Regulator of G-Protein Signaling Subtype 4 Mediates Antihypertrophic Effect of Locally Secreted Natriuretic Peptides in the Heart

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Background—Mice lacking guanylyl cyclase-A (GC-A), a natriuretic peptide receptor, have pressure-independent cardiac hypertrophy. However, the mechanism underlying GC-A–mediated inhibition of cardiac hypertrophy remains to be elucidated. In the present report, we examined the role of regulator of G-protein signaling subtype 4 (RGS4), a GTPase activating protein for G_q and G_i, in the antihypertrophic effects of GC-A.

Methods and Results—In cultured cardiac myocytes, treatment of atrial natriuretic peptide stimulated the binding of guanosine 3′,5′-cyclic monophosphate-dependent protein kinase (PKG) I-α to RGS4, PKG-dependent phosphorylation of RGS4, and association of RGS4 and $G\alpha_q$. In contrast, blockade of GC-A by an antagonist, HS-142-1, attenuated the phosphorylation of RGS4 and association of RGS4 and $G\alpha_q$. Moreover, overexpressing a dominant negative form of RGS4 diminished the inhibitory effects of atrial natriuretic peptide on endothelin-1–stimulated inositol 1,4,5-triphosphate production, [^{3}H]leucine incorporation, and atrial natriuretic peptide gene expression. Furthermore, expression and phosphorylation of RGS4 were significantly reduced in the hearts of GC-A knockout (GC-A-KO) mice compared with wild-type mice. For further investigation, we constructed cardiomyocyte-specific RGS4 transgenic mice and crossbred them with GC-A-KO mice. The cardiac RGS4 overexpression in GC-A-KO mice significantly reduced the ratio of heart to body weight ($P<0.001$), cardiomyocyte size ($P<0.01$), and ventricular calcineurin activity ($P<0.05$) to 80%, 76%, and 67% of nontransgenic GC-A-KO mice, respectively. It also significantly suppressed the augmented cardiac expression of hypertrophy-related genes in GC-A-KO mice.

Conclusions—These results provide evidence that GC-A activates cardiac RGS4, which attenuates $G\alpha_q$ and its downstream hypertrophic signaling, and that RGS4 plays important roles in GC-A–mediated inhibition of cardiac hypertrophy. (***Circulation.*** **2008;117:2329-2339.)**

Key Words: calcineurin ■ hypertrophy ■ natriuretic peptides ■ regulators of G-protein signaling proteins ■ remodeling

Cardiac myocytes respond to mechanical stress and neurohumoral factors by undergoing a hypertrophic response, which is characterized by increases in cell size and protein synthesis, and by activating programs for a specific set of genes, such as atrial natriuretic peptide (ANP), β-myosin heavy chain (MHC), and α-skeletal actin (reviewed in References 1 to 3). Although some of this hypertrophy is adaptive, much of it is maladaptive and can ultimately result in cardiac failure.[4] Calcineurin, a calcium/calmodulin-activated serine-threonine phosphatase that is activated by G-protein–coupled receptor (GPCR) agonists, such as angiotensin (Ang) II and endothelin (ET)-I,[5–7] has emerged as a key mediator of cardiac hypertrophy.

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ANP and brain natriuretic peptide (BNP) are cardiac hormones that act through guanylyl cyclase-A (GC-A) to lower blood pressure (BP), induce diuresis/natriuresis, and dilate blood vessels.[8,9] Cardiac synthesis of ANP and BNP is increased during cardiac hypertrophy associated with various

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cardiovascular diseases.[8] Recently, we reported that in situ activation of cardiac GC-A by locally secreted natriuretic peptides protects the heart from cardiac hypertrophy by guanosine 3′,5′-cyclic monophosphate-dependent protein kinase (PKG)–mediated inhibition of calcineurin and its downstream mediator, nuclear factor of activated T cells (NFAT).[10] However, the molecular mechanism underlying GC-A–mediated inhibition of the calcineurin-NFAT pathway remains to be elucidated.

GTPase-activating proteins for Gα have recently been identified and named regulator of G-protein signaling (RGS) proteins. RGS proteins terminate GPCR signaling by accelerating the rate of GTP hydrolysis by Gα (reviewed in References 11 to 13). Among >30 RGS proteins, RGS2[14–17] and RGS4[18–21] have been implicated in cardiovascular pathophysiology. Interestingly, it has been reported that PKG binds to, phosphorylates, and activates RGS2, attenuating GPCR-mediated vascular contraction.[14,15] In addition, inhibitory effects of RGS proteins on $G\alpha_q$-mediated cardiac hypertrophy have been reported.[16–21] Therefore, it is tempting to speculate that RGS proteins might mediate the effects of PKG in tissues other than blood vessels, especially the heart. In the present study, we investigated the role of RGS in GC-A–mediated inhibition of cardiac hypertrophy.

Methods

Experimental procedures are described in the online-only Data Supplement.

Statistical Analysis

All values are shown as mean±SEM. Statistical significance between the 2 groups was determined with the use of the unpaired *t* test or Mann–Whitney *U* test. For multiple comparisons, the data were subjected to 1-way or 2-way ANOVA followed by Fisher multiple comparisons post hoc tests. Probability values of <0.05 were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Posttranslational and Transcriptional Regulation of RGS4 by ANP

Because inhibitory effects of RGS2 and RGS4 on $G\alpha_q$-mediated cardiac hypertrophy have been reported,[16–21] we first analyzed the localization of RGS2 and RGS4 in the hearts of wild-type (WT) C57BL/6 mice. As shown in Figure 1A, left panel, RGS2 was expressed primarily in coronary artery smooth muscle cells but was rarely observed in cardiac myocytes. In contrast, RGS4 was not expressed in vessels but was observed in cardiac myocytes (Figure 1A, right panel). Therefore, we focused on RGS4 in subsequent experiments.

Using cultured cardiac myocytes, we first examined whether GC-A activation affects the state of RGS4. As shown in Figure 1B, ANP induced binding of PKGIα to RGS4. Because serine phosphorylation of RGS4 is critical for its association with $G\alpha_q$ and for its GTPase activity,[13,22,23] we next evaluated RGS4 phosphorylation. ANP stimulated phosphorylation of RGS4 (142% versus control; Figure 1C). In addition, ANP stimulated RGS4 association with $G\alpha_q$ (149% versus control; Figure 1D). Pretreatment of cardiac myocytes with the PKG inhibitor KT5823 significantly blocked ANP-stimulated RGS4 phosphorylation (Figure 1C) and RGS4 association with $G\alpha_q$ (Figure 1D). ANP significantly elevated RGS4 gene (139% versus control; Figure 1E) and protein (169% versus control; Figure 1F) expression. Pretreatment of cardiac myocytes with the KT5823 significantly blocked ANP-stimulated RGS4 gene (Figure 1E) and protein expression (Figure 1F). In Figure 1E, RNA values were obtained from Northern blot analysis, and these data were corroborated by real-time polymerase chain reaction (data not shown). These results suggest that, in cardiac myocytes, exogenous ANP induces RGS4 phosphorylation, accelerates RGS4 association with $G\alpha_q$, and elevates gene and protein expression of RGS4 through a primarily PKG-dependent mechanism.

Because ANP and BNP are synthesized from the heart,[8] we next investigated the contribution of locally secreted natriuretic peptides on the state of RGS4 using a natriuretic receptor antagonist, HS-142-1. As shown in Figure 1G and 1H, treatment of cardiac myocytes with HS-142-1 significantly reduced RGS4 phosphorylation (47% versus control) and significantly inhibited the association of RGS4 with $G\alpha_q$ (73% versus control). Moreover, as shown in Figure 1I, HS-142-1 treatment significantly decreased the RGS4 protein expression (81% versus control). These results suggest that not only exogenous but also endogenous locally secreted natriuretic peptides are intimately involved in the activation and expression of RGS4.

RGS4 Is Required for the Antihypertrophic Effects of ANP

We next examined whether RGS4 was required for the inhibitory effects of ANP on cardiac hypertrophy induced by ET-1, a potent GPCR agonist. ET-1–mediated activation of $G\alpha_q$ stimulates phospholipase C-β (PLC-β), which leads to production of inositol 1,4,5-triphosphate (IP_3) and mobilization of intracellular calcium.[24] Therefore, we evaluated the contribution of RGS4 to ANP-mediated inhibition of IP_3 production in cultured cardiac myocytes using a dominant negative form of RGS4 (RGS4DN). In advance, we confirmed that ANP inhibits ET-1–stimulated IP_3 production via a PKG-dependent mechanism (Figure I in the online-only Data Supplement). We also confirmed that exogenous expression of a truncated mutant of RGS4 [RGS4(1-58)] works in a dominant negative fashion to RGS4 specifically (Figure I in the online-only Data Supplement). RGS4(1-58) completely blocked WT RGS4-induced suppressive effect of the IP_3 production, whereas it did not block the effect of RGS2,3. As shown in Figure 2A to 2C, the inhibitory effect of ANP on ET-1–stimulated IP_3 production, [^{3}H]leucine incorporation, and elevation of ANP gene expression was abolished in cells overexpressing RGS4DN. These results suggest that endogenous RGS4 is required for ANP to exert its potent antihypertrophic action.

We recently demonstrated that the calcineurin-NFAT pathway contributes importantly to the establishment of cardiac hypertrophy in GC-A knockout (GC-A-KO)

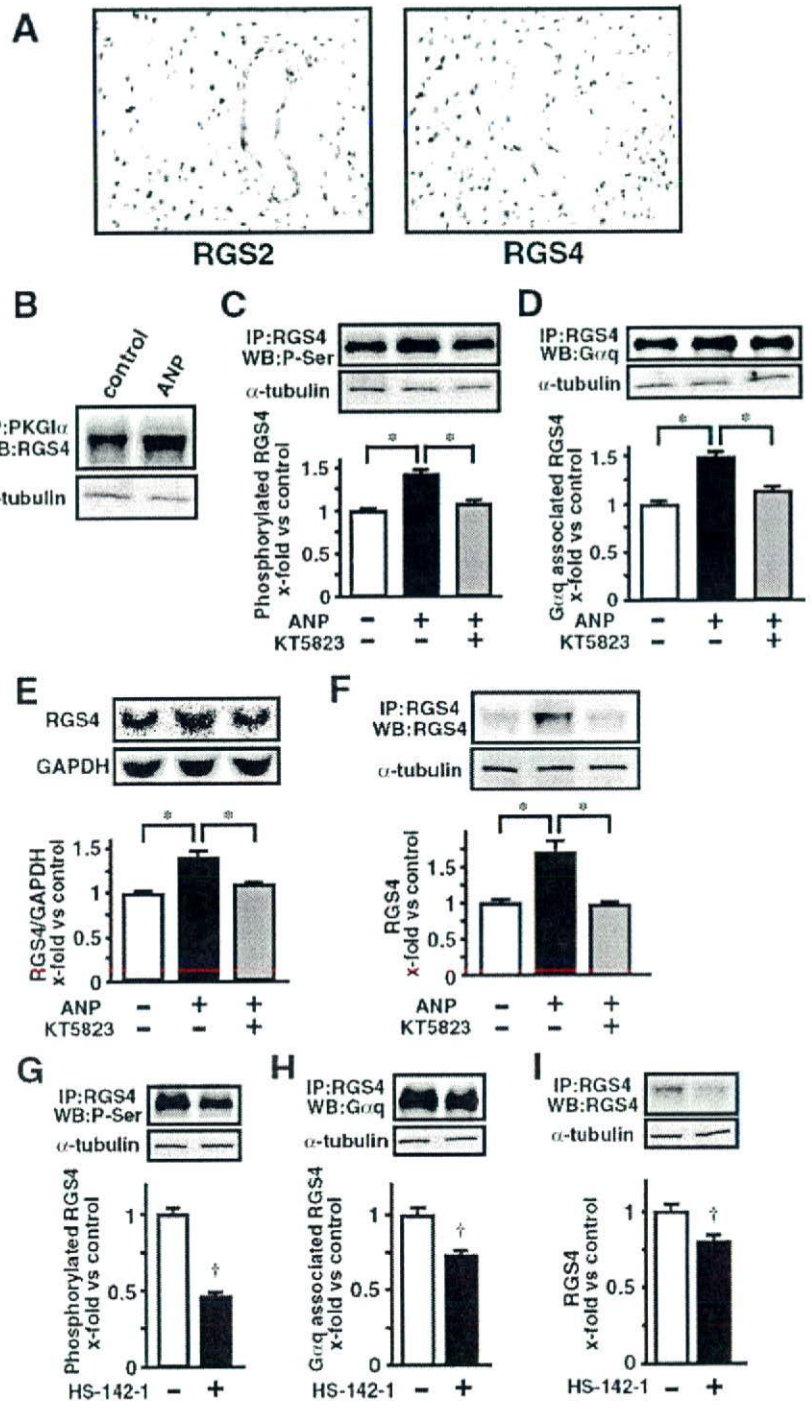

Figure 1. Posttranslational and transcriptional regulation of RGS4 by ANP. A, Representative images of immunohistochemistry of RGS2 (left) and RGS4 (right) at 16 weeks of age in WT mouse heart (magnification ×200). B, Cultured cardiac myocytes were incubated with ANP (10^{-6} mol/L) for 2 minutes. C and D, Cultured cardiac myocytes were preincubated for 30 minutes with or without KT5823 (10^{-6} mol/L), and then ANP (10^{-6} mol/L) was added to the culture medium for 2 minutes. E and F, Cultured cardiac myocytes were incubated for 24 hours with or without ANP (10^{-6} mol/L) in the presence or the absence of KT5823 (10^{-6} mol/L). G to I, Cultured cardiac myocytes were incubated with 100 μg/mL of HS-142-1 for 15 minutes (G, H) and 24 hours (I). In B, C, D, F, G, H, and I, the bands of α-tubulin are shown as loading controls. Values are expressed as mean±SEM of 4 independent assays. *$P<0.05$, †$P<0.05$ vs control.

mice.[10] As shown in Figure 2D, whereas ANP significantly decreased ET-1–mediated elevation of calcineurin activity, the inhibitory effect of ANP was abolished in cells overexpressing RGS4DN. Next, we evaluated the role of RGS4 in the suppressing effect of ANP on dephosphorylation of NFATc3, a direct downstream effector of calcineurin in the heart.[25] As shown in Figure 2E, ANP significantly reversed ET-1–mediated dephosphorylation of NFATc3, and the effect of ANP was abolished in cells overexpressing RGS4DN. The gene expression of modulatory calcineurin-interacting protein (MCIP) 1 (same as RCAN1), which is augmented by NFAT activation,[26] was also increased in cells treated with ET-1, and ANP significantly decreased ET-1–induced elevation of MCIP1 gene expression (Figure 2F). Again, overexpression of RGS4DN abolished the inhibitory effect of ANP (Figure 2F). Thus, these results suggest that RGS4 is necessary for ANP-mediated inhibition of the calcineurin-NFAT pathway in cardiac myocytes. To better visualize the effects of RGS4DN on ANP-mediated actions, the data shown in Figure 2 were expressed as percent inhibition in Figure III in the online-only Data Supplement.

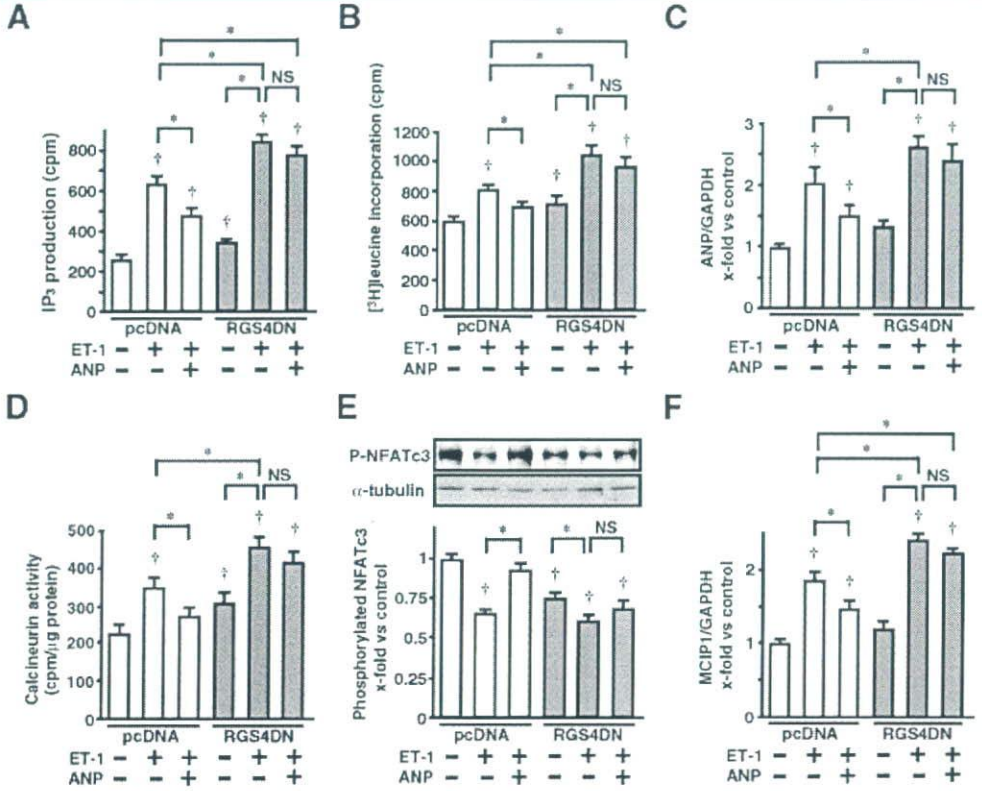

Figure 2. Overexpression of dominant negative RGS4 (RGS4DN) disrupted the antihypertrophic effects of ANP. A, After transfection with plasmid encoding RGS4DN or empty vector (pcDNA3.1), cardiac myocytes were preincubated with or without ANP (10^{-6} mol/L) for 30 minutes. Cells were then stimulated with ET-1 (10^{-7} mol/L) for 15 minutes. B and C, After transfection with plasmid, cells were stimulated with ET-1 (10^{-7} mol/L) in the presence or the absence of ANP (10^{-6} mol/L) for 24 hours. D, After transfection, cells were preincubated for 30 minutes with or without ANP (10^{-6} mol/L) and then stimulated with ET-1 (10^{-7} mol/L) for 2 minutes. E, Transfected cells were preincubated for 30 minutes with or without ANP (10^{-6} mol/L) and then stimulated with ET-1 (10^{-7} mol/L) for 10 minutes. Photo is representative 1 of 4 independent assays. The bands of α-tubulin were shown as loading controls. F, After transfection, cells were stimulated with ET-1 (10^{-7} mol/L) in the presence or absence of ANP (10^{-6} mol/L) for 24 hours. In C and F, gene expressions were evaluated by real-time polymerase chain reaction. Values are expressed as mean±SEM of 4 independent assays. *$P<0.05$, †$P<0.05$ vs control.

Expression and Phosphorylation of RGS4 Were Diminished in Hearts of GC-A-KO Mice

As shown in Figure 3A, at 16 weeks of age, RGS4 expression was significantly diminished in the hearts of GC-A-KO mice compared with WT mice (48% versus WT). As shown in Figure 3B, there was a significant decrease in RGS4 phosphorylation (when assessed as the ratio of phospho-RGS4 to total RGS4) in GC-A-KO ventricles. Total RGS4 protein expression was also significantly diminished in GC-A-KO ventricles compared with WT (Figure 3B, upper middle panel).

Because we previously found that the augmented Ang II system plays an important role in the progress of cardiac remodeling in GC-A-KO mice,[27] we next assessed the regulation of RGS4 expression and phosphorylation in an Ang II–induced cardiac hypertrophy model. Ten days of subcutaneous administration of Ang II (2 mg/kg per day) in 16-week-old WT mice caused significant increase in systolic BP (control, 103±4 versus Ang II, 143±8 mm Hg [$P<0.01$]) and ratio of heart to body weight (control, 3.8±0.3 versus Ang II, 5.2±0.2 [$P<0.01$]). As shown in Figure 3C, top panel, RGS4 expression was significantly diminished in the hearts of Ang II–administrated mice (49% versus control). In contrast, ANP gene expression was significantly augmented (7.7-fold; $P<0.001$; Figure 3C, upper middle panel). As shown in Figure 3D, top panel, there was a significant increase in RGS4 phosphorylation in the hearts of Ang II–administrated mice. However, total RGS4 protein expression was significantly diminished (Figure 3D, upper middle panel). These results suggest that the major cause of downregulation of RGS4 gene expression in the hearts of GC-A-KO mice could likely be a chronic activation of the Ang II system rather than a deficiency of direct positive regulation of endogenous ANP on RGS4 expression and also indicate that the central role of ANP in the regulation of RGS4 status is a posttranslational phosphorylation rather than transcriptional regulation in in vivo pathological situations.

Overexpression of RGS4 Attenuates Cardiac Hypertrophy in GC-A-KO Mice

RGS4 is required for the inhibitory effects of ANP on cardiac hypertrophy and the calcineurin-NFAT pathway (Figure 2). On the other hand, the expression and phosphorylation of RGS4 are significantly downregulated in GC-A-KO mouse hearts (Figure 3). Therefore, we hypothesized that cardiac hypertrophy in GC-A-KO mice is caused at least in part by a reduction of RGS4

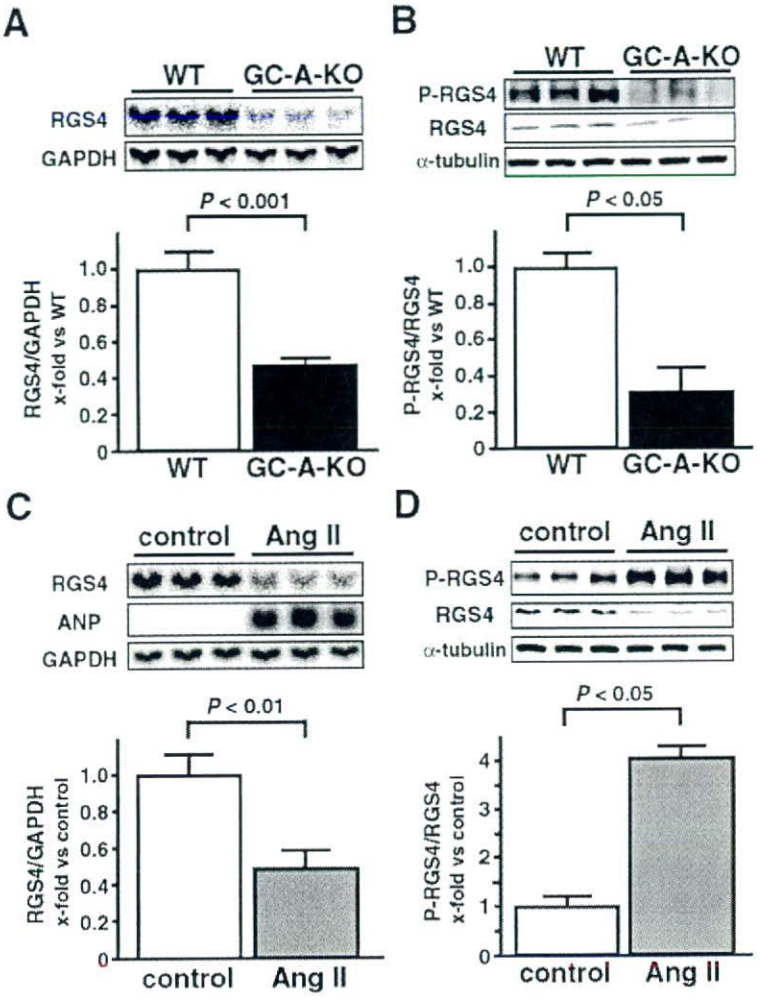

Figure 3. A and C, top, Representative images of Northern blots; bottom, mRNA levels relative to WT (A) or control (C) normalized to GAPDH mRNA levels. n=8 (A) or n=6 (C) animals per group. B and D, Detection of phosphorylated RGS4 and total RGS4 in the hearts; top, images of Western blots; bottom, RGS4 phosphorylation ratio relative to WT (B) or control (D). n=3 animals per group. In B and D, the bands of α-tubulin were shown as loading controls. Values are expressed as mean±SEM.

function, which may lead to the excessive activation of $G\alpha_q$ signaling, including the calcineurin-NFAT pathway. Thus, we sought to determine whether exogenous expression of the RGS4 gene in GC-A-KO mouse heart could inhibit cardiac hypertrophy and the calcineurin-NFAT pathway.

Using the α-MHC promoter, we generated transgenic (Tg) mice expressing mouse RGS4 in a heart-specific manner (Figure 4A).[28] As shown in Figure 4B, 6 lines of Tg mice were successfully established. We used high-expression lines (lines 2, 3, and 4) for the following experiments.

To investigate whether overexpression of RGS4 in the heart could rescue cardiac hypertrophy in GC-A-KO mice, we crossbred RGS4-Tg mice with GC-A-KO mice and also with WT mice. At 16 weeks of age, consistent with a previous report,[19] no significant differences were observed in body weight, systolic BP, or heart rate between RGS4-Tg and control WT mice (Table 1). Likewise, RGS4-Tg/GC-A-KO mice showed no difference in these physiological parameters (Table 1). In the WT background, cardiac overproduction of RGS4 did not affect the ratio of heart to body weight in either male (Figure 4C) or female (Figure 4D) mice. In contrast, cardiac overproduction of RGS4 significantly attenuated the increase in ratio of heart to body weight on the GC-A-KO background in both male (non–Tg-GC-A-KO, 6.13±0.28 versus Tg-GC-A-KO, 4.90±0.33 [Figure 4C]) and female mice (non-Tg-GC-A-KO, 4.96±0.26 versus Tg-GC-A-KO, 4.33±0.31 [Figure 4D]). Because similar tendencies in the attenuation of ratio of heart to body weight by cardiac-specific overproduction of RGS4 were observed in male and female mice on the GC-A-KO background, we performed the following experiments using male mice. As we reported previously,[10] echocardiographic analysis demonstrated an increase in the thickness of the interventricular septum and the left ventricular posterior wall and an increase in the left ventricular diastolic dimension in the GC-A-KO mice compared with WT. Fractional shortening did not differ significantly between these genotypes. Interestingly, on the GC-A-KO background, cardiac overproduction of RGS4 significantly attenuated the increases in thickness of the interventricular septum, thickness of the left ventricular posterior wall, and left ventricular diastolic dimension, whereas it did not affect fractional shortening (Table 2). In contrast, on the WT background, cardiac overproduction of RGS4 did not affect these parameters (Table 2). Representative images of the M-mode echocardiogram are shown in Figure 4E. In hematoxylin and eosin–stained sections, we observed an increase in cross-sectional myocyte area and length in GC-A-KO mice compared with WT mice. Interest-

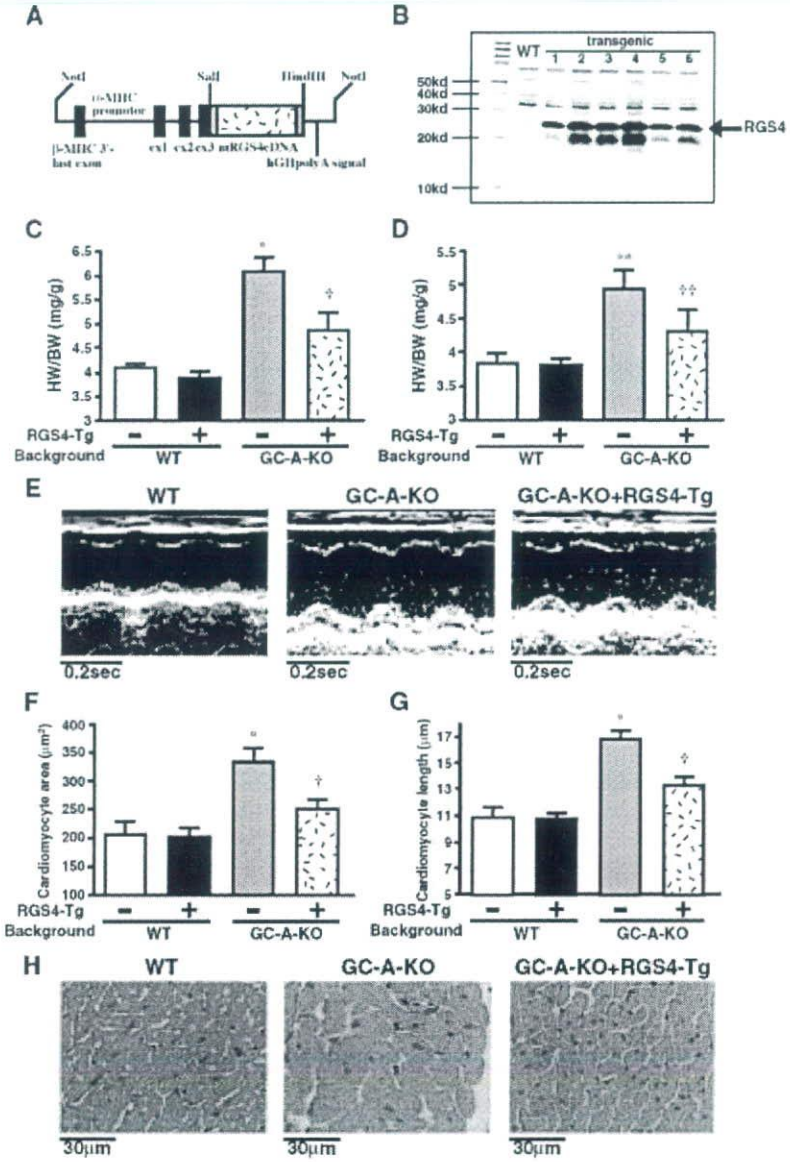

Figure 4. Cardiac myocyte–specific RGS4 transgenic overexpression inhibited hypertrophic remodeling in GC-A-KO mouse hearts. A, Schematic diagram of the transgenic construct used to generate RGS4-Tg mice. The construct contains the α-MHC gene promoter, full-length mouse RGS4 cDNA, and the human growth hormone (hGH) polyadenylation sequence. B, Representative image of Western blot analysis of RGS4 protein from ventricles of WT mice and RGS4-Tg mice (lines 1 to 6). C and D, Transgenic RGS4 overproduction in cardiac myocytes (RGS4-Tg) significantly decreased ratio of heart to body weight (HW/BW) in both male (C) and female (D) GC-A-KO mice. n=8 animals per group. E, Representative images of M-mode echocardiograms. F and G, Transgenic RGS4 overproduction in cardiac myocytes (RGS4-Tg) significantly decreased both cardiomyocyte area (F) and length (G) in GC-A-KO mice. n=4 animals per group. H, Representative photomicrograph of cardiomyocytes in sections stained with hematoxylin and eosin (magnification ×400). Values are expressed as mean±SEM. *$P<0.05$ vs non-Tg WT male; **$P<0.05$ vs non-Tg WT female; †$P<0.05$ vs non-Tg KO male; ††$P<0.05$ vs non-Tg KO female.

ingly, on the GC-A-KO background, the increase was significantly abrogated by cardiac overproduction of RGS4 (Figure 4F and 4G). In contrast, overproduction of RGS4 did not affect myocyte area and length on the WT background. Representative photomicrographs of hematoxylin and eosin–stained sections are shown in Figure 4H.

Effect of Overexpression of RGS4 on Cardiac Hypertrophy–Related Gene Expression in GC-A-KO Mice

As shown in Figure 5, ventricular expression of the MCIP1, ANP, and BNP genes was significantly elevated in GC-A-KO mice compared with WT mice, as we reported

Table 1. Body Weight, Systolic BP, and Heart Rate in Each Experimental Group

	WT Male		WT Female		KO Male		KO Female	
	Non-Tg	Tg	Non-Tg	Tg	Non-Tg	Tg	Non-Tg	Tg
Body weight, g	31.7±1.3	31.2±1.5	26.1±1.3	25.5±1.0	30.9±1.1	31.4±0.9	26.7±0.5	25.4±0.9
Systolic BP, mm Hg	99±2	104±2	103±2	103±2	131±4*	133±3†	115±2‡	114±3§
Heart rate, bpm	619±8	621±5	624±7	629±7	622±8	628±6	618±9	627±8

Data are mean±SEM. Tg indicates α-MHC-RGS4 transgenic; KO, GC-A-knockout. n=8 animals per group.
*$P<0.05$ vs non-Tg WT male.
†$P<0.05$ vs Tg WT male.
‡$P<0.05$ vs non-Tg WT female.
§$P<0.05$ vs Tg WT female.

Table 2. Echocardiographic Characteristics in Each Experimental Group

	WT		KO	
	Non-Tg	Tg	Non-Tg	Tg
IVSth, mm	0.58±0.03	0.53±0.01	0.94±0.04*	0.68±0.03†
LVPWth, mm	0.58±0.01	0.53±0.01	0.88±0.06*	0.66±0.03†
LVEDD, mm	3.86±0.07	3.81±0.08	4.11±0.11*	4.11±0.11†
LVESD, mm	2.55±0.07	2.45±0.09	2.85±0.12*	2.52±0.09†
Fractional shortening, %	33.8±2.4	35.8±1.7	35.3±2.5	38.7±1.8
Heart rate, bpm	287±12	278±8	288±6	297±7

Data are mean±SEM. Tg indicates α-MHC-RGS4 transgenic; KO, GC-A-knockout; IVSth, thickness of the interventricular septum; LVPWth, thickness of the left ventricular posterior wall; LVEDD, left ventricular end-diastolic diameter; and LVESD, left ventricular end-systolic diameter. n=8 animals per group.
*$P<0.05$ vs non-Tg WT.
†$P<0.05$ vs non-Tg KO.

previously.[10] Cardiac-specific overproduction of RGS4 significantly suppressed the expression of MCIP1, ANP, and BNP in GC-A-KO mice, whereas it had no effect in WT mice. In addition, elevated ventricular expression of the α-skeletal actin and β-MHC genes was observed in GC-A-KO mice; this was markedly suppressed by overproduction of RGS4. There were no significant differences in the expression levels of α-MHC and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2 mRNA levels in each group.

Overexpression of RGS4 Rescues Excessive Activation of the Calcineurin-NFAT Pathway in the Absence of GC-A

We next evaluated the effects of RGS4 overexpression on the calcineurin-NFAT pathway in the hearts of GC-A-KO mice. As we reported previously, calcineurin activity, phosphorylation of NFATc3, and GATA4 DNA-binding activity was significantly increased, attenuated, and enhanced in GC-A-KO (non-Tg) mouse hearts compared with WT (non-Tg) mice (Figure 6A through 6C). RGS4 overexpression significantly inhibited calcineurin activity, increased phosphorylated NFATc3, and reduced GATA4 DNA-binding activity on the GC-A-KO background but not on the WT background (Figure 6A through 6C).

Recently, transient receptor potential channel subfamily C (TRPC) members have been reported to promote cardiomyocyte hypertrophy through activation of calcineurin signaling.[29–32] It has also been reported that hypertrophic GPCR agonists stimulate the expression of TRPC3 and TRPC6 through activation of the calcineurin-NFAT pathway.[30,32] Interestingly, as shown in Figure 6D, TRPC3 gene expression was dramatically elevated in GC-A-KO mouse hearts. Cardiac-specific overexpression of RGS4 significantly suppressed the expression of TRPC3 in GC-A-KO mice, whereas it had no effect in WT mice. There were no significant differences in TRPC6 gene expression in each group (data not shown). As shown in Figure 6E, treatment of cardiac myocytes with HS-142-1 significantly elevated TRPC3 gene expression. The concomitant addition of the calcineurin

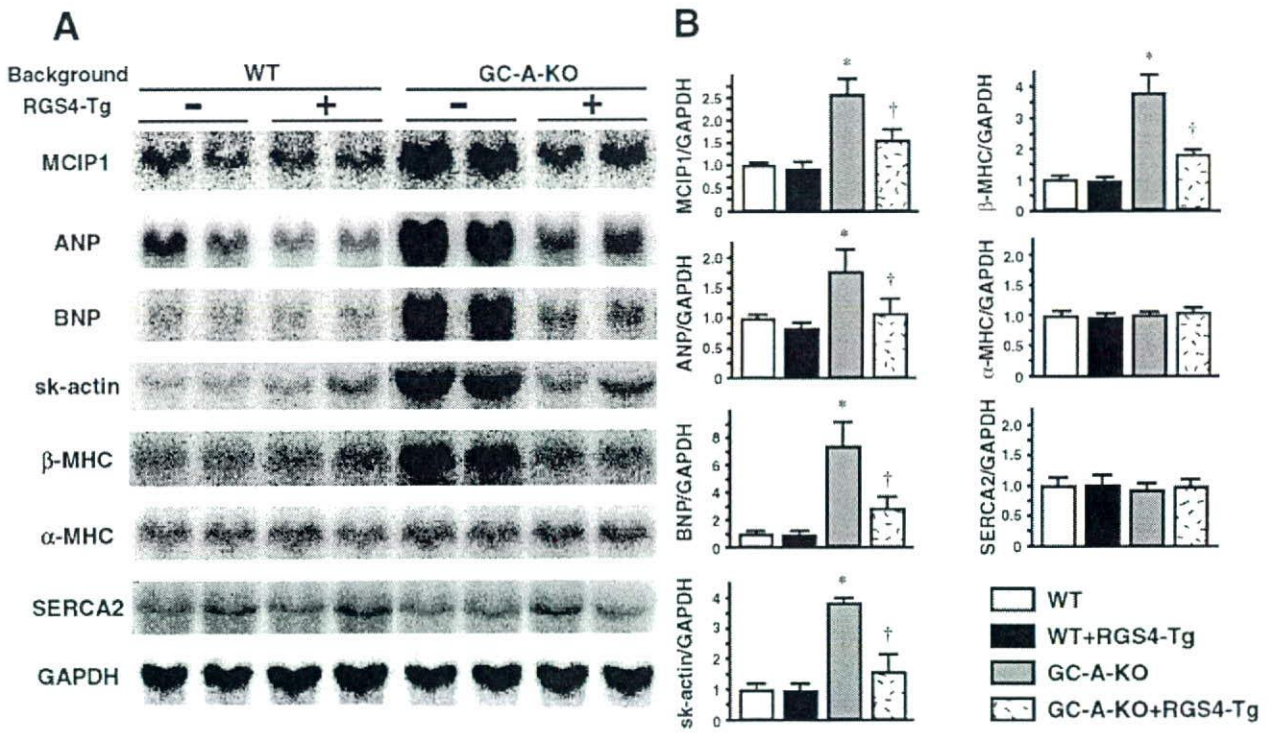

Figure 5. Cardiac myocyte–specific RGS4 transgenic overexpression attenuated overexpression of hypertrophy-related genes in GC-A-KO mice. A, Representative images of Northern blots. B, Quantitative analysis of the Northern blots. Shown are mRNA levels relative to non-Tg WT normalized by GAPDH mRNA levels. n=4 animals per group. Values are expressed as mean±SEM. *$P<0.05$ vs non-Tg WT; †$P<0.05$ vs non-Tg KO.

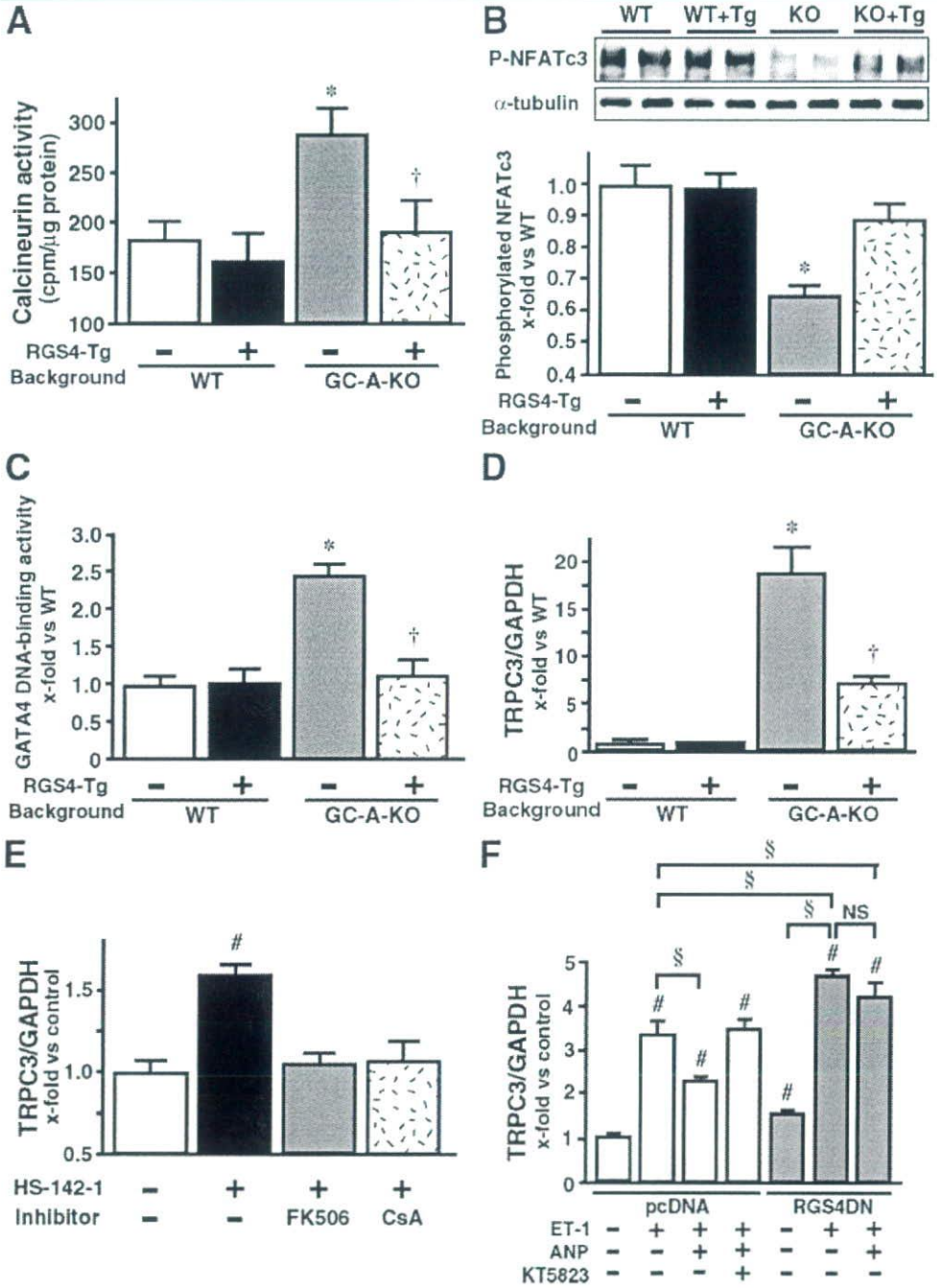

Figure 6. Cardiac myocyte–specific RGS4 transgenic overexpression inhibited calcineurin-NFAT pathway activation in GC-A-KO mice. A, Transgenic overexpression of RGS4 (RGS4-Tg) significantly inhibited calcineurin activity in the hearts of GC-A-KO mice. B, RGS4-Tg significantly augmented NFATc3 phosphorylation in the hearts of GC-A-KO mice. The bands of α-tubulin were shown as loading controls. C, RGS4-Tg reduced DNA binding activity of GATA4 in the heart of GC-A-KO mice. D, Shown are TRPC3mRNA levels relative to non-Tg WT corrected for GAPDH mRNA levels. n=4 animals per group. Values are expressed as mean±SEM. E, Cultured cardiac myocytes were incubated with HS-142-1 (100 μg/mL) in the presence or absence of FK506 (10^{-6} mol/L) or cyclosporine A (CsA; 10^{-6} mol/L) for 24 hours. F, After transfection with plasmid, cardiac myocytes were stimulated with ET-1 (10^{-7} mol/L) with or without ANP (10^{-6} mol/L) in the presence or absence of KT5823 (10^{-6} mol/L) for 24 hours. In D to F, gene expressions were evaluated by real-time polymerase chain reaction. Values are expressed as mean±SEM of 4 independent assays. *$P<0.05$ vs non-Tg WT; †$P<0.05$ vs non-Tg KO; §$P<0.05$, #$P<0.05$ vs control.

inhibitor FK506 or cyclosporine A almost completely blocked HS-142-1–dependent induction of the TRPC3 gene, indicating that locally secreted natriuretic peptides inhibit TRPC3 gene expression through inhibition of the calcineurin-NFAT pathway in an autocrine manner. As shown in Figure 6F, consistent with a previous report,[30] ET-1 significantly elevated TRPC3 gene expression. ANP significantly reduced ET-1–induced elevation of TRPC3 gene expression. The concomitant addition of KT5823 or overexpression of RGS4DN completely abolished the inhibitory effect of ANP,

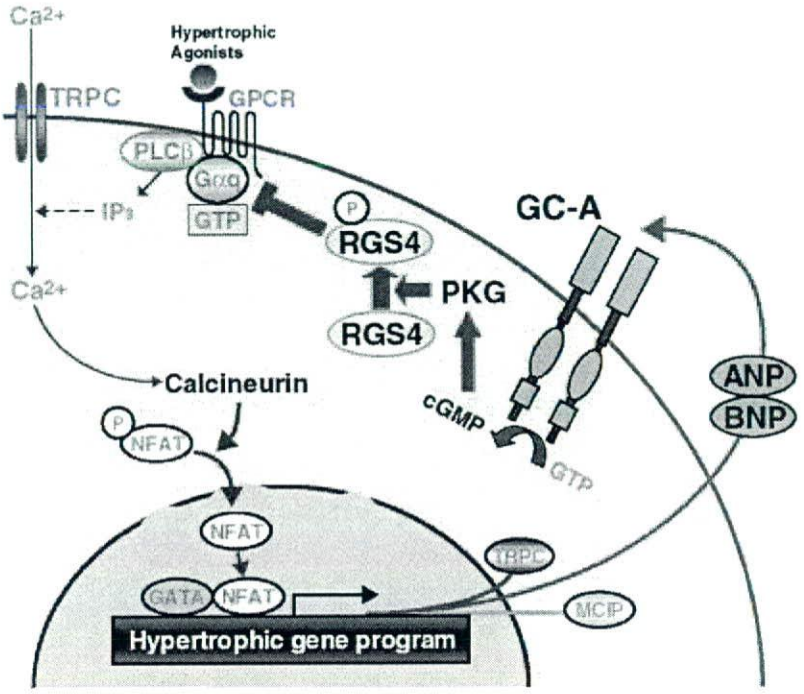

Figure 7. Mechanism of GC-A–mediated inhibition of cardiac hypertrophy suggested from the findings of this and previous studies. Cardiac hypertrophic agonists, such as Ang II and ET-1, stimulate GPCR and activate PLC-β. Subsequent production of IP_3 promotes elevation of intracellular Ca^{2+} levels sufficient to activate the calmodulin-regulated phosphatase calcineurin (recent studies reported that TRPC channel activity may be involved in the activation). Activated calcineurin then dephosphorylates NFAT, which facilitates the nuclear translocation of the transcriptional factor. NFAT and GATA then cooperatively activate transcription of the hypertrophic gene program, including the ANP and BNP genes. The MCIP and TRPC genes are also targets of NFAT and are upregulated in response to calcineurin signaling. We propose that endogenous ANP and BNP mediate antihypertrophic effects through GC-A/PKG signaling–dependent RGS4 phosphorylation and activation, which leads to association of RGS4 with $G\alpha_q$, increase in GTPase activity of $G\alpha_q$, inhibition of calcineurin-NFAT signaling, and suppression of hypertrophy-related gene transcription.

suggesting that the effect of ANP is dependent on PKG-mediated activation of RGS4.

Discussion

As has been reported previously, GC-A plays a primary role in moderating cardiac hypertrophy in vivo independent of its effects on BP regulation.[33–35] We have shown previously that GC-A exerts its antihypertrophic action by antagonizing the calcineurin-NFAT pathway through a PKG-dependent mechanism.[10] Because GPCR agonists such as Ang II provoke calcineurin activation by stimulating $G\alpha_q$,[5,36] it was hypothesized that GC-A signaling negatively interacts with $G\alpha_q$ signaling via the activation of PKG. However, the precise mechanism linking the natriuretic peptide/GC-A/PKG pathway and the $G\alpha_q$ signaling remained to be elucidated.

RGS proteins play key roles in the inhibitory regulation of GPCR signaling by accelerating GTPase activity.[11–13] Mende and coworkers[16] demonstrated an inhibitory effect of RGS2 on ET-1– and phenylephrine-induced cardiac myocyte hypertrophy. However, although they exhibit hypertension, cardiac hypertrophy was not shown in mice deficient for RGS2,[14,15] suggesting only a minor role for RGS2 in the physiological regulation of cardiomyocyte size. On the other hand, Muslin and coworkers[18,37] reported RGS4 gene and protein expression in adult rat heart and found an inhibitory effect of RGS4 on ET-1– and phenylephrine-induced cardiac myocyte hypertrophy.

In this study, we focused on RGS4 and examined its role in GC-A–mediated antihypertrophic action. These results suggest that RGS4 is required for GC-A–mediated antihypertrophic action in the heart and also suggest that the reduced activation of RGS4 causes excessive activation of the calcineurin-NFAT pathway, which results in cardiac hypertrophy in mice deficient for GC-A. A schematic diagram depicting these signaling mechanisms is shown in Figure 7.

In regard to upstream factor(s) that activate the calcineurin-NFAT pathway in GC-A-KO mouse hearts, we suggest that the Ang II system may contribute to calcineurin activation. Like many other calcium-mobilizing GPCRs, Ang II type 1A receptor is coupled to $G\alpha_q$, which activates PLC-β and stimulates IP_3/calcium signaling.[24] It has been reported that the selective Ang II type 1A receptor blocker attenuated cardiac calcineurin activity and the development of cardiac hypertrophy in hypertensive rats, even at a dose that did not lower BP.[38] We previously reported that, in the absence of GC-A, the intracellular signaling downstream of Ang II type 1A receptor becomes hypersensitive to ligand activation.[27] Taking the results herein into account, we suggest that locally secreted natriuretic peptide–induced activation of RGS4 inhibits $G\alpha_q$ signaling coupled to Ang II type 1A receptor and, thereby, downstream hypertrophic transduction.

In the present study, we have demonstrated the importance of RGS4 as a target of cardiac natriuretic peptides to exert antihypertrophic effects. However, our findings do not exclude contributions of other RGS proteins in inhibition of GC-A–mediated inhibition of cardiac hypertrophy. In fact, gene and protein expressions of RGS2, RGS3, and RGS5 have been detected in the heart.[39]

Other molecules downstream of PKG also merit consideration for a role in the antihypertrophic action of natriuretic peptide/GC-A signal transduction. Recently, Kilic et al[40] reported that GC-A moderates cardiac growth response to pressure overload by preventing excessive activation of the Na^+/H^+ exchanger NHE-1 and subsequent increases in Ca^{2+}/calmodulin-dependent kinase II as well as Akt. This demon-

strated that not only calcineurin-NFAT but also other signaling pathways contribute to cardiac hypertrophy in GC-A-KO mice. Nevertheless, the results herein, in conjunction with our previous data, demonstrate a dominant role of reduction in RGS4 function and subsequent excessive activation of the calcineurin-NFAT pathway in cardiac remodeling in the mouse.

Nakayama et al[41] previously described a functional mutation in the 5′-flanking region of the 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 similar to that seen in GC-A-KO mice. In such patients, it is possible that inhibition of the calcineurin-NFAT pathway by RGS4 would be a useful treatment for the prevention of cardiac remodeling.

In conclusion, our findings indicate that GC-A activates RGS4 via PKG in cardiac myocytes; this attenuates $G\alpha_q$ and downstream hypertrophic signaling. These findings provide new insights into endogenous mechanisms for protection of the heart by natriuretic peptide/GC-A signaling and predict that RGS4 is a potential therapeutic target to restrain cardiac remodeling.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiac myocytes respond to mechanical stress and neurohumoral factors by undergoing a hypertrophic response. Although some of this hypertrophy is adaptive, much of it is maladaptive and can ultimately result in cardiac failure. On the other hand, apart from acting as circulating hormones, atrial natriuretic peptide and brain natriuretic peptide have some functionality as autocrine and/or paracrine factors. Recently, we reported that in situ activation of cardiac guanylyl cyclase-A (GC-A), a natriuretic peptide receptor, by locally secreted natriuretic peptides protects the heart from cardiac hypertrophy by guanosine 3′,5′-cyclic monophosphate-dependent protein kinase (PKG)–mediated inhibition of calcineurin and its downstream mediator, nuclear factor of activated T cells. However, the molecular mechanism underlying GC-A–mediated inhibition of the calcineurin–nuclear factor of activated T cells pathway remains to be elucidated. GTPase-activating proteins for $G\alpha$ have recently been identified and named regulator of G-protein signaling (RGS) proteins. In the present study, we investigated the role of RGS in GC-A–mediated inhibition of cardiac hypertrophy. Our findings indicate that GC-A activates RGS4 via PKG in cardiac myocytes; this attenuates $G\alpha_q$ and downstream hypertrophic signaling. Nakayama et al previously described a functional mutation in the 5′-flanking region of the 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 similar to that seen in GC-A knockout mice. In such patients, it is possible that inhibition of the calcineurin–nuclear factor of activated T cells pathway by RGS4 would be a useful treatment for the prevention of cardiac remodeling.

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SHORT COMMUNICATION

INSIG2 gene rs7566605 polymorphism is associated with severe obesity in Japanese

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Abstract The single nucleotide polymorphism (SNP) rs7566605 in the upstream region of the insulin-induced gene 2 (*INSIG2*) is associated with the obesity phenotype in many Caucasian populations. In Japanese, this association with the obesity phenotype is not clear. To investigate the relationship between rs7566605 and obesity in Japanese, we genotyped rs7566605 from severely obese subjects [n = 908, body mass index (BMI) $\geq$ 30 kg/m^2] and normal-weight control subjects (n = 1495, BMI < 25 kg/m^2). A case–control association analysis revealed that rs7566605 was significantly associated with obesity in Japanese. The P value in the minor allele recessive mode was 0.00020, and the odds ratio (OR) adjusted for gender and age was 1.61 [95% confidential interval (CI) = 1.24–2.09]. Obesity-associated phenotypes, which included the level of BMI, plasma glucose, hemoglobin A1c, total cholesterol,

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triglycerides, high-density lipoprotein (HDL) cholesterol, and blood pressure, were not associated with the rs7566605 genotype. Thus, rs7566605 in the upstream region of the *INSIG2* gene was found to be associated with obesity, i.e., severe obesity, in Japanese.

Keywords Insulin-induced gene 2 · Obesity · Japanese population · Association · SNP

Introduction

Obesity has become one of the major issues in public health, medicine, and the economy (Kopelman 2000). Obesity is considered to be important due to its relationship with various complications, such as diabetes mellitus, dyslipidemia, and hypertension. A combination of these dysfunctions is now defined as the metabolic syndrome that significantly increases the risk of cardiovascular disease (Wilson and Grundy 2003). Genetic and environmental factors contribute to the development of obesity (Maes et al. 1997; Barsh et al. 2000; Rankinen et al. 2006). Due to the recent progress in single nucleotide polymorphism (SNP) genotyping techniques, it is possible to conduct genome-wide screens to identify common genetic variants associated with obesity. We conducted a large-scale case–control association study and found that secretogranin III (*SCG3*) (Tanabe et al. 2007) and myotubularin-related protein 9 (*MTMR9*) (Yanagiya et al. 2007) confer susceptibility to the obesity phenotype in the Japanese population. Genome-wide association studies have shown that variations in the upstream region of the insulin-induced gene 2 (*INSIG2*) (Herbert et al. 2006) and in the fat-mass and obesity-associated gene (*FTO*) (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007) are associated with the obesity phenotype. We recently reported the association between variations in the *FTO* gene and severe obesity in Japanese (Hotta et al. 2008). An association between rs7566605 in the upstream region of the *INSIG2* gene and obesity was also found in several Caucasian and Hispanic American populations (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, results from some reports with respect to these associations could not be reproduced (Lyon et al. 2007; Smith et al. 2007; Boes et al. 2008); further, these associations are not observed in the Indian (Kumar et al. 2007), Chinese (Yang et al. 2008) and Japanese populations (Tabara et al. 2008). Thus, the association between rs7566605 in the *INSIG2* gene and obesity in Japanese remains controversial.

To investigate the relationship between the *INSIG2* gene and obesity in Japanese, we performed a case–control association study involving patients with severe adult obesity (BMI $\geq$ 30 kg/m^2) and normal weight controls (BMI $<$ 25 kg/m^2). We found that rs7566605 was significantly associated with severe adult obesity.

Materials and methods

Study subjects

Severely obese subjects were recruited from among the outpatients of medical institutes. Patients with secondary obesity and obesity-related hereditary disorders were excluded from this study, as were patients with medication-induced obesity. Control subjects were recruited from among subjects who had undergone a medical examination for the screening of common diseases. Each subject provided written informed consent, and the protocol was approved by the ethics committee of each institution and that of RIKEN. The sample size for the severely obese subjects (BMI $\geq$ 30 kg/m^2) was 908 (male:female ratio, 418:590; age, 49.1 $\pm$ 14.2 years; BMI, 34.5 $\pm$ 5.4 kg/m^2), whereas that for the normal weight controls (BMI $<$ 25 kg/m^2) was 1,495 (male:female ratio, 672:823; age, 48.1 $\pm$ 16.5 years; BMI, 21.6 $\pm$ 2.1 kg/m^2). Subjects' clinical features are illustrated in Table 1.

DNA preparation and SNP genotyping

Genomic DNA was prepared from the blood samples of each subject with a Genomix kit (Talent Srl, Trieste, Italy). SNP rs7566605 reported in a previous genome-wide association study (Herbert et al. 2006) was genotyped with TaqMan probe (C_29404113_20; Applied Biosystems; Foster City, CA, USA).

Statistical analysis

Genotype or allele frequencise were compared between cases and controls in three different modes. In the first

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Table 1 Clinical characterization of obese and control subjects

	Obese	Control	*P* value[a]
Sample size	908	1495	–
Gender (M/F)	418/490	672/823	–
Age (year)	49.1 ± 14.2	48.1 ± 16.5	0.050
BMI (kg/m^2)	34.50 ± 5.39	21.65 ± 2.07	<0.000001
Glucose (mg/dl)	129.1 ± 49.7	97.7 ± 23.8	<0.000001
HbA1c (%)	6.5 ± 1.8	5.1 ± 0.6	<0.000001
Total cholesterol (mg/dl)	210.1 ± 38.0	201.2 ± 36.4	<0.000001
Triglycerides (mg/dl)	155.6 ± 111.0	104.0 ± 73.1	<0.000001
HDL cholesterol (mg/dl)	53.1 ± 18.8	65.1 ± 15.6	<0.000001
Systolic blood pressure (mmHg)	136.4 ± 18.2	123.4 ± 17.8	<0.000001
Diastolic blood pressure (mmHg)	83.8 ± 12.0	76.0 ± 11.1	<0.000001

Data are mean ± standard deviation

HbA1c hemoglobin A1c, *HDL* high-density lipoprotein

[a] *P* values were analyzed using Mann–Whitney *U* test

mode, i.e, the allele frequency mode, allele frequencies were compared with a 2 × 2 contingency table. In the second mode, i.e., the minor allele recessive mode, frequencies of the homozygous genotype for the minor allele were compared with a 2 × 2 contingency table. In the third mode, i.e., the minor allele dominant mode, frequencies of the homozygous genotype for the major allele were compared with a 2 × 2 contingency table. A test of independence was performed using Pearson's χ^2 method. Odds ratio (OR) and 95% confidence interval (CI) were calculated by Woolf's method. The rs7566605 genotype was transformed to a multidichotomous variable, i.e., homozygosity with C alleles versus the other genotypes. The OR adjusted for age and gender was calculated by multiple logistic regression analysis, with genotype, age, and gender as independent variables. Hardy–Weinberg equilibrium was assessed using the χ^2 test (Nielsen et al. 1998). A simple comparison of clinical data among the different genotypes was performed by one-way analysis of variance (ANOVA). Statistical analyses were performed with StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Case–control association study

We successfully genotyped rs7566605 by the TaqMan assay and performed tests of independence between the phenotype and genotype of obesity in severely obese subjects (BMI ≥ 30 kg/m^2) and normal weight controls (BMI < 25 kg/m^2). The minor allele frequency (MAF) of rs7566605 in the control group was 0.31. This was consistent with data obtained from the haplotype map of the human genome (HapMap). As shown in Table 2, rs7566605 demonstrated significant association with the obesity phenotype [recessive mode, $P = 0.00020$, and the OR (95% CI) was 1.62 (1.26–2.10)]. The rs7566605 genotype was transformed to a multidichotomous variable, i.e., CC homozygote versus the other genotypes. Multiple logistic regression analysis was performed, with genotype, age, and gender as independent variables. The *P* values for age, gender, and genotype were 0.21, 0.51, and 0.00030, respectively. OR (95% CI) was 1.61 (1.24–2.09). Our data indicated that rs7566605 in the *INSIG2* gene was associated with severe obesity in Japanese.

A deviation from the Hardy–Weinberg equilibrium was detected in cases ($P = 0.0015$), because this SNP is associated with obesity and cases were selected by phenotype. Cases were selected on the basis of BMI, and the prevalence of subjects with a BMI ≥30 kg/m^2 is only 2–3% in Japan (Yoshiike et al. 2002). Cases may be biased and not representative of the general population. Thus, it is not unexpected that cases were not in accordance with Hardy–Weinberg equilibrium.

Analysis of various quantitative phenotypes with rs7566605

To investigate whether the genotypes of SNP rs7566605 are associated with the phenotypes of metabolic disorders, we compared the following among the different genotypes in cases, controls, and combined groups: ANOVA results; BMI; levels of fasting plasma glucose; hemoglobin A1c (HbA1c); total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol; and blood pressure. Quantitative phenotypes with respect to BMI and levels of fasting plasma glucose; HbA1c; total cholesterol, triglycerides, and HDL cholesterol; and blood pressure were not found to be significantly associated with the rs7566605 genotypes in either cases or controls (Table 3). Systolic and diastolic blood pressures were significantly lower in the GG homozygote in the control group. Blood pressure was higher in GG homozygote in the obese group. Thus, the rs7566605 genotype was not associated with blood pressure.

Table 2 Association of rs7566605 in the *INSIG2* gene with severe obesity

Sample (sample size)	No. of subjects (%)			No. of chromosomes (%)		HWE test[a]	
	CC	CG	GG	C	G	χ^2	*P* value
Case (n = 908)	127 (14)	365 (40)	416 (46)	619 (34)	1,197 (66)	10.1	0.0015
Control (n = 1495)	136 (9)	664 (44)	695 (46)	936 (31)	2,054 (69)	1.6	0.21

Allele frequency mode[b]			Minor allele recessive mode[b]			Minor allele dominant mode[b]		
χ^2	*P* value	OR[c] (95% CI)	χ^2	*P* value	OR[c] (95% CI)	χ^2	*P* value	OR[c] (95% CI)
4.0	0.046	1.13 (1.00–1.28)	13.9	0.00020	1.62 (1.26–2.10)	0.1	0.75	1.03 (0.87–1.21)

[a] Hardy–Weinberg equilibrium test

[b] Association test was performed in three different modes as described in the "Materials and methods", and the results in the three modes are shown

[c] Odds ratio (OR) with 95% confidence interval (CI)

Table 3 Comparison of various quantitative phenotypes among different genotypes at rs7566605 in obese and control subjects

	Obese[a]			Control[a]		
	CC (n = 127)	CG (n = 365)	GG (n = 416)	CC (n = 136)	CG (n = 664)	GG (n = 695)
Age (year)	48.5 ± 15.2	48.0 ± 14.0	50.0 ± 14.2	47.7 ± 17.4	48.1 ± 16.7	48.4 ± 16.4
P value[b]		0.13			0.89	
BMI (kg/m^2)	35.22 ± 6.91	34.28 ± 5.09	34.52 ± 5.21	21.70 ± 2.06	21.68 ± 2.07	21.60 ± 2.10
P value		0.25			0.73	
Glucose (mg/dl)	127.8 ± 44.1	129.3 ± 50.3	129.1 ± 50.8	102.0 ± 40.6	98.0 ± 21.7	97.0 ± 21.9
P value		0.96			0.23	
HbA1c (%)	6.4 ± 1.7	6.5 ± 1.8	6.5 ± 1.8	5.1 ± 0.8	5.1 ± 0.6	5.1 ± 0.6
P value		0.93			0.81	
Total cholesterol (mg/dl)	209.1 ± 39.3	209.3 ± 37.5	210.4 ± 38.1	204.1 ± 35.0	199.4 ± 36.9	202.2 ± 36.3
P value		0.90			0.23	
Triglycerides (mg/dl)	147.1 ± 85.4	160.1 ± 127.3	153.9 ± 102.7	99.6 ± 58.2	103.0 ± 65.5	105.6 ± 82.3
P value		0.51			0.62	
HDL cholesterol (mg/dl)	51.6 ± 14.1	52.7 ± 15.9	53.8 ± 22.2	67.3 ± 14.8	64.9 ± 15.8	65.0 ± 15.6
P value		0.52			0.45	
SBP[c] (mmHg)	134.8 ± 15.3	136.8 ± 18.7	136.6 ± 18.4	122.9 ± 15.8	125.4 ± 17.9	121.9 ± 18.0
P value		0.57			0.0019	
DBP[d] (mmHg)	82.9 ± 11.1	84.6 ± 11.7	83.4 ± 12.4	76.5 ± 11.3	77.0 ± 11.1	75.1 ± 11.1
P value		0.26			0.008	

Data are mean ± standard deviation

BMI body mass index, *HbA1* hemoglobin A1c, *HDL* high-density lipoprotein

[a] Data of each quantitative phenotype were compared among different genotypes at the rs7566605 in obese and control subjects

[b] *P* values were analyzed using analysis of variance in each group of obese and control subjects

[c] Systolic blood pressure

[d] Diastolic blood pressure

Discussion

Recent genome-wide association studies have shown that rs7566605 in the upstream region of the *INSIG2* gene is associated with obesity (Herbert et al. 2006). Associations between rs7566605 and the obesity phenotype have been observed in many Caucasian subjects (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, these associations were controversial with regard to Asian subjects (Yang et al. 2008; Tabara et al. 2008). The association between rs7566605 and BMI may be hard to be replicated in the Asian general population due to the relatively smaller average BMI value and smaller proportion of obesity with BMI >30 kg/m^2 in Asians compared with

those in Caucasians. Indeed, criteria of obesity is BMI >30 kg/m^2 in Caucasians and BMI >25 kg/m^2 in Japanese. We could show the contribution of rs7566605 to obesity in Japanese using severely obese patients with a BMI ≥30 kg/m^2 as cases. SNP rs7566605 could also contribute to the development of severe obesity in Japanese.

Allele frequency was 0.31–0.34 in Japanese, just as observed in European subjects, and the CC genotype was also associated with severe obesity in Japanese, as previously reported (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, CC genotype was not significantly associated with BMI in obese and control groups, although CC homozygotes had higher BMI. Thus, it is possible that our study did not have sufficient power to detect the association between rs7566605 and BMI. The CC genotype would be thrifty variation and have an advantage for survival before modern times. Subjects with CC genotype would be susceptible to obesity in recent years. As a result, the number of CC homozygotes would increase in severely obese group, leading to a deviation from Hardy–Weinberg equilibrium.

Since rs7566605 exists approximately 10 kb upstream from *INSIG2*, SNPs may affect the transcriptional activity of *INSIG2*. INSIG2 is expressed ubiquitously. It was downregulated by insulin in the liver and involved in fatty acid synthesis (Yabe et al. 2003; Takaishi et al. 2004). INSIG2 also mediates feedback control of cholesterol synthesis (Goldstein et al. 2006). Although serum total cholesterol, HDL cholesterol, and triglycerides were not significantly different among genotypes, it is possible that INSIG2 is related to obesity as it affects lipid metabolism.

In summary, our study indicated that rs7566605 in the upstream region of the *INSIG2* gene may influence the risk of severe obesity in Japanese.

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Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons

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Urinary neutrophil gelatinase-associated lipocalin (Ngal or lipocalin 2) is a very early and sensitive biomarker of kidney injury. Here we determined the origin and time course of Ngal appearance in several experimental and clinically relevant renal diseases. Urinary Ngal levels were found to be markedly increased in lipoatrophic- and streptozotocin-induced mouse models of diabetic nephropathy. In the latter mice, the angiotensin receptor blocker candesartan dramatically decreased urinary Ngal excretion. The reabsorption of Ngal by the proximal tubule was severely reduced in streptozotocin-induced diabetic mice, but upregulation of its mRNA and protein in the kidney was negligible, compared to those of control mice, suggesting that increased urinary Ngal was mainly due to impaired renal reabsorption. In the mouse model of unilateral ureteral obstruction, Ngal protein synthesis was dramatically increased in the dilated thick ascending limb of Henle and N was found in the urine present in the swollen pelvis of the ligated kidney. Five patients with nephrotic syndrome or interstitial nephritis had markedly elevated urinary Ngal levels at presentation, but these decreased in response to treatment. Our study shows that the urinary Ngal level may be useful for monitoring the status and treatment of diverse renal diseases reflecting defects in glomerular filtration barrier, proximal tubule reabsorption, and distal nephrons.

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Neutrophil gelatinase-associated lipocalin (Ngal) is a differentiation inducer for epithelia in embryonic kidney, whose expression is dramatically increased in acute kidney injury (AKI).[1–5] Ngal exerts a spectrum of iron-dependent biological activities,[1–4,6] and administration of Ngal protein mitigates renal injury in mice, suggesting that functional consequence of Ngal upregulation is renoprotection.[2] Ngal mRNA levels in the kidney are increased as much as by 1000-fold during renal ischemia–reperfusion injury in mice.[2,7] Ngal protein starts to accumulate within a few hours in the blood and urine during AKI.[8–11] These characteristics of Ngal have made it a promising biomarker of AKI that is found in the blood and urine.[1,7,12–15] Furthermore, several studies reported that serum and urinary Ngal levels are elevated proportionally to the extent of renal damage in chronic kidney disease,[16,17] but the source and the time course of urinary Ngal concentrations are largely unknown. In this study, we investigated urinary Ngal levels in four types of renal damage caused by distinct mechanism: nephrotic syndrome caused by glomerular disorders, diabetic nephropathy, obstructive nephropathy, and interstitial nephritis. We also examined whether measurement of urinary Ngal is useful for the monitoring of renal damage in mice or humans during the treatment course.

RESULTS

Urinary Ngal excretion is proportional to albumin excretion in mouse models of diabetic nephropathy

As a model of diabetic nephropathy, we first examined urinary Ngal concentrations in A-ZIP/F-1 transgenic mice, which are characterized with lipoatrophic diabetes, fatty liver, hyperlipidemia, severe insulin resistance, and massive proteinuria.[18–20] Urinary Ngal excretion in A-ZIP/F-1 mice at 10 months of age was much larger than that in control FVB/N mice (Figure 1). By Western blot, we observed 30 and 25 kDa bands with Ngal immunoreactivity, and the larger band was found only in the urine from A-ZIP/F-1 mice and

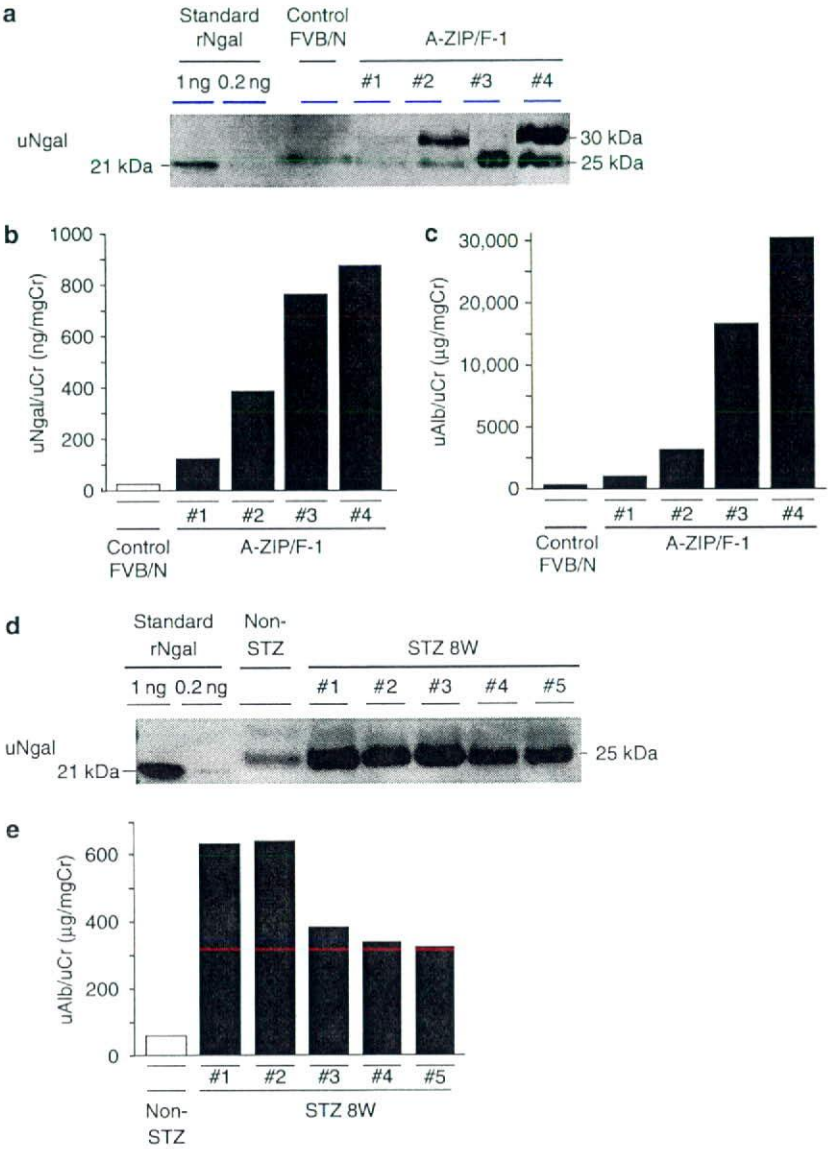

Figure 1 | Urinary Ngal and albumin excretion in two models of diabetic nephropathy. (**a**–**c**) A-ZIP/F-1 diabetic mice at 10 months of age and (**d**, **e**) diabetic mice at 8 weeks after streptozotocin (STZ) injection. (**a**, **d**) Western blot of urine (25 μl each) in individual mice and (**b**, **c**, **e**) urinary levels of Ngal and albumin (Alb) normalized by creatinine (Cr) are shown. (**b**) A-ZIP/F-1 mice, including no. 1, excreted much larger volumes of urine than control FVB/N mice. In three control FVB/N mice, mean urinary Ngal/Cr ratio (± s.e.) was 42 ± 24 ng per mgCr. uNgal, urinary Ngal; rNgal, recombinant Ngal.

not in the urine or tissues from normal, diabetic, or obstructed kidneys of mice with C57BL/6 background (see below). The larger protein may have heavier glycosylation than the smaller protein.[21] When the amounts of two proteins were added, mice with larger urinary Ngal levels tended to have larger urinary albumin levels.

Next, we studied streptozotocin (STZ)-induced diabetes, which manifests with insulin deficiency and microalbuminuria. In STZ mice, urinary albumin excretion increased gradually and, after 8 weeks, reached 7.8-fold of the level before STZ injection (Figure 2). On the other hand, urinary Ngal levels were elevated by 77-fold at 8 weeks. The extent of Ngal and albumin excretion was highly variable among different mice, but urinary albumin levels and log transformation of Ngal levels showed a close linear correlation throughout the course of 12-week observation period.

Elevation of urinary Ngal excretion in STZ mice is not caused by renal synthesis but by reabsorption defect, and treatment with angiotensin receptor blocker reduces urinary Ngal levels

To examine whether local expression of Ngal is increased in STZ mouse kidneys, we studied expression levels of Ngal protein in the whole kidney preparation of STZ and non-STZ control mice and found no significant difference at 8 weeks

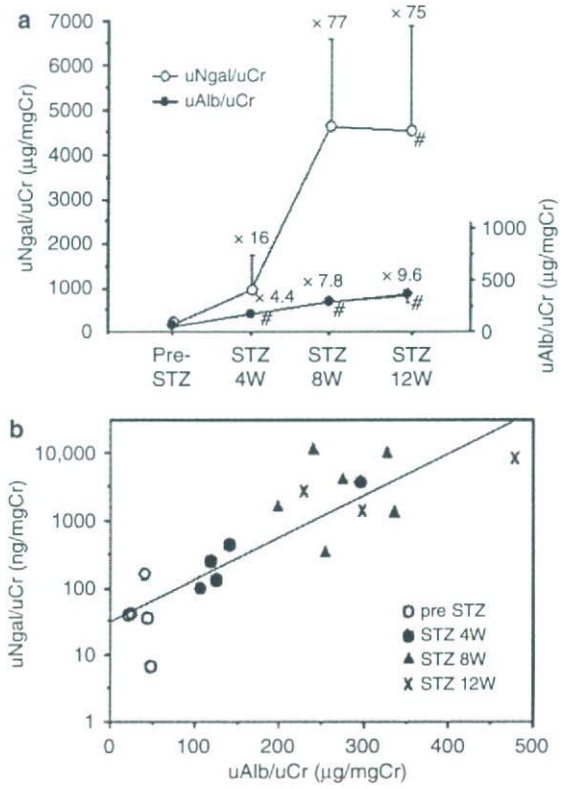

Figure 2 | Time course and correlation of urinary Ngal and albumin excretion in streptozotocin (STZ)-induced diabetic mice. (**a**) Urinary Ngal (uNgal) and albumin (uAlb) levels normalized by urinary creatinine (uCr) were examined before and at 4, 8, and 12 weeks after STZ injection (mean ± s.e.). #, $P<0.05$ versus pre-STZ. Elevation of urinary Ngal levels was significant at 4, 8, and 12 weeks if analyzed after log transformation. (**b**) Correlation between uNgal/uCr and uAlb/uCr; $r=0.86$, $P<0.001$, $n=19$.

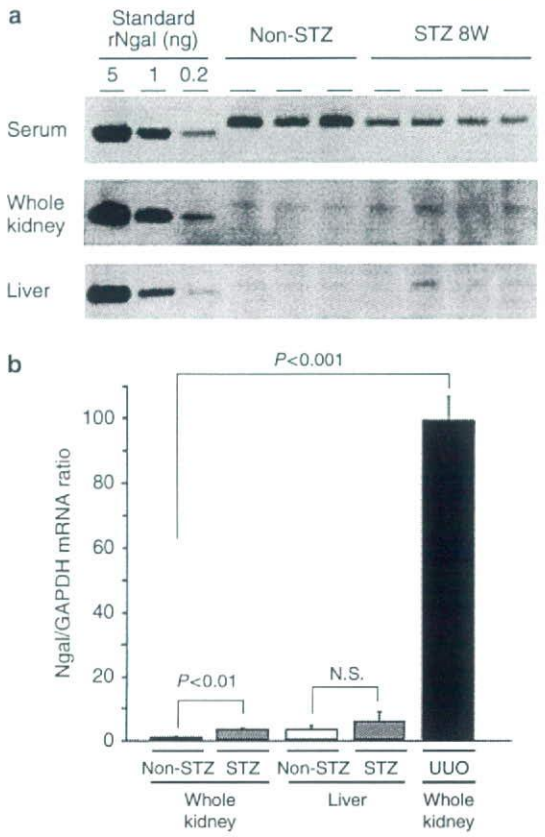

Figure 3 | Ngal protein and mRNA expression in the serum, kidney, and liver of STZ mice. (**a**) Western blot of serum, whole kidney, and liver at 8 weeks after induction of diabetes. Mice without STZ injection served as control (non-STZ). Equal amounts of serum (15 μl) and protein (30 μg) of whole kidney and liver were separated by electrophoresis; rNgal, recombinant Ngal. (**b**) Ngal mRNA expression levels were measured using real-time PCR and normalized by GAPDH expression ($n=4$). The mean Ngal/GAPDH level in non-STZ whole kidney was arbitrarily defined as 1.0. The whole kidneys at 1 day after unilateral ureteral obstruction (UUO) were also examined as a positive control. NS, not significant.

after STZ treatment (Figure 3). We did not find significant alteration of Ngal protein expression in the livers, either. Of note, serum Ngal levels in STZ mice were significantly lower than those in non-STZ mice (23 ± 5 versus 111 ± 22 ng/ml, $n=3$–4, $P<0.01$). We measured Ngal mRNA expression levels in the kidneys and livers of STZ mice, but they were increased only marginally compared to control mice. As a positive control, Ngal mRNA expression was increased by 100-fold in obstructed kidneys after 1 day of ureter ligation ($P<0.001$). These findings suggested that dramatic (nearly 80-fold) increase of urinary Ngal excretion in STZ mice cannot be explained by augmentation of Ngal protein synthesis in the kidney, and led us to investigate tubular reabsorption of Ngal. Injection of histidine-tagged or Alexa Fluor 546-labeled Ngal (21 kDa in size) in the peritoneum of non-STZ mice resulted in glomerular filtration and efficient reabsorption of Ngal at the proximal tubules from the apical side, thus no exogenous Ngal was detected in the urine (Figure 4). In STZ mice, on the other hand, substantial amount of exogenous Ngal was excreted in the urine and reabsorption of labeled Ngal was reduced by 47% ($P<0.01$).

As treatment of diabetic nephropathy with angiotensin receptor blocker (ARB) reduces proteinuria and ameliorates renal injury,[22] we gave the ARB candesartan to STZ mice through drinking water at 10 mg/kg/day (Figure 5). After 1 week, urinary albumin levels were decreased by 14% ($P<0.05$) and urinary Ngal levels were decreased by 77% ($P<0.05$). Serum Ngal levels were not altered by candesartan (20 ± 6 ng/ml). The dose of candesartan used was a subdepressor dose, and did not significantly affect body weights, blood glucose, urea nitrogen and creatinine levels (Table 1). Through these findings, we conclude that increased urinary Ngal excretion in STZ mice was caused mainly by reabsorption defect and treatment with candesartan partially normalized urinary Ngal levels.