endogenous ANP and BNP, focusing in particular on their local effects within the heart.

In Vitro Studies Using Cultured Cardiomyocytes

The role of ANP as an autocrine factor involved in regulating cardiac myocyte growth is suggested by early *in vitro* studies carried out using cultured cardiomyocytes.

Studies from Calderone *et al.* demonstrated the effects of exogenously applied ANP on heart cells [12]. Using cells cultured from neonatal rat heart, they observed that exogenously applied ANP caused concentration-dependent reductions in norepinephrine-stimulated incorporation of [^{3}H]-leucine in myocytes and [^{3}H]-thymidine in fibroblasts [12]. In both cell types, ANP increased intracellular cGMP

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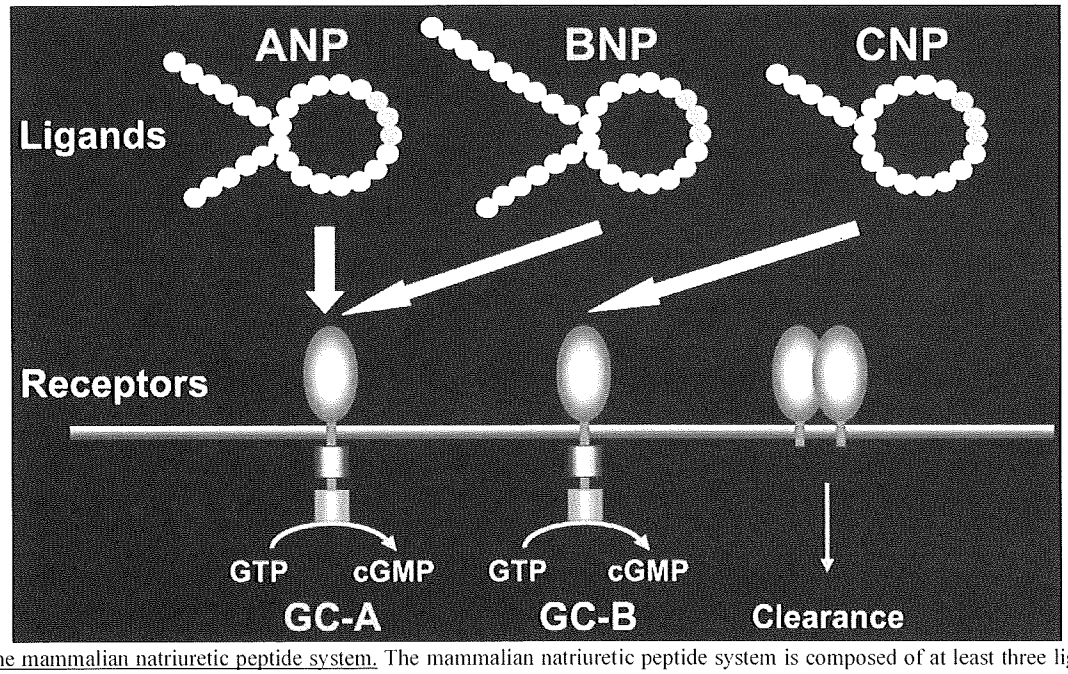

Fig. (1). The mammalian natriuretic peptide system. The mammalian natriuretic peptide system is composed of at least three ligands (ANP, BNP and CNP) and three receptors (GC-A, GC-B and clearance receptor). ANP and BNP are cardiac hormones that are synthesized, processed and secreted by the heart. They exert their biological effects, which include diuresis, natriuresis and vasodilation, *via* a shared receptor, GC-A. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC-A, guanylyl cyclase-A; GC-B, guanylyl cyclase-B.

levels, and 8-bromo-cGMP, a cGMP analogue, mimicked the growth-suppressing effects of ANP. In addition, ANP and 8-bromo-cGMP similarly attenuated the α1-adrenergic receptor-mediated increases in the mRNA level of proANP and decreases in the mRNA level of calcium ATPase in the sarcoplasmic reticulum (SR). The authors concluded that ANP diminishes the effects of norepinephrine on the growth of cardiac myocytes and fibroblasts, most likely *via* cGMP-mediated inhibition of the Ca^{2+} influx stimulated by norepinephrine.

In the opposite direction, we investigated the role of endogenously secreted ANP as an autocrine factor, treating cultured neonatal rat ventricular myocytes with HS-142–1, a non-selective receptor antagonist for GC-A and GC-B [13]. We found that the receptor antagonist increased both basal and phenylephrine-stimulated protein synthesis in a concentration-dependent manner, and these effects were accompanied by a significant increase in myocyte size. In addition, the expression of skeletal actin, β-myosin heavy chain and ANP, markers of hypertrophy, were elevated by treatment with the antagonist under both basal and phenylephrine-stimulated conditions. Conversely, both a cGMP-specific phosphodiesterase inhibitor, zaprinast, and a cGMP analogue suppressed basal and phenylephrine-stimulated protein synthesis. Thus, endogenous secretion of natriuretic peptides (ANP or BNP) from cardiomyocytes appears to inhibit cardiac myocyte hypertrophy under both basal and catecholamine-stimulated conditions, most likely *via* a cGMP-dependent process.

These *in vitro* studies clearly suggest a local function of the natriuretic peptide system in the heart. However, since these studies were carried out using cultured neonatal cells, clarification of the physiological and pathophysiological effects of natriuretic peptides *in vivo* hearts was awaited for further investigation. In addition, although the results of cGMP and HS-142-1 implicated a role of GC-coupled receptors, it remained to be determined which receptor mediates the cardioprotective action of natriuretic peptides.

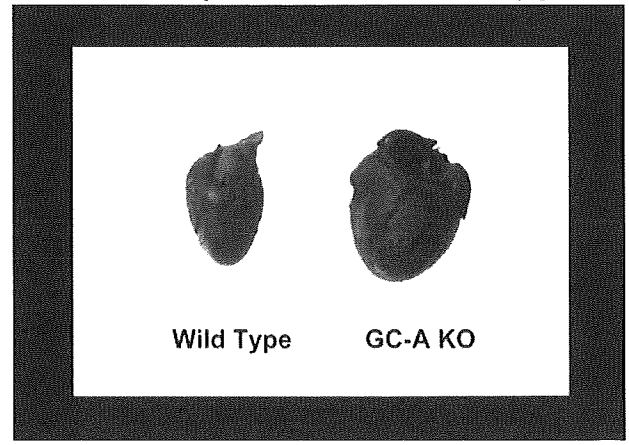

Fig. (2). Cardiac Hypertrophy in GC-A KO. GC-A-deficient mice (GC-A KO) develop cardiac hypertrophy, as shown on the right.

In Vivo Studies Using the Mice Deficient for GC-A

To verify the hypothesis generated by the *in vitro* studies, the cardiovascular effects of endogenous natriuretic peptide signaling *in vivo* have been examined using the genetically engineered mice.

Because ANP and BNP share a common receptor (i.e., GC-A), the absence of one peptide can presumably be

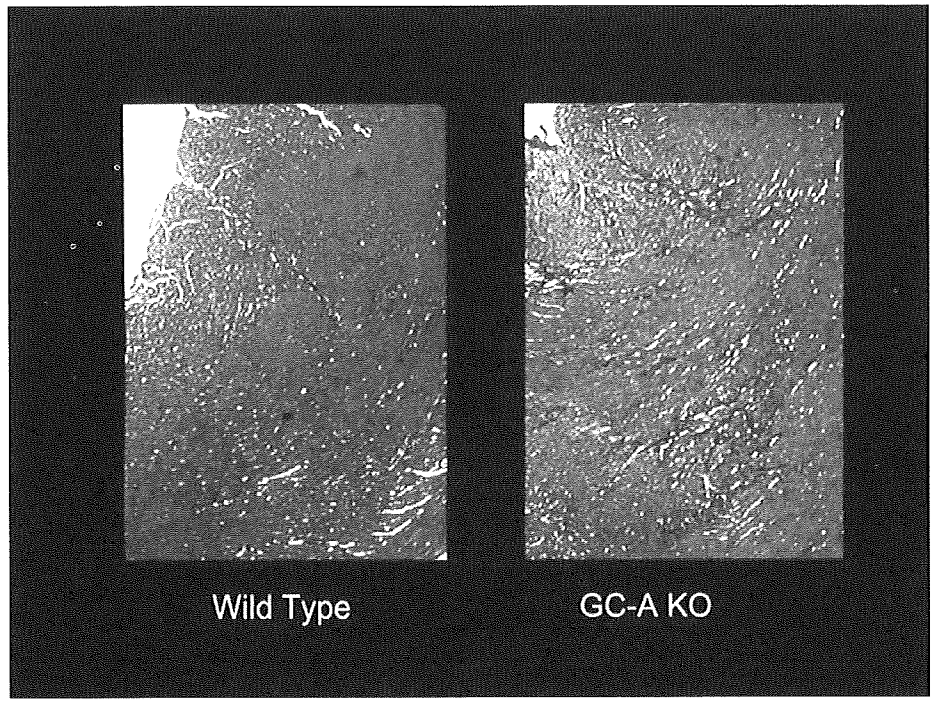

Fig. (3). Cardiac Fibrosis in GC-A KO. GC-A-deficient (GC-A KO) mice develop cardiac fibrosis, as shown in right. Heart tissues were visualized after Masson-Trichrome staining. The fibrotic tissue is seen in blue.

compensated for by the other. Therefore, to reveal the full effects of cardiac natriuretic peptide signaling, we generated mice that lacked endogenous GC-A (GC-A KO), and analyzed the cardiovascular phenotype [14-16]. As shown in Figs. (**2** and **3**), targeted deletion of the GC-A gene resulted in marked cardiac hypertrophy and fibrosis. Although GC-A KO mice also display mild hypertension, the cardiac hypertrophy was disproportionately severe, given the modest rise in blood pressure of the animal. In fact, other animal models that show similar increases in blood pressure do not exhibit the same degree of hypertrophy as GC-A KO mice [17, 18].

Independently, the research group led by Professors Smithies and Maeda at the University of North Carolina showed that mice lacking a functional Npr1 gene, which encodes NPR-A (i.e. GC-A), have elevated blood pressure and marked cardiac hypertrophy with interstitial fibrosis, resembling that seen in human hypertensive heart disease [19]. In their subsequent study, Knowles *et al.* reported that chronic treatment of GC-A KO with an ACE inhibitor, a diuretic, hydralazine or an angiotensin-receptor blocker, which all reduce blood pressure to the similar level as in wild-type mice, had no significant effect on the heart to body weight ratio [20]. Furthermore, in the reverse direction, pressure overload induced by transverse aortic constriction led to greater increases in ANP expression and in left ventricular weight to body weight ratio, in GC-A-KO mice than in wild-type mice [20]. Taken together, the authors concluded that the natriuretic peptide/GC-A system has direct antihypertrophic actions in the heart, independent of its role in blood pressure control. It is reported that targeted deletion of the proANP gene or proBNP gene also resulted in blood pressure-independent biventricular hypertrophy [21] or fibrosis [22], respectively, suggesting that a mechanism other than an increase in cardiac afterload exists for cardiac remodeling to be established in the mice lacking endogenous natriuretic peptides. In addition, since GC-A is expressed in the heart itself [23], it is suggested that this natriuretic peptide system acts in an autocrine/paracrine fashion to exert its cardioprotective effects.

Cardiomyocyte-Specific Overexpression or Deletion of GC-A Gene

Additional insights have also been gained from conditional overexpression or disruption of the GC-A gene.

We generated transgenic mice in which the GC-A transgene was selectively overexpressed in cardiomyocytes [24]. Expression of this gene in the hearts of GC-A KO and wild-type mice did not alter blood pressure or heart rate in either group; however it did reduce the size of both normal myocytes in wild-type mice and hypertrophied myocytes in GC-A KO animals. Coincident with this reduction in myocyte size, cardiac levels of both mRNA and protein levels of ANP were significantly reduced. The genetic model thus separates the systemic regulation of cardiomyocyte size by blood pressure from the local regulation by myocardial effectors.

The moderation of cardiac hypertrophy by local GC-A signaling was also demonstrated in a set of sophisticated experiments performed in the laboratory of Professor Kuhn at the University of Munich in Germany [25]. To test whether local ANP levels modulate cardiomyocyte growth, this group used homologous loxP/Cre-mediated recombination to selectively delete the GC-A gene in cardiomyocytes, thereby circumventing the systemic, hypertensive

phenotype associated with germ line inactivation of GC-A. Mice with the cardiomyocyte-specific GC-A deletion exhibited mild cardiac hypertrophy and a marked increase in the transcription of cardiac hypertrophy markers. Blood pressure levels were 7-10 mmHg below normal, likely reflecting the endocrine actions of the systemically elevated ANP levels. On the other hand, in the mice, cardiac hypertrophic responses to aortic constriction were enhanced and accompanied by marked deterioration of cardiac function, indicating the significant role of cardiomyocyte GC-A in physiological as well as in pathophysiological conditions.

Taken together, these results suggest that the natriuretic peptide system exerts a cardioprotective effect that is independent of its hemodynamic actions and implicate that activation of GC-A signaling has a direct inhibitory effect on cellular hypertrophic signaling within the heart [26].

The Role of the Local Natriuretic Peptide System in Ang II-Induced Cardiac Remodeling

However, the precise mechanism by which activation of GC-A in cardiomyocytes protects the heart from excessive remodeling remained unclear. We previously demonstrated that cardiac hypertrophy and fibrosis in GC-A KO mice can be significantly diminished by targeted deletion of AT1a (double KO for GC-A and AT1a) or by pharmacological blockade of the receptor using a selective antagonist [27]. Conversely, stimulation of AT1a by exogenous application of Ang II, at a dose that does not affect blood pressure (subpressor dose), significantly exacerbated cardiac hypertrophy and dramatically augmented interstitial fibrosis in GC-A-KO mice, but not in wild-type animals. These results suggest that cardiac hypertrophy and fibrosis in GC-A-deficient mice are related, at least in part, to the enhanced cardiac AT1a signaling, and that endogenous GC-A inhibits the excessive activation of AT1a signaling.

The Molecular Mechanism by which Cardiac GC-A Signaling Exerts its Cardioprotective Effect

To identify the differences between the hearts of GC-A KO and wild-type mice on a molecular level, we examined genes whose expression was upregulated in the hearts of GC-A KO mice and found that expression of the product of the Down syndrome critical region gene on chromosome 21, also called modulatory calcineurin-interacting protein 1 (MCIP1, more recently RCAN1), was increased. Because MCIP1 gene is upregulated by calcineurin signaling, we next investigated the role played by calcineurin in the cardiac hypertrophy of GC-A KO mice [28] and found that FK506-mediated blockade of calcineurin activation significantly

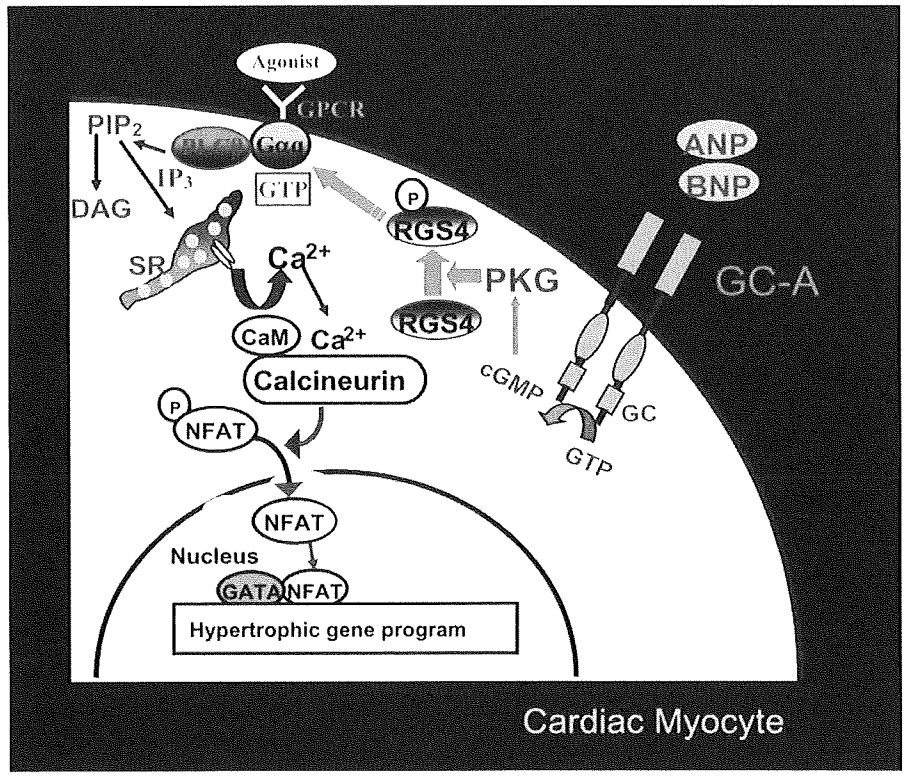

Fig. (4). Schematic diagram depicting the pathway *via* which GC-A signaling inhibits cardiac hypertrophy. Cardiac hypertrophic agonists such as Ang II, norepinephrine and endothelin-1 stimulate G-protein-coupled receptors (GPCR) and activate phospholipase C (PLC). Subsequent production of inositol triphosphate (IP_3) leads to release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum (SR), which raises cytosolic Ca^{2+} to a level sufficient to activate the calmodulin-regulated phosphatase calcineurin. Once activated, calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), which facilitates its nuclear translocation. NFAT and GATA then act cooperatively to activate transcription of the hypertrophic gene program, including the ANP and BNP genes. We propose that endogenous ANP and BNP exert their antihypertrophic effects by stimulating GC-A/PKG-mediated regulator of G protein signaling subtype 4 (RGS4) phosphorylation/activation, which leads RGS4 to associate with Gαq, thereby increasing the GTPase activity of Gαq. This in turn inhibits calcineurin-NFAT signaling and suppresses hypertrophy-related gene transcription. Adopted from reference [29].

reduced the heart weight to body weight ratio, cardiomyocyte size, and collagen volume fraction in GC-A KO mice, though FK506 had no effect on these parameters in wild-type mice. In cultured neonatal cardiomyocytes, pharmacological GC-A inhibition increased both basal and phenylephrine-stimulated calcineurin activities, while stimulation of GC-A by ANP inhibited these activities. We, therefore, suggest that it is by inhibiting calcineurin that cardiac GC-A signaling activated by locally secreted natriuretic peptides protects the heart from excessive cardiac remodeling [28]. To further explore the mechanism of the GC-A-mediated inhibition of calcineurin-induced hypertrophy, we assessed the G protein signaling that is known to occur upstream of calcium-calcineurin signaling and downstream of Ang II signaling [29]. It was recently reported that cGMP-dependent protein kinase binds directly to and phosphorylates/activates regulator of G protein signaling subtype 2 (RGS2), which significantly increases the GTPase activity of Gα(q) and terminates G protein-coupled receptor signaling in vascular smooth muscle [30]. Given that cGMP is an intracellular second messenger for natriuretic peptides, we hypothesized that RGS might mediate the cardioprotective effect of GC-A signaling. To test that idea, we focused on RGS4, which is the dominant RGS in cardiomyocytes [29, 31]. In cultured cardiomyocytes, ANP stimulated the binding of cGMP-dependent protein kinase Iα to RGS4, as well as the phosphorylation of RGS4 and its subsequent association with Gαq [29]. In addition, cardiomyocyte-specific overexpression of RGS4 in GC-A-KO mice significantly reduced the heart weight to body weight ratios, cardiomyocyte size and ventricular calcineurin activity. Conversely, overexpression of a dominant-negative form of RGS4 blocked the inhibitory effects of ANP on endothelin-1-stimulated inositol 1,4,5-triphosphate production, [^{3}H]-leucine incorporation and ANP gene expression. These findings suggest that GC-A activates cardiac RGS4, which then inhibits the activity of Gαq and its downstream hypertrophic effectors, thereby playing a key role in the GC-A-mediated inhibition of cardiac hypertrophy. Fig. (**4**) shows a schematic diagram that depicts the endogenous cardioprotective mechanism meditated by ANP/BNP, GC-A and RGS4.

CLINICAL IMPLICATIONS

The experimental evidence obtained from the various animal models summarized above supports the notion that GC-A signaling plays a key role in the modulation of cardiac remodeling and blood pressure. Several groups have also investigated this relationship in human patients. For example, Nakayama *et al.* identified an 8 nucleotides insertion/deletion mutation at position -60 (60bp upstream of the ATG codon) in the 5'-flanking region (i.e., the promoter region) of the human NPR-A (GC-A) gene [32]. After genotyping 200 subjects with essential hypertension and 200 normotensive control subjects, they found nine individuals with a deletion that reduced the transcriptional activity of the GC-A promoter to less than 30% of the wild-type allele. All nine individuals were heterozygous for the allele and exhibited hypertension, left ventricular hypertrophy or both, suggesting that, in these individuals, this deletion reduces receptor expression and confers increased susceptibility to essential hypertension or left ventricular hypertrophy. In addition, Rubattu *et al.* investigated the relationships between ANP, BNP and GC-A polymorphisms and left ventricular structure in approximately 200 subjects with essential hypertension [33]. Those investigators identified ANP and NPR-A (GC-A) gene variants that were signify-

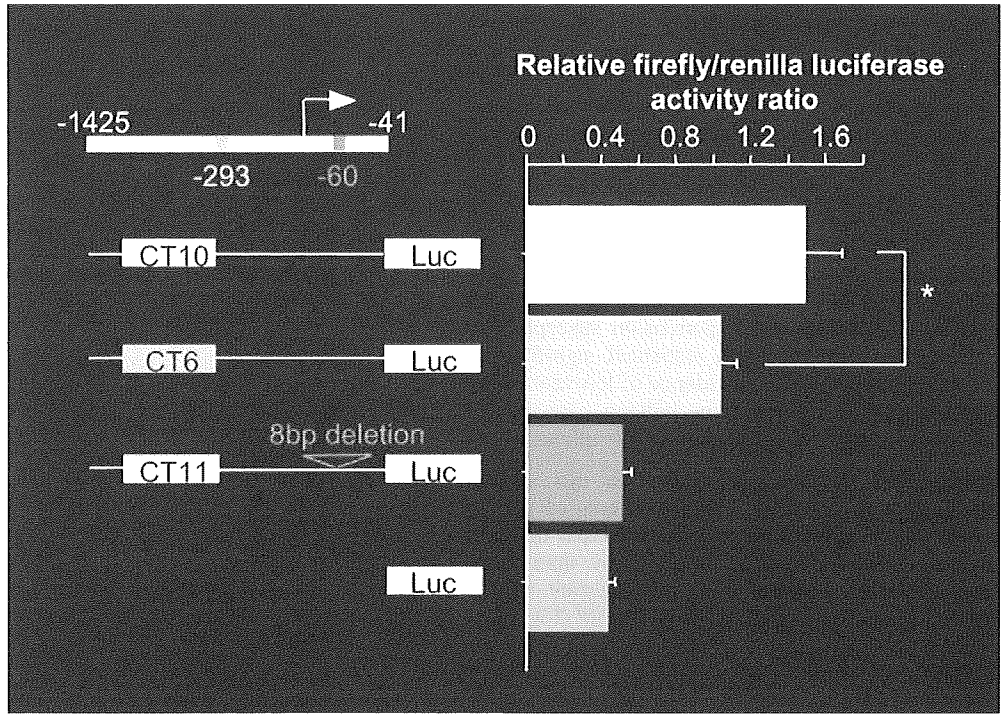

Fig. (5). Transcriptional activities of the indicated (CT)n and 8-bp deletion reporter constructs in human aortic smooth muscle cells. Shown are the mean firefly (Luc)/renilla luciferase activity ratios. Note that the GC-A promoter containing (CT)n=6 or the 8bp deletion drove significantly less transcriptional activity than the promoter containing (CT)n=10. Modified from reference [34].

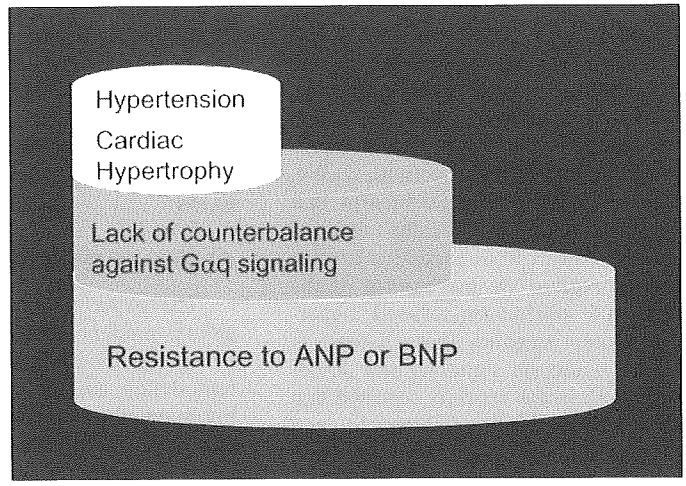

Fig. (6). The patients with GC-A gene mutation are susceptible to cardiovascular diseases. These patients are resistant to ANP/BNP-mediated counter-regulation of Gαq signaling and are therefore more susceptible to cardiovascular diseases such as hypertension and cardiac hypertrophy.

cantly associated with left ventricular mass index and left ventricular septal thickness. By contrast, BNP polymorphisms had no particular effect on cardiac phenotype. They concluded that the ANP/NPRA (GC-A) system contributed significantly to ventricular remodeling in human essential hypertension.

We also examined the association between polymorphisms within the GC-A promoter and essential hypertension in a group of Japanese subjects (177 hypertensive and 170 normotensive) and identified five allele types in which 6, 9, 10, 11 or12 CT dinucleotide repeats around position -293, upstream of the ATG codon [34]. The frequency of the (CT)n=6 allele was significantly higher among hypertensive subjects than among normotensive ones, while the frequencies of the other four allele types did not differ between the two groups. Promoter-reporter analyses carried out in cultured human aortic smooth muscle cells using a luciferase gene fused to the 5'-flanking region of the GC-A gene revealed that, like the promoter containing an 8bp deletion at position -60, the promoter containing (CT)n=6 at position -293 drove less transcriptional activity than the promoter containing (CT)n=10 (control) (Fig. (**5**)). Our results thus define the (CT)n polymorphism in the GC-A promoter as a potent and novel hypertension susceptibility marker. Although it did not reach statistical significance, preliminary data indicate that the incidence of left ventricular hypertrophy tends to be higher among patients carrying the (CT)n=6 allele than among those carrying other alleles.

Collectively, the findings summarized in this section suggest that people carrying the certain variants of human GC-A gene are more susceptible to cardiovascular diseases such as hypertension and cardiac hypertrophy than those who do not, possibly because they are resistant to the counter-regulation of Gαq signaling by ANP/BNP (Fig. (**6**)).

CONCLUSION

Endogenous GC-A signaling protects the heart from excessive remodeling and failure. In addition to the improvement of cardiac pre- and after-load, it is suggested that GC-A-mediated local activation of RGS protein and subsequent suppression of Gαq hypertrophic signaling is involved in the action. Consequently, individuals who express only low levels of GC-A could be genetically prone to cardiac remodeling and hypertension.

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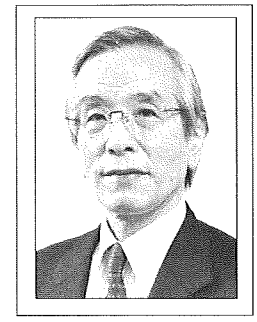
EDITORIAL

Challenge for Novel Peptide Hormones: From Discovery to Therapeutic Application



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Search for Novel Peptide Hormones

Throughout my 30-year academic career, I have been searching for and identifying novel peptide hormones. My interests include the exploration and elucidation of the pathophysiological roles of such peptides, which may open the door to new methods for the diagnosis and treatment of disease. Studies searching for unidentified peptides come into the spotlight only when new substances are discovered. Thus, starting this kind of research project does not necessarily mean it will produce new findings. In many cases, these endeavors result in a succession of failures with little hope of success, requiring sturdy diligence from researchers. While the quest for a novel peptide is no easy task, it may contribute to major scientific breakthroughs when novel peptides are discovered.

Discovery of Natriuretic Peptide Family and Adrenomedullin

We have developed a unique method for exploring peptides. Using this method, we have discovered several important bioactive peptides, including members of the natriuretic peptide family (atrial natriuretic peptide [ANP, 1984], brain natriuretic peptide [BNP, 1988], C-type natriuretic peptide [CNP, 1990]) and adrenomedullin (AM, 1993). By elucidating the pathophysiological roles of these peptides, we developed clinical applications for ANP and BNP in the diagnosis and treatment of heart failure. AM has been shown to possess a variety of physiological functions in the cardiovascular system, suggesting an application in the treatment and prevention of myocardial infarction, heart failure, and pulmonary hypertension, as well as possible roles in regenerative medicine. Clinical studies using AM are now being conducted.

Discovery of Ghrelin

Since the late 1990s, researchers around the world have been competing to discover endogenous ligands of orphan G-protein-coupled receptors (GPCRs) as a front-line target for advanced drug generation. Our laboratory began research into the physiological ligand of the growth hormone secretagogue receptor (GHS-R), an orphan GPCR. As a result, ghrelin was isolated from stomach tissues in 1999. Ghrelin, a 28-amino acid peptide with a unique structure modified with a fatty acid (acylated with n-octanoic acid), potently stimulates growth hormone (GH) secretion. In addition, ghrelin exerts multiple physiological actions related to increasing appetite and food intake, regulating energy metabolism, vasodilation, and cardiovascular protection, and suppressing sympathetic activity. These findings ignited intensive clinical studies assessing the therapeutic potential of ghrelin. Since the early 2000s we have been conducting translational researches into ghrelin; multiple studies have already completed. Clinical trials examining ghrelin have also been started by pharmaceutical companies in Japan and other countries for the treatment of eating disorders (including anorexia nervosa), chronic obstructive pulmonary disease (COPD), and heart failure.

New Therapeutic Applications for Endogenous Peptides

As illustrated above, our research team has been engaged in extensive studies of endogenous bioactive peptides, encompassing their isolation, structure identification, elucidation of their physiological and pathological roles, as well as the development of new therapeutic applications. We have had to overcome several serious obstacles in developing peptide-based drugs.

Peptides have been thought to be unsuitable for drug development; practically, only a limited number of drugs are currently available that include peptide hormone components, such as insulin, luteinizing hormone-releasing hormone (LH-RH), ANP, and BNP. The main reasons pharmaceutical companies have been reluctant to design peptides as drugs include the requirement to administer peptides by injection or infusion, not orally, and the multiple functions of bioactive peptides, in contrast to manufactured compounds that exhibit a narrow pharmacological activity. The fact that living beings synthesize, secrete, and process a trace amount of easily degradable peptides in a well-organized manner suggests hitherto unexplored approaches for utilizing peptides with multiple functions and short half-lives.

For example, ghrelin is activated when serine 3 is modified with octanoic acid (acylated by an ester bond) and inactivated when the ester bond is hydrolyzed by an esterase. Why is such an unstable modification used for the activation of ghrelin? We may argue that a readily hydrolyzable ester structure quickly "turns off" ghrelin activity when its physiological task is completed. In addition, ghrelin's multi-faceted functionality may contribute to enhancing its therapeutic effect when used as a drug.

I contend, from my limited experience, that endogenous bioactive peptides may be excellent therapeutic agents with little or no adverse effects when administered with appropriate timing and dosage. Recently, methods for transnasal and transpulmonary administration of micro particles encompassing peptides have been developed. Now we should recognize the excellent properties of endogenous bioactive peptides and their usefulness in therapy. It is my strong wish that the use of ground-breaking peptide-based drugs and treatment modalities will be realized in the near future.

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Ghrelin-like peptide with fatty acid modification and O-glycosylation in the red stingray, *Dasyatis akajei*

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Abstract

Background: Ghrelin (GRLN) is now known to be an appetite-stimulating and growth hormone (GH)-releasing peptide that is predominantly synthesized and secreted from the stomachs of various vertebrate species from fish to mammals. Here, we report a GRLN-like peptide (GRLN-LP) in a cartilaginous fish, the red stingray, *Dasyatis akajei*.

Results: The purified peptide contains 16 amino acids (GVSFHPQPRS[10]TSKPSA), and the serine residue at position 3 is modified by *n*-octanoic acid. The modification is the characteristic of GRLN. The six N-terminal amino acid residues (GVSFHP) were identical to another elasmobranch shark GRLN-LP that was recently identified although it had low identity with other GRLN peptides. Therefore, we designated this peptide stingray GRLN-LP. Uniquely, stingray GRLN-LP was *O*-glycosylated with mucin-type glycan chains [*N*-acetyl hexosamine $(HexNAc)_3$ hexose$(Hex)_2$] at threonine at position 11 (Thr-11) or both serine at position 10 (Ser-10) and Thr-11. Removal of the glycan structure by *O*-glycanase made the *in vitro* activity of stingray GRLN-LP decreased when it was evaluated by the increase in intracellular Ca^{2+} concentrations using a rat GHS-R1a-expressing cell line, suggesting that the glycan structure plays an important role for maintaining the activity of stingray GRLN-LP.

Conclusions: This study reveals the structural diversity of GRLN and GRLN-LP in vertebrates.

Background

Ghrelin (GRLN), which generally consists of 28 amino acids, was first identified in the stomachs of rats and humans as an endogenous ligand for the growth hormone secretagogue-receptor 1a (GHS-R1a)[1]. The serine residue at position 3 of this peptide (Ser-3) contains a unique octanoyl modification, and the acylation is necessary for the peptide to bind and activate GHS-R1a [1,2]. In mammals, GRLN is an important hormone involves in various physiological events such as pituitary, cardiovascular, steroidogenic, and developmental functions and energy homeostasis [3-6].