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PRE-CLINICAL RESEARCH

Sildenafil Stops Progressive Chamber, Cellular, and Molecular Remodeling and Improves Calcium Handling and Function in Hearts With Pre-Existing Advanced Hypertrophy Caused by Pressure Overload

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Objective This study sought to test the efficacy of phosphodiesterase type 5A (PDE5A) inhibition for treating advanced hypertrophy/remodeling caused by pressure overload, and to elucidate cellular and molecular mechanisms for this response.

Background Sildenafil (SIL) inhibits cyclic guanosine monophosphate–specific PDE5A and can blunt the evolution of cardiac hypertrophy and dysfunction in mice subjected to pressure overload. Whether and how it ameliorates more established advanced disease and dysfunction is unknown.

Methods Mice were subjected to transverse aortic constriction (TAC) for 3 weeks to establish hypertrophy/dilation, and subsequently treated with SIL (100 mg/kg/day) or placebo for 6 weeks of additional TAC.

Results The SIL arrested further progressive chamber dilation, dysfunction, fibrosis, and molecular remodeling, increasing myocardial protein kinase G activity. Isolated myocytes from TAC-SIL hearts showed greater sarcomere shortening and relaxation, and enhanced Ca^{2+} transients and decay compared with nontreated TAC hearts. The SIL treatment restored gene and protein expression of sarcoplasmic reticulum Ca^{2+} uptake adenosine triphosphatase (SERCA2a), phospholamban (PLB), and increased PLB phosphorylation (S16), consistent with improved calcium handling. The phosphatase calcineurin (Cn) and/or protein kinase C-α (PKCα) can both lower phosphorylated phospholamban and depress myocyte calcium cycling. The Cn expression and PKCα activation (outer membrane translocation) were enhanced by chronic TAC and reduced by SIL treatment. Expression of PKCδ and PKCε also increased with TAC but were unaltered by SIL treatment.

Conclusions SIL treatment applied to well-established hypertrophic cardiac disease can prevent further cardiac and myocyte dysfunction and progressive remodeling. This is associated with improved calcium cycling, and reduction of Cn and PKCα activation may be important to this improvement. (J Am Coll Cardiol 2009;53:207–15) © 2009 by the American College of Cardiology Foundation

Heart failure is a leading cause of morbidity and mortality worldwide, commanding a large share of health care resources, and despite recent advances, better treatment options remain sorely needed (1). Hypertension and corresponding hypertrophic heart disease are major risk factors for heart failure, and the therapeutic suppression of hypertrophy is associated with improved mortality (2,3). To date, treatments designed to blunt hypertrophic remodeling and related dysfunction have largely focused on reducing vascular load and neurohormone stimulation. However, intracellular pathways are also now being targeted, which may provide potent benefits by interfering with strategic nodes in more distal signaling processes (1,4).

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Abbreviations and Acronyms

cGMP = cyclic guanosine monophosphate
Cn = calcineurin
ISO = isoproterenol
LV = left ventricular
NOS = nitric oxide synthase
PDE5A = phosphodiesterase 5A
PKCα = protein kinase C-α
PKG = protein kinase G
SERCA 2a = sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase
SIL = sildenafil
SR = sarcoplasmic reticulum
TAC = transverse aortic constriction

An intriguing approach that first arose somewhat as a surprise is the inhibition of cyclic guanosine monophosphate (cGMP)–specific phosphodiesterase 5A (PDE5A) by compounds such as sildenafil (SIL). Such drugs are widely used to treat erectile dysfunction, and have long been thought to have little role in the heart. However, growing evidence supports cardiac expression and functionality of PDE5A (5) physiological effects from its inhibitors. For example, PDE5A inhibition can improve ischemic cardiomyopathy (6), doxorubicin toxicity (7), and pressure overload–induced hypertrophy (8), all thought to be coupled to enhanced protein kinase G activation (9,10). Clinical studies of SIL in heart failure patients have reported improved exercise capacity coupled to reduced pulmonary vascular resistance and better endothelial function (11,12). Based on such findings, the National Institutes of Health recently initiated a multicenter clinical trial of SIL for the treatment of heart failure with a preserved ejection fraction (Clinical Trials.gov identifier: NCT00763867).

We previously reported that SIL exerts antihypertrophic effects against pressure overload when the treatment is initiated at the onset of the stress or shortly (1 week) thereafter (8). In the latter case, hearts first developed concentric hypertrophy with no dilation, and then improved with treatment. However, it is unknown whether SIL can ameliorate more advanced disease, a nontrivial question because PDE5A modulation seems to target nitric oxide–derived cGMP (9,10,13) and nitric oxide synthase (NOS) activity declines with sustained pressure overload (14). Furthermore, the mechanisms of SIL-improved cardiac function in pressure overload hearts (8) and whether they apply to more advanced disease remain unknown. Accordingly, the present study tested the efficacy of delayed SIL treatment of hearts subjected to pressure overload. We show evidence of chamber, cellular, and molecular benefits of PDE5A inhibition in advanced disease, and reveal novel mechanisms for functional improvement related to calcium cycling and its molecular modulation.

Methods

Animal models. Male C57Bl/6 mice (age 9 to 12 weeks, Jackson Labs, Bar Harbor, Maine) were used. Pressure overload was produced by transverse aortic constriction (TAC) (8), with shams undergoing the same operation without aortic constriction. Oral SIL (100 mg/kg/day) was provided in soft diet (Transgenic Dough Diet, Bio-Serv, Frenchtown, New Jersey) (8) starting on week 4 after TAC and continuing for 5 additional weeks. Although this dose is high for humans, the mouse metabolizes SIL at a higher rate (15), and this dose yields a free plasma concentration of 10 to 15 nM, within the specific and therapeutic range for PDE5A (8). Control data for chronic SIL only have been previously reported (8), and the effects were found to be negligible. The Johns Hopkins University Animal Care and Use Committee approved the protocol.

Physiological studies. Cardiac function was assessed by transthoracic echocardiography in conscious mice (Acuson Sequoia C256, Siemens Inc., New York, New York) using a 15-MHz linear-array transducer, and by invasive pressure-volume analysis using the conductance catheter method (SPR-839, Millar Instruments, Inc., Houston, Texas). The catheter was inserted via the left ventricular (LV) apex in open-chest, anesthetized mice, and positioned along the long axis as described (8).

Histology. Formalin (10%)-fixed, paraffin-embedded LV myocardium was sectioned (5 μm) and stained with hematoxylin-eosin or picrosirius red. Myocyte diameter and interstitial and perivascular collagen fraction were determined by digital image analysis (Photoshop version 7.0, Adobe Systems, San Jose, California; Image J, version 1.41, NIH, Bethesda, Maryland), with the observer blinded as to tissue source. Four different hearts in each group, with 5 separate fields of cells (total 50 to 70 cells for each heart) were analyzed.

Isolated myocyte physiologic studies. Sarcomere shortening (Myocam, IonOptix, Milton, Massachusetts) and whole-cell Ca^{2+} transient (Fura-2 AM μmol/l, Invitrogen, Carlsbad, California) was assessed in freshly isolated adult cardiomyocytes (4 hearts per group) at 27°C using 0.5-Hz field stimulation (15 to 20 cells from each heart) as previously described (10). Although lower temperatures can depress adrenergic modulation, we have shown marked concordance of adrenergic regulation by PDE5A inhibitors under various conditions between such in vitro conditions and in vivo hearts (9,10). Data at rest and after isoproterenol (ISO) (10 nmol/l) stimulation were obtained.

Protein and gene expression. Protein and messenger ribonucleic acid (mRNA) isolates were prepared from LV tissue flash frozen in liquid nitrogen, and expression was assessed using standard techniques (Online Appendix).

Immunofluorescent histology. Protein kinase C-α (PKCα) myocyte localization was examined with antisera by confocal fluorescent immunohistochemistry as described (10) (Online Appendix).

Statistical analysis. Data are presented as mean ± SEM. Differences between groups were assessed by 1- or 2-way analysis of variance (with or without repeated measures, as required) followed by a Tukey multiple comparisons test. In instances in which within-group variance differed substantially, a nonparametric Kruskal-Wallis test and Bonferroni correction was used.

Results

SIL inhibits progressive hypertrophy and dilation in mice subjected to TAC. After 3-week TAC, hearts had a +135% increase in LV mass, chamber end-systolic (+91%) and end-diastolic (+10%) dimensions, and reduced fractional shortening (−42%) (Fig. 1A). Subsequent treatment with SIL fully arrested progressive remodeling, whereas control hearts further dilated and hypertrophied after 9-week TAC. Post-mortem analysis confirmed that both heart and lung weight, normalized to tibia length, was lower with SIL treatment (Fig. 1B). Myocyte cross-sectional dimension and interstitial and perivascular fibrosis was also reduced in SIL-treated myocardium (Fig. 1C).

A- and B-type natriuretic peptide gene expression increased markedly after 3-week TAC. Both increased 30% to 50% after 9-week TAC in nontreated hearts but remained at 3-week levels in those receiving SIL (Fig. 2A). β-myosin heavy chain also increased, but this was not reduced in SIL-treated hearts.

Modulation of protein kinase G (PKG) activity and fetal gene expression. We next assessed the activation of cGMP-stimulated PKG in hearts with or without delayed SIL treatment. Enzyme activity assessed by S-239-vasodilator-stimulated phosphoprotein phosphorylation and in vitro kinase assay both showed increases after 9-week TAC that were further enhanced in SIL-treated animals (Fig. 2B). TAC resulted in increased PKG-1α (primary cardiac isoform) protein expression (Fig. 2C), but this declined to normal levels with SIL treatment, supporting post-translational (cGMP stimulation) mechanisms in this setting. The PDE5A protein expression was unaltered among the various conditions.

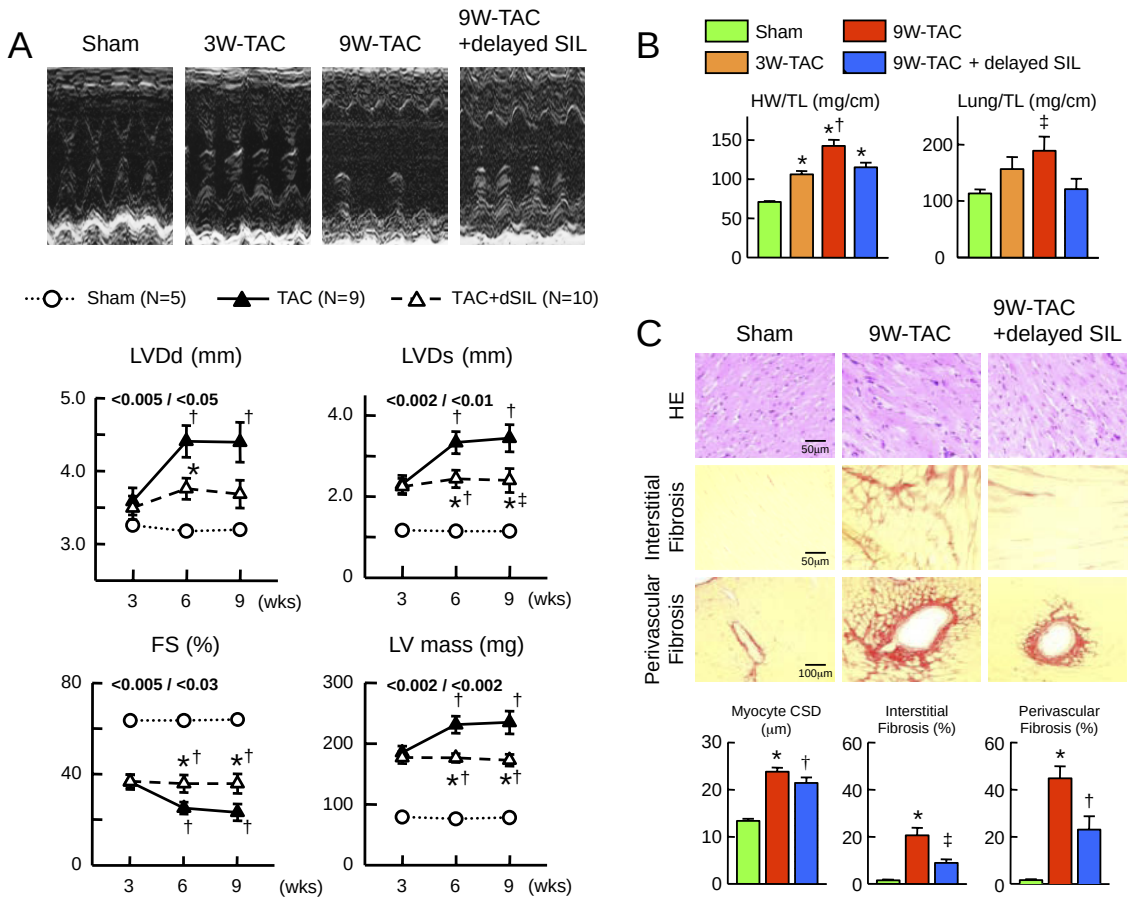

Figure 1 **Delayed Sildenafil Treatment Suppresses Progressive Cardiac Dilation, Dysfunction, Fibrosis, and Hypertrophy in Hearts Subjected to Sustained Pressure Overload**

(A) Representative echocardiographic images and summary analysis in sham controls and transverse aortic constriction (TAC) mice with or without delayed sildenafil treatment (dSIL). The p values are for 2-way analysis of variance (ANOVA); first value for all 3 groups, second comparing 9-week (9W) TAC ± sildenafil (SIL). Between-group comparisons (Tukey test) are denoted by symbols: *$p < 0.05$ versus 9W TAC. †$p < 0.01$ versus sham. ‡$p < 0.05$ versus sham. **(B)** Heart weight (HW) and lung weight normalized by tibial length (TL). *$p < 0.0001$ versus sham. †$p < 0.007$ versus 3-week (3W) TAC and 9W TAC + SIL. **(C)** Myocardium stained with hematoxylin and eosin (HE) and picrosirius red (PSR) showing reduced interstitial and perivascular fibrosis and myocyte hypertrophy by SIL treatment. $p < 0.001$ for ANOVA in each panel. *$p < 0.01$ versus sham and 9W TAC + SIL. ‡$p < 0.01$ versus sham. ‡$p = 0.08$ versus sham. CSD = cross-sectional diameter; FS = fraction shortening; LVDd = left ventricular systolic diastolic dimension; LVDs = left ventricular systolic dimension.

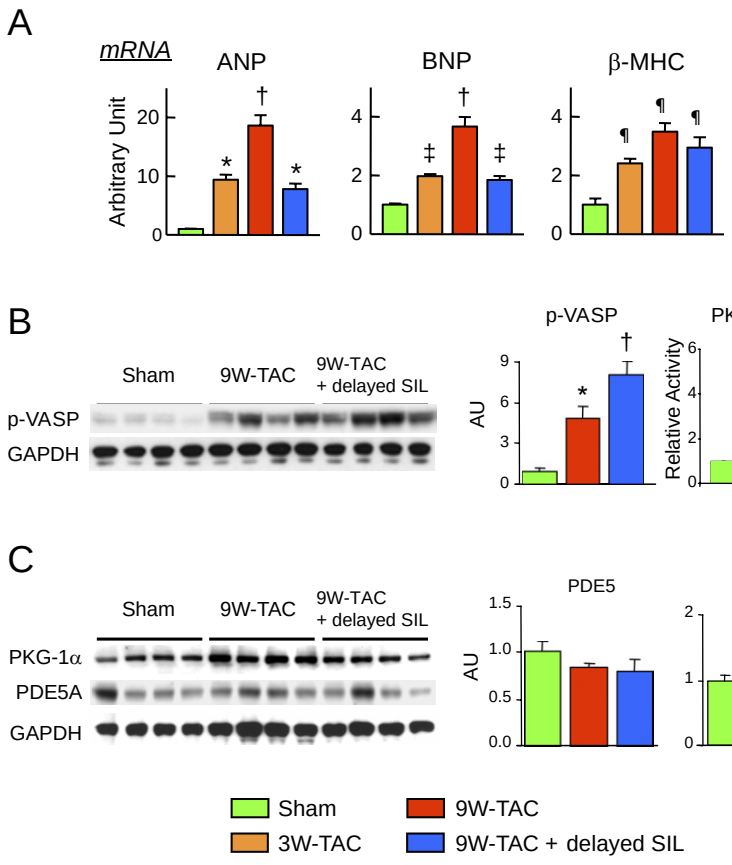

Figure 2 **Delayed Sildenafil Treatment Prevents Progressive Increases in Fetal Gene Re-Expression and Enhances the Activation of PKG**

Fetal **(A)** ANP, BNP, and β-MHC messenger ribonucleic acid (mRNA) expression levels normalized to GAPDH mRNA expression in whole-heart isolates (n = 3 to 5 per group; $p < 0.001$ for ANOVA in each). *$p < 0.01$ versus sham. †$p < 0.001$ versus all other groups. ‡$p = 0.06$ versus sham. ¶$p < 0.001$ versus sham. **(B)** Western blots of phospho-VASP protein expression (summary data to **right**) and in vitro PKG activity in sham and 9-week TAC ± SIL hearts ($p < 0.001$ for ANOVA for each). **(C)** Western blot of PKG-1α and PDE5A protein expression (summary data to **right**). *$p < 0.02$ versus sham. †$p = 0.05$ versus 9W TAC + SIL, $p < 0.001$ versus sham. ‡$p < 0.005$ versus sham and 9W TAC. ¶$p < 0.03$ versus sham and 9W TAC + SIL. ANP = A-type natriuretic peptide; β-MHC = beta-myosin heavy chain, BNP = B-type natriuretic peptide; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MHC = myosin heavy chain; PDE5A = phosphodiesterase 5A; PKG = protein kinase G; pVASP = phosphorylated vasodilator-stimulating phosphoprotein; other abbreviations as in Figure 1.

SIL treatment improves cardiac contractility and relaxation in vivo and in vitro. Figure 3A shows the results of LV pressure-volume analyses. After 3-week TAC, hearts dilated and had increased end-systolic elastance and contractility (dP/dt_{max}/IP and maximal power index), as reported in humans with hypertrophic heart disease (16). More sustained TAC reduced contractility and prolonged relaxation in untreated hearts, but this was prevented by SIL.

To identify mechanisms for improved cardiac function, LV myocytes were studied (Fig. 3B). Compared with sham controls, 9-week TAC myocytes had depressed sarcomere shortening and prolonged relaxation, and SIL treatment restored both to control levels. These changes were accompanied by improved calcium handling. The peak Ca^{2+}-transient was not significantly altered after 9-week TAC, but the time for 50% decline was prolonged. The latter improved toward control values with SIL treatment, whereas peak amplitude increased. Contraction, relaxation, and Ca^{2+}-transients all improved similarly with ISO in each group (p value for interaction by 2-way analysis of variance range: 0.2 to 0.94); thus, this cascade was not differentially affected by SIL treatment.

SIL treatment modifies sarcoplasmic reticulum (SR) Ca^{2+} handling protein expression and phosphorylation. Because Ca^{2+} cycling improved in SIL-treated TAC hearts, we examined SR handling proteins that might be modified by the therapy. Gene expression of SR-Ca^{2+} adenosine triphosphatase (SERCA2a) and phospholamban declined after 3-week TAC (Fig. 4A), and this persisted after 9-week TAC. Protein expression was decreased at this time as well. The SIL treatment increased both SERCA2a and phospholamban expression, and enhanced PLB s-23 phosphorylation (increasing Ca^{2+}-uptake) in comparison with nontreated TAC.

SIL suppresses calcineurin (Cn) and PKCα activation. Increased PLB phosphorylation by SIL is consistent with

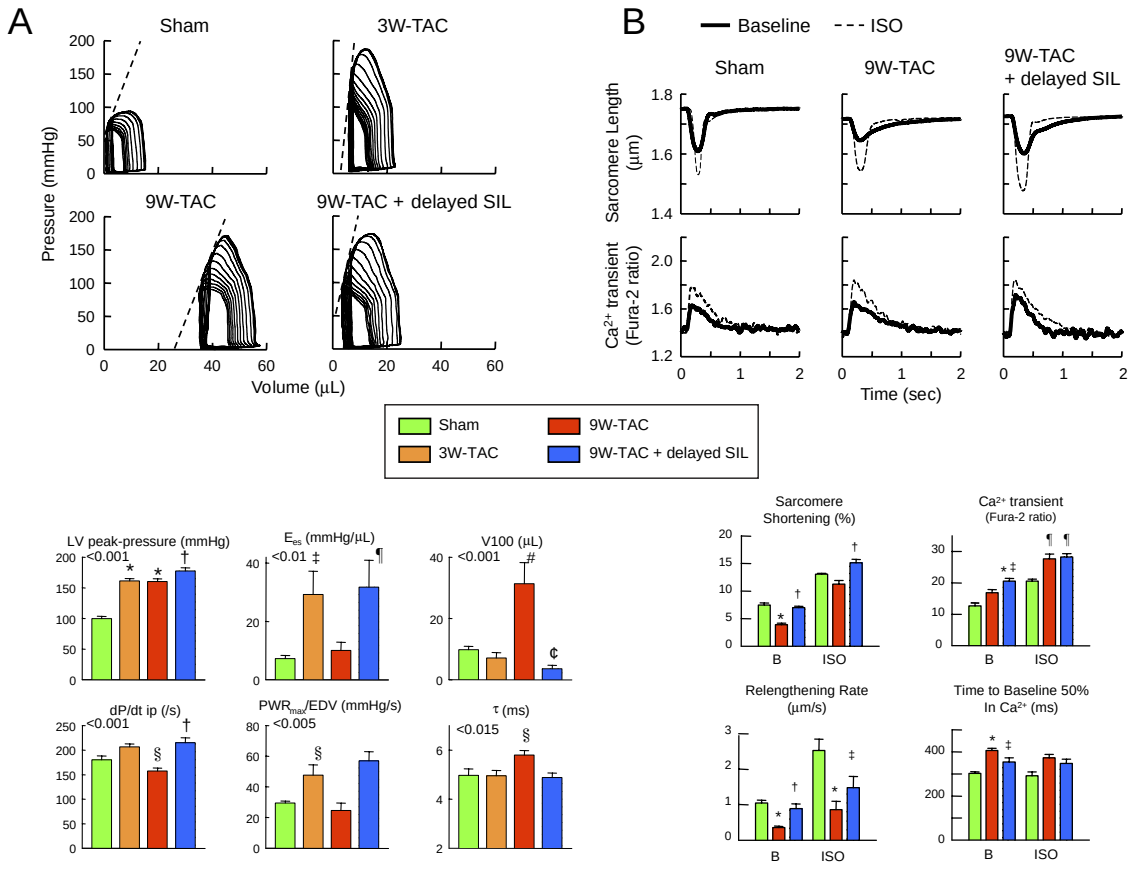

Figure 3 **Delayed Sildenafil Treatment in Hearts Subjected to Pressure Overload Improves Cardiac Systolic and Diastolic Function, Myocyte Shortening, and Calcium Handling**

(A) Representative in vivo pressure-volume loops and summary analysis. The 3-week TAC hearts were somewhat dilated with a higher end-systolic elastance and contractility (dP/dt_{max}) normalized to instantaneous developed pressure; peak power divided by end-diastolic volume (PWR_{max}/EDV), and preserved relaxation (tau). Contractility declined and relaxation prolonged to levels at or below control after 9W TAC, and hearts showed marked dilation/remodeling. The SIL treatment prevented this functional deterioration; p value in each panel shows ANOVA result or for end-systolic elastance and V100, Kruskal-Wallis test; post hoc tests: *$p < 0.001$ versus sham. †$p = 0.06$ versus 9W TAC. ‡$p < 0.04$ versus sham. ¶$p < 0.1$ versus 9W TAC and sham. #$p < 0.001$ versus other groups. ¢$p < 0.05$ versus sham and 3W TAC. §$p < 0.001$ versus 3W TAC and 9W TAC + SIL. **(B)** Isolated myocyte sarcomere shortening (SS), relengthening rate, peak calcium transient, and time for 50% transient decay measured at rest and in response to isoproterenol (ISO) stimulation ($n = 4$ hearts for each experimental group, 15 to 20 myocytes per heart). Results of 2-way ANOVA: $p < 0.001$ for ISO and for group effect, $p > 0.2$ for interaction effect for all parameters; within group ANOVA: *$p < 0.005$ versus sham. †$p < 0.005$ versus 9W TAC. ‡$p < 0.02$ versus 9W TAC. ¶$p < 0.001$ versus sham. Abbreviations as in Figure 1.

enhanced PKG activity, but it could also result from suppressed phosphatase activity. One mechanism involves calcineurin (Cn), which inhibits protein phosphatase inhibitor-1 activity, resulting in the disinhibition of the phosphatase protein phosphatase-1 to lower PLB phosphorylation (17,18). Cn protein expression and activity (indexed by regulator of calcineurin 1 mRNA) increased after 3-week TAC by 3.2-fold and 2.5-fold, respectively (both $p < 0.01$) (Online Fig. 1), and remained elevated at 9-week TAC in nontreated hearts but declined with SIL (Fig. 4C). Another mechanism for I-1 inactivation is its phosphorylation by PKCα at S67 and T75, which also enhances PP1 activity and thus PLB dephosphorylation (19). PKCα (and δ and ε isoforms) protein expression increased markedly after 9-week TAC (Fig. 4D), and SIL treatment did not reduce them. However, PKCα activity is coupled to its translocation to the outer membrane, and this was observed after both 3 and 9 weeks of TAC (Fig. 5A). PKCα returned to a diffuse distribution consistent with reduced activation after SIL treatment. Confocal analysis was confirmed by cell fractionation/immunoblot analysis (Fig. 5B). To test whether SIL directly targeted PKCα activation, freshly isolated cardiomyocytes were incubated with the PKC activator PMA; PMA induced rapid PKCα membrane translocation (Fig. 5C) but SIL coincubation did not suppress it, suggesting that the in vivo effect was more likely indirect.

Discussion

Cardiac hypertrophy and attendant myocardial remodeling and myocyte and chamber dysfunction remain major causes

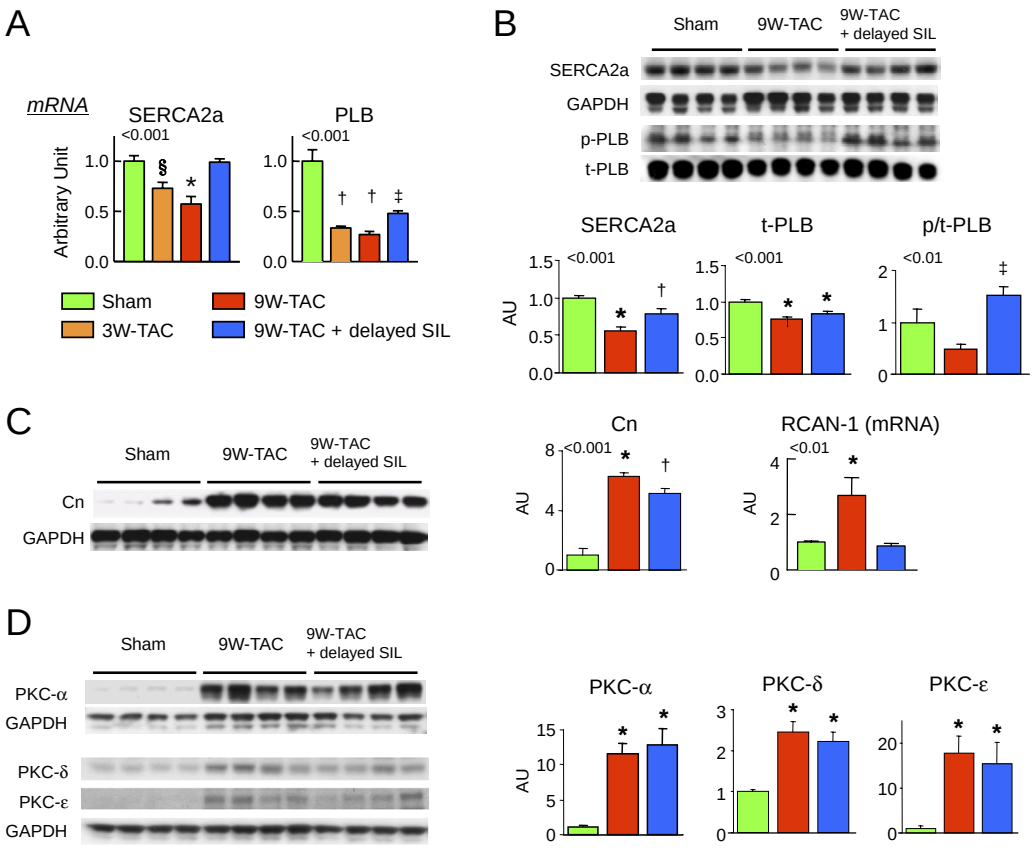

Figure 4 **Delayed Sildenafil Treatment in Pressure Overload Hearts Restores SR Calcium-Handling Proteins and Phosphorylation, Reduces Calcineurin Expression and Activity, but Does Not Reduce Total PKC α, δ, or ε Isoform Expression**

(A) The SERCA2a and phospholamban (PLB) mRNA expression normalized to GAPDH levels in 4 experimental groups (ANOVA p values shown; post hoc tests: *p < 0.005 versus sham and 9W TAC + SIL; †p < 0.001 versus sham; ‡p < 0.01 versus 9W TAC; §p < 0.04 single comparison between 3W TAC and sham control). **(B)** Western blots of SERCA2a, total PLB, and phosphorylated PLB expression. *p < 0.005 versus sham. †p < 0.05 versus 9W TAC. ‡p < 0.01 versus 9W TAC. p values for ANOVA are shown. **(C)** Calcineurin (Cn) protein expression (**left,** Western blot; **right,** summary data) and gene expression of RCAN-1 (n = 4 per group). *p < 0.02 versus sham. †p < 0.05 versus sham and 9W TAC (Kruskal-Wallis test). **(D)** The PKC α, δ, and ε isoform protein expression in TAC hearts ± SIL (p < 0.03 for Kruskal-Wallis test for each. *p < 0.05 versus control; Bonferroni test). RCAN-1 = regulator of calcineurin 1; SERCA2a= sarcoplasmic reticulum–Ca^{2+} adenosine triphosphatase; other abbreviations as in Figures 1 and 2.

of morbidity and mortality worldwide, and new approaches to combat this pathophysiology are needed. In a prior study, we first showed that PDE5A inhibition coupled with activation of PKG may offer a novel approach to treating this disorder (8). The present results substantially extend this finding. First, therapy was initiated only after the hypertrophic disease process was far more established, yet improvements in function, remodeling, and molecular signaling were achieved. Second, isolated myocytes were studied, revealing enhanced myocyte contraction/relaxation and Ca^{2+} handling under both rest and beta-adrenergic receptor stimulated conditions. Third, we extended prior mechanistic analysis, showing improvement of SR calcium handling proteins coupled with suppression of both Cn and PKCα activation. These findings further support a translational potential for PDE5A inhibitors in established hypertrophic heart disease.

Treating hypertrophy and cardiac failure via a cGMP/PKG/PDE5 pathway. Although the potential for cGMP/PKG signaling to suppress cardiac hypertrophy has been recognized for some time, it has been difficult to translate into an effective therapy. Prior studies have focused on increasing cGMP synthesis via natriuretic peptides or nitric oxide, but this remains compromised by peripheral vasodilation and tachyphylaxis in part because of feedback inhibition by PDEs (20,21). Even in genetically engineered animals with natriuretic peptide or NOS pathways modulated (22,23), TAC-induced hypertrophy changes have been modest, and no study has examined a situation in which the disease was already well established. Suppression of cGMP hydrolysis provides an alternative approach. Of 3 PDE species identified in the heart to date (5), 2 are dual substrate (PDE1 and PDE2), the former requiring Ca^{2+}-calmodulin activation and the latter also acting as a cGMP-

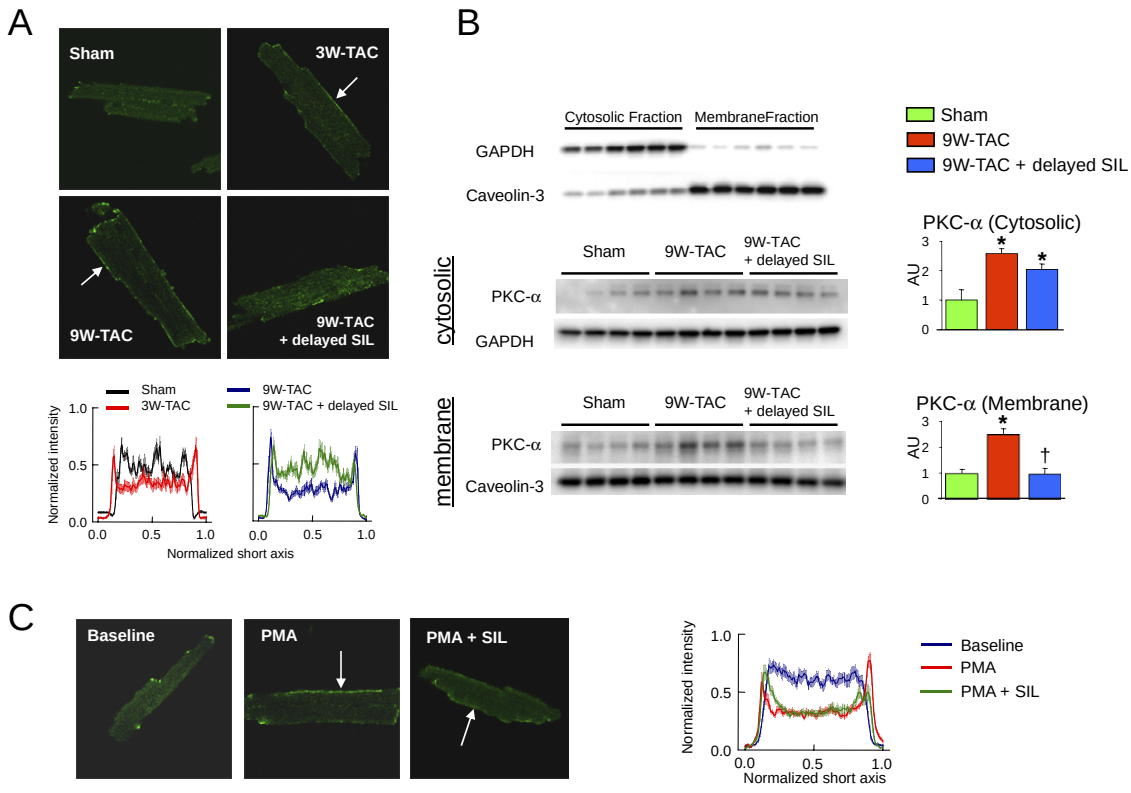

Figure 5 **Sildenafil Treatment Suppresses Outer Membrane Translocation (Activation) of PKCα Stimulated by Sustained Pressure Overload**

(A) Representative confocal microscopy images of myocytes stained for PKCα. Cells were from the same pool of myocytes used to perform the physiologic studies. The PKCα at baseline was cytosolic, whereas it translocated to the outer membrane at 3W and 9W TAC. The TAC + SIL treatment resulted in reduction in membrane localization. Summary line-scan densitometry is displayed below (mean ± SD), showing differences from cells in each group (n = 10). Sham differed from 3W and 9W TAC, and 9W TAC from 9W TAC + SIL (all $p < 0.01$). **(B)** Cell membrane versus cytosolic fractionation analysis of PKCα protein expression. **Top panel** shows separation controls, with caveolin-3 (CAV-3) for membrane, and GAPDH for cytosol. **Lower panels** show expression changes for each fraction, increasing with TAC in both, and declining in the membrane fraction with SIL treatment ($p < 0.006$ for ANOVA in both; *$p < 0.05$ vs. control. †$p < 0.02$ vs. TAC). **(C)** The PKCα translocation to the outer membrane in freshly isolated myocytes after 2-h PMA incubation is not blocked by cotreatment with SIL. Summary line-scan densitometry histograms shown to the **right.** PMA = phorbol myristate acetate; other abbreviations as in Figures 1 and 2.

stimulated cyclic adenosine monophosphate hydrolytic enzyme. Their role in physiologic cardiac cGMP regulation remains largely unknown. The first selective cGMP-PDE discovered was PDE5A, and it remains the best characterized (5). Although first thought to have little role in the heart, growing evidence supports its regulation of a localized cGMP pool that can potently modulate cardiac stress responses (5–8), and the current data strongly supports this further.

Hydrolysis of cGMP by PDE5A is compartmentalized and seems to particularly target NOS-generated cGMP (9,10,24). However, sustained pressure overload results in functional NOS uncoupling, reducing its synthesis of NO and increasing its generation of superoxide (14). The fact that SIL remains effective in such a setting is important and may relate to observations that PDE5A inhibition reduces NO-reactive oxygen species interaction decline in nitrotyrosine and improves NOS coupling and activity in TAC hearts (13). The mechanism for this is presently under study. It is also worth noting that therapies targeting NO→sGC→cGMP→PKG (sGC is soluble guanylate cyclase) do not necessarily lead to the same end modulation (i.e., PKG activation). For example, hearts exposed to sustained pressure overload but then treated with tetrahydrobiopterin, an essential cofactor required for NOS coupling and thus normal function, also display suppression of progressive remodeling, dysfunction, hypertrophy, and fibrosis, yet unlike SIL, do not have heightened PKG activity (25).

PDE5A inhibition and cardiac contractility. Acute PDE5A inhibition minimally alters resting heart or myocyte function because basal cGMP-cyclase activity is low (9,10); however, it can substantially blunt β-adrenergic stimulation, which coactivates the cyclase (10,26). Likewise, chronic SIL (same dose used in the present study) also minimally affects the normal heart (8), yet improves function in hearts and myocytes subjected to sustained pressure overload. The apparent paradox between acute negative yet

chronic positive contractility responses shares some similarity to β-blockade. Acute β-blockade is negatively inotropic, but this effect depends on the level of adrenergic tone, whereas chronic blockade in a stressed/failing heart enhances contraction, likely by suppressing sustained catecholamine cytotoxicity (27). However, β-blockers are far less potent in suppressing pressure overload hypertrophy and their utility in hypertensive heart disease has been questioned (28). By contrast, PDE5A inhibitors target more distal signaling and suppress hypertrophy and maladaptive molecular changes triggered by mechanical and neurohumoral stress even without altering blood pressure. By blocking pathways that otherwise depress Ca^{2+} handling, myocyte function, and remodeling, the net chronic effect positively impacts contractility.

Our results contrast to those of a recent study performed in chronic right ventricular hypertrophied rat hearts, in which PDE5A inhibition acutely augmented contractility linked to an increase in cAMP (not cGMP) and activation of protein kinase A (not PKG) (29). In this study, myocyte shortening was equally enhanced by isoproterenol, SIL, or their combination, suggesting that the same pathway was activated by both. Right ventricular hypertrophy reduced PKG activity (and phosphorylated VASP) and increased PDE5A expression. In contrast, we found that ISO augmented LV myocyte shortening similarly in mice subjected to TAC ± SIL, hypertrophy enhanced PKG activity (and pVASP) and SIL increased it further, and PDE5A expression was unchanged. In our prior study, myocardial cAMP was also similar in hearts subjected to chronic TAC ± SIL (8). The difference may lie between chronic versus acute interventions, right versus left ventricle, or conceivably species. With regard to the latter, PDE5A inhibitors blunt β-adrenergic stimulation in humans(26) similar to their effect in mice(10).

PDE5A inhibition and calcium cycling regulation. Sildenafil acutely depresses ISO-stimulated contraction without altering the whole cell Ca^{2+} transient (10). This may reflect a balance between a PKG-mediated decline in L-type Ca^{2+} current (30), reduced myofilament sensitivity (31), and PLB phosphorylation (32). With chronic pressure overload, however, other regulators seem involved. Reduced PLB phosphorylation is found in experimental and human heart failure (32), and both Cn and PKCα are thought play a role. PKCα is the dominant isoenzyme in heart and translocates to the outer sarcolemmal membrane on stimulation (33). It phosphorylates and thus inhibits I-1 (inhibitor of the phosphatase PP1), reducing PLB phosphorylation. Genetic (19) and pharmacologic (34) blockade of PKCα improves function, although its impact on hypertrophy is less marked. Whereas SIL did not alter PKCα (or other PKC isoforms) protein expression, it shifted it away from the outer membrane consistent with de-activation. Also, I-1 can be inhibited by Cn via dephosphorylating sites normally stimulated by PKA (Thr-35) (17,35). Future studies are needed to directly prove the role of I-1 modulation to SIL-modified cardiac function.

Conclusions

Delayed SIL treatment inhibits the progression of hypertrophy, fibrosis, and chamber remodeling, and improves basal and β-stimulated contractility and relaxation in a model of established pressure overload–induced hypertrophic heart disease. Functional improvement is coupled to enhanced Ca^{2+} handling, and increased expression of SR Ca^{2+} uptake proteins and PLB phosphorylation likely contribute. The capacity of SIL to block maladaptive remodeling even when administered later in the disease process provides important experimental support for current clinical studies, where disease will already be present.

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REFERENCES

1. Mudd JO, Kass DA. Tackling heart failure in the twenty-first century. Nature 2008;451:919–28.
2. Wachtell K, Okin PM, Olsen MH, et al. Regression of electrocardiographic left ventricular hypertrophy during antihypertensive therapy and reduction in sudden cardiac death: the LIFE Study. Circulation 2007;116:700–5.
3. Devereux RB, Wachtell K, Gerdts E, et al. Prognostic significance of left ventricular mass change during treatment of hypertension. JAMA 2004;292:2350–6.
4. McKinsey TA, Kass DA. Small-molecule therapies for cardiac hypertrophy: moving beneath the cell surface. Nat Rev Drug Discov 2007;6:617–35.
5. Kass DA, Champion HC, Beavo JA. Phosphodiesterase type 5: expanding roles in cardiovascular regulation. Circ Res 2007;101:1084–95.
6. Salloum FN, Abbate A, Das A, et al. Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. Am J Physiol Heart Circ Physiol 2008;294:H1398–406.
7. Fisher PW, Salloum F, Das A, Hyder H, Kukreja RC. Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. Circulation 2005;111:1601–10.
8. Takimoto E, Champion HC, Li M, et al. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. Nat Med 2005;11:214–22.
9. Takimoto E, Belardi D, Tocchetti CG, et al. Compartmentalization of cardiac beta-adrenergic inotropy modulation by phosphodiesterase type 5. Circulation 2007;115:2159–67.
10. Takimoto E, Champion HC, Belardi D, et al. cGMP catabolism by phosphodiesterase 5A regulates cardiac adrenergic stimulation by NOS3-dependent mechanism. Circ Res 2005;96:100–9.
11. Lewis GD, Lachmann J, Camuso J, et al. Sildenafil improves exercise hemodynamics and oxygen uptake in patients with systolic heart failure. Circulation 2007;115:59–66.
12. Guazzi M, Samaja M, Arena R, Vicenzi M, Guazzi MD. Long-term use of sildenafil in the therapeutic management of heart failure. J Am Coll Cardiol 2007;50:2136–44.
13. Kass DA, Takimoto E, Nagayama T, Champion HC. Phosphodiesterase regulation of nitric oxide signaling. Cardiovasc Res 2007;75:303–14.
14. Takimoto E, Champion HC, Li M, et al. Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. J Clin Invest 2005;115:1221–31.

15. Walker DK, Ackland MJ, James GC, et al. Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man. Xenobiotica 1999;29:297–310.
16. Pak PH, Maughan WL, Baughman KL, Kieval RS, Kass DA. Mechanism of acute mechanical benefit from VDD pacing in hypertrophied heart: similarity of responses in hypertrophic cardiomyopathy and hypertensive heart disease. Circulation 1998;98:242–8.
17. El Armouche A, Bednorz A, Pamminger T, et al. Role of calcineurin and protein phosphatase-2A in the regulation of phosphatase inhibitor-1 in cardiac myocytes. Biochem Biophys Res Commun 2006;346:700–6.
18. MacDonnell SM, Kubo H, Harris DM, et al. Calcineurin inhibition normalizes beta-adrenergic responsiveness in the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 2007;293:H3122–9.
19. Braz JC, Gregory K, Pathak A, et al. PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nat Med 2004;10:248–54.
20. Forfia PR, Lee M, Tunin RS, Mahmud M, Champion HC, Kass DA. Acute phosphodiesterase 5 inhibition mimics hemodynamic effects of B-type natriuretic peptide and potentiates B-type natriuretic peptide effects in failing but not normal canine heart. J Am Coll Cardiol 2007;49:1079–88.
21. Kim D, Rybalkin SD, Pi X, et al. Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. Circulation 2001;104:2338–43.
22. Holtwick R, van Eickels M, Skryabin BV, et al. Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-A. J Clin Invest 2003;111:1399–407.
23. Zahabi A, Picard S, Fortin N, Reudelhuber TL, Deschepper CF. Expression of constitutively active guanylate cyclase in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts. J Biol Chem 2003;278:47694–9.
24. Castro LR, Verde I, Cooper DM, Fischmeister R. Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. Circulation 2006;113:2221–8.
25. Moens AL, Takimoto E, Tocchetti CG, et al. Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. Circulation 2008;117:2626–36.
26. Borlaug BA, Melenovsky V, Marhin T, Fitzgerald P, Kass DA. Sildenafil inhibits beta-adrenergic-stimulated cardiac contractility in humans. Circulation 2005;112:2642–9.
27. Brodde OE. Beta-adrenoceptor blocker treatment and the cardiac beta-adrenoceptor-G-protein(s)-adenylyl cyclase system in chronic heart failure. Naunyn Schmiedebergs Arch Pharmacol 2007;374:361–72.
28. Bangalore S, Messerli FH, Kostis JB, Pepine CJ. Cardiovascular protection using beta-blockers: a critical review of the evidence. J Am Coll Cardiol 2007;50:563–72.
29. Nagendran J, Archer SL, Soliman D, et al. Phosphodiesteras type 5 (PDE5) is highly expressed in the hypertrophied human right ventricle and acute inhibition of PDE5 improves contractility. Circulation 2007;116:238–48.
30. Yang L, Liu G, Zakharov SI, Bellinger AM, Mongillo M, Marx SO. Protein kinase G phosphorylates Cav1.2 alpha1c and beta2 subunits. Circ Res 2007;101:465–74.
31. Layland J, Li JM, Shah AM. Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. J Physiol 2002;540:457–67.
32. MacLennan DH, Kranias EG. Phospholamban: a crucial regulator of cardiac contractility. Nat Rev Mol Cell Biol 2003;4:566–77.
33. Jalili T, Takeishi Y, Song G, Ball NA, Howles G, Walsh RA. PKC translocation without changes in Galphaq and PLC-beta protein abundance in cardiac hypertrophy and failure. Am J Physiol 1999;277:H2298–304.
34. Hambleton M, Hahn H, Pleger ST, et al. Pharmacological- and gene therapy-based inhibition of protein kinase Calpha/beta enhances cardiac contractility and attenuates heart failure. Circulation 2006;114:574–82.
35. MacDonnell SM, Kubo H, Harris DM, et al. Calcineurin inhibition normalizes beta-adrenergic responsiveness in the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 2007;293:H3122–9.

Key Words: PDE5 ■ pressure overload ■ hypertrophy ■ myocyte ■ cardiac function.

APPENDIX

For a supplementary Methods section and a figure showing calcineurin protein expression and activity increase after 3 weeks of transaortic constriction, please see the online version of this article.