

Roles of cGMP-dependent protein kinase I (cGKI) and PDE5 in the regulation of Ang II-induced cardiac hypertrophy and fibrosis

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Conflicting results have been reported for the roles of cGMP and cGMP-dependent protein kinase I (cGKI) in various pathological conditions leading to cardiac hypertrophy and fibrosis. A cardioprotective effect of cGMP/cGKI has been reported in whole animals and isolated cardiomyocytes, but recent evidence from a mouse model expressing cGKI β only in smooth muscle (β RM) but not in cardiomyocytes, endothelial cells, or fibroblasts has forced a reevaluation of the requirement for cGKI activity in the cardiomyocyte antihypertrophic effects of cGMP. In particular, β RM mice developed the same hypertrophy as WT controls when subjected to thoracic aortic constriction or isoproterenol infusion. Here, we challenged β RM and WT (Ctr) littermate control mice with angiotensin II (AII) infusion (7 d; 2 mg·kg⁻¹·d⁻¹) to induce hypertrophy. Both genotypes developed cardiac hypertrophy, which was more pronounced in Ctr animals. Cardiomyocyte size and interstitial fibrosis were increased equally in both genotypes. Addition of sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, in the drinking water had a small effect in reducing myocyte hypertrophy in WT mice and no effect in β RM mice. However, sildenafil substantially blocked the increase in collagen I, fibronectin 1, TGF β , and CTGF mRNA in Ctr but not in β RM hearts. These data indicate that, for the initial phase of AII-induced cardiac hypertrophy, lack of cardiomyocyte cGKI activity does not worsen hypertrophic growth. However, expression of cGKI in one or more cell types other than smooth muscle is necessary to allow the antifibrotic effect of sildenafil.

PKGI | PDE | cardiac failure | hypertension | NO/cyclic GMP system

Cyclic GMP-dependent protein kinase I (cGKI) is expressed in a wide variety of cells including cardiomyocytes (CMs), cardiac myofibroblasts (CMFs), cardiac fibroblasts (CFs), endothelial cells (ECs), and smooth muscle cells (SMCs) (1). Increased cGKI activity has been reported to protect against cardiac hypertrophy induced by pressure overload (2, 3). For example, mice that carry a mutated form of cGKI unable to interact with downstream targets, develop increased pathologic hypertrophy, accelerated mortality, and congestive heart failure when subjected to thoracic aorta constriction (TAC) (4).

Hypertrophy induced by angiotensin II (AII) is thought to be attenuated by cGKI, because AII signaling through G_{q/11} is abrogated by the regulator of G-protein signaling protein (RGS), a known substrate of cGKI (5). However, selective deletion of the AII receptor 1 (AT-R1) in the kidney ameliorated AII-induced cardiac hypertrophy, suggesting that the cardiac AT-R1 is dispensable for the induction of this response (6), whereas transgenic mice that overexpress the human AT-R1 specifically in CMs develop hypertrophy, fibrosis, dysfunctions, and early death (7). Furthermore, activation of TRPC channels followed by elevated [Ca²⁺]_i may contribute to the induction of the cardiac

hypertrophy gene response (8–11). Again, TRPC3 and TRPC6 channels are negatively regulated by cGKI (12–14).

It also has been reported that particulate guanylyl cyclase A, the major receptor for atrial natriuretic peptide (ANP) in the heart, couples to and directly activates the TRPC3/C6 channels in chronic cardiac hypertrophy, thus bypassing cGMP and cGKI (15). It was reported that cardiac cGMP can affect cardiac properties by modulating cAMP levels, bypassing again cGKI (16, 17). Thus, the site(s) of action of cGMP/cGKI are not entirely clear (18) and interpretation of cGMP/cGKI effects on cardiac hypertrophy may be quite complicated.

Sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, elevates cardiac cGMP at high doses (100 mg·kg⁻¹·d⁻¹), increases cGKI activity, and has been reported to reverse TAC-induced cardiac hypertrophy (2). This effect is postulated to be caused by an increased activity of RGS2 (19), and in part by the direct regulation of TRPC3/6 conductance by cGKI (11, 14) mentioned above. However, two clinical studies did not report positive therapeutic results after prolonged treatment of diastolic dysfunction with sildenafil (20, 21).

We (22) and others (23) have tested the hypothesis that CM-cGKI attenuates cardiac hypertrophy by using the cGKI β rescue mouse line (β RM). These animals express the cGKI β isozyme under the SM22 α promoter, which is active principally in SMCs and activated CMFs, but do not express cGKI β in other cell types

Significance

It has been reported that elevation of cGMP and activation of cGMP-dependent protein kinase I (cGKI) by inhibition of PDE5 activity with sildenafil prevents cardiac hypertrophy. We studied the roles of cGKI and PDE5 inhibition on angiotensin (AII)-induced hypertrophy and fibrosis using control (Ctr) mice and mice expressing cGKI only in cells where the smooth muscle SM22 α promoter is active (β RM). In Ctr mice, sildenafil did not reduce the AII-induced increase of cardiomyocyte (CM) size or myocyte hypertrophic markers but did reduce fibrosis. In β RM mice, sildenafil had little or no effect on markers of fibrosis. These results show that the sildenafil/cGMP/cGKI cascade can have an inhibitory effect on cardiac fibrosis but not on CM hypertrophy.

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(24). Chronic infusion of isoproterenol or TAC induced in β RM mice a cardiac hypertrophy that was identical to that of WT littermate controls (22), a result that argues against the hypothesis that CM, EC, or CF cGKI is responsible for the antihypertrophic effects of cGMP in the heart. To examine the role(s) of sildenafil to inhibit cardiac hypertrophy and fibrosis, we now extend these experiments using AII-induced hypertrophy and high concentrations of sildenafil (0.6 mM) in the drinking water. Once again, the lack of cGKI in CM and CF did not cause an increased hypertrophic response as predicted (2). Moreover, little or no antihypertrophic effect of sildenafil was observed in the WT mice and no effect was seen in the β RM mice. However, a large effect of sildenafil was observed on fibrosis in WT but not the β RM mice, suggesting that cGKI present in cardiac SMCs or activated CMFs is an important regulator of cardiac fibrosis.

Results

The cGKI β rescue mouse line (β RM) expresses the cGKI β isozyme under the SM22 α promoter, which is active principally in SMCs and activated CMFs, but does not express cGKI β in other cell types (24). As previously reported (25), β RM mice are leaner than their control (Ctr) littermates (Fig. S1). However, the lack of cGKI in tissues other than smooth muscle does not affect the basic morphology and function of the heart of β RM mice (see below; Fig. S1 and refs. 22 and 24).

All Induces Cardiac Hypertrophy in the Absence and Presence of Sildenafil. We exposed β RM and littermate Ctr mice to a 7-d continuous infusion of AII (2 mg·kg⁻¹·d⁻¹), a concentration known to establish chronic hypertension and cardiac hypertrophy (26). In addition, a subgroup of animals was treated with the PDE5 inhibitor sildenafil, administered through the drinking water at a concentration of 400 mg/L (0.6 mM). In some animals of both genotypes, systemic blood pressure was monitored before and during AII treatment using in vivo telemetry (Ctr: from 105

to 148 mmHg after AII, $n = 5$; β RM: from 105 to 153 mmHg after AII, $n = 5$; Fig. 1A). Median arterial blood pressure increased to the same extent within the first 24 h after implantation of the AII-delivering minipumps (Fig. 1B).

Cardiac hypertrophy was evaluated at the end of the 7-d AII infusion. Fig. 1C (see also Table S1) shows the total heart weight/tibia length ratio in the different groups of Ctr and β RM mice. As expected, infusion of AII triggered an increase in the cardiac mass in both genotypes. In the β RM mice, which have no cGKI in their CMs, this increase was not greater but if anything slightly less prominent. This lack of an increase in the β RM mice also can be seen when the heart mass of the AII-treated animals is normalized on the average mass of the corresponding control group (Fig. 1D). This result suggests, in agreement with our previous findings (22), that the absence of cGKI in the CMs and ECs of the heart does not increase the extent of cardiac hypertrophy, at least under normal physiological conditions.

Coadministration of sildenafil (0.6 mM) in the drinking water during the AII infusion had no effect in β RM mice and only slightly reduced the hypertrophic response in WT mice, without reaching statistical significance (Fig. 1C). This concentration yields an approximate plasma level of 70 nM sufficient to greatly inhibit PDE5 (27). Application of the same dose mixed in the food yielded plasma levels of 10 nM and reversed TAC-induced cardiac hypertrophy (2). A parallel experiment was conducted on a separate cohort of WT mice that were infused with AII and treated with sildenafil by single daily administration via oral gavages (100 mg·kg⁻¹·d⁻¹). In this group, nearly identical results were seen (Fig. S2C).

β RM mice are more fragile and about 40% of the β RM mice died during the 7-d AII infusion, whereas almost all Ctr mice survived. Coadministration of sildenafil did not alter the survival trends (Fig. S24). Control groups for both β RM and WT animals, which received infusion of vehicle solution, also did not show any difference in survival rate, which were always above

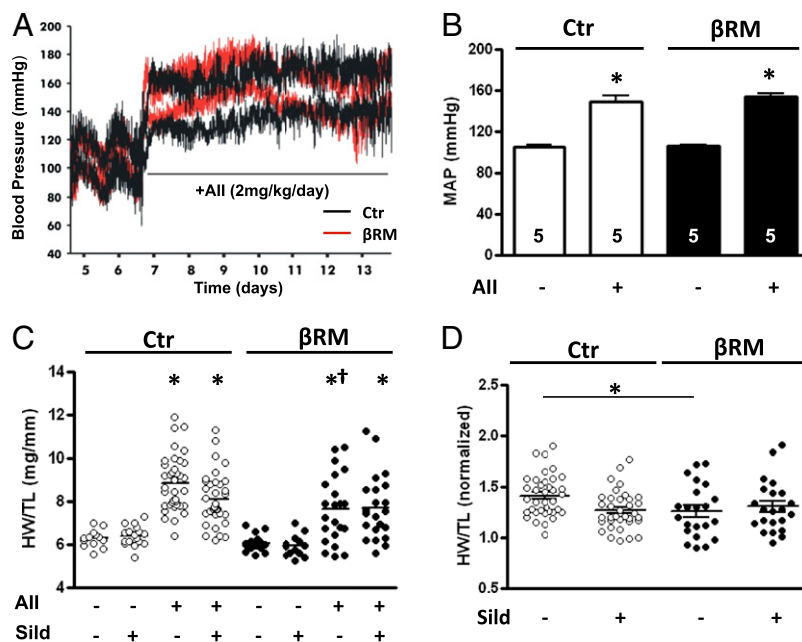


Fig. 1. Effects of AII and sildenafil administration. (A) Example of systolic and diastolic blood pressure measurement by telemetry recording and (B) average median arterial pressure (MAP) before and after AII infusion ($n = 5$ in all groups; $*P < 0.05$ vs. Ctr group, one-way ANOVA). (C) Scatter plot diagram of measured heart mass as total heart weight/tibia length (HW/TL) ($*P < 0.05$ vs. corresponding Ctr group; $^{\dagger}P < 0.05$, β RM All vs. Ctr All; one-way ANOVA with Tukey's multiple-comparison test; bars and whiskers represent median and SEM, respectively). For this ANOVA test, all eight groups were included. ANOVA test only for the four Ctr groups yielded a significance of $P < 0.01$ for Ctr plus All vs. Ctr plus All plus sildenafil. The same ANOVA test for β RM mice did not change the significance value. (D) The same values had been normalized on the average respective control group value ($*P < 0.05$, one-way ANOVA with Tukey's multiple-comparison test).

90% both in the presence or absence of sildenafil. Several different animal models were tested to unravel the cause of the death of the β RM mice during the AII infusion (see *SI Text* for details and *Figs. S2–S4*). The most likely cause was that hypertension activated platelets and thrombus formation (28, 29) and led to intravascular thrombi.

All Infusion Induces Similar CM Size and Interstitial Fibrosis and Does Not Affect Cardiac Contractility in WT and β RM Mice. We studied the functional consequences of the experimental conditions on cardiac activity by monitoring heart contraction with echocardiography on the seventh day of AII treatment. Fractional shortening (%FS) did not vary significantly between genotypes either before AII treatment (FS in percentage: Ctr, 39.90 ± 1.87 , vs. β RM, 40.97 ± 1.70 ; *Fig. 2A*) or after the 7-d AII infusion (*Fig. 2B*). The %FS showed a tendency toward an increase in Ctr animals infused with AII that did reach significance in the sildenafil-treated group (for Ctr AII-infused group ANOVA gave a *P* value of 0.053). A more clear effect of increased blood pressure was measured on systolic and diastolic left ventricle diameters, both of which were significantly reduced in Ctr AII-treated groups (*Fig. 2C* and *D*). In β RM mice, ventricle diameters did not change upon AII treatment and consequently %FS did not vary. Sildenafil treatment showed no clear effect in any of the treated groups.

We then analyzed additional cardiac tissue samples. Ventricle CM cross-sectional area was measured after staining of plasma membranes with fluorophore-labeled wheat germ agglutinin. As shown in *Fig. 3*, AII infusions induced a marked increase in CM area, in a similar fashion for all treated groups. Concomitant sildenafil administration did not affect this increase, suggesting that the slight reduction in heart mass caused by sildenafil in Ctr mice is not due to a block of myocyte hypertrophy, and therefore may be caused by less intercellular matrix production. To test this hypothesis, another group of heart sections were stained with Sirius red to detect collagen deposition. Image analysis of the stained sections showed that AII infusion caused a marked but comparable deposition of interstitial collagen fibers in both

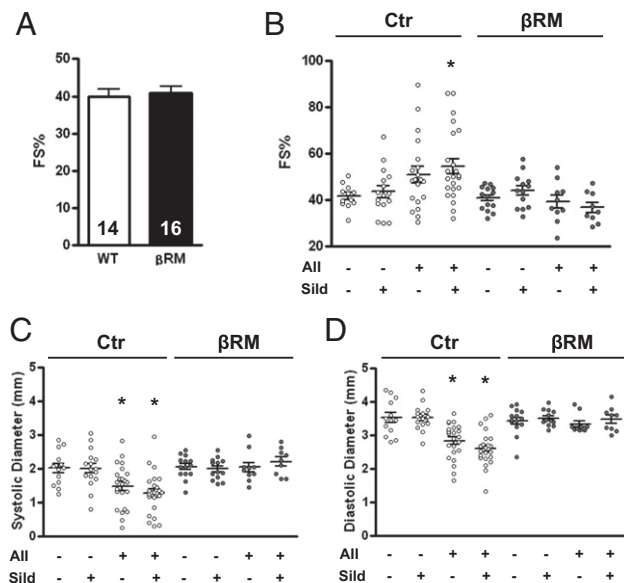


Fig. 2. Physiological parameters of the animals before and after treatment. Cardiac contractility as measured by echocardiography and expressed as percentage fractional shortening (FS%) in basal conditions (*A*) and in the experimental groups (*B*). Left ventricle chamber diameter during systole (*C*) and diastole (*D*) (**P* < 0.05 vs. corresponding Ctr group, one-way ANOVA with Tukey's multiple-comparison test).

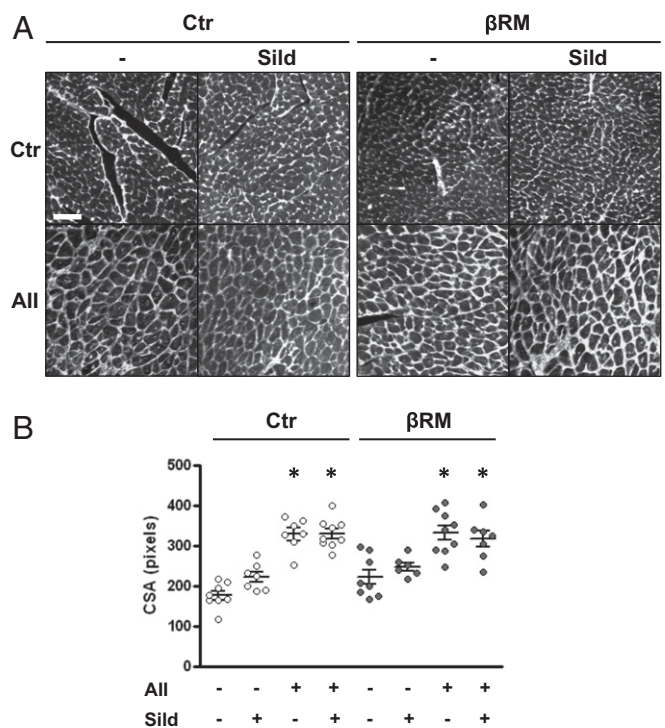


Fig. 3. CM cross-sectional area (CSA). Representative images from heart sections (left ventricle at 20 \times magnification) stained with wheat germ agglutinin (WGA) (*A*), and scatter plot of CSA (*B*). Bar and whiskers represent median and SEM, respectively. **P* < 0.05 vs. respective Ctr group; one-way ANOVA with Tukey's multiple-comparison test. (Scale bar: 50 μ m.) One pixel of the analyzed images is equivalent to 0.45 μ m.

Ctr and β RM mice (*Fig. 4*). Sildenafil appeared to reduce the average amount of collagen in Ctr hearts, although the difference did not reach statistical significance (*P* = 0.076) in unpaired *T* test or ANOVA. In β RM hearts, more collagen staining was seen in nearly all sections, but sildenafil had no effect on collagen deposition (*Fig. 4B*), suggesting that a nonsmooth muscle cell type (i.e., CMs, CFs, or ECs) are responsible for cGMP/cGKI-dependent regulation of AII-induced collagen deposition.

Sildenafil Treatment Decreases Fibrotic Gene Markers. Next, quantitative real-time PCR was performed on total heart RNA samples to measure transcription of several genes associated with hypertrophy and fibrosis (primers are shown in *Table S2*) (*Fig. 5*). ANP mRNA levels, a classic marker of maladaptive cardiac hypertrophy, were equally increased in both genotypes by AII, and this increase was maintained in the presence of sildenafil (*Fig. 5A*). Again, the lack of cGKI in the CM did not increase this marker of hypertrophy. Similarly, the β -myosin heavy-chain gene transcript was elevated by AII in Ctr and β RM mice, although in β RM the increase was statistically significant only in the sildenafil-treated group (*Fig. 5B*). PDE5 and α -myosin transcript levels also were comparable in all of the groups (*Fig. S5*). These data strongly suggest that CM cGMP/cGKI does not directly regulate CM hypertrophy under these AII-induced conditions.

Analysis of the transcription of four genes involved in the fibrotic response [two profibrotic hormones, namely connective tissue growth factor (CTGF), and transforming growth factor β (TGF- β), and two extracellular matrix proteins, fibronectin1 (Fn1) and collagen 1] showed a significant increase both in Ctr and β RM hearts. Sildenafil treatment lowered this increase in Ctr, but not in β RM mice (*Fig. 5C–E*). These findings indicate

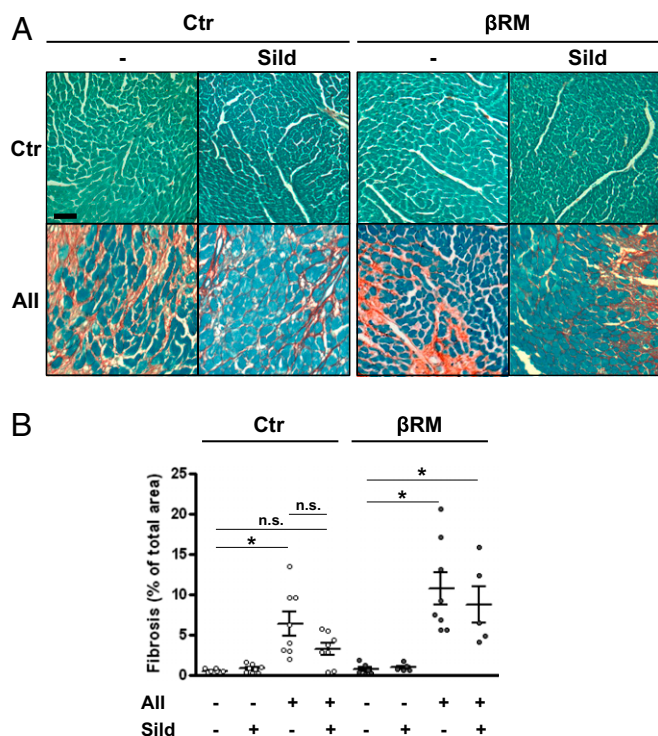


Fig. 4. All infusion-induced fibrosis in cardiac tissue. Representative images from heart sections (left ventricle at 20× magnification) stained with Fast Green and Sirius red to detect collagen deposition (A). The percentage fibrosis over the total section surface was determined by software-assisted image analysis (B). * $P < 0.05$, one-way ANOVA with Tukey's multiple-comparison test against value minus All. (Scale bars: 50 μm .)

that cGKI in CMs or CFs, or ECs is necessary for the inhibitory effect of sildenafil on the transcription of these profibrotic genes.

Discussion

It is known that chronically elevated AII levels and hypertension induce cardiac hypertrophy and fibrosis (26). This study investigated the effects of activation of cGKI on the initial adaptation of heart muscle to pressure overload. We challenged mice that were genetically engineered to ablate cGKI expression in all cells except those expressing the smooth muscle SM22 α promoter, because global KO of cGKI results in premature death of the animals in the first weeks after birth (30). Although it is very unlikely, we cannot rule out completely that some results are caused by undetected compensatory mechanisms.

The presented results can be summarized as follows: (i) AII infusion resulted in augmented cardiac mass in both Ctr and βRM mice. In contrast to expectation (2), the hypertrophy was not augmented in βRM mice (Fig. 1B). The $\beta\text{RM}/\text{AII}$ infusion model allows us to conclude that lack of cGKI in CMs, ECs, and CFs does not prevent the basal AII-induced hypertrophic response. (ii) Sildenafil has a modest effect in reducing AII-induced cardiac hypertrophy in Ctr mice and little or no effect in βRM mice. This implies that some cell type other than the smooth muscle that still expresses significant amounts of cGKI is required for a sildenafil response. (iii) Sildenafil treatment suppressed the AII-dependent elevated transcript levels of two profibrotic hormones and two extracellular matrix proteins in Ctr but not in βRM mice. This result strongly suggests the major effects of sildenafil on these parameters are due to effects outside of the SMCs and CMFs. (iv) Similarly to what has been observed with isoproterenol infusion and aortic constriction (22), AII-induced hypertrophy was not augmented and, if anything,

slightly reduced in βRM compared with WT mice despite an increase in fibrosis (Fig. 4). The mechanism leading to cardiac hypertrophy at this early stage was not elucidated (see *SI Text* and Figs. S5–S9).

In a previously published study (22), our group showed that βRM mice subjected to isoproterenol infusion (1 wk) or TAC (3 wk) develop the same degree of cardiac hypertrophy as their Ctr littermate controls. These observations and the results in this paper are in apparent contrast with the established hypothesis in the literature that activated cGKI serves as a general anti-hypertrophic brake in CMs. One likely possible explanation may be that the “basal” activity state of cGMP/cGKI has little effect as a break on hypertrophy unless there is also a strong tone of guanylyl cyclase. If so, it would imply that any therapy based on increased cGMP/cGKI will need to include a guanylyl cyclase activation component.

In this study, we treated mice with a high concentration of sildenafil, as used also by others (2, 3, 23). Although sildenafil had little effect on initial AII-induced cardiac hypertrophy, it showed a large effect on the profibrotic response as indicated by reductions of profibrotic and interstitial matrix gene expression in Ctr mice. It is well established that TGF- β is a key player in the profibrotic response of the heart to pressure overload and has an important role in the cross talk between CMs and CFs (31).

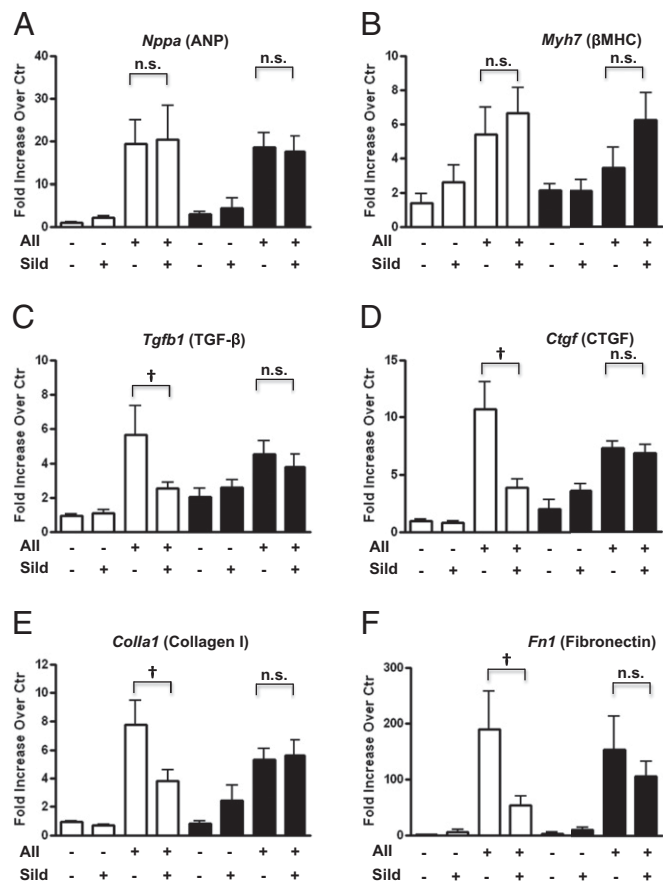


Fig. 5. mRNA expression levels of *Nppa* (ANP) (A), *Myh7* (βMHC) (B), *Tgfb1* (TGF- β) (C), *Ctgf* (CTGF) (D), *Colla1* (Collagen I) (E), and *Fn1* (Fibronectin) (F). mRNA expression level was normalized to 18s rRNA and then to corresponding Ctr group average, assessed by real-time RT-PCR. The white bars refer to Ctr groups, and the black bars to βRM groups. Values for All-treated animals are significantly different from Ctr animals at $P < 0.05$. † $P < 0.05$ vs. Ctr All group; n.s., nonsignificant. Statistics was done with one-way ANOVA with Tukey's multiple-comparison test. $n = 5\text{--}7$ for each group.

cGKI activity has been shown to suppress TGF- β -induced CF proliferation and differentiation into myofibroblasts (32). CTGF, whose expression is induced by TGF- β , is another important intracellular messenger that is essential for TGF- β -induced collagen synthesis by CFs (33). The antifibrotic effects of sildenafil were only observed in Ctr mice and not in β RM mice, which lack cGKI in CFs.

Both CMs and CFs can be a source of TGF- β , although CFs likely represent the main source in heart (34). It is therefore possible that a suppressive effect of sildenafil on TGF- β production is occurring in either or both of these cell types. However, it seems most likely that the main effect is occurring in CFs, since they express high levels of PDE5 (22), and at this early time point of the hypertrophic growth response, an induction of PDE5 has not yet occurred (Fig. S6). Sildenafil had no obvious effect in the β RM mice that express cGKI in SMCs.

Finally, other cell types such as resident macrophages and ECs might also be important in this regard. ECs express PDE5 and cGKI in Ctr mice but no cGKI in β RM mice. They are a particularly interesting candidate for the source of the antifibrotic response because they respond to increased cGMP with production of C-type natriuretic peptide (CNP), which is a known cardiac protective agent (35, 36). For all of these reasons, the data do not allow us to determine which cell type is most involved in the sildenafil effect other than to say it is not SMCs.

In conclusion, this study suggests that ablation of cGKI in CMs, CFs, and/or ECs does not by itself potentiate the

hypertrophic/fibrotic effect of AII under normal physiological conditions. The data also suggest that the major effect of sildenafil to reduce AII-induced hypertrophy/fibrosis is almost entirely on the fibrosis component of the response. Moreover, this antifibrotic effect is mediated by actions of sildenafil on one or more of the nonsmooth muscle cell types that express cGKI in the Ctr but not the β RM mice.

Materials and Methods

Experimental Animals. The strategy to create the β RM mouse line was described earlier (24). For all of the experiments performed, 10- to 17-wk-old male β RM mice (genotype: SM22 $\alpha^{+/b}$; cGKI $^{-/-}$) and littermate controls (Ctr; genotype SM22 $\alpha^{+/b}$; cGKI $^{+/+}$ or SM22 $\alpha^{+/+}$; cGKI $^{+/+}$) were used. All experimental procedures were conducted according to the local government's committee on animal care and welfare in Munich.

Statistical Analysis. GraphPad (Prism) software, version 4, was used for statistical analysis. If not otherwise indicated, all values are presented as the mean \pm SEM. To assess statistical significance, comparisons between groups, genotypes, and/or stimulation conditions were performed by using ANOVA or unpaired Student *t* test. In scatter plot diagrams, bar and whiskers represent mean value and SEM, respectively.

An extended and detailed materials and methods section is present in *SI Text*.

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