Sustained Soluble Guanylate Cyclase Stimulation Offsets Nitric-Oxide Synthase Inhibition to Restore Acute Cardiac Modulation by Sildenafil

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ABSTRACT

Phosphodiesterase type 5 (PDE5) inhibitors are used to treat erectile dysfunction, and growing evidence supports potential cardiovascular utility. Their efficacy declines with reduced nitric-oxide synthase (NOS) activity common to various diseases. We tested whether direct soluble guanylate cyclase (sGC) stimulation restores in vivo cardiovascular modulation by PDE5 inhibition despite acute or chronically suppressed NOS activity. Mice (C57/B16; n = 62) were studied by in vivo pressure-volume analysis to assess acute modulation by the PDE5 inhibitor sildenafil (SIL; 100 μg/kg/min) of the cardiac response to isoproterenol (ISO) with or without NOS inhibition [N^\text{\textbeta}-nitro-L-arginine methyl ester (L-NAME)] and cotreatment by the sGC stimulator BAY 41-8543 (4-morpholino[3,4-b]pyrimidine-4,6-diamine). SIL induced mild vasodilation but no baseline cardiac effects and markedly blunted ISO-stimulated contractility. Acute BAY 41-8543 at a dose lacking cardiovascular effects did not alter ISO responses. However, after acute L-NAME, SIL ceased to influence cardiovascular function, but adding BAY 41-8543 fully restored SIL effects. After 1 week of L-NAME, neither SIL nor SIL + BAY 41-8543 acutely induced vasodilation or blunted ISO responses. However, sustained BAY 41-8543 despite concurrent NOS inhibition restored the cardiovascular efficacy of SIL. The disparity between acute and chronic NOS inhibition related to diffusion of PDE5 away from myocyte z-bands coupled with reduced protein kinase G activation. Both were restored by sustained sGC costimulation. Thus, PDE5 regulation of adrenergic reserve and systemic vasodilation depends upon NOS-induced cGMP/protein kinase G and can be enhanced by sustained low-level stimulation of sGC. This may prove beneficial for enhancing the efficacy of PDE5 inhibitors in conditions with chronically reduced NOS activity.

Phosphodiesterase type 5 (PDE5) is a cGMP-selective phosphodiesterase whose inhibition by pharmacological agents such as sildenafil is widely used to treat erectile dysfunction (Ghofrani et al., 2006). Although the cardiovascular effects of PDE5 inhibitors were first thought to be minor (Jackson et al., 2006), many studies have revealed their potential utility for a variety of conditions (for review, see Kass et al., 2007a), including pulmonary hypertension (Galiè et al., 2005), endothelial dysfunction (Gori et al., 2005; Guazzi et al., 2007b), suppression of ischemia/reperfusion injury (Kukreja et al., 2005), exertional intolerance in heart failure patients (Guazzi et al., 2007a; Lewis et al., 2007), and reduction of hypertrophy and cardiac dysfunction from sustained pressure overload (Takimoto et al., 2005b). Acute cardiac modulation has also been reported, with PDE5 inhibition (PDE5-I) blunting ß-adrenergic-stimulated contractility in several species (Senzaki et al., 2001; Takimoto et al., 2005a), including humans (Borlaug et al., 2005).

The mechanism for these effects is generally thought to involve suppression of cGMP hydrolysis, thereby activating protein kinase G (PKG). This requires sufficient basal cyclase activity and may explain why PDE5-I is more influential under stress than rest conditions. Studies have further found that cGMP generated by nitric-oxide synthase (NOS) is par-
particularly important for PDE5 cardiac (Takimoto et al., 2005a; Castro et al., 2006) and vascular (Piggott et al., 2006) regulation. Syndromes where NOs activity is compromised, such as diabetes and heart failure, blunt the efficacy of PDE5-I for inducing vasodilation (Bivalacqua et al., 2004) and altering cardiac function (Senzaki et al., 2001). In the heart, this interaction is further dictated by PDE5 myocyte localization, being normally concentrated at the z-band but becoming more diffuse if NOs is genetically absent or chronically inhibited (Takimoto et al., 2005a; Kass et al., 2007b). In such settings, PDE5-I has little impact on acute β-stimulation and acute exogenous NO cannot restore this (Takimoto et al., 2005a). Although chronic NO donors might be effective, these have been considered counterindicated, being limited by both hypotension and tachyphylaxis.

Another approach is to directly stimulate soluble guanylate cyclase (sGC), a key downstream target of NO. A new class of drugs has been recently developed to achieve this (Eugenov et al., 2006; Stasch et al., 2006; Boerrigter et al., 2007). These agents induce dose-dependent vasodilation (Stasch et al., 2002a,b) by NO-independent sGC activation and an effect that is synergistic with NO-sGC stimulation (Priviero et al., 2005; Dumitrascu et al., 2006). This suggests one might be able to administer a nondilating dose of an sGC stimulator with minimal basal effects that could potentiate the efficacy of PDE5-I even when NOs activity was blunted. This might also reverse dislocated PDE5 and, thus, its cardiac regulation. Understanding this physiology has taken on increased relevance with the planned initiation of a clinical trial of PDE5-I (sildenafil) on rest and cardiac regulation. Understanding this physiology has taken on increased relevance with the planned initiation of a clinical trial of PDE5-I (sildenafil) on rest and acute exogenous NO cannot restore this (Takimoto et al., 2005a). Although chronic NO donors might be effective, these have been considered counterindicated, being limited by both hypotension and tachyphylaxis.

Materials and Methods

Pharmaceuticals and Reagents. Sildenafil citrate (SIL; gift from Pfzer Central Research, Sandwich, Kent, UK), isoproterenol (Sigma-Aldrich, St. Louis, MO), BAY 41-8543 (Bayer AG, Wuppertal, Germany), and Nω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) were used in this study. In Vivo Studies. Male adult mice (n = 62; C57Bl6, 8 weeks; The Jackson Laboratory, Bar Harbor, ME) were anesthetized, underwent thoracotomy, and instrumented for pressure-volume analysis (SPR-839 PV; Millar Instruments Inc., Houston, TX) as described previously (Georgakopoulos and Kass, 2001; Takimoto et al., 2005a). Isoproterenol (ISO; 20 ng/kg/min) with or without SIL (100 μg/kg/min) or BAY 41-8543 (5 μg/kg/min) was infused into a central jugular vein. The SIL dose yields a 37 ± 5.2 nM free plasma concentration (Takimoto et al., 2005a) similar to peak levels in humans after a therapeutic 100-mg dose (Borlaug et al., 2005). The BAY 41-8543 dose was selected as having negligible effects on resting hemodynamics based on an initial dose-response curve (Supplemental Table 1).

Heart function was assessed by pressure-volume (PV) loops at a fixed heart rate (550/min) achieved by atrial pacing. The acute protocol consisted of assessing PV data at baseline, after ISO stimulation, re-baseline, drug intervention (either SIL, BAY 41-8543, or both), and then drug intervention with a second ISO exposure. Previous studies have confirmed high reproducibility of repeated ISO infusion studies alone (Takimoto et al., 2005a). In separate studies, animals were pretreated with the NOs inhibitor L-NAME (50 mg/kg i.p.) and 30 min later underwent the same pharmacological protocol. Other animals underwent chronic NOs inhibition (L-NAME, 1 mg/ml in drinking water x 1 week; 4–6 ml/day). This dose reverses the cardiovascular phenotype of mice overexpressing endothelial NOS (Ohashi et al., 1998) and inhibits NOx in WT mice (Ozaki et al., 2002). Mice were then studied acutely as above or studied after a 2nd week with either L-NAME discontinued or continued but with BAY 41-8543 (30 mg/kg/day p.o.) added during the 2nd week. Four to eight mice were studied for each protocol.

Analysis of PDE5 Localization. To assess myocyte distribution of PDE5, immunohistochemistry was performed with either commercial antisera (1:250 dilution; Cell Signaling Technology Inc., Danvers, MA) or fluorescent histology employing a transfected fusion protein linking mouse PDE5 with aDs-Red. This probe was cloned from PDE5 coding sequence cDNA obtained from mouse lung (forward primer, 5′-ACGAACTCGAGTTGAAAGCAGGCCCGC-3′; reverse primer, 5′-ATAATCCGGGGTCTGTCGTCCCGCTTGCCCT-3′), and the polymerase chain reaction (PCR) product was used to construct a shuttle vector (TA cloning kit; Invitrogen, Carlsbad, CA). Xhol was used to subclone PDE5 into the pDsRed-C1 vector (Clontech, Mountain View, CA) to produce an in-frame fusion protein of DsRed monomer and PDE5. The DsRed-PDE5 fusion construct was then subcloned into an adeno virus vector. Cells were imaged on a Zeiss inverted epifluorescence microscope (Zeiss 510) with an argon-krypton laser confocal scanning system (UltraVIEW; PerkinElmer Life and Analytical Studies, Waltham, MA). Localization of the red-PDE5 was assessed by standard fluorescence microscopy (n = 5–10 cells/heart).

Analysis of PDE5 and Phosphovasoactive-Stimulated Phosphoprotein (p-VASP). Protein levels of PDE5 and p-VASP were assessed in fresh frozen myocardium. Tissue homogenized in lysis buffer (Cell Signaling Technology Inc.) containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) was centrifuged at 12,000 g for 20 min, and protein was quantified by bicinchoninic acid assay (Pierce Chemical, Rockford, IL). NuPAGE lithium dodecyl sulfate sample buffer was added, and lysates (50 μg) were electrophoresed on NuPAGE 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes and probed with primary antibodies, anti-p (Ser239)-VASP (1:1000; Alexis Corporation, Lausen, Switzerland), PDE5 (1:1000; Cell Signaling Technology Inc.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:3000; Cell Signaling Technology Inc.), followed by horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG1; Santa Cruz Biotechnology). Protein bands were detected by chemiluminescence and quantified using NIH ImageJ software (http://rsb.info.nih.gov/ij/). Results are expressed normalized to GAPDH.

Gene expression of PDE5 was assessed by quantitative PCR (TaqMan). PCR primers and probes specific for mRNA sequence of PDE5 and GAPDH (Applied Biosystems, PDE5; Mm00463177_m1, GAPDH; Mm99999915_g1) were used. PCR samples (25 ng) were run in duplicate, and GAPDH content was used to normalize PDE5 content of different samples. Reactions were performed with 900 nM of the specific primer pairs and 250 nM of specific probe for 40 cycles.
of amplification (denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s).

**Statistical Analysis.** Data are presented as mean ± S.E.M. Comparisons of ISO response with or without SIL and/or BAY 41-8543, and basal effect of SIL and/or BAY 41-8543 were performed by paired Student’s t test. Between-group comparisons were performed by one-way ANOVA with a Tukey multiple comparisons test. p Values < 0.05 were considered significant. The investigation conforms with guidelines for the care and use of laboratory animals as published by the National Research Council (1996).

**Results**

**Effects of PDE5 Inhibition, sGC Stimulation, and Both on Rest and ISO-Stimulated Function.** Summary hemodynamic data for control (and L-NAME treatment groups) are provided in Table 1, and effects of acute SIL, BAY 41-8543, or both on selected rest parameters are displayed in Fig. 1. In control mice, SIL slightly lowered systolic pressure and reduced arterial vascular resistance; however, cardiac function assessed using pressure-volume relations was not significantly altered (Fig. 1, left bars). End-diastolic volume also did not change significantly (data not shown). BAY 41-8543 alone had little impact on cardiac or vascular function, as expected given the dose chosen, and combining SIL + BAY 41-8543 (BAY) induced vasodilation similar to SIL alone but still did not significantly alter cardiac function (Fig. 1).

Left ventricular responses to ISO stimulation are shown in Fig. 2A. ISO enhanced systolic function reflected by the rise in ejection fraction and peak rate of pressure rise (dP/dt\text{max}) and end-systolic elastance (E\text{es}), and SIL inhibited this response as previously reported (Senzaki et al., 2001; Borlaug et al., 2005b; Takimoto et al., 2005a). BAY 41-8543 itself did not alter the ISO response, and the combination again behaved like SIL alone. Summary data for contractility parameters are provided in the lower panels. ISO increased E\text{es} by ~75% in controls and BAY 41-8543-treated hearts, but this was markedly reduced by SIL with or without concurrent sGC stimulation by BAY 41-8543. Similar findings were obtained with dP/dt\text{max}.

**Synergy of cGC Stimulation and PDE5-I in Animals with Acute NOS Inhibition.** We next tested whether a decline in SIL modulation due to acute NOS inhibition was reversible by coadministration of an sGC stimulator. Acute L-NAME (Table 1) increased systemic vascular resistance (p < 0.05) with a borderline increase in peak systolic pressure (p = 0.06). dP/dt\text{max} both declined (p < 0.001). Under this condition (Fig. 1, middle bars), SIL induced less arterial vasodilatation, whereas BAY 41-8543 effects remained similar as in controls. Neither pharmaceutical alone significantly altered systolic function. However, their acute combination reversed these hemodynamic effects of acute L-NAME, lowering arterial resistance and systolic pressure and increasing dP/dt\text{max}.

Acute-LNAME also prevented SIL-mediated suppression of ISO-stimulated contractility (Fig. 2B). BAY itself also did not alter this response, but an antiadrenergic effect was recovered when BAY was combined with SIL. Because the combination itself raised contractility (Fig. 1), one could question whether this obviated further changes with ISO. However, acute L-NAME itself increased the ISO contractile response 2-fold over control hearts (probably reflecting release of NO-cGMP negative feedback), and the basal rise in contractility with SIL + BAY was well below this ISO response, leaving substantial reserve that was still blocked by coadministration.

**Influence of Chronic NOS Inhibition.** We next tested whether modulation of cardiovascular function by PDE5 inhibition was similarly observed in mice subjected to chronic NOS inhibition. After 1 week of L-NAME, systolic pressure and resistance remained elevated and dP/dt\text{max} less diminished than with acute L-NAME (p = 0.05) and baseline (p < 0.03; Table 1). LV mass was unchanged (heart weight/tibial length, 71 ± 1.4 in controls, 74 ± 1.5 mg/cm with chronic L-NAME, p = N.S.). In this condition, SIL and, importantly, the combination of SIL + BAY 41-8543 no longer significantly reduced systemic resistance, peak systolic pressure, or altered dP/dt\text{max} (Fig. 1, right bars). ISO-stimulated cardiac function was not inhibited by SIL, BAY, or their combination (Fig. 2C). Both systemic resistance and cardiac ISO modulation responses with the combined treatment significantly differed between chronic L-NAME-treated animals and the other two groups.

To test whether the chronic L-NAME condition was reversible, mice were first administered L-NAME for 1 week, and then studied a week after its discontinuation (washout). In these animals, SIL effects on resting and ISO-stimulated contraction were restored to normal (Fig. 3A).

Although acute BAY 41-8543 administration did not recover PDE5-I regulation in hearts exposed to chronic L-NAME, we hypothesized that sustained sGC stimulation might be more effective. To test this, L-NAME was adminis-

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**TABLE 1**

Baseline hemodynamics in control and with acute and chronic L-NAME treatment

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 17)</th>
<th>L-NAME</th>
<th>Chronic (n = 16)</th>
<th>ANOVA (p)</th>
</tr>
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<tbody>
<tr>
<td>LV peak-systolic pressure (mm Hg)</td>
<td>91.8 ± 2.0</td>
<td>99.2 ± 2.9*</td>
<td>103.4 ± 2.4*</td>
<td>0.006</td>
</tr>
<tr>
<td>SVR (mm Hg/ml/s)</td>
<td>572 ± 40</td>
<td>802 ± 77*</td>
<td>689 ± 47*</td>
<td>0.03</td>
</tr>
<tr>
<td>LVEDV (μl)</td>
<td>28.6 ± 2.2</td>
<td>30.3 ± 3.0</td>
<td>33.0 ± 2.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>SV (μl)</td>
<td>17.2 ± 1.6</td>
<td>13.8 ± 1.1</td>
<td>17.3 ± 0.8</td>
<td>N.S.</td>
</tr>
<tr>
<td>E\text{es} (mm Hg/μl)</td>
<td>9.1 ± 0.6</td>
<td>8.6 ± 0.7</td>
<td>7.7 ± 0.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>dP/dt\text{max} (mm Hg/s)</td>
<td>12343 ± 621</td>
<td>8371 ± 515*</td>
<td>10574 ± 387*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>65 ± 3</td>
<td>48 ± 3*</td>
<td>54 ± 2*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1. LV, left ventricular; systemic vascular resistance; LVEDV, left ventricular end diastolic volume; SV, stroke volume; N.S., not significant.
2. Post hoc comparison, p < 0.05 vs. control.
3. Post hoc comparison, p = 0.06 vs. control.
4. Post hoc comparison, p < 0.001 vs. control.
5. Post hoc comparison, p = 0.054 vs. acute L-NAME.
tered for 2 weeks, and BAY 41-8543 was provided concurrently (30 mg/kg/day p.o.) during only the 2nd week. Baseline function was similar to controls (Table 2), but animals now displayed normal acute SIL suppression of basal vascular tone and ISO-stimulated cardiac contraction (Fig. 3B). Thus, chronic but not acute sGC stimulation restored PDE5-I cardiovascular modulation despite continued NOS inhibition.

Relation of Antiadrenergic Action of SIL to Myocyte PDE5 Localization and PKG Activation. Prior studies have shown that PDE5 adrenergic contractile regulation is associated with changes in the myocyte localization of the protein, with normal positioning at the myocyte z-band being lost in myocytes where NOS is inactivated (Takimoto et al., 2005a). Figure 4A shows this was fully reversible if NOS function was restored after 1-week L-NAME wash-out. This was further tested in myocytes transfected in vivo with a fusion DsRed-PDE5 construct, avoiding the need for antisera. As shown in Fig. 4B, PDE5 localized to z-bands (colo-

<table>
<thead>
<tr>
<th>Time ((days))</th>
<th>Baseline</th>
<th>Sildenafil</th>
<th>BAY 41-8543</th>
<th>Sildenafil+BAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>25</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 2. Left ventricular PV loops for baseline and ISO stimulation under control conditions and in mice coadministered SIL, BAY, or both. Top panels show pressure-volume loops and corresponding end-systolic pressure-volume relations (ESPVRs). Bottom graphs display summary data (n = 5–6 for each) for absolute change in Ees (slope of ESPVR) and peak rate of pressure rise, dP/dt max. Data are shown for control mice (A), mice pretreated acutely with the NOS inhibitor, L-NAME (B), and mice pretreated with L-NAME for 1 week (C). See text for details. *p < 0.05 versus the corresponding control response (without drug).

Fig. 1. Influence of SIL, sGC stimulation (BAY 41-8543; BAY), or both on resting systemic vascular resistance (SVR), peak systolic blood pressure (SBP), and peak ventricular dP/dt (dP/dt max). Results are shown for three groups: control, acute L-NAME (A-LNM), and chronic L-NAME (C-LNM) and are displayed as percent change from baseline. The absolute baseline values for these and other parameters are provided in Table 1. *p Values shown beneath each set of bars are for one-way ANOVA (within group) for drug effects (SIL, BAY, or their combination). Symbols below individual bars show post hoc (Tukey) test for between-treatment comparisons: †, p < 0.004 versus combined, p = 0.08 versus SIL; ‡, p < 0.005 versus BAY and SIL; and §, p < 0.05 versus combined. Symbols shown above the bars identify comparisons between groups (e.g., control, acute, or chronic L-NAME) for a given treatment: *, p < 0.05 versus control; †, p < 0.002 versus other two groups.
calizing with α-actinin) in cells transfected with the construct in vivo, became diffuse with NOS inhibition, and relocalized at z-bands after L-NAME washout. We further examined whether similar relocalization occurred with chronic sGC stimulation despite sustained NOS inhibition and found that it did (Fig. 3B, bottom). This was probably important for the recovery of PDE5-adenorenergic modulation despite sustained NOS inhibition by chronic BAY 41-8543 treatment.

We next tested whether chronic NOS inhibition blocked myocardial PKG activation and whether this was restored by sustained sGC stimulation. Experiments were repeated as performed for the hemodynamic studies, and hearts were rapidly harvested for protein analysis. Under basal conditions, PKG activation reflected by VASP phosphorylation was minimal but stimulated by the combination of SIL + ISO (Fig. 5A). With chronic l-NAME, phosphorylation declined, whereas it was restored by either l-NAME washout or chronic l-NAME with concomitant BAY 41-8543 treatment. Thus, antiadrenergic effects of SIL for each condition also correlated with activation of PKG. Gene and/or protein expression of PDE5 did not seem to contribute to the different responses (Fig. 5, C and D).

### Discussion

The physiologic effectiveness of PDE5 inhibition depends upon sufficient generation of cGMP that can theoretically be derived from natriuretic peptide or NOS stimulation pathways. Here, we show that the regulation of arterial vasodilatory and cardiac β-adrenergic reserve by PDE5-I targets cGMP from soluble GC stimulation. The cGMP generated by this pathway was also shown to be important to a normal myocyte localization of PDE5 and to PKG activation induced by PDE5-I. Bypassing NOS and acutely stimulating sGC restored PDE5-I arterial and cardiac modulation when NOS was acutely but not chronically inhibited, the latter being associated with loss of normal myocyte PDE5 localization. However, sustained sGC stimulation was effective in offsetting the impact of chronic NOS inhibition. Given the expanding use of PDE5-I use for diseases in which NOS activity is chronically depressed, these findings suggest a therapeutic approach that may enhance their efficacy.

### PDE5 Regulation of Cardiovascular Function

PDE5 is expressed in vascular smooth muscle and endothelium, with high levels in the corpus cavernosum and pulmonary

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**TABLE 2**

Hemodynamic effects of SIL in the heart with chronic l-NAME washout and chronic l-NAME plus delayed BAY

<table>
<thead>
<tr>
<th>Chronic l-NAME Washout (n = 4)</th>
<th>Chronic l-NAME + BAY (n = 8)</th>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>SVR (mm Hg/ml/s)</td>
<td>507 ± 30</td>
</tr>
<tr>
<td>LVEDV (ml)</td>
<td>27.4 ± 3.9</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>19.2 ± 1.3</td>
</tr>
<tr>
<td>$E_v$ (mm Hg/µl)</td>
<td>11.3 ± 1.6</td>
</tr>
<tr>
<td>dP/dt$_{max}$ (mm Hg/s)</td>
<td>12818 ± 592</td>
</tr>
</tbody>
</table>

LV, left ventricular; LVEDV, left ventricular end diastolic volume; SV, stroke volume; N.S., not significant.
vessels. Its cardiac expression is approximately 100-fold less than in lung (Takimoto et al., 2005a), and for many years, it was not thought to be an important role in the heart or cardiac disease. However, recent studies indicate involvement in a variety of myocardial stress responses (Salloum et al., 2003; Kukreja et al., 2005; Takimoto et al., 2005b) and acute β-adrenergic stimulation (Senzaki et al., 2001; Borlaug et al., 2005; Takimoto et al., 2005a). Basal cyclase activity and, thus, cGMP is low in normal resting heart, and PDE5-I has little impact under these conditions. Although two pathways can generate cGMP in vascular smooth muscle and cardiac myocytes, recent data suggest they are not interchangeable (Castro et al., 2006; Fischmeister et al., 2006; Takimoto et al., 2007b) and that NOS-NO stimulation is more selectively targeted by PDE5, at least in normal cells. The current findings showing that sGC stimulation rescues SIL-modulation despite acute NOS inhibition stand in marked contrast to a recent study where we showed ANP costimulation was totally ineffective in this regard (Takimoto et al., 2007b). Reduction of NOS activity, by genetic knockout, pharmacological inhibition, or diseases such as diabetes or heart disease.
failure, suppresses the vasoactivity and cardiac modulation of PDE5 inhibitors (Zhao et al., 2001; Bivalacqua et al., 2004; Champion et al., 2005). In the vasculature, reduced NOS activity manifests as a diminished erectile (Bivalacqua et al., 2004) or pulmonary vasodilator (Zhao et al., 2001) response, whereas in the heart, it blunts the negative modulation of β-adrenergic stimulation (Senzaki et al., 2001; Takimoto et al., 2005a) and, in preliminary studies, an ability to suppress pressure overload hypertrophy (Takimoto and Kass, 2005). Between 30 and 40% of patients with erectile dysfunction display little response to PDE5-I (Burnett, 2006), and although similar data for pulmonary hypertension or potential heart indications have yet to be reported, it seems probable that insufficient cGMP generation will apply to these syndromes as well. Thus, developing a method to circumvent this limitation is important.

The new drug class of sGC stimulators presents a potential solution. First developed in the mid-1990s (Evgenov et al., 2006), agents such as YC-1 (an indazole derivative) were found to be heme-dependent NO-independent sGC stimulators (Ko et al., 1994). BAY 41-8543, used in the current study, is based on the YC-1 structure, but is 2 orders of magnitude more potent (Stasch et al., 2002a,b). Its activity requires the sGC heme to be in its normally reduced form, and its effectiveness in the experiments reported here suggests this was indeed the form that was present. These drugs act to enhance NO-sGC stimulation, and because l-NAME does not fully inhibit NOS activity, their capacity to offset reduced but not absent NO probably played a role. However, this should apply similarly to acute and chronic NOS inhibition, yet for the latter, acute BAY 41-8543 + SIL was less effective because other mechanisms such as altered PDE5 localization/regulation applied. Another factor that may play a role in chronic hypertension or hypertrophy is sGC oxidation, which renders the enzyme less responsive to NO. In such cases, BAY 41-8543 would have less impact (Evgenov et al., 2006), whereas BAY 58-2667, which activates heme-oxidized or heme-free sGC, might be more effective (Evgenov et al., 2006; Stasch et al., 2006).

**Contrasting Effects of Acute and Chronic NOS Inhibition.** Chronic NOS inhibition led to a change in PDE5 distribution from z-bands, whereas this does not occur with acute inhibition (Takimoto et al., 2005a), and this localization seems to be important for PDE5-I to modulate adrenergic stimulation. The current study extends prior work by showing reversible localization changes, with concordance of results relying upon or independent of antisera binding specificity. Prior work identified NOS activity as central to this behavior (Takimoto et al., 2005a), and the current results further clarify cGMP synthesis rather than NO per se to be the primary signal. cGMP can bind to regulatory GAF domains in the N terminus of the PDE to stimulate hydrolytic activity, and PKG phosphorylation at S92 further activates the enzyme (Corbin et al., 2000; Smith et al., 2000; Zoraghi et al., 2005). It is intriguing to note that PKG can bind to troponin T via a leucine-zipper motif, and this has been proposed to be required for PKG phosphorylation of troponin I and subsequent desensitization of the myofilament to calcium (Yuasa et al., 1999). Loss of PDE5 localization at this site could then prevent its action of sarcomeric cGMP/PKG levels and, thus, contractile function, even if acutely fed the correct substrate.

Somewhat similar behavior was also observed in the systemic vasculature. Acute NOS inhibition prevented peripheral vasodilation by SIL, and this was restored by sGC stimulation. However, more prolonged NOS inhibition was not similarly countered, although more sustained sGC stimulation was effective. PDE5 may also be compartmentalized in vascular smooth muscle because studies suggest regional control of cGMP in these cells as well (Piggott et al., 2006). Our data indicate an additional impediment that prevents acute sGC stimulation from being effective if NOS is chronically inhibited. Oxidation of sGC with chronic NOS inhibition might be one factor, and altered compartments could be another.

**Study Limitations.** The focus of our study was on cardiac contractile and hemodynamic modulation by PDE5 inhibitors. Given the multiple conditions and comparisons, we limited the analysis to one dose of SIL and BAY 41-8543, the former chosen for its effectiveness and the latter for its lack of demonstrable basal effects in controls. It is certainly possible that higher doses of BAY 41-8544 would itself offset systemic vascular effects from NOS inhibition, as previously reported in rats (Zanfolin et al., 2006). However, whether such stimulation alone would fully mimic the effects of its combination with PDE5-I is unlikely. First, inhibition of cGMP hydrolytic regulation is compartmentalized (Fischmeister et al., 2006; Takimoto et al., 2007a), so that inhibiting a specific PDE may result in different targeted effects from those derived by cyclase activation. Second, PDE5 activity seems to increase in a number of diseases (Murray et al., 2002; Forfia et al., 2007; Nagendran et al., 2007) and has been linked to reduced efficacy of cGMP synthesis pathways. Although frequently used as a model, NOS inhibition (e.g., l-NAME) does not truly mimic reduced NOS activity present in cardiovascular diseases. First, pharmacological inhibition is broad, whereas dysfunction in disease is probably more organ targeted. Second, it blocks multiple NOS isoforms. Third, it does not replicate functional NOS uncoupling, where the enzyme generates oxidants and less NO, a state thought important in many diseases. Although chronic NOS inhibition can induce myocardial fibrosis when administered for 4 weeks or more, and sGC stimulation may also affect this and/or other features from reduced NOS; however, such analysis was beyond the scope of our investigation.

Last, we did not assess levels of myocardial cGMP but rather focused on PKG activity. This is based on prior studies where we showed that both acute and chronic cardiac cGMP levels seemed to be unaltered by PDE5 inhibitors despite activation of PKG, and this is thought likely due to the low levels of compartmentalized cGMP involved with the signaling.

**Potential Therapeutic Implications.** Direct stimulation of sGC has been proposed for treating diseases such as heart failure (Boerrigter et al., 2007), angiotensin II-stimulated hypertension (Masuyama et al., 2006), and 5/6 nephrectomy-induced renal disease (Kalk et al., 2006). The present findings suggest another potential use; enhancing the action of PDE5-I in settings where critical sGC-generated cGMP is inadequate. Chronic sGC stimulation may effectively restore the normal regulatory activity of PDE5, repair its myocyte distribution, and provide substrate for its regulation. Importantly, it seems possible to achieve this without amplifying systemic blood pressure lowering. Future translational stud-
Cardiac Regulated by PDE5 Inhibition and sGC

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