Inhibition of TRPC6 Channel Activity Contributes to the Antihypertrophic Effects of Natriuretic Peptides-Guanylyl Cyclase-A Signaling in the Heart

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Rationale: Atrial and brain natriuretic peptides (ANP and BNP, respectively) exert antihypertrophic effects in the heart via their common receptor, guanylyl cyclase (GC)-A, which catalyzes the synthesis of cGMP, leading to activation of protein kinase (PK)G. Still, much of the network of molecular mediators via which ANP/BNP-GC-A signaling inhibit cardiac hypertrophy remains to be characterized.

Objective: We investigated the effect of ANP-GC-A signaling on transient receptor potential subfamily C (TRPC)6, a receptor-operated Ca^{2+} channel known to positively regulate prohypertrophic calcineurin–nuclear factor of activated T cells (NFAT) signaling.

Methods and Results: In cardiac myocytes, ANP induced phosphorylation of TRPC6 at threonine 69, the PKG phosphorylation site, and significantly inhibited agonist-evoked NFAT activation and Ca^{2+} influx, whereas in HEK293 cells, it dramatically inhibited agonist-evoked TRPC6 channel activity. These inhibitory effects of ANP were abolished in the presence of specific PKG inhibitors or by substituting an alanine for threonine 69 in TRPC6. In model mice lacking GC-A, the calcineurin-NFAT pathway is constitutively activated, and BTP2, a selective TRPC channel blocker, significantly attenuated the cardiac hypertrophy otherwise seen. Conversely, overexpression of TRPC6 in mice lacking GC-A exacerbated cardiac hypertrophy. BTP2 also significantly inhibited angiotensin II–induced cardiac hypertrophy in mice.

Conclusions: Collectively, these findings suggest that TRPC6 is a critical target of antihypertrophic effects elicited via the cardiac ANP/BNP-GC-A pathway and suggest TRPC6 blockade could be an effective therapeutic strategy for preventing pathological cardiac remodeling. (*Circ Res.* 2010;106:1849-1860.)

Key Words: natriuretic peptides ■ calcium ■ ion channels ■ hypertrophy

In response to pathological stimuli such as prolonged mechanical stress, massive tissue injury, or abnormal neurohumoral activation, hearts show hypertrophic growth and remodeling, which is characterized by an increase in myocyte cell size, assembly of sarcomere proteins, interstitial fibrosis, and reexpression of fetal cardiac genes. Although the hypertrophic response is initially compensatory, it ultimately causes heart failure, which is now a leading cause of morbidity and mortality around the world. Diverse intracellular signaling pathways exerting pro- or antihypertrophic effects have been shown to play important roles in the complex processes of cardiac remodeling,[1,2] but the details of the molecular mechanisms mediating the crosstalk among these signaling pathways remain uncertain. Unraveling those details should give us a better understanding of the molecular processes underlying the establishment of cardiac hypertro-

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Non-standard Abbreviations and Acronyms	
Ang II	angiotensin II
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
GC-A	guanylyl cyclase-A
HW/BW	heart weight/body weight
KO	knockout
LTCC	L-type voltage-dependent Ca^{2+} channel
NFAT	nuclear factor of activated T cells
PKG	protein kinase G
RCAN	regulator of calcineurin
RGS	regulator of G-protein signaling
siRNA	small interfering RNA
TRPC	transient receptor potential subfamily C
WT	wild type

phy and heart failure, which could ultimately lead to the discovery of novel therapeutic targets for prevention of pathological cardiac remodeling.

The heart regulates cardiovascular homeostasis in part by secreting 2 peptide mediators, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP).[3,4] ANP and BNP bind to their common receptor, guanylyl cyclase (GC)-A (also called NPR-A and NPR1), which then catalyzes the synthesis of cGMP, leading to the activation of protein kinase (PK)G.[5,6] Under pathological conditions in the heart, there is a significant increase in the ventricular expression of both ANP and BNP,[4,7,8] which then act as both endocrine and local antihypertrophic factors.[9] Indeed, ANP/BNP have been shown to exert antihypertrophic effects on cardiac myocytes in vitro and in vivo[10–14] by counteracting multiple prohypertrophic signaling pathways, including the MEK1–ERK1/2 (mitogen-associated/extracellular regulated kinase 1–extracellular regulated kinase 1/2) pathway, the Ca^{2+}/calmodulin-dependent kinase II pathway, the Akt pathway, and the calcineurin-nuclear factor of activated T cell (NFAT) pathway.[1,2,15–17] ANP/BNP reportedly antagonize prohypertrophic signaling by inhibiting receptor-mediated Ca^{2+} influx into cells through activation of regulator of G-protein signaling (RGS) proteins and inhibition of L-type voltage-dependent Ca^{2+} channels (LTCCs) and the Na^+/H^+ exchanger.[10,17–20] Still, much of the network of molecular mediators via which ANP/BNP inhibit cardiac hypertrophy remains to be characterized.

The serine-threonine phosphatase calcineurin functions as a Ca^{2+}-dependent regulator of cardiac hypertrophy and the fetal gene program.[21] Calcineurin dephosphorylates NFAT family transcription factors and induces their translocation to the nucleus, where they bind to the regulatory regions of cardiac genes in conjunction with other cardiac transcription factors and promote hypertrophic growth.[21] Transient receptor potential subfamily C (TRPC)3 and -6 reportedly serve as positive upstream regulators of the calcineurin-NFAT signaling pathway.[22–24] TRPC3 and 6 form homo- and heteromultimeric cation channels that are activated directly by diacylglycerol[25,26] and function to couple receptor-phospholipase C activity to Ca^{2+} influx, which in turn activates calcineurin-NFAT signaling pathways and possibly other Ca^{2+}-dependent signaling pathways.[26] NFAT also activates TRPC6 gene transcription, thereby accelerating the calcineurin-NFAT prohypertrophic signaling loop.[23] It was recently shown that TRPC3 and 6 activities are greatly attenuated by PKG-catalyzed phosphorylation of Thr11 and Ser263 in TRPC3 and Thr69 in TRPC6, which are well conserved among mouse, rat and human.[27,28]

In the present study, we examined the functional crosstalk between the ANP/BNP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways during the process of cardiac hypertrophy and characterized its biological significance in cardiac pathophysiology. Our findings demonstrate that TRPC6 is a direct target of ANP/BNP-GC-A-cGMP-PKG antihypertrophic signaling and suggest that inhibition of TRPC6 could represent a novel therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

Methods

Plasmid Construction

Regulator of calcineurin (RCAN)1-luciferase (RCAN1-luc), in which RCAN1 intron 3 containing 15 NFAT sites was inserted upstream of the luciferase gene, was kindly provided by B. A. Rothermel (University of Texas, Southwestern Medical Center, Dallas).[29] Expression vectors encoding wild-type (WT) and mutant (T69A) mouse TRPC6 were described previously.[28]

Cell Culture

Primary neonatal rat ventricular myocytes were isolated and grown as described previously.[30]

Patch Clamp Studies

The details of the patch clamp recording and data analysis were essentially the same as described previously.[31]

Animal Experiments

The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine. Beginning at 12 weeks of age, GC-A knockout (KO) mice (C57BL/6 background) were left untreated (control) or were treated for 4 weeks with BTP2 (20 mg/kg per day PO) or nitrendipine (40 mg/kg per day PO). BTP2 was dissolved in methylcellulose (Shin-Etsu Chemical) to a concentration of 3.0 mg/mL and was given daily via gastric gavage adjusted to the individual body weight of each mouse. The same amount of 0.5% methylcellulose was given to the other treatment groups in the same manner. Nitrendipine was given as described previously.[32]

Echocardiographic Analysis

Echocardiography was carried out as described previously[33,34] using a Toshiba Power Vision 8000 echocardiography system equipped with a 12-MHz imaging transducer.

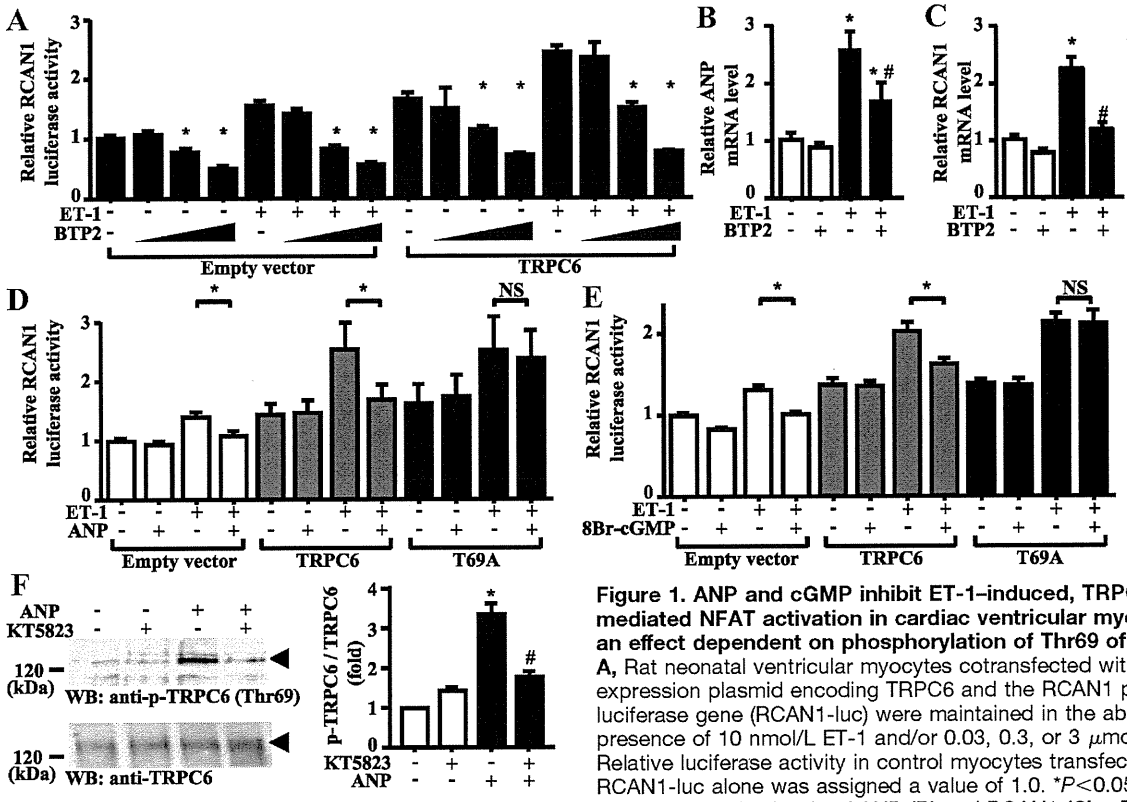

Figure 1. ANP and cGMP inhibit ET-1–induced, TRPC6-mediated NFAT activation in cardiac ventricular myocytes, an effect dependent on phosphorylation of Thr69 of TRPC6. **A,** Rat neonatal ventricular myocytes cotransfected with an expression plasmid encoding TRPC6 and the RCAN1 promoter-luciferase gene (RCAN1-luc) were maintained in the absence or presence of 10 nmol/L ET-1 and/or 0.03, 0.3, or 3 μmol/L BTP2. Relative luciferase activity in control myocytes transfected with RCAN1-luc alone was assigned a value of 1.0. *P<0.05 vs cells treated without BTP2 in each group. **B and C,** Real-time RT-PCR analysis of the relative levels of ANP **(B)** and RCAN1 **(C)** mRNA in cultured ventricular myocytes treated with or without 10 nmol/L ET-1 and/or 3 μmol/L BTP2. The relative mRNA level in control myocytes was assigned a value of 1.0. *P<0.05 vs control; #P<0.05 vs myocytes treated with ET-1 alone. **D and E,** Ventricular myocytes cotransfected with an expression plasmid encoding WT TRPC6 or a TRPC6 T69A mutant and RCAN1-luc were maintained in the absence or presence of 10 nmol/L ET-1 and/or 100 nmol/L ANP **(D)** or 100 μmol/L 8Br-cGMP **(E).** Relative luciferase activity in control myocytes transfected with RCAN1-luc alone was assigned a value of 1.0. *P<0.05; NS: P=NS. In all graphs **(A through E),** values are shown as means±SEM. **F,** Representative Western blots of Thr69-phosphorylated TRPC6 **(top)** and total TRPC6 **(bottom)** in cardiac myocytes treated for 1 hour with or without 100 nmol/L ANP and/or 1 μmol/L KT5823 are shown at **left.** Graphs at the **right** show the relative levels of Thr69-phosphorylated TRPC6 in cardiac myocytes treated for 1 hour with or without 100 nmol/L ANP and/or 1 μmol/L KT5823. The relative level of Thr69-phosphorylated TRPC6 in control cardiac myocytes was assigned a value of 1.0. Values are shown as means±SEM (n=3 each). *P<0.001 vs control myocytes; #P<0.05 vs myocytes with ANP alone.

Statistical Analysis

Data are presented as means±SEM. Unpaired t tests were used for comparisons between 2 groups, and ANOVA with post hoc Fisher tests was used for comparisons among groups. Values of P<0.05 were considered significant.

Results

Signaling via the ANP-cGMP-PKG Pathway Inhibits Endothelin-1–Induced, TRPC6-Mediated Activation of Calcineurin-NFAT Signaling in Cardiac Myocytes

In previous reports, TRPC family ion channels (TRPC3 and TRPC6) were shown to play a central role in activating calcineurin-NFAT signaling in the ventricular myocardium during the process of pathological cardiac hypertrophy.[22–24,35] Consistent with those reports, we found that in cultured neonatal rat ventricular myocytes, BTP2, a selective TRPC inhibitor,[36–40] significantly and dose-dependently inhibited endothelin (ET)-1-induced activation of the NFAT-dependent *RCAN1* promoter, with and without overexpression of TRPC6 (Figure 1A). BTP2 also significantly attenuated ET-1–induced hypertrophic responses, including increased expression of *ANP*, *RCAN1* and *TRPC6* mRNA and enlargement of cultured cardiac myocytes (Figure 1B and 1C; and Online Figure I, A through C, in the Online Data Supplement, available at http://circres.ahajournals.org). To determine whether ANP inhibits calcineurin-NFAT signaling through TRPC6 inhibition, we examined the effect of ANP on ET-1–induced, TRPC6-mediated activation of calcineurin-NFAT signaling in cultured ventricular myocytes transfected with a *RCAN1* promoter-reporter gene and/or an expression vector for TRPC6. Even without overexpression of TRPC6, ET-1 induced *RCAN1* promoter activity in ventricular myocytes, and this activity was significantly inhibited by ANP (Figure 1D). With overexpression of TRPC6, basal *RCAN1* promoter activity was higher than without it, and ET-1 increased the promoter's activity still further (Figure 1D). ANP then significantly inhibited the ET-1–induced, TRPC6-enhanced *RCAN1* promoter activity. Moreover, when cells were transfected with a TRPC6 T69A mutant, in which the PKG-phosphorylation site, Thr69, was substituted with an Ala, the basal and ET-1–induced activities of the *RCAN1* promoter were comparable to those obtained with overexpression of WT TRPC6, but the inhibitory effects of ANP were almost completely abolished (Figure 1D). 8Br-cGMP, a membrane-permeant cGMP analogue, also significantly in-

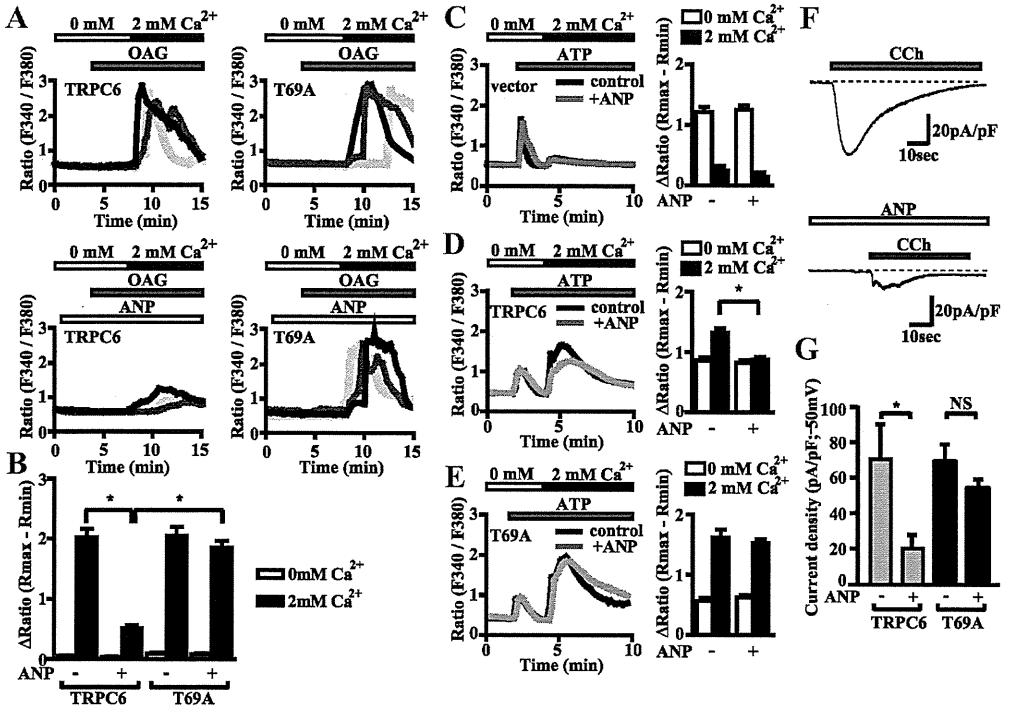

Figure 2. ANP inhibits agonist-evoked Ca^{2+} influx through phosphorylation of Thr69 in TRPC6. A, Three different representative time courses of OAG-induced Ca^{2+} influx via TRPC6 **(left graphs)** or the TRPC6 T69A mutant **(right graphs)** in HEK293 cells treated without **(top graphs)** or with **(bottom graphs)** 100 nmol/L ANP. **B,** OAG-evoked Ca^{2+} influx via TRPC6 in HEK293 cells treated with or without ANP in Ca^{2+}-free **(white bar)** and Ca^{2+}-containing (2 mmol/L) external solution **(black bar).** $^*P<0.05$. **C,** Representative time courses of ATP-evoked Ca^{2+} influx in control HEK293 cells treated without **(black line)** or with **(gray line)** ANP. Graphs at **right** show ATP-induced Ca^{2+} influx in control HEK293 cells treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing (2 mmol/L) external solution **(black bar). D and E,** Representative time courses of ATP-induced Ca^{2+} influx via TRPC6 or the TRPC6 T69A mutant in HEK293 cells treated without **(black line)** or with **(gray line)** ANP. Graphs at **right** show ATP-induced Ca^{2+} influx via TRPC6 or the TRPC6 T69A mutant in HEK293 cells treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing (2 mmol/L) external solution **(black bar).** $^*P<0.05$. In all graphs, values are shown as means±SEM. **F,** Inward cation currents (I_{TRPC6}) activated by the muscarinic receptor agonist carbachol (CCh; 100 μmol/L) in the absence **(top)** and presence **(bottom)** of 100 nmol/L ANP. Murine TRPC6-expressing HEK293 cells were voltage-clamped at a holding potential of -60 mV. Bath: normal PSS; pipette: Cs-aspartate internal solution. ANP was added to the bath 10 minutes before application of CCh. **G,** Current density of CCh-induced I_{TRPC6} in HEK 293 cells expressing WT or mutant (T69A) TRPC6, with or without ANP; n=12 in TRPC6 without ANP, 7 in TRPC6 with ANP, 7 in T69A without ANP, 5 in T69A with ANP. Values are shown as means±SEM. $^*P<0.05$ vs cells without ANP. NS: P=NS.

hibited ET-1–induced RCAN1 promoter activation in cardiac myocytes cotransfected with WT TRPC6 or not, but it failed to inhibit the response in myocytes cotransfected with the TRPC6 T69A mutant (Figure 1E). This suggests that signaling in the ANP-GC-A-cGMP-PKG pathway leads to phosphorylation of TRPC6 on Thr69, which inhibits receptor-mediated TRPC6 channel activation and, in turn, activation of calcineurin-NFAT signaling in cardiac myocytes. Indeed, using a specific antibody against phosphorylated Thr69, we confirmed that ANP stimulates phosphorylation of TRPC6 Thr69 in cardiac myocytes, and that the phosphorylation was blocked in the presence of the selective PKG inhibitor KT5823 (Figure 1F).

ANP-cGMP-PKG Signaling Inhibits TRPC6 Ion Channel Activity

We next examined the effects of ANP-cGMP-PKG signaling on TRPC6 ion channel activity. In HEK293 cells expressing WT TRPC6, the membrane-permeant diacylglycerol analogue oleoyl-2-acetyl-*sn*-glycerol (OAG) induced a significant increase in Ca^{2+} influx (Figure 2A, top left) that was dramatically inhibited by prior application of ANP (Figure 2A, bottom left). By contrast, in HEK293 cells expressing the TRPC6 T69A mutant, OAG-induced Ca^{2+} influx was unaffected by ANP, indicating that ANP directly inhibits OAG-induced TRPC6 channel activity through the PKG-phosphorylation site (Figure 2A, right graphs, and 2B).

To evaluate the effect of ANP on receptor-mediated activation of TRPC6, we next stimulated HEK293 cells using ATP and examined the effect of ANP on ATP-induced TRPC6 activation. In control cells, which did not express TRPC6, ATP did not induce sustained Ca^{2+} influx, and ANP had no effect (Figure 2C). In cells expressing WT TRPC6, by contrast, ATP induced sustained Ca^{2+} influx, which was significantly inhibited by ANP (Figure 2D). Moreover, mutation of the PKG-phosphorylation site (T69A) in TRPC6 abolished the ANP-induced inhibition of ATP-evoked Ca^{2+} influx (Figure 2E). This means that ANP acts in a PKG-dependent manner to inhibit TRPC6 activation via Gq-coupled receptors. RGS2 and RGS4 are reportedly involved in the cGMP-mediated inhibition of Gq-coupled receptor signaling pathways, including the calcineurin-NFAT pathway.[20,41,42] Because RGS2 and 4 are known to block activa-

tion of Gq upstream of TRPC6, we assessed the endogenous expression of *RGS2* and *RGS4* in HEK293 cells and confirmed expression of both *RGS2* and *RGS4* mRNA (Online Figure I, D). Thus our finding that ATP-induced activation of the PKG-resistant TRPC6 T69A mutant in the presence of ANP was similar to seen with WT TRPC6 in the absence of ANP, despite endogenous expression of RGS2 and 4, indicates the existence of an RGS2/4-independent pathway via which ANP inhibits Gq-mediated TRPC6 activation (Figure 2D and 2E).

We also measured cationic currents induced by carbachol in HEK293 cells expressing TRPC6 (Figure 2F) and found that they were significantly inhibited in the presence of ANP (Figure 2F and 2G). Furthermore, the inhibitory effect of ANP was substantially blunted in cells expressing the TRPC6 T69A mutant, again confirming that ANP inhibits Gq-coupled receptor-mediated TRPC6 activation directly through the PKG-phosphorylation site (Figure 2G). Likewise cationic currents carried by TRPC6 channels activated by GTPγs were also inhibited by ANP (Online Figure I, E), and this inhibitory effect was significantly blunted in the presence of DT-3, a selective PKG Iα inhibitor, or by mutation (T69A) of TRPC6 (Online Figure I, F). All of these data indicate that signaling via the ANP-cGMP-PKG pathway inhibits TRPC6 ion channel activity through direct phosphorylation of TRPC6.

ANP Inhibits Agonist-Induced Ca^{2+} Influx Into Ventricular Myocytes

We next examined the inhibitory effect of ANP on Ca^{2+} signaling in cardiac myocytes. When we used Fura-2 to measure the frequency of Ca^{2+} oscillations induced by ET-1 or angiotensin II (Ang II) in cultured neonatal ventricular myocytes, we found that both increased the frequency of Ca^{2+} oscillation in the cells, that this effect was significantly inhibited by ANP (Figure 3A and 3B), and that the inhibitory effect of ANP was almost completely abolished in the presence of KT5823 (Figure 3C and 3D). ET-1– or Ang II–induced increases in Ca^{2+} oscillation were also significantly inhibited when TRPC3 and 6 were simultaneously knocked down using small interfering (si)RNAs (Figure 3E and 3F, and Online Figure II, A, D, F, and I).[24] Moreover, knocking down either TRPC3 or 6 had a similar effect (Online Figure II, A, B, C, E, F, G, H, and J). This suggests that TRPC3 and 6 act in concert to mediate ANP-sensitive, ET-1– or Ang II–induced increases in Ca^{2+} oscillation in cardiac myocytes. ET-1 and Ang II also induced Ca^{2+} influx into cardiac myocytes that was significantly inhibited by ANP (Figure 4A and 4B). We previously showed that knocking down either TRPC3 or 6 significantly reduced Ang II–induced Ca^{2+} influx into cardiac myocytes.[24] In the present study, we found that knocking down TRPC3 and 6 significantly inhibited ET-1– and Ang II–induced Ca^{2+} influx and abolished the inhibitory effect exerted by ANP on this Ca^{2+} influx, which suggests ANP inhibits TRPC3/6-mediated Ca^{2+} influx into cardiac myocytes (Figure 4C and 4D; Online Figure III, A through D).

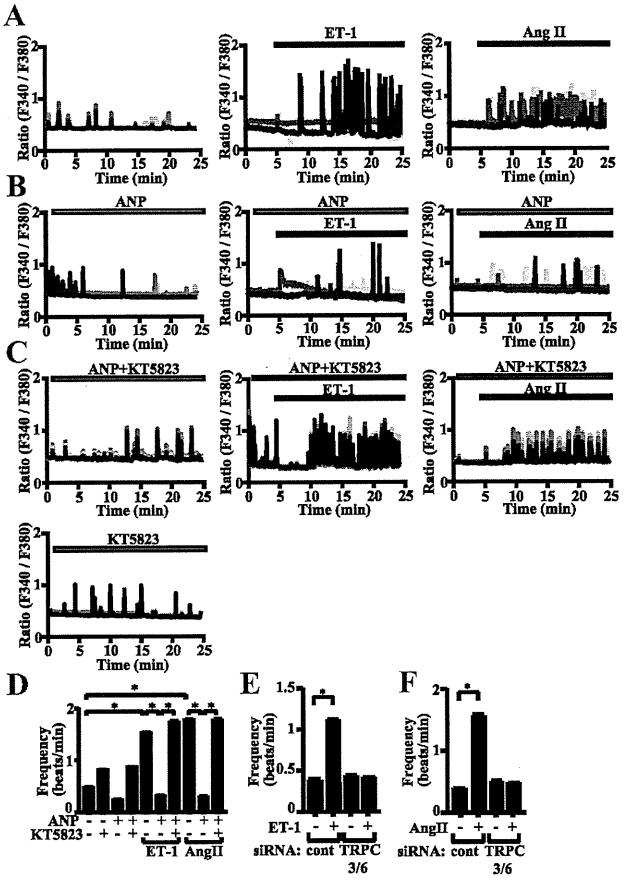

Figure 3. ANP inhibits agonist-induced increases in Ca^{2+} oscillation in cardiac ventricular myocytes. A through C, Three representative traces showing Ca^{2+} oscillations in cultured cardiac myocytes treated with vehicle, ET-1 or Ang II in the absence **(A)** or presence of ANP **(B)** or ANP+KT5823 **(C)**. **D,** Graph shows the frequencies of the Ca^{2+} oscillations under the above conditions. Values are shown as means±SEM. *$P<0.05$. **E and F,** Effects of double knockdown of TRPC3 and 6 on the frequencies of Ca^{2+} oscillations in cultured cardiac myocytes treated with ET-1 (E) or Ang II (F). KT5823: a selective PKG inhibitor (200 nmol/L). Values are shown as means±SEM. *$P<0.05$.

To further determine whether TRPC channels or LTCCs were responsible for the ANP-induced inhibition of agonist-induced Ca^{2+} influx in cultured ventricular myocytes, we tested the effects of nitrendipine, a selective LTCC inhibitor, and BTP2 on the Ca^{2+} influx. Although nitrendipine reduced the overall Ca^{2+} influx induced by ET-1 or Ang II, the inhibitory effect of ANP was preserved (Figure 5A and 5B). By contrast, ANP-induced inhibition of agonist-evoked Ca^{2+} influx was almost completely blocked in the presence of BTP2 (Figure 5C). The dose of BTP2 we used in this study did not affect KCl-induced, nitrendipine-sensitive Ca^{2+} influx (Figure 5D). These findings further confirm that inhibition of TRPCs, not LTCCs, is a critical component of the inhibitory effect of ANP on ET-1 and Ang II–induced Ca^{2+} influx into cultured ventricular myocytes.

We also assessed the contribution made by RGS2 and RGS4 to ANP-induced inhibition of Gq-coupled receptor-mediated Ca^{2+} influx into cardiac myocytes by using siRNAs

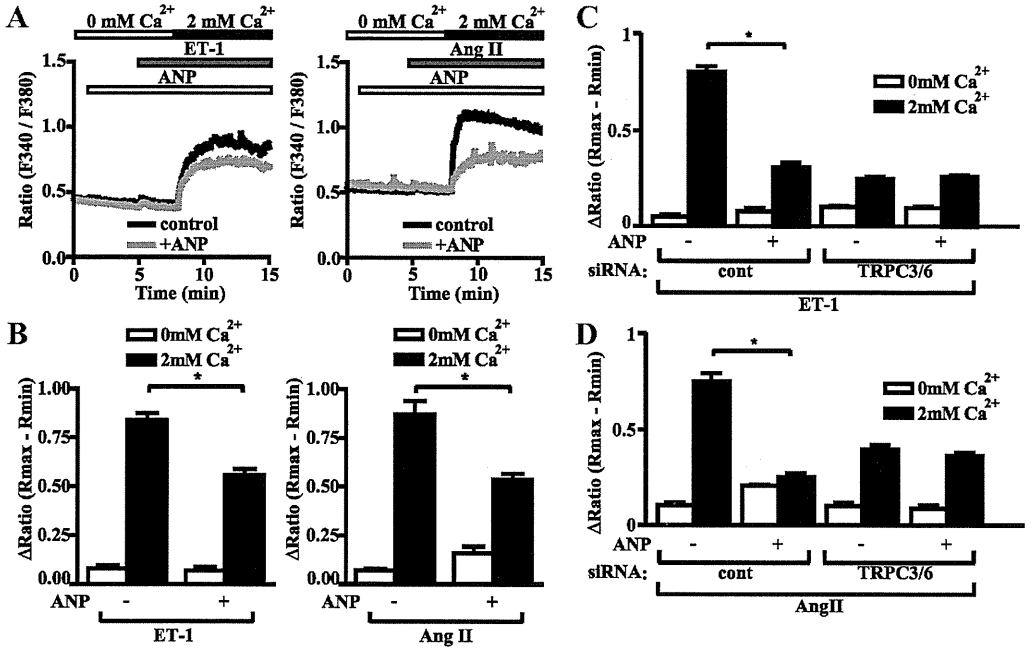

Figure 4. ANP inhibits agonist-evoked Ca^{2+} influx into cardiac ventricular myocytes. A, Representative time courses of ET-1– or Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with **(gray line)** or without **(black line)** ANP. **B,** ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes cells treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar).** Values are shown as means±SEM. *$P<0.05$. **C and D,** Effects of double knockdown of TRPC3 and 6 on Ca^{2+} influx in cultured cardiac myocytes treated with ET-1 **(C)** or Ang II **(D)** in the presence or absence of ANP. In all of these experiments, 10 nmol/L ET-1, 100 nmol/L Ang II, 100 nmol/L ANP, and 2 mmol/L external Ca^{2+} were used.

to simultaneously knock down their expression. We initially confirmed that the *RGS2* and *RGS4* siRNAs efficiently and specifically knocked down *RGS2* and *RGS4* mRNA levels to 18% and 27%, respectively, of those seen with control siRNA in ventricular myocytes (Figure 5E). In ventricular myocytes cotransfected with *RGS2* and *RGS4* siRNAs, ANP still significantly inhibited ET-1– and Ang II–induced Ca^{2+} influx, but this inhibitory effect was significantly attenuated (Figure 5F and 5G; Online Figure IV, A and B). In addition, type1a Ang II receptor densities and the expression levels of mRNAs and proteins related to Gq-coupled receptor-mediated Ca^{2+} influx were not significantly altered (Online Figure IV, C through E). Collectively then, these results support our notion that ANP inhibits ET-1– and Ang II–induced Ca^{2+} influx into cardiac myocytes in a manner that is, at least in part, TRPC-dependent.

TRPC Channels Play a Pivotal Role in Cardiac Hypertrophy in GC-A KO Mice

ANP increases intracellular cGMP via its receptor, GC-A, a particulate type of guanylyl cyclase. GC-A KO mice, which lack GC-A, exhibit reduced plasma cGMP levels, salt-resistant hypertension and cardiac hypertrophy.[43–45] Activation of calcineurin-NFAT signaling is reportedly involved in the development of the cardiac hypertrophy seen in GC-A KO mice,[2,15] and because TRPC6 forms a positive regulatory circuit with the calcineurin-NFAT pathway,[23] we examined whether TRPC6 gene expression is induced in the ventricles of GC-A KO mice. Real-time RT-PCR analysis clearly showed a significant increase in the expression of TRPC6, ANP, BNP and TRPC3 mRNA in GC-A KO ventricles (Figure 6A), which is consistent with the notion that GC-A negatively regulates calcineurin-NFAT prohypertrophic signaling. The levels of TRPC3 and 6 protein were also significantly higher in GC-A KO ventricles than WT ventricles (Online Figure V, A and B). To evaluate the contribution made by TRPC6 and 3 to the development of cardiac hypertrophy, we treated GC-A KO mice with BTP2. Although BTP2 did not affect blood pressure or heart rate in GC-A KO mice (Figure 6B), it significantly reduced cardiac hypertrophy assessed based on heart weight (HW), heart weight/body weight (HW/BW) ratios, and myocardial cell diameters measured in histological samples (Figure 6C and 6D). By contrast, nitrendipine did not reduce cardiac hypertrophy in GC-A KO mice, though it modestly reduced blood pressures (WT without nitrendipine, 94.5±0.5 mm Hg; WT with nitrendipine, 89.7±3 mm Hg; GC-A KO without nitrendipine, 119.8±2.1 mm Hg; GC-A KO with nitrendipine, 116.2±1.5 mm Hg; Online Figure V, C through E; Online Table I). BTP2 also reduced cardiac fibrosis in GC-A KO mice (Figure 6E). Consistent with these findings, echocardiographic analysis showed that BTP2 reduced posterior wall thickness and restored left ventricular end-diastolic dimension in *GC-A* KO ventricles without affecting % fractional shortening (Figure 6F). In addition, the increased ventricular expression of *RCAN1* and such hypertrophy marker genes as *ANP*, *BNP*, β-myosin heavy chain (*βMHC*), and skeletal α-actin in *GC-A* KO ventricles was dramatically attenuated by BTP-2 (Figure 7A), though expression of α-myosin heavy chain (*αMHC*) and cardiac α-actin was not (Figure 7A). Likewise, the increased

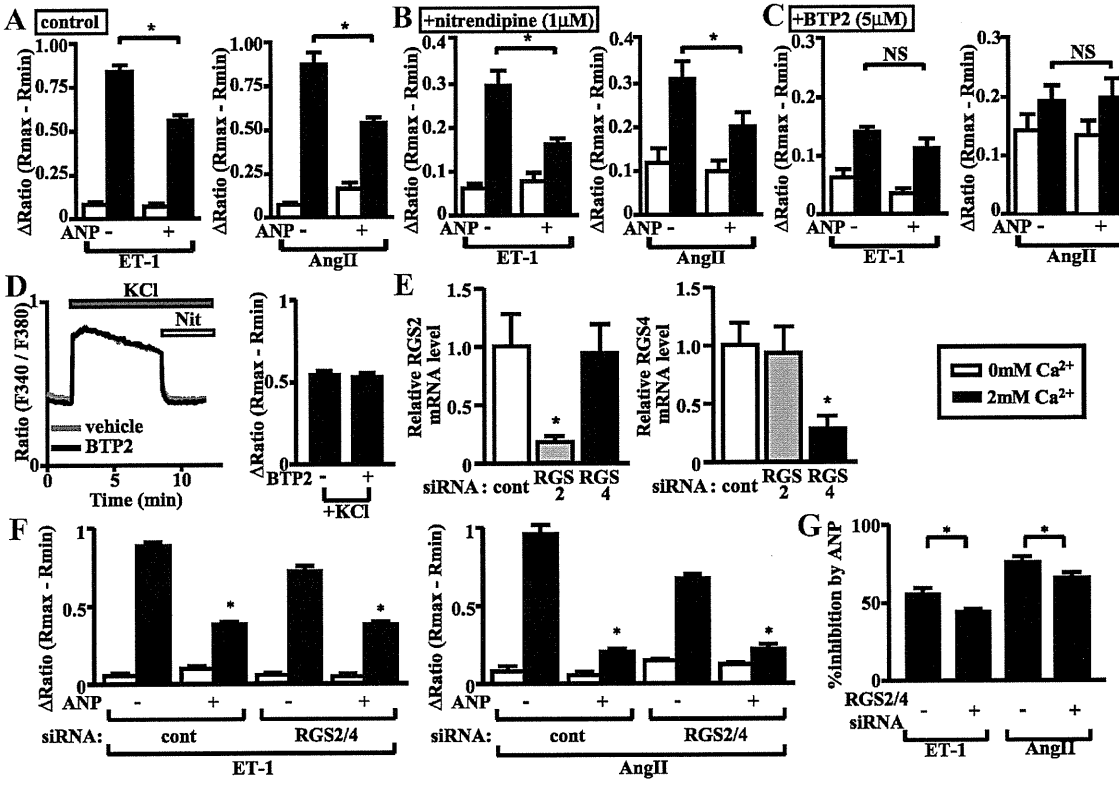

Figure 5. TRPC channels are involved in ANP-induced inhibition of receptor-mediated Ca^{2+} entry into cardiac ventricular myocytes. A, ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar). B and C,** Effect of nitrendipine (1 μmol/L) **(B)** or BTP2 (5 μmol/L) **(C)** on ET-1– and Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar). D,** Representative traces showing the effect of BTP2 (5 μmol/L) on KCl-induced (5 mmol/L), nitrendipine-sensitive (1 μmol/L) Ca^{2+} influx into cardiac myocytes. Graphs at **right** show the effect of BTP2 on KCl-induced Ca^{2+} influx into cardiac myocytes. **E,** Effect of RGS2 and RGS4 siRNA on expression of RGS2 **(left)** and RGS4 **(right)** mRNA in cultured ventricular myocytes (n=4 each). $^*P<0.05$ vs control siRNA. **F,** Effect of RGS2 and RGS4 double knockdown on ET-1–evoked **(left)** and Ang II–evoked **(right)** Ca^{2+} influx into cultured ventricular myocytes treated with or without ANP in Ca^{2+}-free **(white bar)** or Ca^{2+}-containing external solution **(black bar). G,** Effect of RGS2 and RGS4 double knockdown on the inhibitory effects of ANP in ET-1– or Ang II–evoked Ca^{2+} influx into cultured ventricular myocytes. In all graphs, values are shown as means±SEM $^*P<0.05$. In all these experiments, 10 nmol/L ET-1, 100 nmol/L Ang II, 100 nmol/L ANP, and 2 mmol/L external Ca^{2+} were used.

expression of *TRPC6* and *TRPC3* seen in GC-A KO ventricles was diminished by BTP2 treatment (Figure 7A). Clearly, TRPC-mediated signaling is significantly involved in the development of pathological cardiac hypertrophy induced by a genetic deletion of GC-A.

Exaggerated Cardiac Hypertrophy in Mice With Cardiac Overexpression of TRPC6 Against a GC-A–Null Background

To further confirm the negative functional interaction of the ANP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways, we next crossed transgenic mice cardio-selectively expressing TRPC6 (TRPC6 Tg; previously referred to as line 16) with GC-A KO mice.[23] As previously reported, 12-week-old TRPC6 Tg mice did not show cardiac hypertrophy, as compared to WT mice, whereas GC-A KO mice showed a significant increase in blood pressure, HW and HW/BW ratios (Figure 7B and 7C).[23,44] Moreover, TRPC6 Tg mice with a GC-A-null background showed significantly greater HW/BW ratios than GC-A KO mice, without an increase in blood pressure, which is indicative of the hypersensitivity of GC-A KO mice to TRPC6-mediated prohypertrophic signaling (Figure 7B and 7C). Consistent with this finding, echocardiographic analysis showed ventricular wall thicknesses to be greater in GC-A; TRPC6 Tg mice than GC-A KO mice, without a change in systolic function (Figure 7D).

TRPC Inhibition Prevents Ang II–Induced Cardiac Hypertrophy

The notion that TRPC6 inhibition is a critical component of the antihypertrophic effects exerted via the ANP-GC-A-cGMP-PKG pathway suggests that direct inhibition of TRPC6 could be a novel therapeutic approach to preventing pathological cardiac hypertrophy. Indeed, BTP2 significantly inhibited the cardiac hypertrophy otherwise seen in GC-A KO mice (Figure 6C through 6F and Figure 7A). To further test this hypothesis using models of cardiac hypertrophy in which GC-A-cGMP-PKG signaling is genetically intact, we examined the effects of BTP2 on Ang II–induced cardiac hypertrophy. When we chronically infused Ang II using a subcutaneously implanted osmotic

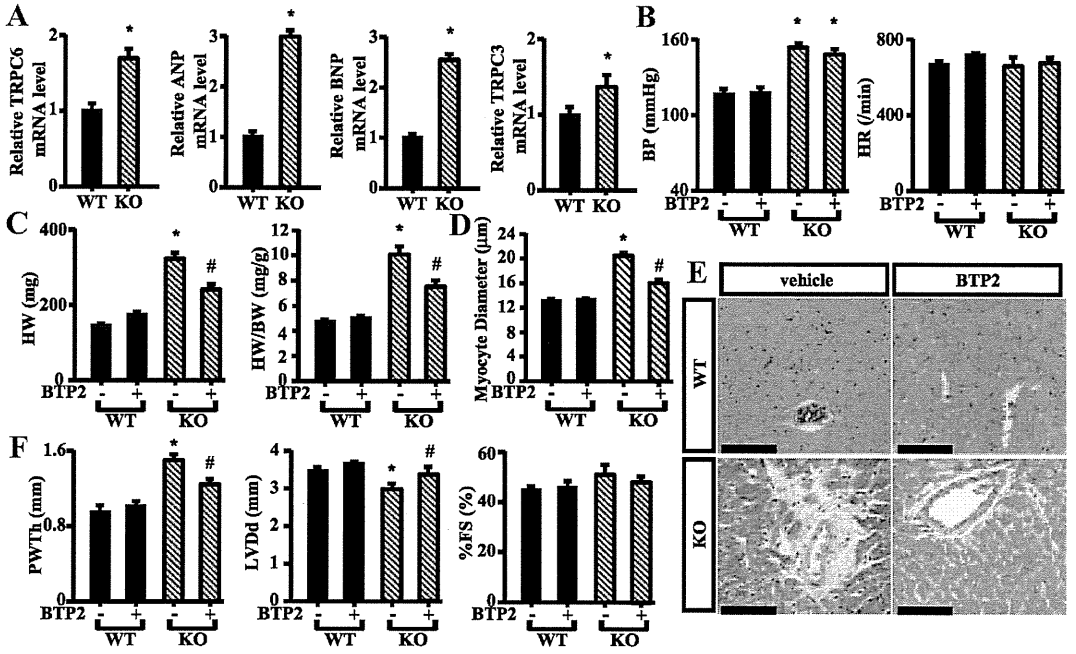

Figure 6. TRPC6 plays an important role in cardiac hypertrophy in GC-A KO mice. A, Real-time RT-PCR analysis of the relative expression of TRPC6, ANP, BNP, and TRPC3 mRNA in ventricular myocardium from WT and GC-A KO (KO) mice at 12 weeks of age. Relative mRNA levels in WT were assigned a value of 1.0. *P<0.05 vs WT. Values are shown as means±SEM. *P<0.05. **B,** Blood pressure (BP) and heart rate (HR) in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). **C,** Heart weight (HW) and HW/BW (mg/g) ratios in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). Values are shown as means±SEM. *P<0.05 vs WT without BTP2; #P<0.05 vs KO without BTP2. **D,** Myocardial cell diameters in left ventricles were measured for 200 cells in each group. Values are shown as means±SEM. *P<0.05 vs WT without BTP2; #P<0.05 vs KO without BTP2. **E,** Histology of 16-week-old WT and GC-A KO ventricles treated for 28 days with or without BTP2. **Scale bars:** 100 mm. **F,** Echocardiographic parameters in WT and KO mice treated for 28 days with or without BTP2 (20 mg/g per day). Graphs show posterior wall thickness (PWTh) (mm), left ventricular end diastolic dimension (LVDd) (mm), and percentage fractional shortening (%FS). Values are shown as means±SEM. *P<0.05 vs WT without BTP2; #P<0.05 vs KO without BTP2.

minipump, we observed a significant increase in blood pressure and HW/BW ratios (Figure 8A and 8B). Administration of BTP2 significantly inhibited Ang II–induced cardiac hypertrophy assessed based on HW/BW ratios, without affecting blood pressure (Figure 8A and 8B). Echocardiographic analysis confirmed that BTP2 inhibited the Ang II–induced hypertrophic response in the heart (Figure 8C and Online Table II), without affecting systolic function (Online Table II). In addition, the increase in the cardiac expression of the hypertrophy marker genes *RCAN1*, *BNP*, *βMHC*, and skeletal α-actin, which was observed in Ang II–treated mice, was significantly inhibited by BTP2 treatment (Figure 8D), whereas expression of cardiac α-actin, *TRPC6*, and *TRPC3* was not significantly affected in mice treated with or without Ang II and/or BTP2 (Figure 8D; Online Figure V, F and G).

Discussion

Characterization of the crosstalk among the cardiac signaling pathways that promote or antagonize hypertrophic responses should lead to a better understanding of molecular processes underlying the establishment of cardiac hypertrophy and heart failure, which could ultimately lead to the discovery of novel therapeutic approaches to preventing pathological cardiac remodeling. In the present study we unraveled the functionally negative crosstalk between the ANP-GC-A-cGMP-PKG and TRPC6-calcineurin-NFAT pathways in cardiac myocytes using in vitro culture systems and in vivo genetically engineered models. ANP acted via the cGMP-PKG pathway to directly inhibit TRPC6 activity, which in turn suppressed prohypertrophic signaling. Cardiac hypertrophy was significantly attenuated by the selective TRPC inhibitor BTP2 in GC-A KO mice, which were hypersensitive to hypertrophic signaling caused by overexpression of TRPC6. Likewise, BTP-2 significantly inhibited the cardiac hypertrophy induced by chronic Ang II infusion. Our study thus demonstrates that inhibition of TRPC6 activity mediates the antihypertrophic effects of ANP/BNP, and suggests that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

It was recently reported that RGS4 mediates the antihypertrophic effects of GC-A-catalyzed signaling in the heart.[20] RGS2 also reportedly mediates the antihypertrophic effects of inhibiting phosphodiesterase 5, which enhances activity in the NO-cGMP pathway.[41] It is thus suggested that RGS proteins mediate the antihypertrophic effects exerted by cGMP. In our study, we confirmed that RGS2 and 4 are significantly involved in the ANP-induced inhibition of agonist-induced Ca^{2+} influx (Figure 5G). PKG-catalyzed phosphorylation of TRPC6 on Thr69 is significantly involved in ANP-induced inhibition of receptor-mediated TRPC6 activity, despite the expression of *RGS2* and *RGS4* mRNA in HEK293 cells, however (Figure 2D, 2E, and 2G; Online Figure I, D).

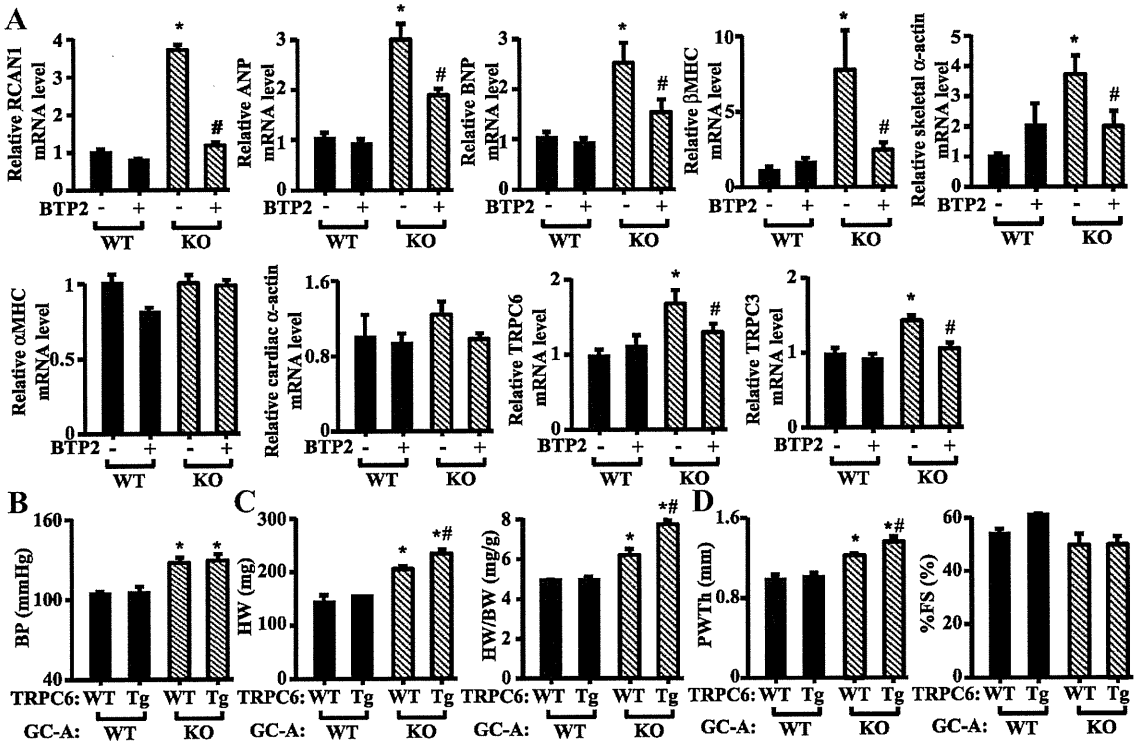

Figure 7. Inhibition of TRPC attenuates hypertrophic gene reprogramming in GC-A KO mice, whereas overexpression of TRPC6 exacerbated cardiac hypertrophy. A, Real-time RT-PCR analysis of relative expression of RCAN1, ANP, BNP, βMHC, skeletal α-actin, αMHC, cardiac α-actin, TRPC6, and TRPC3 mRNA in ventricular myocardium from 16-week-old WT and GC-A KO (KO) mice treated for 28 days with or without BTP2 (20 mg/g per day). Relative mRNA levels in WT without BTP2 were assigned a value of 1.0. Values are shown as means±SEM. *P<0.05 vs WT without BTP2; #P<0.05 vs KO without BTP2. **B and C,** Blood pressure (BP) **(B),** heart weight (HW) **(C, left)** and HW/BW ratios **(C, right)** in WT, TRPC6 Tg, GC-A KO, and TRPC6 Tg; GC-A KO mice. Values are shown as means±SEM. *P<0.05 vs WT; #P<0.05 vs GC-A KO. **D,** Echocardiographic parameters in WT, TRPC6 Tg GC-A KO, and TRPC6 Tg; GC-A KO mice. Graphs show posterior wall thickness (PWTh) (mm) and percentage fractional shortening (%FS). Values are shown as means±SEM. *P<0.05 vs WT; #P<0.05 vs GC-A KO.

Agonist-induced activation of the TRPC6 T69A mutant in the presence of ANP was around 80% to 90% of that seen with WT TRPC6 in the absence of ANP. Furthermore, even when we knocked down both RGS2 and RGS4, the significant inhibitory effect of ANP on agonist-induced Ca^{2+} influx was preserved in cardiac myocytes (Figure 5E through 5G; Online Figure IV, A and B). Thus, RGS-independent mechanisms also contribute to the antihypertrophic effects of cGMP-dependent signaling. This is not surprising, as multiple mechanisms would be expected to participate in the antihypertrophic effects exerted via the ANP/BNP-GC-A-cGMP-PKG pathway. In that regard, PKG also reportedly inhibits LTCC activity and calcineurin-NFAT signaling.[18] Although under our experimental conditions treatment with the LTCC blocker nifedipine did not significantly attenuate the inhibitory effect of ANP on agonist-induced Ca^{2+} influx into cardiac myocytes, inhibition of LTCCs can contribute to the antihypertrophic effects of ANP. Indeed, activation of TRPC3/6 has been shown to lead to LTCC activation,[24] and ANP may inhibit that activation, thereby inhibiting calcineurin-NFAT signaling. It therefore seems likely that ANP/BNP-GC-A-cGMP-PKG signaling inhibits prohypertrophic signaling pathways at multiple steps. ANP and BNP are already administered clinically to patients with acute heart failure.[46,47] But because, currently, these drugs are only administered intravenously, they are not available for use in the treatment of chronic pathological conditions such as cardiac hypertrophy and chronic heart failure. Development of nonpeptide GC-A agonists that can be administered orally may lead to the development of new therapeutic agents for preventing pathological cardiac hypertrophy and remodeling.

It has been shown in separate studies that GC-A is desensitized in failing human hearts[48] and that Ang II and ET-1 act to desensitize GC-A.[49] This makes it unlikely that under pathological conditions, endogenous cardiac ANP/BNP-GC-A-cGMP signaling would be sufficient to block the pathological signaling activity. Such disruption of the balance between anti- and prohypertrophic signaling would lead to further activation of TRPC3/6-dependent prohypertrophic signaling, thereby promoting the pathological cardiac remodeling. Thus inhibition of TRPC3/6 channel activity could be an effective therapeutic strategy for preventing cardiac remodeling under these pathological conditions. Indeed, our finding that BTP2 attenuated cardiac hypertrophy in GC-A KO and Ang II–infused mice may support this notion. In that regard, Pyr3 is a pyrazole compound recently identified as a specific inhibitor for TRPC3 that blocks Ca^{2+} influx carried by TRPC3/6 heteromeric complexes and by TRPC3 homomeric complexes. Moreover, Pyr3 also inhibited Ang II–induced hypertrophic responses in cultured cardiac myocytes more

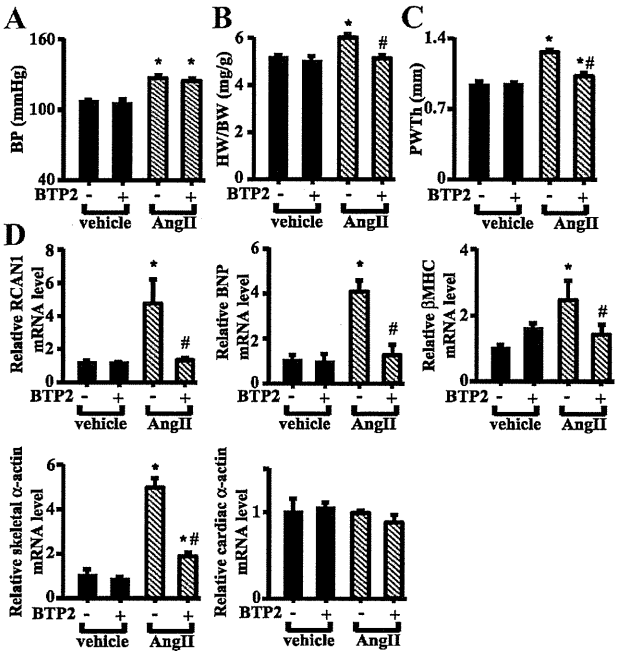

Figure 8. Inhibition of TRPC attenuates angiotensin II-induced cardiac hypertrophy in mice. A, Blood pressure (BP) in mice treated with or without Ang II (0.6 mg/kg per day) and/or BTP2 (20 mg/g per day). Values are shown as means±SEM. *P<0.05 vs control. **B,** HW/BW ratio (mg/g) in mice treated with or without Ang II (0.6 mg/kg per day) and BTP2 (20 mg/g per day). Values are shown as means±SEM. *P<0.05 vs control; #P<0.05 vs mice treated with Ang II and without BTP2. **C,** Posterior wall thickness (PWTh) (mm) evaluated by echocardiography in mice treated with of without Ang II (0.6 mg/kg per day) and BTP2 (20 mg/g per day). Values are shown as means±SEM. *P<0.05 vs control; #P<0.05 vs mice treated with Ang II and without BTP2. **D,** Real-time RT-PCR analysis of relative expression of RCAN1, BNP, βMHC, skeletal α-actin, and cardiac α-actin mRNA in ventricular myocardium from 10-week-old mice treated for 14 days with or without Ang II (0.6 mg/kg per day) and/or BTP2 (20 mg/g per day). Relative mRNA levels in control mice without Ang II and BTP2 were assigned a value of 1.0. Values are shown as means±SEM. *P<0.05 vs control; #P<0.05 vs mice treated with Ang II and without BTP2.

potently than BTP2.[37] In our study, knocking down either TRPC3 or -6 in cardiac myocytes significantly inhibited ET-1– and Ang II–induced increases in Ca^{2+} oscillation to levels comparable to those seen when both TRPC3 and -6 were knocked down simultaneously (Online Figure II, A through J). This raises the possibility that a TRPC3/6 heteromeric complex plays a key role in mediating agonist-induced prohypertrophic signaling in cardiac myocytes. Development of highly specific TRPC6 inhibitors could lead to the development of more potent and safer agents with which to prevent pathological cardiac remodeling and heart failure.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Under pathological conditions, the ventricular expression of 2 peptide mediators, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), is increased in the heart. These peptides act as both endocrine and local antihypertrophic factors. However, the molecular mechanisms by which ANP/BNP inhibit cardiac hypertrophy remain unclear.
- Transient receptor potential subfamily C (TRPC)3 and -6 form homo- and heteromultimeric cation channels that are activated directly by diacylglycerol following receptor activation and reportedly serve as positive upstream regulators of the pathological calcineurin-NFAT signaling pathway in cardiac myocytes.

What New Information Does This Article Contribute?

- We demonstrate that ANP/BNP acts via the guanylyl cyclase (GC)-A-cGMP-protein kinase (PK)G pathway to inhibit TRPC6 channel activity, which in turn suppresses the subsequent activation of the prohypertrophic calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway.
- The present study suggests that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy and remodeling.

Characterization of the crosstalk among the cardiac signaling pathways that promote or antagonize cardiac hypertrophy should lead to a better understanding of molecular processes underlying the development of heart failure and ultimately to the discovery of novel therapeutic approaches for preventing pathological cardiac remodeling and heart failure. The cardiac hormones ANP and BNP reportedly exert antihypertrophic effects on the heart via their common receptor, GC-A, which catalyzes the synthesis of cGMP, leading to activation of PKG. Details of molecular mechanisms via which ANP/BNP-GC-A signaling inhibit cardiac hypertrophy are not well understood. The present study demonstrates that ANP/BNP-GC-A-cGMP-PKG signaling pathway inhibits TRPC6 activity by phosphorylation of threonine 69, which in turn suppresses prohypertrophic calcineurin-NFAT signaling. In mice lacking GC-A, BTP2, a selective TRPC channel blocker, significantly attenuated the cardiac hypertrophy otherwise seen. Conversely, overexpression of TRPC6 in mice lacking GC-A exacerbated cardiac hypertrophy. BTP2 also significantly inhibited angiotensin II-induced cardiac hypertrophy in mice. The present study reports the novel finding that inhibition of TRPC6 contributes to the antihypertrophic effects exerted by ANP/BNP-GC-A-cGMP-PKG signaling. These results suggest that inhibition of TRPC6 could be an effective therapeutic strategy for preventing pathological cardiac hypertrophy.

Circulating C-Type Natriuretic Peptide (CNP) Rescues Chondrodysplastic CNP Knockout Mice from Their Impaired Skeletal Growth and Early Death

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C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth through a subtype of membranous guanylyl cyclase receptor, GC-B. Although its two cognate natriuretic peptides, ANP and BNP, are cardiac hormones produced from heart, CNP is thought to act as an autocrine/paracrine regulator. To elucidate whether systemic administration of CNP would be a novel medical treatment for chondrodysplasias, for which no drug therapy has yet been developed, we investigated the effect of circulating CNP by using the CNP transgenic mice with an increased circulating CNP under the control of human serum amyloid P component promoter (*SAP-Nppc-Tg* mice). *SAP-Nppc-Tg* mice developed prominent overgrowth of bones formed through endochondral ossification. In organ culture experiments, the growth of tibial explants of *SAP-Nppc-Tg* mice was not changed from that of their wild-type littermates, exhibiting that the stimulatory effect on endochondral bone growth observed in *SAP-Nppc-Tg* mice is humoral. Then we crossed chondrodysplastic CNP-depleted mice with *SAP-Nppc-Tg* mice. Impaired endochondral bone growth in CNP knockout mice were considerably and significantly recovered by increased circulating CNP, followed by the improvement in not only their longitudinal growth but also their body weight. In addition, the mortality of CNP knockout mice was greatly decreased by circulating CNP. Systemic administration of CNP might have therapeutic potential against not only impaired skeletal growth but also other aspects of impaired growth including impaired body weight gain in patients suffering from chondrodysplasias and might resultantly protect them from their early death. **(*Endocrinology* 151: 4381–4388, 2010)**

Recent studies have elucidated that C-type natriuretic peptide (CNP) is a crucial regulator of endochondral bone growth (1, 2). The biological actions of CNP are thought to be mediated by the production of intracellular second-messenger cGMP through a subtype of membranous guanylyl cyclase receptor, guanylyl cyclase (GC)-B (3). We have exhibited that both CNP and GC-B are expressed in the proliferative and prehypertrophic chondrocyte layers of the growth plate (1) and that CNP or GC-B knockout mice develop severely short stature phenotype owing to their impaired endochondral bone growth (1, 4). On the contrary, mice with targeted overexpression of CNP in the growth plate by using type II collagen promoter exhibit prominent skeletal overgrowth (5, 6).

After these discoveries, we planned to translate this strong stimulatory effect of the CNP/GC-B system on bone growth into clinical treatment for patients suffering from diseases with impaired skeletal growth. Chondrodysplasias are a group of genetic disorders characterized by impaired skeletal growth. The many different forms of chondrodysplasias add to produce a significant number of affected individuals with significant morbidity and mortality (7). Nevertheless, no efficient drug therapy has been developed to date for the treatment of chondrodysplasias.

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Abbreviations: CNP, C-type natriuretic peptide; DIG, digoxigenin; GC, guanylyl cyclase; HE, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; SAP, serum amyloid P.

In our previous report, we achieved targeted overexpression of CNP in the growth plate of a mice model of achondroplasia (8), the most common form of chondrodysplasias with a constitutive active mutation in the fibroblast growth factor receptor 3 gene (9), and successfully treated its impaired skeletal growth and short stature phenotype (5).

In contrast to atrial natriuretic peptide and brain natriuretic peptide, the two cognate natriuretic peptides of CNP that act as cardiac hormones produced predominantly from atrium and ventricle of heart, respectively (10, 11), CNP is thought to be an autocrine/paracrine regulator, rather than an endocrine regulator (12, 13). Because we have to evaluate the effect of circulating CNP on endochondral bone growth in case we use CNP as a drug for chondrodysplasias via systemic administration, we generated CNP transgenic mice with increased circulating CNP as a model of systemic administration of CNP (14): these transgenic mice carried the human serum amyloid P (SAP) component promoter/mouse CNP fusion gene (*SAP-Nppc-Tg*), and the expression of the transgene was targeted to the liver (15). *SAP-Nppc-Tg* mice exhibited prominent overgrowth of bones formed through endochondral ossification (14), and furthermore, we successfully rescued achondroplastic model mice from their impaired bone growth by crossing them with *SAP-Nppc-Tg* mice (16).

In the present study, we further investigated the effect of circulating CNP by using *SAP-Nppc-Tg* mice. At first, to certify the humoral effect of the overexpressed CNP in *SAP-Nppc-Tg* mice on endochondral bone growth, we performed organ culture experiments by using tibial explants from *SAP-Nppc-Tg* mice and compared them with those from cartilage-targeted CNP transgenic mice under the control of type II collagen promoter (*Col2-Nppc-Tg* mice) (5). Then we studied the effects of circulating CNP on the chondrodysplastic CNP knockout ($Nppc^{-/-}$) mice by crossing them with *SAP-Nppc-Tg* mice.

Materials and Methods

Animals

Generation of CNP transgenic mice under the control of human SAP component promoter (*SAP-Nppc-Tg* mice) was reported previously (14). These mice carried the human SAP/mouse CNP fusion gene, and CNP overexpression in these mice was targeted to the liver (15). *SAP-Nppc-Tg* mice were intended to have increased circulating CNP levels, and plasma CNP concentrations measured by RIA were 84% higher in *SAP-Nppc-Tg* mice than in wild-type mice (14). Generation of CNP transgenic mice under the control of mouse type II collagen promoter (*Col2-Nppc-Tg* mice) (5) and CNP knockout mice ($Nppc^{-/-}$ mice) (1) was also described previously.

To generate $Nppc^{-/-}$ mice carrying *SAP-Nppc* transgene, male $Nppc^{+/-}$ mice were mated with female *SAP-Nppc-Tg* mice, and female F1 offspring heterozygous for both the transgene and the *Nppc* allele ablation were mated with male F1 offspring heterozygous only for the *Nppc* allele ablation to generate $Nppc^{-/-}$ mice with the transgene expression ($Nppc^{-/-}$/*SAP-Nppc-Tg* mice). For generation of homozygous *SAP-Nppc-Tg* mice, male and female heterozygous *SAP-Nppc-Tg* mice were mated, and the genotype of the resultant transgenic mice was determined by quantifying *SAP-Nppc* transgene using StepOnePlus real-time PCR systems (Applied Biosystems Inc., Foster City, CA).

The care of the animals and all experiments were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine.

Organ culture

Tibias from fetal *SAP-Nppc-Tg* mice and their wild-type littermates (on d 16 of pregnancy), newborn *Col2-Nppc-Tg* mice and their wild-type littermates, and newborn $Nppc^{-/-}$/*SAP-Nppc-Tg* mice and their $Nppc^{-/-}$ littermates were dissected out and cultured for 4 d in Biggers, Gwatkin, Judah tissue culture medium for bone and cartilage (Invitrogen, Carlsbad, CA) with BSA (6 mg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan), ascorbic acid (150 μg/ml; Wako), and penicillin/streptomycin (10,000 U/ml; Wako) in 12-well plates. Tibias from newborn $Nppc^{-/-}$ mice were incubated with vehicle or CNP at the dose of 10^{-9}, 10^{-8}, or 10^{-7} M for 4 d. At the end of the culture period, the longitudinal length of tibial explants was measured using a linear ocular scale mounted on a dissecting microscope at ×10 magnification.

Skeletal analysis

Mice were subjected to soft x-ray analysis (30 kVp, 5 mA for 1 min; Softron type SRO-M5; Softron, Tokyo, Japan), and the lengths of bones were measured on the soft x-ray film.

Histological analysis

For light microscopy, sections were cut from paraffin-embedded specimens. For Alcian Blue-hematoxylin and eosin (HE) staining, sections were deparaffinized with xylene and rehydrated through an ethanol series and distilled water. The sections were treated with 3% acetic acid for 3 min and Alcian Blue (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 min. Then they were treated with hematoxylin (Muto) for 2 min, eosin alcohol (Muto) for 1 min, dehydrated, and then mounted with malinol (Muto).

As for *in situ* hybridization analyses for type II and type X collagens, 414- and 658-bp DNA fragments corresponding to the nucleotide positions 138-551 and 2893-3550 of mouse *Col2a1* and *Col10a1* cDNA (GenBank accession no. NM_031163 and 009925), respectively, were subcloned into pGEMT-Easy vector (Promega, Madison, WI) and were used for the generation of sense or antisense RNA probes. Digoxigenin (DIG)-labeled RNA probes were prepared with DIG RNA labeling mix (Roche, Stockholm, Sweden). Paraffin-embedded sections were hybridized with DIG-labeled RNA probes at 60 C for 16 h. The bound label was detected using 4-nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt, an alkaline phosphate color substrate. The sections were counterstained with Kernechtrot (Muto).

For immunohistochemical detection of proliferating cell nuclear antigen (PCNA), tissue sections were incubated with mouse

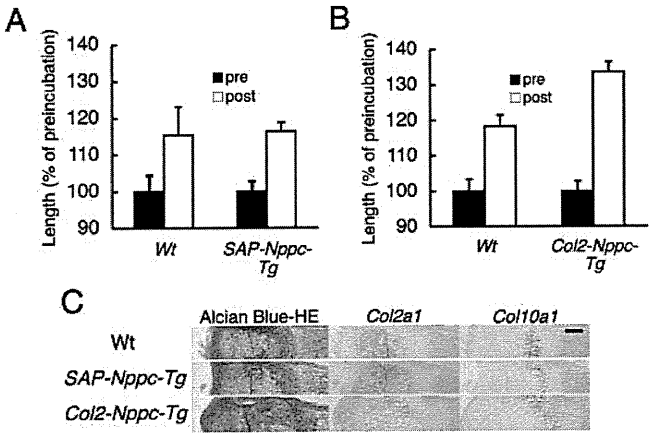

FIG. 1. Organ culture experiments using tibial explants from two different CNP transgenic mice. A and B, Graphs indicating percent of the longitudinal lengths of tibial explants at the end of incubation (*white bars*) compared by those of tibial explants at the beginning of incubation (*black bars*). Fetal (GD16) wild-type (Wt) *vs. SAP-Nppc-Tg* explants (A), and neonatal wild-type (Wt) *vs. Col2-Nppc-Tg* explants (B) are shown. C, Histological pictures of the growth plates of tibial explants at the end of 4-d culture period. From *top* to *bottom*, pictures of wild-type (Wt), *SAP-Nppc-Tg*, and *Col2-Nppc-Tg* explants are shown. *Left three panels* exhibit Alcian Blue-hematoxylin and eosin (HE) staining, and *middle three* and *right three panels* show *in situ* hybridization analyses for type II collagen (*Col2a1*) and type X collagen (*Col10a1*), respectively. *Scale bar*, 100 μm.

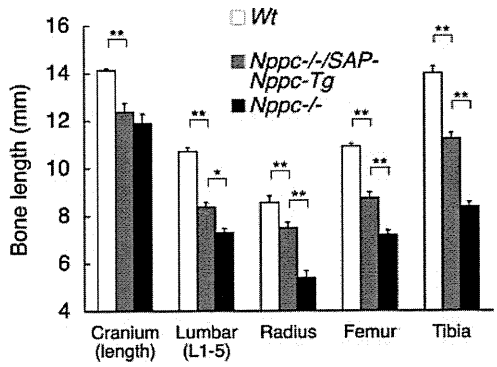

FIG. 3. Bone lengths of mice at the age of 3 wk measured on soft x-ray films. *White bars*, Wild-type mice; *gray bars*, $Nppc^{-/-}$/*SAP-Nppc-Tg* mice; *black bars*, $Nppc^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$.

monoclonal anti-PCNA antibody (Dako, Glostrup, Denmark), and immunostaining was performed using Histofine mouse stain kit (Nichirei Corp., Tokyo, Japan) according to the manufacturer's instructions. Under the microscope (×400), three visual fields in the proliferative chondrocyte zone of the growth plate were randomly selected, and all cells and PCNA-positive cells in each field were counted. Then labeling index was calculated as the mean of these three values. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining was performed using *in situ* apoptosis detection kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instruction.

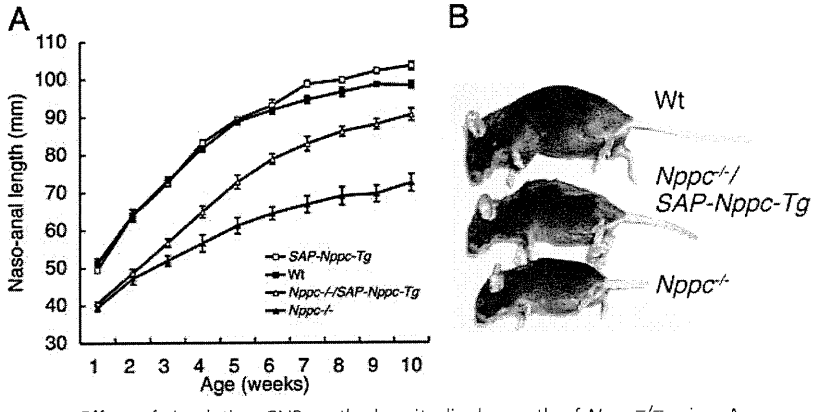

FIG. 2. Effect of circulating CNP on the longitudinal growth of $Nppc^{-/-}$ mice. A, Growth curves of nasoanal length of *SAP-Nppc-Tg* (*open square*), wild-type (*closed square*), $Nppc^{-/-}$/*SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice. B, Gross appearance of wild-type (Wt), $Nppc^{-/-}$/*SAP-Nppc-Tg*, and $Nppc^{-/-}$ mice at the age of 15 wk.

Statistical analysis

Data are expressed as means ± SE. The statistical significance of differences in mean values was assessed by Student's *t* test. The difference in survival rates among genotypes was assessed by Kaplan-Meier analysis.

Results

Organ culture experiments using tibial explants from *SAP-Nppc-Tg mice*

We generated two lines of CNP transgenic mice under the control of an SAP promoter, and both of them exhibited prominent skeletal overgrowth phenotype (14). We used one of them with milder skeletal phenotype as the *SAP-Nppc-Tg* mice for further experiments. To confirm whether the effect of *SAP-Nppc*-transgene on skeletal growth is humoral, we performed organ culture experiments by using tibias from *SAP-Nppc-Tg* mice and compared them with those from CNP transgenic mice with targeted overexpression of CNP in the cartilage by using mouse type II collagen promoter (*Col2-Nppc-Tg* mice) (5).

At the end of the 4-d culture period, the length of tibial explants from *SAP-Nppc-Tg* mice was not changed from that from their wild-type littermates, whereas the length of tibial explants from *Col2-Nppc-Tg* mice was about 13% larger than that from their wild-type littermates (Fig. 1, A and B). Histological analyses revealed that the widths of both nonhypertrophic and hypertrophic chondrocyte layers of the growth plates in *SAP-Nppc-Tg* explants, shown to express type II and type X collagens by *in situ* hybridization analyses, respectively, were not changed from those in wild-type explants, whereas they were larger in *Col2-Nppc-Tg* explants (Fig. 1C).

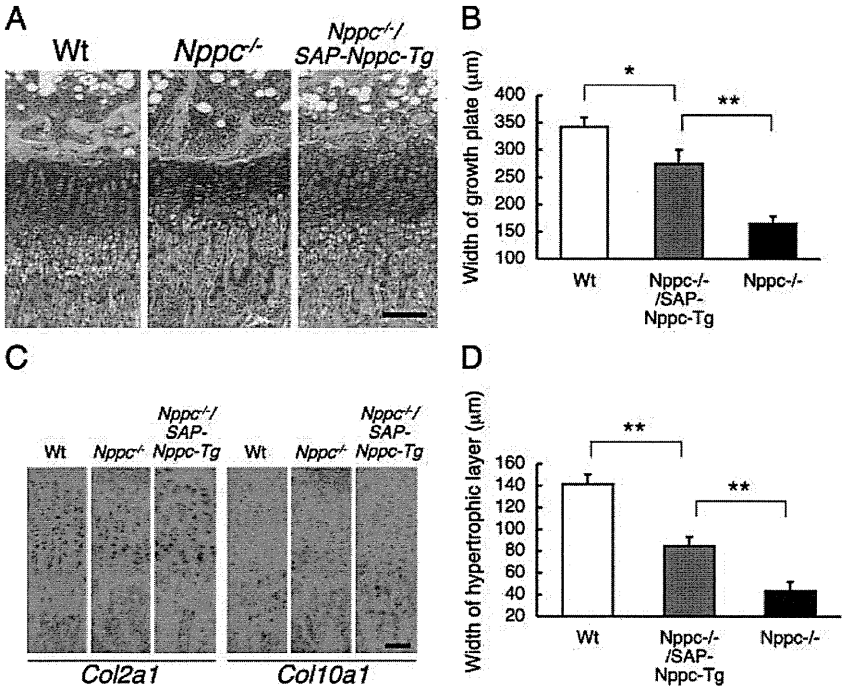

FIG. 4. Histological analyses of tibial growth plates of wild-type (Wt), *Nppc*$^{-/-}$, and *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice at the age of 3 wk. A, Histological pictures stained with Alcian Blue-hematoxylin and eosin (HE). *Scale bar*, 100 μm. B, Width of growth plates of tibias from wild-type (*white bar*), *Nppc*$^{-/-}$*/SAP-Nppc-Tg* (*gray bar*), and *Nppc*$^{-/-}$ (*black bar*) mice. *, $P < 0.05$; **, $P < 0.01$. C, Pictures of *in situ* hybridization analyses for type II collagen (*Col2a1*, *left three panels*) and type X collagen (*Col10a1*, *right three panels*). *Scale bar*, 50 μm. D, Width of hypertrophic chondrocyte layers of the growth plates of tibias from wild-type (*white bar*), *Nppc*$^{-/-}$*/SAP-Nppc-Tg* (*gray bar*), and *Nppc*$^{-/-}$ (*black bar*) mice. **, $P < 0.01$.

In addition, *in situ* hybridization analyses exhibited that the patterns and intensities of the staining for type II and type X collagens as the differentiation markers for nonhypertrophic and hypertrophic chondrocytes, respectively, were not different between in *SAP-Nppc-Tg* and wild-type explants. Furthermore, the proliferation of the growth plate chondrocytes in *SAP-Nppc-Tg* explants, estimated by immunohistochemical staining for PCNA, was almost the same as that in wild-type explants (labeling index: 60.4 ± 3.4 *vs.* 60.0 ± 2.4%). These results exhibit that CNP generated by *SAP-Nppc*-transgene affects endochondral bone growth in an endocrine manner.

The impaired endochondral bone growth of *Nppc*$^{-/-}$ mice was recovered by circulating CNP

Next we investigated the effect of circulating CNP on the chondrodysplastic phenotype of CNP knockout mice by crossing them with *SAP-Nppc-Tg* mice. Because *Nppc*$^{-/-}$ mice are thought to be infertile, we crossed *Nppc*$^{+/-}$ mice with *SAP-Nppc-Tg* mice and obtained *Nppc*$^{+/-}$*/SAP-Nppc-Tg* mice. Then these *Nppc*$^{+/-}$*/SAP-Nppc-Tg* mice were crossed with *Nppc*$^{+/-}$ mice to generate *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice.

At the first week after birth, *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice were smaller than their wild-type littermates, and the nasoanal length of *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice was almost the same as that of *Nppc*$^{-/-}$ mice (Fig. 2A). But they gradually became larger than *Nppc*$^{-/-}$ mice and became close to their wild-type littermates (Fig. 2, A and B). The nasoanal length of *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice was significantly larger than that of *Nppc*$^{-/-}$ mice at the age of 3 wk in male and at the age of 4 wk in female (male: 56.6 ± 1.1 mm and 51.9 ± 1.3 mm, respectively, n = 15 and 11 each, $P < 0.01$, and female: 63.3 ± 1.2 mm and 53.8 ± 0.7 mm, respectively, n = 10 and 10 each, $P < 0.01$). In accordance with the above observation, most bones formed through endochondral ossification in *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice grew longer than those in *Nppc*$^{-/-}$ mice. At the age of 3 wk, lumbar spine, radius, femur, and tibia of *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice were significantly longer than those of *Nppc*$^{-/-}$ mice, although they were still significantly shorter than those of their wild-type littermates (Fig. 3).

Histological analysis revealed that the width of the growth plate of tibias from *Nppc*$^{-/-}$*/SAP-Nppc-Tg* mice was significantly larger than that from *Nppc*$^{-/-}$ mice and was comparable with that from wild-type mice (Fig. 4, A and B). Width of every zone of the growth plate, especially that of hypertrophic chondrocyte zone expressing type X collagen as shown by *in situ* hybridization analysis, was significantly larger in *Nppc*$^{-/-}$*/SAP-Nppc-Tg* tibia than that in *Nppc*$^{-/-}$ tibia and was comparable with that in wild-type tibia (Fig. 4, A, C, and D).

The intensities or patterns of the staining for both type II and type X collagens by *in situ* hybridization were not different between that in *Nppc*$^{-/-}$*/SAP-Nppc-Tg* and that in *Nppc*$^{-/-}$ tibias, indicating that the differentiation for nonhypertrophic and hypertrophic chondrocytes in *Nppc*$^{-/-}$ growth plate was not affected by circulating CNP (Fig. 4C). Furthermore, immunohistochemical detection of PCNA revealed that the rate of PCNA-positive chondrocytes in *Nppc*$^{-/-}$*/SAP-Nppc-Tg* growth plate was not changed from that in *Nppc*$^{-/-}$ growth plate (labeling index: 23.0 ± 7.3 *vs.* 25.4 ± 1.4%), exhibiting that the proliferation of the chondrocytes in *Nppc*$^{-/-}$ growth plate was not altered by circulating CNP. In addition, we

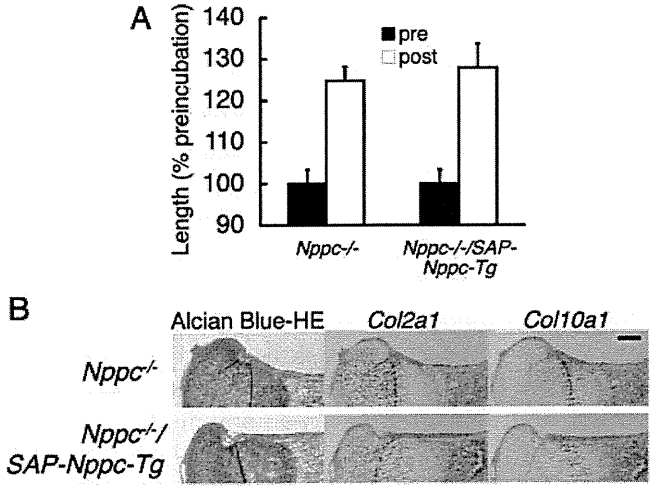

FIG. 5. Organ culture experiments using tibial explants from neonatal $Nppc^{-/-}$ and $Nppc^{-/-}$/*SAP-Nppc-Tg* mice. A, The graph indicating percent of the longitudinal length of tibial explants at the end of incubation (*white bars*) compared with that of tibial explants at the beginning of incubation (*black bars*). B, Histological analyses of the tibial explants at the end of the 4-d culture period. *Upper panels* show histological pictures of the growth plates of $Nppc^{-/-}$ explants, and *lower panels* show those of $Nppc^{-/-}$/*SAP-Nppc-Tg* explants. *Left panels* exhibit Alcian Blue-hematoxylin and eosin (HE) staining, and *middle* and *right panels* show *in situ* hybridization analyses for type II collagen (*Col2a1*) and type X collagen (*Col10a1*), respectively. *Scale bar*, 100 μm.

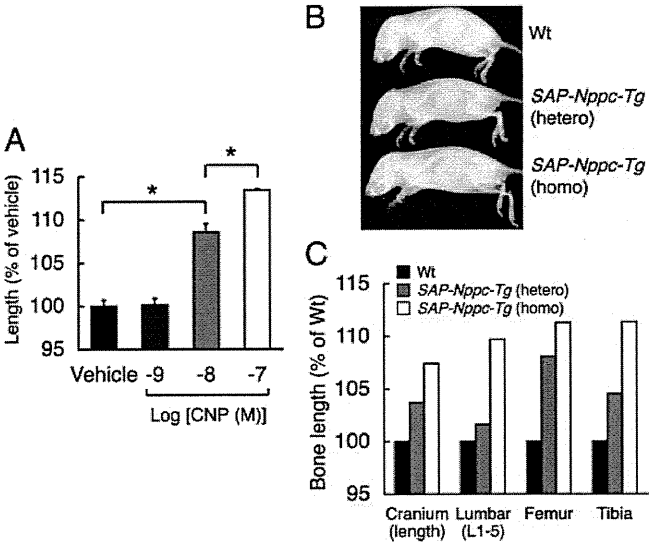

FIG. 6. Dose-dependent effect of circulating CNP on endochondral bone growth. A, Dose-dependent effect of addition of CNP on the growth of tibial explants from $Nppc^{-/-}$ mice in organ culture. The graph indicates percent of the longitudinal length of tibial explants incubated with indicated doses of CNP compared with that with vehicle, at the end of the 4-d culture period. *, $P < 0.05$. B, Soft x-ray picture of 3-wk-old wild-type (Wt) and *SAP-Nppc-Tg* mice with heterozygous (hetero) and homozygous (homo) *SAP-Nppc-Tg* transgene. Note that the nasoanal length is increased in accordance with the copy number of the transgene. C, The graph indicating percent of the length of each bone of heterozygous (*gray bar*) or homozygous (*white bar*) *SAP-Nppc-Tg* mice compared with that of wild-type mice (*black bar*) [n = 2 (Wt), four (hetero), and four (homo), each].

could scarcely find out the difference in the state of apoptosis of the growth plate chondrocytes between that in $Nppc^{-/-}$/*SAP-Nppc-Tg* and that in $Nppc^{-/-}$ tibias by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining (data not shown).

To further confirm whether the *SAP-Nppc*-transgene product humorally affects the endochondral bone growth in $Nppc^{-/-}$ mice, organ culture experiments using tibial explants from neonatal $Nppc^{-/-}$/*SAP-Nppc-Tg* and $Nppc^{-/-}$ mice were performed. At the end of the 4-d culture period, longitudinal length of tibial explants from $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was not changed from that from $Nppc^{-/-}$ mice (Fig. 5A). Histological analyses revealed that the widths of both nonhypertrophic and hypertrophic chondrocyte layers of the growth plate, expressing type II and type X collagens, respectively, were not different between in $Nppc^{-/-}$/*SAP-Nppc-Tg* and $Nppc^{-/-}$ explants (Fig. 5B). Neither the differentiation (estimated by *in situ* hybridization analyses for type II and type X collagens, Fig. 5B) nor the proliferation (evaluated by PCNA analysis, labeling index: 41.0 ± 3.3 *vs.* 44.9 ± 3.0%) of the growth plate chondrocytes was different between that in $Nppc^{-/-}$/*SAP-Nppc-Tg* and that in $Nppc^{-/-}$ explants.

To investigate whether the stimulatory effect of circulating CNP on the endochondral bone growth of $Nppc^{-/-}$ mice is dose dependent, we studied the effect of CNP on the growth of tibial explants from neonatal $Nppc^{-/-}$ mice in organ culture experiment. As shown in Fig. 6A, the growth of tibial explants from $Nppc^{-/-}$ mice was stimulated by addition of CNP in a dose-dependent manner. Furthermore, we generated *SAP-Nppc-Tg* mice with homozygous *SAP-Nppc* transgene to confirm a dose-dependent effect of circulating CNP on endochondral bone growth *in vivo*.

At the age of 3 wk, soft x-ray analyses revealed that the longitudinal body length and the growth of every bone formed through endochondral bone growth were promoted in accordance with the copy number of *SAP-Nppc* transgene, indicating the dose-dependent effect of circulating CNP on endochondral bone growth *in vivo* (Fig. 6B). Collectively, these results suggest that circulating CNP would cure the impaired skeletal growth of $Nppc^{-/-}$ mice in a dose-dependent manner *in vivo*.

Effects of increased circulating CNP on the body weight gain and the survival rate of $Nppc^{-/-}$ mice

We also investigated the effects of circulating CNP on other aspects of the impaired growth of chondrodysplastic $Nppc^{-/-}$ mice. The body weight of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice was smaller than that of their wild-type littermates and was comparable with that of their $Nppc^{-/-}$ littermates at the age of 1 wk (Fig. 7A). However, $Nppc^{-/-}$/*SAP-Nppc-Tg* mice gradually became heavier

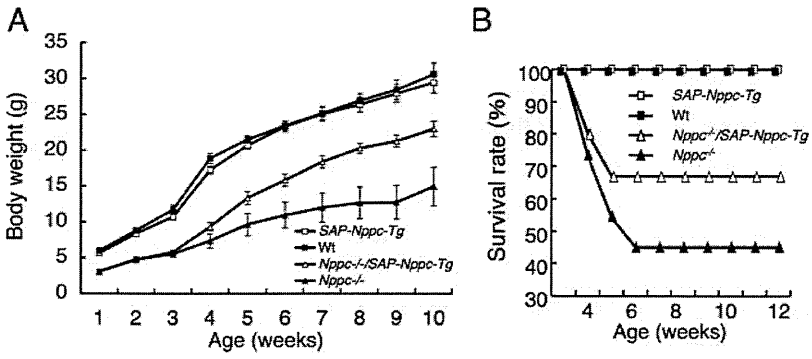

FIG. 7. Effect of circulating CNP on the body weight and the survival rate of $Nppc^{-/-}$ mice. A, Growth curves of body weight of *SAP-Nppc-Tg* (*open square*), wild-type (Wt; *closed square*), $Nppc^{-/-}$*/SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice. B, Survival curves of *SAP-Nppc-Tg* (*open square*), wild-type (*closed square*), $Nppc^{-/-}$*/SAP-Nppc-Tg* (*open triangle*), and $Nppc^{-/-}$ (*closed triangle*) mice.

than their $Nppc^{-/-}$ littermates (Fig. 7A), and the body weight of $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was significantly larger than their wild-type littermates at the age of 4 wk in males and 3 wk in females (males: 9.3 ± 0.5 g and 7.3 ± 0.9 g, respectively, n = 12 and 7 each, $P < 0.05$, and females: 5.4 ± 0.1 g and 4.7 ± 0.2 g, respectively, n = 11 and 12 each, $P < 0.05$). On the other hand, there was no difference in body weight between the *SAP-Nppc-Tg* and wild-type mice, albeit *SAP-Nppc-Tg* mice became larger than the wild-type mice in nasoanal length (Figs. 2A and 6).

We have previously reported that the survival rate of $Nppc^{-/-}$ mice greatly drops before adulthood, albeit the genotype ratio of $Nppc^{-/-}$ mice on d 16.5 of pregnancy is in accord with Mendelian proportion (1). In this study, analysis of intercrosses between $Nppc^{+/-}$*/SAP-Nppc-Tg* mice and $Nppc^{+/-}$ mice revealed that the genotype ratios of wild type to $Nppc^{+/-}$ to $Nppc^{-/-}$ and *SAP-Nppc-Tg* to $Nppc^{+/-}$*/SAP-Nppc-Tg* to $Nppc^{-/-}$*/SAP-Nppc-Tg* at weaning (3 wk of age) are 1:2.78:1 and 1:2.71:1.24 (total n = 104 and 110), respectively, indicating expected Mendelian proportions. As have we previously reported, the survival rate of $Nppc^{-/-}$ mice dropped to about 40% before adulthood (Fig. 7B). However, the survival rate of $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was greatly improved compared with that of $Nppc^{-/-}$ mice (Fig. 7B).

Discussion

In the present study, we investigated the endocrine effects of CNP on chondrodysplastic CNP knockout mice by using *SAP-Nppc-Tg* mice.

In the organ culture experiments, the growth of *SAP-Nppc-Tg* tibias was not changed from that of wild-type tibias, whereas the growth of *Col2-Nppc-Tg* tibias was strongly promoted compared with that of wild-type tibias. This result confirms that the growth stimulating effect of bones formed through endochondral ossification in *SAP-Nppc-Tg* mice is not autocrine/paracrine but endocrine effect of CNP, which is produced by the *SAP-Nppc* transgene. Because we expected that we would observe the effect of circulating CNP on endochondral bone growth clearly in a state without basal CNP effect, we then investigated whether or not elevation of circulating CNP could recover the impaired endochondral bone growth caused by depletion of CNP in mice *in vivo*. Decreased width of the growth plate observed in $Nppc^{-/-}$ mice was recovered in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice, and accordingly, impaired endochondral bone growth observed in $Nppc^{-/-}$ mice was considerably and significantly recovered in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice.

The endocrine effect of CNP produced by the *SAP-Nppc* transgene in $Nppc^{-/-}$*/SAP-Nppc-Tg* mice was further confirmed by the organ culture experiments in that the growth of $Nppc^{-/-}$*/SAP-Nppc-Tg* tibias was not changed from that of $Nppc^{-/-}$ tibias. These results clearly indicate that CNP can humorally affect endochondral bone growth. Furthermore, the result of the organ culture experiment using $Nppc^{-/-}$ bones (Fig. 6A) and the gene-dose effect of *SAP-Nppc* transgene on bone growth *in vivo* (Fig. 6B, C) suggest that the endocrine effect of CNP on endochondral bone growth is dose dependent.

Chondrodysplasia is composed of many different forms of genetic disorders characterized by impaired endochondral bone growth (7, 17). Because the CNP/GC-B system plays a crucial role in endochondral bone growth, loss of function mutations in the genes coding for molecules related to the CNP/GC-B system could cause chondrodysplasia. In fact, recent studies have revealed that mutations in the gene encoding human GC-B cause one form of chondrodysplasia, acromesomelic dysplasia type Maroteaux (18, 19).

In mice, loss of function mutations in the GC-B gene cause impaired skeletal growth in spontaneous mutant *cn/cn* and short-limbed dwarfism (*slw/slw*) mice (20, 21). As for spontaneous mutations in other genes related to the CNP/GC-B system, a mutation in the gene coding for cGMP-dependent protein kinase type II, an important downstream mediator of the CNP/GC-B system, causes impaired endochondral bone growth in Komeda miniature rat Ishikawa (22, 23). Furthermore, recent studies have elucidated that a spontaneous loss of function mutation in the murine CNP gene causes impaired skeletal growth observed in the long bone abnormality (*lbab/lbab*) mice (24–26).

Just as in the case with rodents, any forms of human chondrodysplasia might be caused by mutations in the cGMP-dependent protein kinase type II or CNP gene, albeit they are not yet discovered. In case a form of human chondrodysplasia caused by a mutation in the CNP gene is discovered in future, CNP knockout mice would be a novel mice model of human chondrodysplasia. On the other hand, spontaneous GC-B mutant (*cn/cn* and *slw/slw*) mice and GC-B knockout mice are regarded as mice models of acromesomelic dysplasia type Maroteaux, and impaired skeletal growth of these mice would not be recovered by crossing them with *SAP-Nppc-Tg* mice. This notion is supported by the result of the organ culture experiment, in which tibial explants from fetal GC-B knockout mice are not increased in length by addition of CNP (4).

We previously reported that the impaired skeletal growth of achondroplastic model mice was almost completely recovered by crossing them with *SAP-Nppc-Tg* mice (16). The impairment of skeletal growth of the achondroplastic model mice that we used in our previous study was considerably mild compared with that of $Nppc^{-/-}$ mice: the nasoanal length of the achondroplastic model mice was about 10% shorter than that of wild-type mice at the age of 10 wk (14), whereas the nasoanal length of $Nppc^{-/-}$ mice was about 30% shorter than that of wild-type mice. The reason that the impaired skeletal growth of $Nppc^{-/-}$ mice was not completely rescued in $Nppc^{-/-}$/*SAP-Nppc-Tg* mice in our present study might be because the low graded elevation of the plasma CNP concentrations in *SAP-Nppc-Tg* mice (about 1.8 times higher than those in wild-type mice) was not sufficient for the complete rescue of severe skeletal phenotype of $Nppc^{-/-}$ mice, whereas it was enough to cure the mild skeletal impairment of the achondroplastic model mice. Although about 2 times of elevation of plasma CNP concentrations can stimulate bone growth in *SAP-Nppc-Tg* mice (14) or human with a chromosomal translocation (27), higher plasma concentration of CNP might be needed for the complete treatment of impaired bone growth in chondrodysplasia.

As for the mechanism of the skeletal rescue of CNP knockout mice by circulating CNP, the differentiation and the proliferation of the growth plate chondrocytes of $Nppc^{-/-}$/*SAP-Nppc-Tg* mice were not changed from those of $Nppc^{-/-}$ mice. This result coincides with our previous observation that CNP does not so strongly affect differentiation and proliferation of the growth plate chondrocytes *in vivo* (5, 14). On the other hand, proteoglycan synthesis is greatly increased in the growth plate of *SAP-Nppc-Tg* mice (14), so we speculate that the shortened $Nppc^{-/-}$ growth plate is restored by circulating CNP in $Nppc^{-/-}$/*SAP-Nppc-Tg* mice through the recovery of matrix synthesis, resulting in the recovery of endochondral bone growth.

The impaired growth of $Nppc^{-/-}$ mice was recovered in not only longitudinal length but also body weight, and furthermore, the mortality of $Nppc^{-/-}$ mice was greatly decreased, by circulating CNP. Together with our previous results that targeted overexpression of CNP in the cartilage of $Nppc^{-/-}$ mice improved not only their impaired longitudinal growth but also their impaired body weight gain and that prolonged their survival (1), we consider that the recovery from the impaired endochondral bone growth in $Nppc^{-/-}$ mice by circulating CNP resulted in the recovery of overall growth and also in longevity. The mechanisms through which recovery in skeletal growth results in the recovery of overall growth and the prolonged survival are not yet elucidated. One of the possibilities is that the malformation in the maxillofacial region of $Nppc^{-/-}$ mice, which is caused by impaired endochondral ossification, may disturb their teeth coming together correctly: this condition may prevent them from eating enough and lead them to malnutrition. Further investigation of the craniofacial phenotype of $Nppc^{-/-}$ mice is now ongoing in our laboratory (Nakao, K., Y. Okubo, N. Koyama, K. Osawa, M. Miura, A. Yasoda, K. Nakao, and K. Bessho, manuscript in preparation).

In conclusion, we have revealed that circulating CNP rescues the impaired growth and early death of chondrodysplastic CNP knockout mice through the recovery of endochondral bone growth. We have started to apply the strong stimulatory effect of the CNP/GC-B system on endochondral bone growth to the treatment of chondrodysplasias (16) for those no effective drug therapy is available to date. The results of our present paper suggest that systemic administration of CNP or its analog, which would stimulate GC-B, might have therapeutic potential against not only impaired skeletal growth but also other aspects of impaired growth including impaired body weight gain in patients suffering from chondrodysplasias and might resultantly protect them from their early death.

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