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Phosphodiesterase 5 inhibition blocks pressure overload-induced cardiac hypertrophy independent of the calcineurin pathway

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Aims Cyclic GMP (cGMP)-specific phosphodiesterase 5 (PDE5) inhibition by sildenafil (SIL) activates myocardial cGMP-dependent protein kinase G (PKG) and blunts cardiac hypertrophy. To date, the only documented target of PKG in myocardium is the serine–threonine phosphatase calcineurin (Cn), which is central to pathological cardiac hypertrophy. We tested whether Cn suppression is necessary in order to observe anti-hypertrophic effects of SIL.

Methods and results Mice lacking the Cn-Aβ subunit ($CnA\beta^{-/-}$) and wild-type (WT) controls were subjected to transverse aorta constriction (TAC) with or without SIL (200 mg/kg/day, p.o.) for 3 weeks. TAC-induced elevation of Cn expression and activity in WT was absent in $CnA\beta^{-/-}$ hearts, and the latter accordingly developed less cardiac hypertrophy (50 vs. 100% increase in heart weight/tibia length, $P < 0.03$) and chamber dilation. SIL remained effective in $CnA\beta^{-/-}$ mice, increasing PKG activity similarly as in WT, suppressing hypertrophy and fetal gene expression, and enhancing heart function without altering afterload. TAC-stimulated calcium–calmodulin kinase II, Akt, and glycogen synthase kinase 3β in both groups (the first rising more in $CnA\beta^{-/-}$ hearts), and SIL also suppressed these similarly. Activation of extracellular signal-regulated kinase observed in WT-TAC but not $CnA\beta^{-/-}$ hearts was also suppressed by SIL.

Conclusion PDE5A inhibition and its accompanying PKG activation blunt hypertrophy and improve heart function even without Cn activation. This occurs by its modulation of several alternative pathways which may result from concomitant distal targeting, or activity against a common proximal node.

1. Introduction

Cardiac hypertrophy induced by sustained pressure-overload is a major risk factor for heart disease and cardiovascular mortality world wide.[1] Such pathological hypertrophy is regulated by multiple cascades of kinase and phosphatase signalling,[2,3] and a prominent contributor is the calcium–calmodulin-dependent serine–threonine phosphatase calcineurin (Cn).[4,5] Cn consists of a 19 kDa regulatory subunit (CnB) and a 57–61 kDa catalytic subunit (CnA), and when activated it de-phosphorylates and thus triggers nuclear translocation of the transcription factor NFAT (nuclear factor of activated T-cells) stimulating hypertrophy and remodelling.[6,7] The mammalian heart expresses two isoforms of Cn, CnAα and CnAβ, with the latter regulating 80% of enzyme activity and the dominant mediator of hypertrophic signalling.[8,9]

Cn is negatively controlled by several enzymes that counter its pro-growth/hypertrophic effects, including the muscle-specific F-box protein atrogen-1,[10] z-band-binding protein calsarcin-1,[11,12] and regulator of calcineurin-1 (RCAN-1).[13,14] Another mechanism for Cn suppression occurs by the activation of cyclic GMP (cGMP)-dependent kinase (protein kinase G, PKG).[15] This pathway is particularly intriguing given the clinical availability of safe and effective drugs to stimulate it, such as nitric oxide donors, natriuretic peptides (NP), or inhibitors of cGMP catabolism.[16] Genetic deletion of NP-coupled cGMP synthesis exacerbates stress-induced hypertrophy[17] and enhances Cn activation,[18] whereas pharmacological or genetic activation of PKG in neonatal myocytes suppresses Cn-dependent NFAT activation and cellular hypertrophy.[15]

Recently, we reported that PKG activation can also be potently achieved in hearts subjected to sustained

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pressure-overload by inhibiting phosphodiesterase type 5 (PDE5) to blunt cGMP catabolism.[19] Such drugs (e.g. sildenafil, SIL) are widely used clinically to treat erectile dysfunction[20] and pulmonary hypertension,[21,22] but have not been previously thought to be important in heart muscle itself.[23–25] However, SIL therapy leads to a marked suppression of pathological hypertrophy/fibrosis and improves cardiac function, coupled to the downregulation of Cn and NFAT activation among other factors.[19] Although this supports prior cell-based evidence for a PKG suppressive effect on Cn signalling,[15] the centrality of this pathway *in vivo* remains unknown. Clarification of the mechanisms for PDE5 inhibitory effects has taken on greater importance given the multi-centre NIH-sponsored heart failure trial that was initiated in September 2008 (RELAX; http://clinicaltrials.gov/ct2/show/NCT00763867).

In this study, we tested the role of Cn suppression by SIL in a model of sustained pressure-overload, using mice lacking the *CnA*β gene with reduced Cn expression and activity.[8]

2. Methods

2.1 Animal models

Male wild-type (WT) C57BL6 mice and mice harbouring a global CnAβ subunit gene deletion (*CnA*$\beta^{-/-}$) on a C57BL6 background (4–6 months old) were used. Details of the genetic model were previously reported.[8] WT ($n = 10$) and *CnA*$\beta^{-/-}$ ($n = 13$) mice were exposed to pressure-overload with ($n = 5$ in WT, $n = 7$ in *CnA*$\beta^{-/-}$) or without ($n = 5$ in WT, $n = 6$ in *CnA*$\beta^{-/-}$) SIL treatment. Sham controls ($n = 5$ for each genotype) were subjected to the same surgery without aortic banding. Animals were studied 3 weeks following the surgery (echocardiography, haemo-dynamic study, histology, and molecular assays). Pressure-overload was produced by transverse aorta constriction (TAC) around a 26-gauge needle as described.[19] Vehicle or PDE5 inhibitor (SIL, 200 mg/kg/day) was mixed into rodent chow (Bioserv; 4–6 g/day).[19] The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

2.2 Echocardiography

In vivo cardiac morphology was assessed by transthoracic echocardiography (Acuson Sequoia C256, 13 MHz transducer, Siemens) in conscious mice.[19] M-mode left ventricular (LV) end-systolic and end-diastolic dimensions were averaged from 3–5 beats. LV mass was calculated as described.[19] Wall thickness of the lateral free wall and intra-ventricular septum were averaged. The studies were performed blinded as to heart condition or treatment.

2.3 *In vivo* haemodynamics

In vivo LV function was assessed by pressure–volume (PV) catheter method.[19,26,27] Briefly, mice were anaesthetized with 1–2% isoflurane, urethane (750–100 mg/kg, i.p.), etomidate (5–10 mg/kg, i.p.), and morphine (1–2 mg/kg, i.p.), underwent tracheostomy, and were ventilated with 6–7 μL/g tidal volume and 130 breaths/min. The LV apex was exposed through an incision between the seventh and eighth ribs, and a 1.4 Fr PV catheter (SPR 839, Millar Instruments, Inc.) advanced through the apex to lie along the longitudinal axis. Absolute volume was calibrated, and pressure-volume data measured at steady state as reported.[27]

2.4 Tissue histology

Formalin-fixed (10%) myocardium was analysed for myocyte hypertrophy and fibrosis.[19] Tissue was paraffin-embedded, sectioned into 5–8 μm slices, and stained with H&E or Masson's trichrome. Photomicrographs were quantified to assess mean cardiomyocyte diameter and interstitial collagen fraction using computer-assisted image analysis (Adobe Photoshop 5.0; Adobe, NIH ImageJ). Average data reflect results from four hearts in each group (more than 50 cells per heart).

2.5 Protein kinase G activity assay

PKG activity was assayed by colourimetric analysis (CycLex) using whole-heart lysates according to the manufacturer's instructions.[19]

2.6 Protein analysis

Protein was prepared from snap-frozen heart tissue as described previously.[19] Protein extracts were run on 4–12% Bis–Tris NuPage (Invitrogen) gels, blotted onto nitrocellulose membranes, and probed with primary antibodies: calcineurin Aα (CnAα), calcineurin Aβ (CnAβ), Ser473-phospho-Akt (Santa Cruz), Pan-CnA (Chemicon), Thr286-phospho-CaM kinase II (Affinity BioReagents), GSK3β, Ser9-phospho-GSK3β, ERK, Thr202/Thr204-phospho-ERK, Akt, and GAPDH (Cell Signaling Technology). Antibody binding was visualized by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescense (GE Healthcare).

Total protein (ERK, CaMKII, Akt, GSK3β) was assessed after stripping the same membrane used to detect phospho-protein. Blots were quantified using NIH ImageJ software, and ratios of phosphorylated/total protein calculated and normalized by WT-sham results.

2.7 Quantitative real-time polymerase chain reaction

Total RNA was extracted from snap-frozen heart tissue using TRIzol reagent (Invitrogen). The yield and purity of RNA were estimated

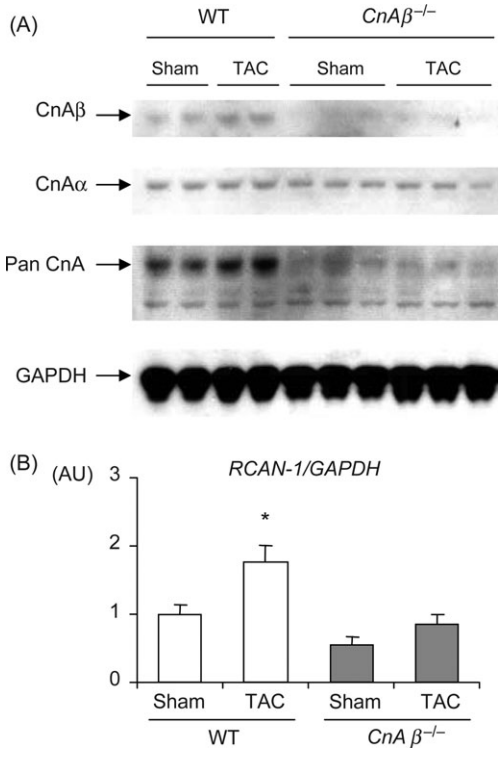

Figure 1 Upregulation of calcineurin (Cn) expression and activity by 3 weeks of transverse aorta constriction (TAC) in wild-type (WT) but not *CnA*$\beta^{-/-}$ hearts. (*A*) Western blot analysis using antibodies against the CnAα and CnAβ subunits, Pan Cn, and GAPDH. (*B*) *RCAN-1* mRNA expression normalized to *GAPDH* in wild-type sham. $n = 4$ per group. *$P < 0.05$ vs. wild-type sham.

spectrophotometrically using the A260/A280 ratio. One microgram of RNA was reverse transcribed into cDNA using SuperScript first-strand synthesis system (Applied Biosystems). Twenty-five nanograms of cDNA was subjected to polymerase chain reaction (PCR) amplification using TaqMan RT–PCR Master Mix reagent (Applied Biosystems). TaqMan primers and probes for *CnAα*, *CnAβ*, *RCAN-1*, and *GAPDH* were purchased from Applied Biosystems. Atrial natriuretic peptide (*ANP*) and beta-myosin heavy chain (*β-MHC*) mRNA gene expression was assessed using SYBRgreen primers as described.[28]

2.8 Statistical analysis

All values were expressed as mean ± SEM. Statistical analyses between the experimental groups were performed using Student's *t*-test or one-way ANOVA when comparing multiple groups. Two-way ANOVA was employed to analyse interaction between the genotypes. Significance was defined as $P < 0.05$.

3. Results

3.1 Upregulation of calcineurin expression and activity in wild-type but not *CnAβ*$^{-/-}$ mice by transverse aorta constriction

We first determined whether TAC-stimulated Cn expression and activity were indeed largely absent in *CnAβ*$^{-/-}$ mice. Though reduced basal expression had been previously demonstrated with this model,[8] whether it remained so after TAC had not been reported. TAC stimulated a

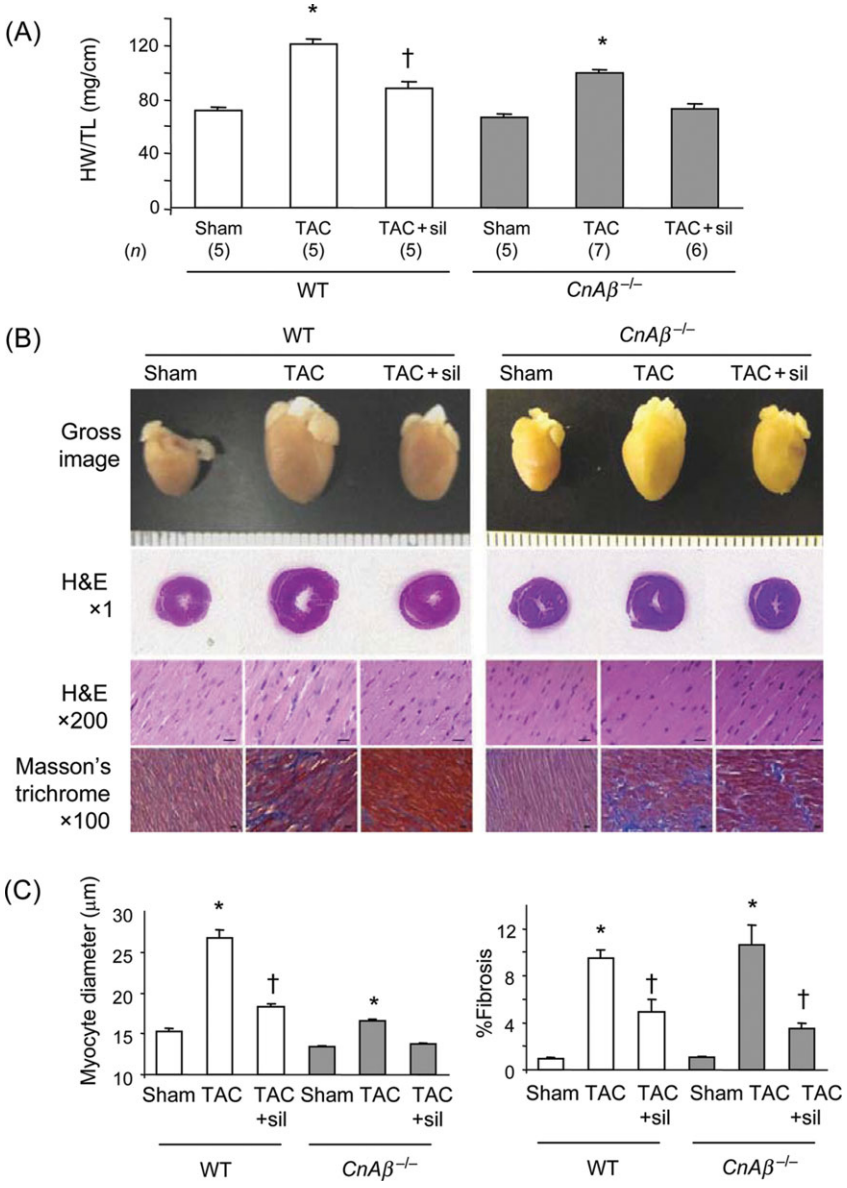

Figure 2 *CnAβ*$^{-/-}$ hearts develop a diminished form of hypertrophy that is inhibited by phosphodiesterase 5 inhibition. (*A*) Heart weight/tibial length ratios after 3 weeks of transverse aorta constriction (TAC) with or without sildenafil (sil) co-treatment. *n* indicates the number of animals. Two-way ANOVA [genotype: wild-type (WT) or *CnAβ*$^{-/-}$ × condition: sham or transverse aorta constriction] shows significant difference ($P < 0.03$). *$P < 0.001$ vs. sham and transverse aorta constriction + sildenafil within the same genotype, †$P < 0.01$ vs. sham (the same genotype). (*B*) Representative gross heart pictures (upper panels), H&E staining of heart cross-sections at ×1 and ×200 (middle panels), and representative Masson's trichrome staining of heart cross-sections (lower panels). Ruler scale, 1 mm; Black scale bar, 20 μm. (*C*) Summary quantification results of myocyte diameter and collagen fraction (bar graphs). $n = 4$ hearts per group, more than 50 myocytes per heart. Two-way ANOVA (genotype: wild-type or CnAβ$^{-/-}$ × condition: sham or transverse aorta constriction) shows significant difference ($P < 0.001$) in myocyte diameter, but not in fibrosis ($P = 0.6$). *$P < 0.01$ vs. sham and transverse aorta constriction + sildenafil (the same genotype), †$P < 0.01$ vs. sham (the same genotype).

two-fold increase in CnAβ protein expression in controls, but protein was barely detectable at rest and remained low after TAC in *CnA*$\beta^{-/-}$ mice (*Figure 1A*). TAC did not alter the expression of the other catalytic subunit (CnAα) in either genotype. The disparity in CnA expression was further confirmed using pan-sensitive antisera (*Figure 1A*). Cn activity was assessed by its stimulation of *RCAN-1* expression,[29] a Cn modulator stimulated by Cn-NFAT. *RCAN-1* mRNA rose nearly two-fold in controls but was not significantly altered in *CnA*$\beta^{-/-}$ mice (*Figure 1B*) ($n = 4$ per group).

3.2 Phosphodiesterase 5 inhibition blocks pressure-overload-induced hypertrophy in *CnA*$\beta^{-/-}$ hearts

To test whether PDE5-modulated suppression of Cn activation was necessary to observe anti-hypertrophic effects from the treatment, WT and *CnA*$\beta^{-/-}$ mice were subjected to 3 weeks of TAC. Consistent with earlier reports,[8] TAC induced half the magnitude of cardiac hypertrophy in *CnA*$\beta^{-/-}$ mice compared with controls ($P < 0.03$) (*Figure 2A*, $n = 5$–7 per group). However, SIL still was effective in suppressing this residual hypertrophic response. It blocked ~60% of the increase in cardiac mass in WT, and nearly 100% in the *CnA*$\beta^{-/-}$ mice. This was due both to decline in myocyte size and to interstitial fibrosis (*Figure 2B* and *C*, $n = 4$ hearts per group), the latter being similarly induced by TAC in *CnA*$\beta^{-/-}$ despite low Cn expression/activity, and equally suppressed by SIL (*Figure 2C*).

Echocardiography was performed in both groups (*Figure 3*, $n = 4$–7 per group), confirming a reduced hypertrophic response to TAC in *CnA*$\beta^{-/-}$ mice, but similar improvement from SIL treatment in both models (*Figure 3A* and *B*). *CnA*$\beta^{-/-}$ hearts responded to TAC with minimal chamber dilation (increase in end-diastolic volume), whereas WT controls developed significant dilation, which was inhibited by SIL (*Figure 3B*). *CnA*$\beta^{-/-}$ TAC hearts had reduced

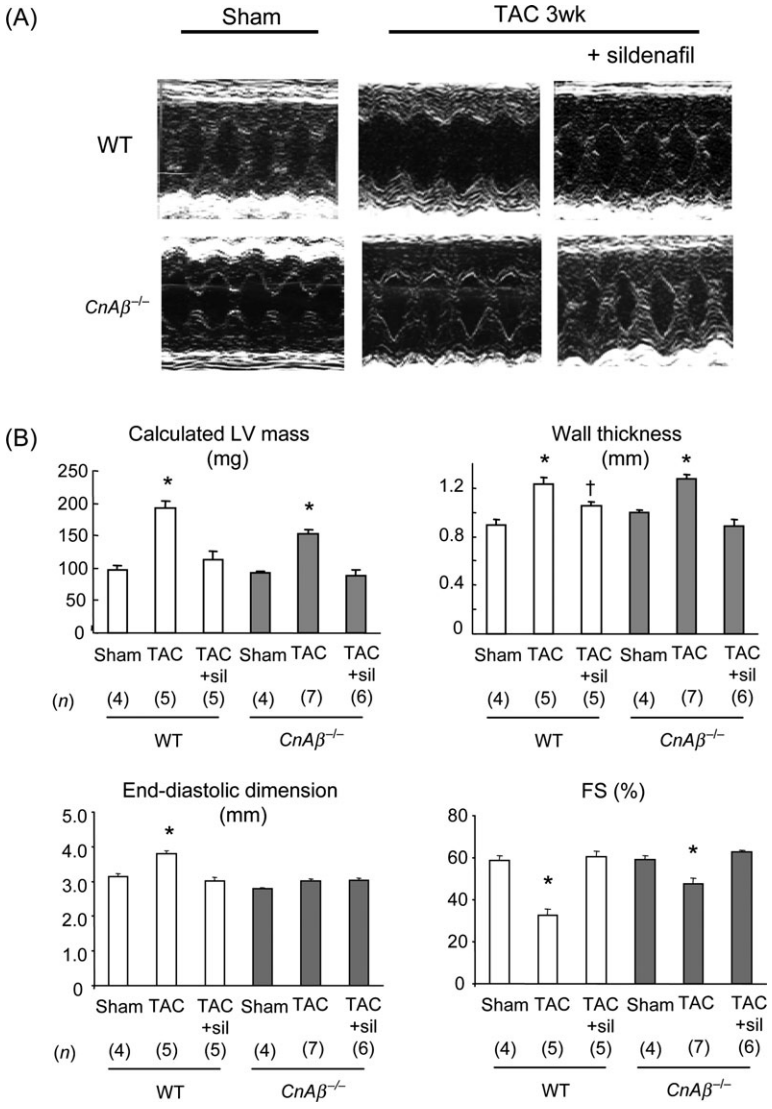

Figure 3 (*A*) Representative M-mode echocardiographic images after 3 week transverse aorta constriction (TAC) with or without sildenafil. (*B*) Parameters from echocardiographic studies. *n* indicates the number of animals. LV mass, left ventricular wall thickness, and fractional shortening (FS) are calculated as described in Methods. Two-way ANOVA [genotype: wild-type (WT) or CnA$\beta^{-/-}$ × condition: sham or transverse aorta constriction] shows significant difference ($P < 0.05$) in left ventricular mass, end-diastolic dimension, and fractional shortening, but not in left ventricular wall thickness ($P = 0.7$). *$P < 0.01$ vs. sham and transverse aorta constriction + sildenafil (the same genotype), †$P < 0.05$ vs. sham (the same genotype).

fractional shortening though this was less marked than in WT-TAC. SIL improved this functional change in both genotypes (*Figure 3B*).

SIL reduced TAC-induced myocardial *CnA*β gene but not *CnA*α expression in WT controls (the latter unchanged by TAC, *Figure 4A*; $n = 4$ per group). To confirm that SIL activated PKG in both models, myocardial PKG activity was assessed (*Figure 4B*; $n = 4$ per group). In controls, TAC resulted in the doubling of PKG activity, and SIL increased this further to nearly five-fold above baseline. In *CnA*$\beta^{-/-}$ hearts, there was a modest but significant rise in PKG activity with TAC alone, and similar net response (five-fold rise) with SIL. Thus, loss of Cn signalling did not alter the upregulation of PKG activity by SIL.

3.3 Phosphodiesterase 5 inhibition improves cardiac function in *CnA*$\beta^{-/-}$ hearts

To more specifically evaluate cardiac function, invasive PV analysis was performed (*Figure 5A* and *B*; $n = 3$–5 per group). Peak systolic pressure rose similarly with TAC in both groups and was unaltered by SIL (*Figure 5B*). This occurs as the primary mechanism contributing to ventricular afterload in the transverse aortic band, and any modest systemic vasodilation that can accompany PDE5 inhibition does not affect this. In WT hearts, TAC led to a right-ward shift of the loop to higher volumes, which was reversed by SIL (*Figure 5A*). There was less dilation in *CnA*$\beta^{-/-}$ mice, though SIL treatment still resulted in a left shift of end-systolic volumes, consistent with enhanced contractility (*Figure 5A*). Systolic function parameters such as cardiac output (CO) and the load-independent parameter (d*P* d*t*/IP) were enhanced despite persistent afterload increase by SIL in both groups (*Figure 5B*). Relaxation time constant (tau) was prolonged by TAC and normalized by SIL in both groups as well (*Figure 5B*).

3.4 Effect of sildenafil on hypertrophic signalling in wild-type vs. *CnA*$\beta^{-/-}$ hearts

Pressure-overload activates a gene transcription programme that includes fetal genes such as *ANP* and β-*MHC*. Both were suppressed by SIL treatment in normal hearts (*Figure 6A*, $n = 4$ per group). *CnA*$\beta^{-/-}$ hearts exposed to TAC displayed a blunted increase in *ANP* but similar rise in β-*MHC* expression. Both were also suppressed by SIL.

The blunted yet still significant hypertrophic response in *CnA*$\beta^{-/-}$ hearts exposed to TAC suggested that alternative pathways might be activated. Given prior data demonstrating the efficacy of SIL to suppress Akt, GSK3β, and ERK1/2 signalling,[19] we examined each of these, as well as Ca^{2+}/calmodulin-dependent kinase-II (CaMK-II). All of these kinases have been shown to contribute to hypertrophy development.[30–33] Importantly, basal phosphorylation of all four were similar in both control and *CnA*$\beta^{-/-}$ hearts (*Figure 6B* and *D*, $n = 4$ per group). Each became more phosphorylated after 3 week TAC in WT mice, and this was suppressed by SIL treatment (*Figure 6C* and *D*; $n = 4$ per group). In *CnA*$\beta^{-/-}$ hearts, CaMK-II was even more activated by TAC (10 vs. 2-fold), whereas Akt and GSK3β were similarly phosphorylated (*Figure 6C* and *D*). In contrast, ERK1/2 was not activated in *CnA*$\beta^{-/-}$ hearts, consistent with studies coupling its pathological activation to Cn.[34] It did increase in WT controls and was suppressed by SIL. These results show that despite the lack of Cn, pressure-overload still potently activates alternative cascades and these remain targeted by a PDE5 inhibition–PKG activation pathway.

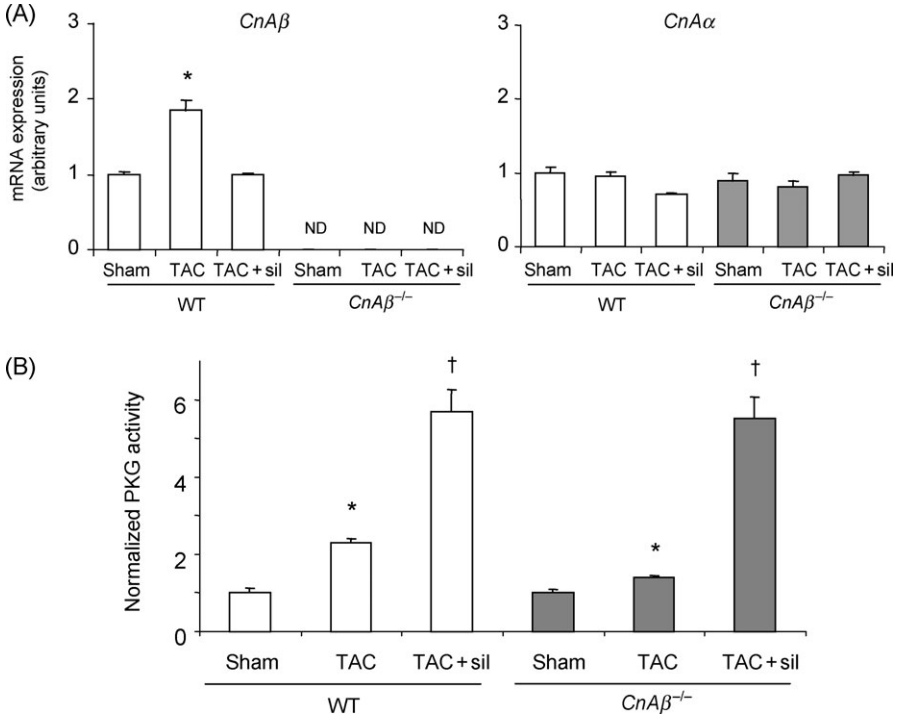

Figure 4 *CnA*α and *CnA*β mRNA expression and myocardial protein kinase G activity. (*A*) mRNA levels for *CnA*β and *A*α subunits, normalized to *GAPDH*, in wild-type (WT) and *CnA*$\beta^{-/-}$ hearts after sham, 3 weeks of transverse aorta constriction (TAC) with or without sildenafil co-treatment. $n = 4$ per group. *$P < 0.001$ vs. sham and transverse aorta constriction + sildenafil. ND, not detected. (*B*) Relative myocardial protein kinase G activity (normalized to sham). $n = 4$ per group. *$P < 0.05$ vs. sham (the same genotype), †$P < 0.01$ vs. transverse aorta constriction (the same genotype).

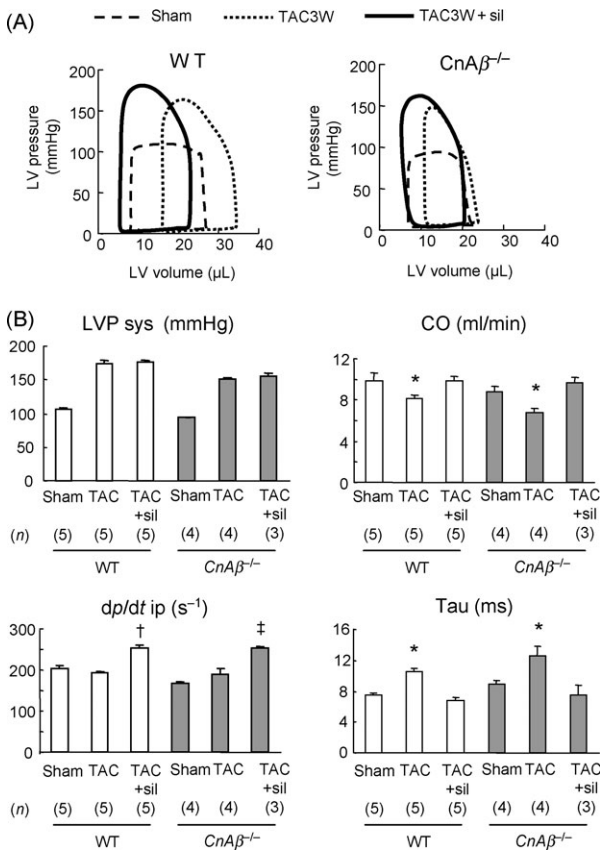

Figure 5 Phosphodiesterase 5 inhibition improves cardiac function in both wild-type (WT) and *CnA*$\beta^{-/-}$ hearts subjected to transverse aorta constriction (TAC). (*A*) Representative steady-state pressure–volume (PV) loops (average of 10 beats) in sham (dotted line), 3 week transverse aorta constriction (fine dotted line), and 3 week transverse aorta constriction + sildenafil animals (solid line). (*B*) LVP sys (peak systolic left ventricular pressure), CO (cardiac output), d*p* d*t* mx/IP (peak rate of pressure rise normalized to instantaneous pressure, or contractility), and tau (relaxation time constant) in wild-type and *CnA*$\beta^{-/-}$ mice subjected to sham or transverse aorta constriction with and without sildenafil. *n* indicates the number of animals. Two-way ANOVA (genotype: wild-type or CnA$\beta^{-/-}$ × condition: sham or transverse aorta constriction) shows significant difference ($P < 0.05$) in d*p* d*t* mx/IP, but not other parameters (LVP sys, CO, tau). $^{*}P < 0.05$ vs. sham and transverse aorta constriction + sildenafil (the same genotype), $^{\dagger}P < 0.05$ vs. sham and transverse aorta constriction (the same genotype), $^{\ddagger}P < 0.05$ vs. sham (the same genotype).

4. Discussion

Although cGMP-PKG signalling negatively regulates cardiac hypertrophy, the underlying mechanisms have remained unclear. Thus far, studies identified Cn as a primary target for anti-hypertrophic effects.[15] Here, we provide the first *in vivo* evidence that enhanced cGMP-PKG signalling can inhibit hypertrophy development even in hearts effectively lacking Cn activity.

Despite substantially reduced Cn activation by the deletion of the CnAβ subunit, hearts in our study developed a ∼50% increase in cardiac hypertrophy with TAC, about half that induced in controls. These results are consistent with earlier reports,[8] though the even greater suppression of hypertrophy in *CnA*$\beta^{-/-}$ mice previously observed may relate to a less severe stimulus employed (25% increase by pressure-overload in control animals). In the current study, *CnA*$\beta^{-/-}$ hearts developed non-dilative hypertrophy, but cardiac function was mildly though still significantly depressed and accompanied by differential activation of signalling cascades compared with WT hearts. For example, CaMKII activation was much more than in WT, suggesting a potentially greater role of this pathway in the absence of Cn activity.

SIL improved cardiac function in hearts subjected to TAC both with and without *CnA*β. Recent evidence has shown that Cn is not only a hypertrophic mediator but also a direct modulator of contractility via the modulation of phospholamban (PLB).[35,36] In normal mouse myocytes, Cn opposes PKA-mediated PLB phosphorylation, reducing intracellular and SR Ca^{2+}.[36] Acute Cn inhibition improves β-adrenergic responsiveness in myocytes isolated from the spontaneous hypertensive rat by improving PLB phosphorylation.[35] We have also found improved function and calcium transients in myocytes from TAC hearts treated by SIL, where Cn activity is also reduced.[37] However, the current study reveals SIL could enhance function even without targeting Cn, indicating alternative mechanisms also apply. One could be its inhibition of CaMKII. This would suppress hypertrophy by reducing CaMKII-stimulated histone deacetylase 4 nuclear export and subsequent MEF2 transcriptional activation,[38,39] but CaMKII also phosphorylates cardiac Ca^{2+} cycling proteins including the L-type Ca^{2+} channel, ryanodine receptor, IP3 receptor, and PLB,[40] and Cn overexpressing hearts also show activated CaMKII, which has been proposed to explain depressed function in this model.[41] Our data would support a potential role for CaMKII, but show its activation and de-activation can occur independent of Cn.

Akt and GSK3β, but not ERK1/2, are phosphorylated in *CnA*$\beta^{-/-}$ hearts in response to pressure-overload. These pathways contribute to physiological hypertrophy,[2,3] though with higher levels of phosphorylation as from sustained TAC, they can also lead to cardiac dysfunction and dilation. It is currently unknown whether inhibition of these pathways by SIL is direct or indirect reflection of its targeting against other pathological kinases. The cause for the lack of ERK1/2 phosphorylation in *CnA*$\beta^{-/-}$ hearts exposed to TAC is consistent with several prior studies that have coupled Cn and ERK1/2 activation. For example, hearts overexpressing Cn display ERK activation,[42] and sustained isoproterenol stimulation also activates ERK via the Cn pathway to induce cardiomyocyte hypertrophy.[43] Here we show the opposite: that TAC applied to hearts largely lacking Cn results in minimal ERK1/2 activation.

On the basis of the present findings, the mechanisms for PKG modulation of hypertrophy should be expanded beyond that linked to Cn signalling. PKG has been shown to modify calcium signalling by effects on the single L-type Ca^{2+} channel, though this was revealed in cell studies using an exogenous diffusible PKG activator.[15] Another mechanism may relate to proximal suppression of Gαq stimulation by PKG activation of the regulator of G-protein-signalling (RGS) proteins.[44] Recent data support such a mechanism for ANP-mediated anti-hypertrophic effects, targeting the signal via RGS4.[45] Whether PDE5-inhibition-mediated anti-hypertrophy also shares RGS4 mechanism remains to be clarified, given intracellular cGMP pool responding to PDE5 inhibition and ANP stimulation might be different.[27] Lastly, PKG can phosphorylate and blunt RhoA activation in smooth muscle,[46,47] and we have found similar suppression in the TAC-stressed hearts

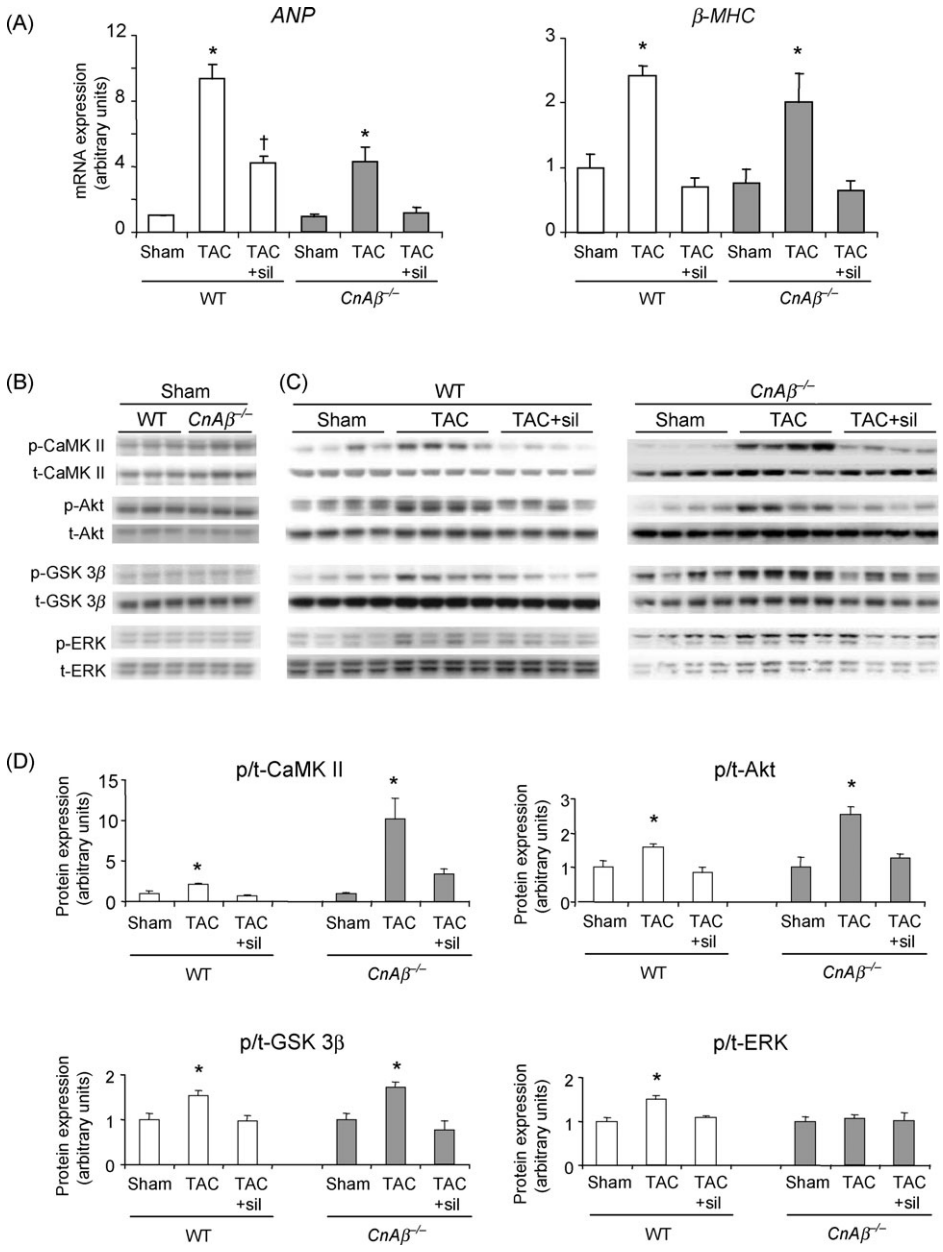

Figure 6 (*A*) *ANP* and *β-MHC* gene re-expression in wild-type (WT) and *CnA*$\beta^{-/-}$ hearts subjected to sham or transverse aorta constriction (TAC) surgery with and without sildenafil ($n = 4$ per group). Two-way ANOVA (genotype: wild-type or CnA$\beta^{-/-}$ × condition: sham or transverse aorta constriction) shows significant difference ($P < 0.05$) in *ANP*, but not in *β-MHC*. *$P < 0.05$ vs. sham and transverse aorta constriction + sildenafil (the same genotype). †$P < 0.01$ vs. sham (the same genotype). (*B*) Phosphorylation of CaMKII (Thr286), Akt (Ser473), GSK3β (Ser9), and ERK1/2 (Thr202/Thr204) in wild-type sham- and *CnA*$\beta^{-/-}$ sham-operated hearts is comparable, allowing for comparisons between wild-type and *CnA*$\beta^{-/-}$ blots by normalizing to respective sham levels ($n = 3$ per group). (*C*) Western blots of phospho- (p-) and total- (t-) CaMKII, Akt, GSK3β, and ERK1/2 in wild-type and *CnA*$\beta^{-/-}$ sham and transverse aorta constriction hearts with and without sildenafil. (*D*) Quantification results of phospho/total (p/t) protein ratios. $n = 4$ per group. Two-way ANOVA (genotype: wild-type or CnA$\beta^{-/-}$ × condition: sham or transverse aorta constriction) shows significant difference ($P < 0.05$) in CaMKII and ERK1/2. *$P < 0.05$ vs. sham and transverse aorta constriction + sildenafil (the same genotype).

treated with PDE5 inhibitors.[48] However, the importance of the Rho–Rho kinase pathway to cardiac hypertrophy remains unclear, with recent evidence showing effects on fibrosis, but less impact on hypertrophic remodelling.[49]

Our study has several limitations. One technical limitation was the use of myocyte diameter to estimate myocyte hypertrophy, as more accurate characterizations use cell volume. However, myocyte diameter can appropriately reflect cardiomyocyte hypertrophy, and the method has been used in this same model in several prior studies.[17,19] We did not test alternative hypertrophy models such as infusion of angiotensin II or isoproterenol. Although potentially interesting, prior studies using the *CnA*$\beta^{-/-}$ mouse found little-to-no hypertrophy induced by these models in this model.[8] In this case, further anti-hypertrophic efficacy of PDE5 inhibition could not be tested.

In summary, we have shown that potent cardiac anti-hypertrophic and functional effects of cGMP-PKG activation via PDE5A inhibition are still observed in hearts with substantial knock-down of functional Cn. This indicates their effects include suppression of alternative cascades in addition to blunting the Cn/NFAT pathway as reported

previously. To achieve this, cGMP-PKG either targets multiple distal pathways or functions strategically at proximal nodes that can control many of them. Ongoing studies should better elucidate these proximal targets and clarify the nature of PKG stress-modulation in the diseased heart.

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Conflict of interest: none declared.

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