Sildenafil and B-Type Natriuretic Peptide Acutely Phosphorylate Titin and Improve Diastolic Distensibility In Vivo

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Background—In vitro studies suggest that phosphorylation of titin reduces myocyte/myofiber stiffness. Titin can be phosphorylated by cGMP-activated protein kinase. Intracellular cGMP production is stimulated by B-type natriuretic peptide (BNP) and degraded by phosphodiesterases, including phosphodiesterase-5A. We hypothesized that a phosphodiesterase-5A inhibitor (sildenafil) alone or in combination with BNP would increase left ventricular diastolic distensibility by phosphorylating titin.

Methods and Results—Eight elderly dogs with experimental hypertension and 4 young normal dogs underwent measurement of the end-diastolic pressure-volume relationship during caval occlusion at baseline, after sildenafil, and BNP infusion. To assess diastolic distensibility independently of load/extrinsic forces, the end-diastolic volume at a common end-diastolic pressure on the sequential end-diastolic pressure-volume relationships was measured (left ventricular capacitance). In a separate group of dogs (n=7 old hypertensive and 7 young normal), serial full-thickness left ventricular biopsies were harvested from the beating heart during identical infusions to measure myofilament protein phosphorylation. Plasma cGMP increased with sildenafil and further with BNP (7.31±2.37 to 26.9±10.3 to 70.3±8.1 pmol/mL; P<0.001). Left ventricular diastolic capacitance increased with sildenafil and further with BNP (51.4±16.9 to 53.7±16.8 to 60.0±19.4 mL; P<0.001). Changes were similar in old hypertensive and young normal dogs. There were no effects on phosphorylation of troponin I, troponin T, phospholamban, or myosin light chain-1 or -2. Titin phosphorylation increased with sildenafil and BNP, whereas titin-based cardiomyocyte stiffness decreased.

Conclusion—Short-term cGMP-enhancing treatment with sildenafil and BNP improves left ventricular diastolic distensibility in vivo, in part by phosphorylating titin. (Circulation. 2011;124:2882-2891.)

Key Words: cyclic GMP-dependent protein kinases ■ heart failure, diastolic ■ natriuretic peptide, brain ■ sildenafil

Half of heart failure (HF) patients have preserved ejection fraction (HFpEF), and age and hypertension are potent risk factors for HFpEF.1 Although the pathophysiology of HFpEF is complex, reduced left ventricular (LV) diastolic compliance or distensibility is often present in HFpEF and elderly hypertensive patients2 and may be due to hypertrophy, increased extracellular matrix, or changes in cardiomyocyte function.2,3 Transcriptional (isoform distribution) and post-translational (phosphorylation state) modifications of titin alter its tensile properties and, in turn, passive myofiber stiffness.4-9 Titin exists in 2 isoforms, the longer and more compliant isoform N2BA and the shorter, stiffer isoform N2B, both of which contain a common N2-B unique sequence (N2-Bus). Phosphorylation of the N2-Bus by cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) decreases passive tension in skinned cardiac strips and isolated cardiomyocytes in vitro.4-7,9 PKG-mediated reductions in passive tension were linked to phosphorylation and increased the compliance of the N2-Bus region of titin.6 Furthermore, hypophosphorylation of titin in myocardium harvested from humans with dilated cardiomyopathy has been reported.6 Thus, titin hypophosphorylation may contribute to diastolic dysfunction in HF, and cGMP-activating therapies may phosphorylate titin and improve diastolic function.

Clinical Perspective on p 2891

Nitric oxide (NO) activates soluble guanylyl cyclase and natriuretic peptides activate particulate guanylyl cyclase to
produce cGMP,10 cGMP signaling is terminated by cGMP-hydrolyzing phosphodiesterase (PDE) enzymes.10 PDE-5A, which is inhibited by sildenafil, selectively hydrolyzes cGMP. The potential for cGMP-activating therapies to modulate diastolic function in vivo is also supported by earlier studies in variable patient populations in which agents that increase myocardial NO resulted in downward shifts in the end-diastolic pressure (EDP)-volume (EDV) relationship (EDPVR; increased distensibility) represented by increases in EDV at matched EDP (increased capacitance).11–13 However, in these elegant human studies, concomitant effects on load or extrinsic forces were difficult to exclude, and myofilament protein phosphorylation was not investigated.

We hypothesized that agents that increase cGMP may enhance titin compliance by phosphorylation, leading to improved LV diastolic stiffness or distensibility in vivo. Thus, we tested the effect of a PDE-5A inhibitor (sildenafil) alone or in combination with a natriuretic peptide (B-type natriuretic peptide [BNP]) on LV diastolic function in elderly canines with experimental hypertension (OH dogs) and normal young adult canines (YN dogs). Furthermore, we defined associated alterations in phosphorylation of titin and other sarcomeric proteins.

Methods

Study Design

The study included 11 YN and 15 OH dogs. All experimental procedures were designed in accordance with National Institutes of Health guidelines and approved by the Mayo Institutional Animal Care and Use Committee. Dogs were euthanized by intravenous potassium chloride overdose in the conscious state before and 8 weeks after surgery. Animals were frozen in liquid nitrogen within seconds and stored at −80°C until use. Protein phosphorylation states were analyzed by 1-dimensional and, in a subset of dogs, 2-dimensional SDS-PAGE with Western blotting, phosphoprotein staining, or autoradiography as previously described.4,6,17 Passive force measurements were performed in mechanically isolated, skinned, single cardiomyocytes as described previously. Single cardiomyocytes (2–5 per dog) from 3 OH and 3 YN dogs obtained at baseline and after each serial treatment were attached between a force transducer and a motor. Their sarcomere length was adjusted to 2.2 µm, and passive force (Fpassive) was measured in relaxing buffer. See the online-only Data Supplement for details.

Hemodynamic Study

Short-term hemodynamic studies were performed in 4 YN and 8 OH dogs 8 weeks after renal wrapping or sham surgery. Animals were anesthetized (fentanyl 0.25 mg/kg followed by 0.18 mg·kg⁻¹·h⁻¹ and midazolam 0.75 mg/kg followed by 0.59 mg·kg⁻¹·h⁻¹), intubated, ventilated, and given maintenance saline infusion (3 mL·kg⁻¹·min⁻¹). Thoracotomy and pericardiotomy were performed. Under fluoroscopic guidance, animals were instrumented with an ECG, a pulmonary artery catheter, an LV integrated pressure-conductance catheter (Millar), a left atrial and central aortic high-fidelity pressure transducer (Millar), a pneumatic occluding device around the thoracic inferior vena cava, and an atrial lead for pacing at 10 to 20 bpm above sinus rate. The conductance catheter was calibrated with the measurement of blood conductance (ρ), thermodilution stroke volume, and parallel conductance (hypertonic saline method) as previously described.14 All dogs received autonomic blockade with atropine (1 mg) and propranolol (2 mg/kg). Steady-state and inferior vena cava occlusion (in triplicate) data were collected at suspended end expiration at baseline (after autonomic blockade). Sildenafil (2 mg/kg intravenous bolus) was then administered and data collection was repeated 30 minutes later. Next, BNP was administered (2-µg/kg bolus and 0.01-µg·kg⁻¹·min⁻¹ infusion) with data collection 30 minutes later. Blood for plasma cAMP and cGMP concentrations by radioimmunoassay was collected at each experimental period. After each stage of drug treatment, hemodynamic data collection or plasma/tissue biopsy specimen collection was completed in 5 to 10 minutes; then the next treatment was started.

Pressure-Volume Analysis

Data were collected and analyzed with Sonoview and Cardiosoft software (Sonometrics Corp). The end-systolic pressure (ESP)-volume (ESV) relationship (ESPVR) was defined as ESP=Ees(ESV,Vi), where Ees (end-systolic elastance) is the slope of ESPVR and Vi is its volume axis intercept. The ESPVR data points were fit to the monoexponential equation ESP=−e⁻⁵.3·EDV−Vi, using least-squares nonlinear regression.15 To assess LV diastolic distensibility independently of load/extrinsic pericardium-mediated forces, the EDV at a common EDP on the sequential ESPVRs was measured (LV diastolic capacitance) with the EDP midway down the baseline ESPVR used as the comparator (Figure 1). Similarly, we measured systolic capacitance on the sequential ESPVRs measuring ESV at a common ESP defined at the midpoint of the baseline ESPVR.

Ventricular Biopsy and Biochemical and Mechanical Measurements

Although sarcomeric protein phosphorylation status may display transmural variation,16 we assessed average phosphorylation on full-thickness LV biopsies. Because the biopsy and hemostatic sutures would alter chamber diastolic properties, serial samples were harvested from different regions of the anterior or anterior lateral wall from 7 YN and 7 OH dogs subjected to an identical experimental protocol without collection of hemodynamic data. Biopsy samples were frozen in liquid nitrogen within seconds and stored at −80°C until use. Protein phosphorylation states were analyzed by 1-dimensional and, in a subset of dogs, 2-dimensional SDS-PAGE with Western blotting, phosphoprotein staining, or autoradiography as previously described.4,6,17 Passive force measurements were performed in mechanically isolated, skinned, single cardiomyocytes as described previously.4 Single cardiomyocytes (2–5 per dog) from 3 OH and 3 YN dogs obtained at baseline and after each serial treatment were attached between a force transducer and a motor. Their sarcomere length was adjusted to 2.2 µm, and passive force (Fpassive) was measured in relaxing buffer. See the online-only Data Supplement for details.

Statistics

All continuous variables are presented as mean±SD. The Student t test was used to compare echocardiographic characteristics between OH and YN dogs at baseline and to assess change (paired t test) from baseline to 8 weeks after surgery. Two-way ANOVA with repeated measures was used to test for significant changes during serial treatment and to determine whether changes with sildenafil and BNP differed between YN and OH dogs. Normal distribution is difficult to confirm in the relatively small experimental groups used in these types of translational studies. Thus, data are also reported as median (25th and 75th percentiles), and significance of changes across and between experimental periods was confirmed by the Friedman test, followed by Wilcoxon signed-rank test for individual group comparisons (Tables I and II in the online-only Data Supplement). To test for interaction between serial treatment and OH versus YN status with a nonparametric method, we performed the Scheirer-Ray-Hare extension of the Kruskal-Wallis test.18 Relationships between variables were tested with the Spearman rank correlation. Statistical significance was set at P<0.05 without adjustment for multiple comparisons. Statistical analyses use SPSS (version 19.0, SPSS Inc, Chicago, IL).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Before surgery, OH dogs had similar body weight and EF but higher LV mass and LV/body weight ratio than YN dogs (Table 1 and Table I in the online-only Data Supplement). At 8 weeks after surgery, systolic, diastolic, mean, and pulse pressures were higher in OH than in YN dogs. Furthermore, LV mass had increased by ~25% in OH but remained stable in YN dogs. There was no significant change in EF in either group.

Table 1. Echocardiography at Baseline (Before Surgery) and at 8 Weeks

<table>
<thead>
<tr>
<th>Variables</th>
<th>YN Dogs (n=4)</th>
<th>OH Dogs (n=8)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>25.40±1.34</td>
<td>26.83±3.78</td>
<td>0.43</td>
</tr>
<tr>
<td>Baseline LV mass, g</td>
<td>104±5</td>
<td>147±25</td>
<td>0.002</td>
</tr>
<tr>
<td>LV mass at 8 wk, g</td>
<td>106±5</td>
<td>188±46†</td>
<td>0.0008</td>
</tr>
<tr>
<td>LV/BW at 8 wk, g/kg</td>
<td>4.11±0.24</td>
<td>5.52±0.85</td>
<td>0.003</td>
</tr>
<tr>
<td>LV mass change, %</td>
<td>0.01±0.02</td>
<td>0.27±0.16</td>
<td>0.004</td>
</tr>
<tr>
<td>Systolic blood pressure at 8 wk, mm Hg</td>
<td>167±12</td>
<td>220±25</td>
<td>0.0005</td>
</tr>
<tr>
<td>Diastolic blood pressure at 8 wk, mm Hg</td>
<td>94±6</td>
<td>126±21</td>
<td>0.005</td>
</tr>
<tr>
<td>Mean arterial pressure at 8 wk, mm Hg</td>
<td>122±7</td>
<td>159±20</td>
<td>0.002</td>
</tr>
<tr>
<td>Pulse pressure at 8 wk, mm Hg</td>
<td>73±7</td>
<td>94±15</td>
<td>0.01</td>
</tr>
<tr>
<td>Baseline ejection fraction, %</td>
<td>74±3</td>
<td>70±3</td>
<td>0.1</td>
</tr>
<tr>
<td>Ejection fraction at 8 wk, %</td>
<td>74±2</td>
<td>68±11</td>
<td>0.25</td>
</tr>
</tbody>
</table>

YN indicates young normal; OH, old hypertensive; LV, left ventricular; and BW, body weight.

*Student t test.
†P<0.05 versus baseline.

Effects of Sildenafil and BNP on Ventricular Function

Plasma cAMP and cGMP levels were similar in OH and YN dogs at baseline. Plasma cAMP did not change significantly with sildenafil or BNP treatment (Table 2 and Table II in the online-only Data Supplement). Plasma cGMP levels increased serially in both OH and YN dogs during treatment with sildenafil and BNP.

In all dogs, diastolic capacitance increased with sildenafil and increased further with BNP but did so similarly (P=0.37 for interaction) in OH and YN dogs (Figures 1 and 2, Table 2, and Table II in the online-only Data Supplement). Representative EDPVRs in Figure 1 show all data points from the triplicate inferior vena cava occlusions during each experimental period. There was a rightward shift in the EDPVR without consistent changes in the shape of the curve (no statistically significant change in β and α), indicative of an increase in distensibility.

The change in diastolic distensibility occurred in the setting of decreases in steady-state arterial and LV systolic pressure, which were higher in OH dogs at baseline (LV ESP 116.1±22.6 versus 95.0±6.5 mm Hg; P<0.05) but decreased with sildenafil and BNP in both groups. As LV ESP decreased and stroke volume increased, afterload (arterial elastance [Ea]) decreased. Despite decreases in afterload, steady-state LV EDV increased (significantly during BNP infusion) without an increase in LV EDP, whereas mean left atrial pressure decreased progressively, consistent with improved intrinsic LV distensibility. τ Was higher in OH than YN dogs (47±10 versus 38±3 ms; P<0.05) and did not change overall with sildenafil or after BNP.

In all dogs, systolic capacitance increased with sildenafil and increased further with BNP but did so similarly (P=0.94 for interaction) in OH and YN dogs (Figures 1 and 2, Table 2, and Table II in the online-only Data Supplement). There

Figure 1. End-diastolic (EDPVR) and end-systolic (ESPVR) pressure-volume relationships. Top, Representative EDPVRs from an old hypertensive (OH) and a young normal (YN) dog defined during acute caval occlusion at baseline and after sequential treatment with sildenafil and Burkhoff. The dotted line shows the point of intersection of each curve with the mid left ventricular end-diastolic pressure (LVEDP) of the PVR at baseline. Bottom, ESPVRs from an OH and a YN dog at baseline and after sequential treatment with sildenafil and B-type natriuretic peptide (BNP). The dotted line shows the point of intersection of each curve with the mid left ventricular end-systolic pressure (LVESP) of the PVR at baseline. There is a rightward shift in the PVRs with serial therapy.
was a rightward shift in the ESPVR without consistent changes in the slope of the relationship (no change in Ees but an increase in Vp). Similarly, the stroke work–EDV relationship (preload-recruitable stroke work) was shifted rightward (increased volume intercept) without a change in slope. However, there was no change in load-dependent indexes of contractility (EF and peak dP/dt). Despite the rightward shift in the ESPVR, the steady-state ESV did not increase as a result of the concomitant decrease in steady-state ESP.

There was no correlation between changes in afterload (Ea) and changes in systolic (P=0.36) and diastolic (P=0.71) capacitance during sequential therapy.

**Passive Force of Isolated Skinned Cardiomyocytes**

At baseline, Fpassive was higher in cardiomyocytes isolated from OH dogs than in those from YN dogs (Figure 3). In both OH and YN dogs, Fpassive was lower in cardiomyocytes obtained from

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**Table 2. Hemodynamic and Plasma Evaluation (n=12)**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Sildenafil</th>
<th>BNP + Sildenafil</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Steady state hemodynamics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Heart rate, bpm</td>
<td>105±11</td>
<td>105±10</td>
<td>105±12</td>
<td>0.16</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>97.0±24.1</td>
<td>92.4±22.8†</td>
<td>87.8±22.6†</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean left atrial pressure, mm Hg</td>
<td>8.6±3.5</td>
<td>6.2±2.1†</td>
<td>5.6±3.9†</td>
<td>0.048</td>
</tr>
<tr>
<td>LV end-systolic pressure, mm Hg</td>
<td>103.5±23.0</td>
<td>99.9±21.9</td>
<td>91.8±21.3‡</td>
<td>0.002</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>8.5±3.0</td>
<td>8.1±3.4</td>
<td>8.1±3.6</td>
<td>0.57</td>
</tr>
<tr>
<td>LV end-systolic volume, mL</td>
<td>36.8±17.0</td>
<td>35.4±16.1</td>
<td>38.1±18.2</td>
<td>0.55</td>
</tr>
<tr>
<td>LV end-diastolic volume, mL</td>
<td>66.6±16.8</td>
<td>66.4±14.6</td>
<td>72.2±18.1‡</td>
<td>0.006</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>3.1±0.9</td>
<td>3.2±0.9</td>
<td>3.6±0.8‡</td>
<td>0.003</td>
</tr>
<tr>
<td>Stroke volume, mL</td>
<td>29.9±8.4</td>
<td>31.0±8.7</td>
<td>34.1±8.1‡</td>
<td>0.001</td>
</tr>
<tr>
<td>Ea, mm Hg/mL</td>
<td>3.9±2.0</td>
<td>3.7±1.8</td>
<td>3.0±1.4‡</td>
<td>0.003</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>46.5±13.8</td>
<td>48.3±15.2</td>
<td>49.2±14.1</td>
<td>0.22</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg/s</td>
<td>1795±459</td>
<td>1841±385</td>
<td>1834±442</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>ESPVR analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ees, mm Hg/mL</td>
<td>2.16±0.78</td>
<td>2.43±1.15</td>
<td>2.22±0.85</td>
<td>0.37</td>
</tr>
<tr>
<td>Ees Vp, mL</td>
<td>−22.3±19.9</td>
<td>−17.3±23.0</td>
<td>−14.2±20.8†</td>
<td>0.007</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>57.9±17.7</td>
<td>57.5±15.2</td>
<td>60.7±12.5</td>
<td>0.2</td>
</tr>
<tr>
<td>PRSW, Vo, mL</td>
<td>10.0±21.9</td>
<td>13.4±18.1</td>
<td>19.3±18.6†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic capacitance, mL</td>
<td>22.9±15.3</td>
<td>25.1±16.2†</td>
<td>31.0±16.7†</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>EDPVR analysis</strong></td>
<td></td>
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</tr>
<tr>
<td>Diastolic capacitance, mL</td>
<td>51.4±16.9</td>
<td>53.7±16.8†</td>
<td>60.0±19.4‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic stiffness coefficient, β, mm Hg/mL</td>
<td>0.053±0.04</td>
<td>0.069±0.06</td>
<td>0.059±0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Curve-fitting constant, α</td>
<td>1.39±1.84</td>
<td>1.04±1.65</td>
<td>0.88±1.50</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Plasma cyclic nucleotides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP, pmol/mL</td>
<td>7.31±2.37</td>
<td>26.9±10.3†</td>
<td>70.3±8.1‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cAMP, pmol/mL</td>
<td>47.6±16.8</td>
<td>43.5±19.2</td>
<td>61.1±47.7</td>
<td>0.07</td>
</tr>
</tbody>
</table>

BNP indicates B-type natriuretic peptide; LV, left ventricular; Ea, arterial elastance; ESPVR, end-systolic pressure-volume relationship; Ees, end-systolic elastance; PRSW, preload-recruitable stroke work; EDPVR, end-diastolic pressure-volume relationship.

*Repeated measures ANOVA.
†P<0.05 versus baseline; ††P<0.05 vs sildenafil.

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**Figure 2.** Diastolic and systolic capacitance. Left, Group data for diastolic capacitance during sequential therapy indexed to baseline (BL) for 8 old hypertensive and 4 young normal dogs. Right, Group data for systolic capacitance during sequential therapy indexed to baseline. P<0.05 (ANOVA) for the effect of serial therapy on diastolic and systolic capacitance. BNP indicates B-type natriuretic peptide. †P<0.05 vs baseline; ††P<0.05 vs sildenafil.
LV tissue after sildenafil and BNP treatment compared with cardiomyocytes obtained from LV tissue at baseline.

Sarcomeric Protein Phosphorylation

In the LV tissue samples obtained after autonomic blockade, serine 23/24 phosphorylation of troponin I (TnI) did not change with treatment, and the proportion of monophosphorylated and diphosphorylated TnI isoelectric variants remained unaltered (Figure 4). Troponin T (TnT) was predominantly phosphorylated, but there was no change in phosphorylation status after therapy (Figure 5). Myosin light chain (MLC)-1 was primarily nonphosphorylated and did not change after therapy (Figure 5). MLC-2 was ~40% monophosphorylated but did not change during treatment (Figure 5). There was nearly complete dephosphorylation of the serine 16 site of phospholamban (after autonomic blockade), and this did not change during treatment (Figure 6).

The ratio of phosphorylated N2B and N2BA isoforms (ProQ Diamond stain) to total titin (Sypro Ruby Stain) increased ~100% with sildenafil compared with baseline, and the higher level of phosphorylation was maintained with BNP (Figure 7A through 7C). The ratio of the phosphorylated N2B isoform to the phosphorylated N2BA isoform increased with sildenafil and remained elevated after BNP treatment (Figure 7A and 7C). To confirm the findings with the ProQ phosphostain, we also performed back-phosphorylation assays in which skinned fibers were either phosphorylated by adding PKG or first dephosphorylated with a phosphatase and then treated with PKG to assess inherent phosphorylation as the difference in the phosphorylation signal between PKG and phosphatase plus PKG (Figure 7D through 7F). Inherent phosphorylation was found to be higher with sildenafil and remained elevated after treatment with BNP in the N2B titin isoform (Figure 7D and 7E). In contrast, inherent phosphorylation levels remained unaltered in the T2 titin species, which is considered a degraded form containing the A-band region but little of the elastic I-band region (Figure 7D and 7F). Hence, the effect of sildenafil/BNP on PKG-mediated titin phosphorylation is mainly an effect on I-band titin.

Discussion

Our primary finding is that sildenafil and sildenafil plus BNP increased diastolic and systolic capacitance in association with increases in total titin phosphorylation and decreases in titin-based myocyte passive stiffness. In these autonomically blocked canines, sildenafil or sildenafil plus BNP did not alter phosphorylation of other sarcomeric proteins. These findings confirm and extend previous in vitro studies in which incubation of myocardial preparations with cGMP-PKG increased phosphorylation of titin and reduced passive diastolic myofiber stiffness. These data suggest that therapies elevating cGMP may provide benefit in the short- or long-term treatment of HFpEF.
Effect of Sildenafil and BNP on Diastolic Distensibility

Improvement in passive LV diastolic properties may include decreases in diastolic stiffness (decreased β, slope of EDPVR) or increased distensibility in which the EDPVR shifts downward without changes in shape. In vivo, sildenafil and BNP resulted in acute increases in LV diastolic capacitance unassociated with consistent changes in β or α, suggesting an acute effect on LV distensibility. Short-term increases in LV distensibility may be due to release of extrinsic forces, altered extracellular matrix, or changes in the cardiomyocyte.2,3,14,15,19–21 Diastolic capacitance was measured in the open-chest state and in the absence of the pericardium during acute preload reduction to diminish the influence of extrinsic forces. cGMP-enhancing therapies have not been shown to acutely alter fibrosis or collagen cross-links. Thus, short-term changes in the posttranslational state of sarcomeric proteins in response to increases in cGMP provide the most likely explanation of the observed effects as discussed below.

The enhanced diastolic distensibility associated with cGMP augmentation was associated with increases in steady-state LV EDV without increased steady-state LV EDP and with decreased mean left atrial pressure, all changes consistent with improved diastolic function. The shift indicates that, for a particular LV volume, the LV operates at lower LV pressure, which is reflected in lower left atrial pressure, suggesting potential relief of symptoms and other manifestations of pulmonary congestion.

In these anesthetized, instrumented, open-chest, autonomically blocked canines, EDP was normal at baseline. Thus, we cannot exclude the possibility that changes in the shape of the EDPVR (altered diastolic chamber stiffness constant) would have been evident if data had been collected over a larger range of EDV, providing a more accurate (less extrapolation) characterization of the monoexponential EDPVR. Furthermore, detection of changes in shape of the EDPVR may be obscured by concomitant and reciprocal changes in the stiffness and curve-fitting constants.15 Improvement in diastolic distensibility has similarly been reported during intracoronary nitroprusside, NO, and substance P infusion in humans.11–13 Although the presence of the pericardium in these human studies increased the potential for altered right ventricular loading and ventricular interdependence to mediate shifts in the EDPVR, it is notable that our findings (in the absence of the pericardium and with measurements in the midportion of the EDPVR) are similar. In human subjects with dilated cardiomyopathy, N-monomethyl-L-
arginine, an NO synthase inhibitor, impaired diastolic compliance.\(^{22}\) The present findings with sildenafil and BNP are consistent with these previous studies in which cGMP was increased via NO and soluble guanylyl cyclase.

**Effect of Sildenafil and BNP on Systolic Capacitance**

Sildenafil and BNP increased systolic capacitance, as exhibited by the rightward shift in the ESPVRs with increased ESV at a common ESP and an increase in the volume axis intercept of the ESPVR \((V_0)\) without a change in the slope of the ESPVR \((E_{es})\). Similarly, the stroke work–EDV relationship (preload-recruitable stroke work) was shifted rightward without a change in slope. Although these changes are consistent with a negative inotropic effect, more load-dependent indexes of systolic performance \((EF, peak dP/dt)\) did not change with sequential treatment, likely owing to the concomitant decrease in afterload.

It is now well understood that the ESPVR is not linear, that the degree and nature of nonlinearity vary with inotropic state, and that the volume intercept \((V_0)\) and slope \((E_{es})\) may change with changes in inotropic state (reviewed by Burkhoff et al\(^ {15}\)). Furthermore, the ESPVR is not truly load independent because the inotropic state varies directly with afterload impedance, an effect attributed to load-dependent changes in actin-myosin interactions and calcium sensitivity of the troponin regulatory complex.\(^ {15}\) Thus, sildenafil- and sildenafil plus BNP-mediated decreases in afterload could contribute to the rightward shift in the ESPVR,\(^ {14,22}\) although the changes in afterload were subtle (≈10-mm Hg change in ESP) and there was no correlation between change in afterload and changes in systolic capacitance. Although we believe that the decrease in the passive tensile properties of cardiomyocytes after sildenafil and BNP infusion explains the increased systolic capacitance, a contribution of afterload reduction, particularly seen after BNP infusion, cannot be excluded.

Some\(^ {24,25}\) but not all\(^ {26,27}\) in vivo studies have reported a subtle but detectable negative inotropic effect of cGMP-activating compounds, although subject characteristics, methodology, and experimental conditions in these studies varied and none investigated the combination of PDE-5A inhibition and natriuretic peptides.

Although still controversial, several recent reports have supported a contribution from titin-based passive tension to length-dependent activation (Frank-Starling relationship) in the cardiac sarcomere.\(^ {28,29}\) However, despite decreased passive tension in isolated cardiomyocytes, we have not detected a clear change in preload-recruitable stroke work or Ees with sildenafil or the addition of BNP.

**Mechanism of Sildenafil- and BNP-Mediated Effects on Myocardial Function**

cGMP may alter myocardial function via PKG-mediated phosphorylation of sarcomeric or regulatory proteins or by...
modulating cyclic nucleotide signaling through cGMP-stimulated (PDE-2 and -5) or cGMP-inhibited (PDE-3) PDEs. Ex vivo studies of myocytes or multicellular preparations have shown that cGMP stimulators or analogs produce an acceleration of relaxation, an increase in resting cell length, and reduced inotropy and that these effects occur in the absence of changes in calcium transient amplitude or kinetics. Although NO-mediated increases in cGMP blunt the inotropic response to β-adrenergic stimulation, the negative inotropic effect of cGMP is PKG-dependent and can occur independently of PKA signaling. The mechanisms whereby PKG alters calcium sensitivity are unclear, although PKG-mediated phosphorylation of TnI has been implicated in vitro studies.

Phospholamban is phosphorylated by PKA and PKG, and modulation of phospholamban phosphorylation alters relaxation and potentially inotropic function, although the circumstances in which PKG- versus PKA-mediated phosphorylation affects phospholamban phosphorylation status in vivo are poorly understood. Titin, MLC-1, and MLC-2 are not known to be phosphorylated by PKG, but this has not been widely studied. Importantly, in our autonomically blocked dogs, neither sildenafil nor sildenafil plus BNP altered the phosphorylation status of TnI, phospholamban, TnT, MLC-1, or MLC-2. Of note, TnI and particularly phospholamban were highly dephosphorylated, likely owing to the presence of autonomic blockade. Consistent with the lack of change in TnI or phospholamban phosphorylation status, there was no consistent effect on the speed of relaxation with sildenafil or sildenafil plus BNP. Although sildenafil and sildenafil plus BNP had no effect on the regulatory proteins noted above, titin phosphorylation was increased with sildenafil and sildenafil plus BNP with preferential phosphorylation of the springy I-band region. In vitro studies show that passive tension in the physiological range of sarcomere length is critically influenced by titin. Relative expression of the N2BA titin isoform increases in proportion to body size in mammals and allows longer operating sarcomere lengths without increases in passive tension.

Phosphorylation of titin by incubation with PKA and PKG acutely decreases passive tension in cardiomyocytes and myofibers in vitro, most dramatically after initial dephosphorylation. Both kinases phosphorylate titin at the N2-Bus segment, and this posttranslational event enhances titin compliance and thus reduces myofiber passive stiffness. We observed that titin phosphorylation did not increase further after BNP was added to sildenafil despite a substantial increase in plasma cGMP. However, the 80% inherent phosphorylation after sildenafil administration, as measured with back-phosphorylation assays, suggests potential saturation.

We used sildenafil, a PDE-5A inhibitor, and BNP to enhance cGMP signaling. Recently, there has been uncertainty regarding the expression of PDE-5A in cardiomyocytes. However, the presence of sildenafil-induced physiological effects in cardiomyocytes is supported by studies demonstrating sildenafil stimulation of cGMP-associated electric currents in isolated rat cardiomyocytes transfected with cGMP-gated ion channels. Sildenafil may also have nonselective inhibitory effects on a different PDE known to be abundantly expressed in the heart, PDE-1. Regardless of the mechanism for cGMP enhancement by sildenafil in the heart, the short-term hemodynamic and titin phosphorylation effects of sildenafil in our experiments are unequivocal.

The ratio of phosphorylated titin to total titin increased after sildenafil therapy with no further increase with BNP treatment. Most changes in titin phosphorylation appeared to occur within the I-band region. Moreover, the ratio of phosphorylated N2B to N2BA titin isoforms increased after sildenafil treatment with no further change after the addition of BNP. Fukuda et al showed that the extent of reduction in passive tension during phosphorylation of titin by PKA is highest in myocardial tissue with more of the N2B isoform. They postulated that passive force reduction is due to a reduction in the ratio of the length of the phosphorylated segment to the length of the titin isoform. Thus, phosphorylation of the shorter N2B isoform will be associated with a larger passive force reduction. Although phosphorylation of the N2-Bus segment is linked to changes in titin elasticity, there are other PKG-sensitive phosphorylation sites on titin. Thus, correlation between overall titin phosphorylation and changes in diastolic function is not likely to be linear.

We measured $F_{\text{passive}}$ in isolated skinned cardiomyocytes ex vivo, which was lower after treatment with sildenafil and maintained after the addition of BNP. $F_{\text{passive}}$ has a strong correlation with LVEDP and other indexes of diastolic function, including radial myocardial stiffness modulus (E) and circumferential LV end-diastolic wall stress ($\sigma$). Human subjects with diastolic HF have higher $F_{\text{passive}}$ compared with normal subjects. Because the cardiomyocytes were treated in a manner that disrupts the sarcolemma and other membranes and with experiments performed in nonactivating (Ca$^{2+}$-free) buffer, altered calcium handling is not responsible for the observed changes in $F_{\text{passive}}$. Because we excluded posttranslational changes in myofilament-associated proteins (TnI, TnT, MLC-1, and MLC-2), titin phosphorylation changes are most likely to explain the change in $F_{\text{passive}}$. The lack of improvement in $F_{\text{passive}}$ after the addition of BNP to sildenafil despite an increase in plasma cGMP may be secondary to saturation of titin phosphorylation.

The EDPVR represents a summary of the PVR at the end of diastole in each cardiac cycle and is affected by any intervention or physiological process that contributes to active relaxation and passive stiffness properties of the ventricular chamber. Impairment or incomplete relaxation (at higher heart rates) and increased passive diastolic tone will contribute to changes in the EDPVR. Impaired relaxation may be due to posttranslational changes in myofilament-associated proteins (TnI, TnT, MLC-1, and MLC-2) regulating calcium sensitivity or calcium transients (phospholamban), which were not affected in our experiments with sildenafil or sildenafil plus BNP.

We found a short-term increase in LV distensibility reflected in diastolic LV capacitance and the rightward shift in the EDPVR during treatment with sildenafil that was further enhanced with the addition of BNP. However, titin phosphorylation and cardiomyocyte $F_{\text{passive}}$ did not change further after the addition of BNP. There are several potential explanations.
for the improved diastolic distensibility with BNP other than posttranslational changes in titin. Changes in systemic afterload may influence systolic and diastolic stiffness or distensibility.22,32 Ludbrook et al47 observed a downward shift of the EDPRV with no appreciable alteration in the stiffness constant with a vasodilator (16% decrease in mean arterial pressure) in human HF. Although PDE-5 is thought to be specific for NO-soluble guanylyl cyclase–derived cGMP,43 pulmonary arterial vasodilation in response to BNP is markedly accentuated in the presence of sildenafil.48 Thus, despite pericardiotomy, we cannot exclude that an effect of BNP is to reduce ventricular interdependence via effects on right ventricular load. Finally, sildenafil- and BNP-mediated reduction in coronary perfusion pressure may enhance diastolic distensibility.49 Unfortunately, we did not measure right ventricular or coronary perfusion pressure.

Limitations
The inability to collect myocardial samples and hemodynamics in the same animals limits the ability to correlate changes in phosphorylation with changes in LV function, and differential dose-dependent and time course effects were not explored. The order of infusions was not altered, so we are unable to determine whether BNP alone phosphorylates titin or alters distensibility. Studies were conducted in the presence of autonomic blockade, and this may influence the interaction of PKG– and PKA-mediated phosphorylation. However, the use of ß-blockers and age-related decrements in adrenergic responsiveness are ubiquitous in HFpEF and hypertension. A study of the effect of alternate particulate or soluble guanylyl cyclase activators alone and in combination with PDE inhibitors would be of interest.

Conclusions
Diastolic dysfunction is thought to contribute to the pathophysiology of HFpEF, but to date, no therapy has been demonstrated to improve diastolic dysfunction, symptoms, or outcomes in HFpEF. In vitro studies suggest dramatic effects of cGMP-PKG titin phosphorylation on diastolic function. We show here for the first time that therapies known to enhance cGMP enhance diastolic distensibility in association with enhanced titin phosphorylation and reduced titin-based passive stiffness in vivo in a clinically relevant large mammalian model. Although evidence of a negative inotropic effect was also apparent, this was subtle and not associated with decreases in EF or cardiac output. These data provide support for ongoing trials of sildenafil in HFpEF (Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure [RELAX] trial; www.clinicaltrials.gov, NCT00763867) and for future investigation of combining sildenafil and natriuretic peptide therapy to treat HFpEF.

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Disclosures
None.

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Sildenafil and B-Type Natriuretic Peptide Acutely Phosphorylate Titin and Improve Diastolic Distensibility In Vivo
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Supplemental Methods

Preload Recruitable Stroke Work, PRSW

The preload recruitable stroke work relationship was determined using the method of Glower et al. Preload was varied by inferior vena-caval occlusion. Global stroke work over each cardiac cycle was calculated as the integral of left ventricular pressure and volume as described by the formula:

\[ SW = - \int_{EDV}^{ESV} P(t)dV \]

Stroke work from each cardiac cycle was then plotted against end diastolic LV chamber volume resulting in a highly linear relationship. PRSW is the slope for this relationship. PRSW-Vo is the volume axis intercept for this relationship. See supplemental figures.

Sarcomeric protein phosphorylation studies

To determine titin isoform phosphorylation, samples were homogenized in a modified Laemmli buffer containing 8 M urea, 2 M thiourea, 3% SDS (w/v), 75 mM DTT, 0.03% (w/v) bromophenol blue, 10% (v/v) glycerol, and 50 mM Tris-HCl, pH 6.8, and resolved by agarose-strengthened SDS-PAGE (2% polyacrylamide concentration) followed by staining with SYPRO Ruby and Pro-Q diamond phosphoprotein stains. Each sample was loaded in triplicate. N2B and N2BA isoform protein and phosphorylation signals were quantified by densitometry. Phospho-titin to total-titin and phospho-N2B to phospho-N2BA ratios were calculated and indexed to signals at baseline.

Proteins for the remaining studies were extracted by homogenization on ice using 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% 3-10 immobilized pH
gradient buffer, protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). For Western blotting, protein extracts were resolved by 29:1 12% SDS-PAGE (TnI) or 10% tricine SDS-PAGE (phospholamban) and then transferred to Hybond PVDF membrane (GE Healthcare, Piscataway, NJ). Antibodies used identified cardiac muscle TnI (1:1000 dilution, Fitzgerald Industries, Concord, MA), serine 23/24 phosphorylated TnI (1:1333 dilution, Cell Signalling Technology, Danvers, MA), phospholamban (1:2000 dilution, Millipore, Temecula, CA) and serine 16 phosphorylated phospholamban (1:1600 dilution, Millipore, Temecula, CA). A horseradish peroxidase-conjugated anti-rabbit IgG (37.5 ng per blot, Jackson Immunoresearch, West Grove, PA) was used as secondary antibody to detect serine 16 phosphorylated phospholamban with bound antibody complexes visualized using ECL (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Temecula, CA). Cy3 labeled anti-mouse IgG secondary antibody (200 ng per blot, Jackson Immunoresearch, West Grove, PA) was used to detect phospholamban and TnI, while Cy5 labeled anti-rabbit IgG secondary antibody (200 ng per blot, Piscataway, NJ) was used to detect serine 23/24 phospho-TnI. Total protein and phosphorylation signals quantified by densitometry were normalized to a LV homogenate sample from a single dog harvested after sacrifice to standardize for comparison across gels.

Two dimensional SDS-PAGE was used to resolve TnT, MLC-1 and MLC-2 isoelectric forms. Homogenates were first processed using a 2D-Clean-Up Kit prior to resolution in the first dimension (Amersham Biosciences, Piscataway, NJ). For resolution of acidic proteins (TnT, MLC-1, MLC-2), 3-5.6 NL IPG strips
were rehydrated overnight with protein homogenate in acidic protein rehydration buffer (7M Urea, 2M Thiourea, 2% CHAPS, 0.5% 3.5-5.0 IPG buffer, 0.002% bromophenol blue and protease inhibitor). The first dimension focusing was performed with an Ettan IPGphor 3 Isoelectric Focusing Unit. For resolution of the basic TnI phosphoforms, 7-11 NL IPG gel strips were rehydrated overnight in basic rehydration solution (7M Urea, 2M Thiourea, 2% CHAPS, 0.5% 7-11 NL IPG buffer, 0.002% bromophenol blue, 12 µL/mL Destreak reagent and protease inhibitor). For sample loading, protein homogenates were dissolved in basic rehydration buffer and loaded at the anode using a sample cup. After first dimension focusing, all gel strips were equilibrated for 15 minutes in 6 M urea, 50 mM Bis-Tris, pH 6.4, 30% glycerol, 2% SDS, and 0.002% bromophenol blue containing first 10 mM dithiothreitol for 15 minutes and then 2.5% iodoacetamide for 15 minutes. The second dimension SDS-PAGE resolution was performed using 29:1 11% polyacrylamide gels. Resolved gels were stained with Deep Purple total protein fluorescent stain. Densitometry was performed using a Typhoon 9410 scanner and accompanying ImageQuant TL software.

**Titin phosphorylation by ^32^P-autoradiography**

In addition to using the Pro-Q Diamond /Sypro Ruby system, protein phosphorylation of titin was probed by standard autoradiography following 2% SDS-PAGE with concentrations of 2% polyacrylamide, as previously reported. Skinned fibers from biopsies were either directly phosphorylated by adding PKG (1.68 × 10^-5 U/µl), cGMP, and [γ ^32^P]ATP for 1 hour at 36°C or were first de-phosphorylated by Alkaline Phosphatase (0.3 U/µl) for 2 hours at 30°C before
incubation with PKG. After a washing step with relaxing solution to remove the phosphatase the fibers were incubated with cGMP-PKG for 1 hour at 36°C in relaxing solution supplemented with phosphatase inhibitor cocktail (Sigma-Aldrich) and [γ^{32}P]ATP (specific activity, 250 μCi/μM). Fibers in relaxing solution to which [γ^{32}P]ATP but no PKG was added, served as controls.

The fibers were denatured, dissolved, electrophoresed on 2% SDS-polyacrylamide gels and titin protein bands were stained with coomassie blue. Gels were dried before exposure to autoradiographic film usually for up to 24 h at room temperature. \(^{32}\)P-incorporation was visualized by LAS-4000 Image Reader and signals were analyzed with Multi Gauge V3.2 software. Signal from N2BA band was too faint to allow for a meaningful analysis. However phosphorylation of the more abundant N2B titin isoform and titin degradation product band T2 was compared.

**Passive Tension in Isolated Cardiomyocytes**

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously.\(^5,6\) Biopsies were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 145; EGTA, 2; MgATP, 4; imidazole, 10; pH7.0), mechanically disrupted and incubated for 5 minutes in relaxing solution supplemented with 0.5% Triton X-100 to remove all membrane structures. Subsequently, cells were washed twice in relaxing solution, after which single cardiomyocytes were attached with silicone adhesive between a force transducer and a motor. Sarcomere length of isolated cardiomyocytes was adjusted to 2.2 μm and the passive tension (\(F_{\text{passive}}\)) of cardiomyocytes was measured in relaxing
solution (pCa 9). All force values were normalized to cardiomyocyte cross-sectional area.
**Supplementary Table 1: Echocardiography at baseline (prior to surgery) and at 8 weeks, n=12**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Young Normal Dogs</th>
<th>Old Hypertensive Dogs</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=4</td>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>Body weight, kg, median (IQR)</td>
<td>26.0(24.0,27.0)</td>
<td>28.5(24.3,32.0)</td>
<td>0.283</td>
</tr>
<tr>
<td>Baseline LV Mass, gram, median (IQR)</td>
<td>105.8(96.8,106.1)</td>
<td>132.8(129.2,166.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>8 Week LV Mass, gram, median (IQR)</td>
<td>105.1(97.2,109.9)</td>
<td>161.8(154.6,202.4)*</td>
<td>0.004</td>
</tr>
<tr>
<td>8 Week LV/BW, g/kg median (IQR)</td>
<td>4.05(3.89,4.16)</td>
<td>6.45(5.36,7.07)</td>
<td>0.004</td>
</tr>
<tr>
<td>LV Mass Change, %, median (IQR)</td>
<td>2.13(1.38,2.64)</td>
<td>21.6(19.7,22.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>8 Week Systolic Blood Pressure, mmHg, median (IQR)</td>
<td>155.0(153.4,174.2)</td>
<td>215.4(193.9,233.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>8 Week Diastolic Blood Pressure, mmHg, median (IQR)</td>
<td>90.0(87.6,94.4)</td>
<td>125.7(111.2,140.7)</td>
<td>0.048</td>
</tr>
<tr>
<td>8 Week Mean Arterial Pressure, mmHg, median (IQR)</td>
<td>116.0(113.2,127.6)</td>
<td>157.9(141.2,170.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>8 Week Pulse Pressure, mmHg, median (IQR)</td>
<td>65.8(65.0,76.2)</td>
<td>94.6(79.4,106.2)</td>
<td>0.025</td>
</tr>
<tr>
<td>Baseline Ejection Fraction, %, median (IQR)</td>
<td>72.0(69.0,74.0)</td>
<td>72.0(68.8,73.0)</td>
<td>0.368</td>
</tr>
<tr>
<td>8 Week Ejection Fraction, %, median (IQR)</td>
<td>73.0(70.0,77.0)</td>
<td>72.5(69.0,73.0)</td>
<td>0.461</td>
</tr>
</tbody>
</table>

† Group comparisons by Wilcoxon rank-sum test (Mann-Whitney U test); * P<0.05 Vs Baseline using Wilcoxon signed-rank test.
Supplementary Table 2: Hemodynamic studies and plasma hormone evaluation, n=12

<table>
<thead>
<tr>
<th>Steady State Hemodynamics</th>
<th>Baseline</th>
<th>Sildenafil</th>
<th>BNP + Sildenafil</th>
<th>P-value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate, bpm (IQR)</td>
<td>106.6(97.9,113.2)</td>
<td>106.5(97.5,112.9)</td>
<td>106.4(97.5,115.5)</td>
<td>0.779</td>
</tr>
<tr>
<td>Mean Aortic Pressure, mmHg (IQR)</td>
<td>87.4(79.7,117.6)</td>
<td>85.7(75.4,111.7)*</td>
<td>85.2(71.9,100.4)*</td>
<td>0.002</td>
</tr>
<tr>
<td>Mean Left Atrial Pressure, mmHg (IQR)</td>
<td>7.8(5.7,11.7)</td>
<td>5.7(5.0,7.0)*</td>
<td>5.8(3.2,7.1)*</td>
<td>0.05</td>
</tr>
<tr>
<td>LV End Systolic Pressure, mmHg (IQR)</td>
<td>95.2(84.2,120.8)</td>
<td>94.2(81.6,118.4)</td>
<td>88.4(73.7,102.8)*</td>
<td>0.009</td>
</tr>
<tr>
<td>Mean Aortic Pressure, mmHg (IQR)</td>
<td>8.2(6.2,10.6)</td>
<td>7.6(5.7,10.1)</td>
<td>6.7(5.3,10.4)</td>
<td>0.338</td>
</tr>
<tr>
<td>LV End Systolic Volume, ml (IQR)</td>
<td>33.5(23.3,46.5)</td>
<td>35.4(22.0,45.9)</td>
<td>32.9(22.5,53.0)</td>
<td>0.472</td>
</tr>
<tr>
<td>LV End Diastolic Volume, ml</td>
<td>62.3(52.8,74.2)</td>
<td>62.0(54.2,76.0)</td>
<td>67.2(55.9,85.4)*</td>
<td>0.005</td>
</tr>
<tr>
<td>Cardiac Output, L/min (IQR)</td>
<td>3.1(2.4,3.6)</td>
<td>3.4(2.5,3.9)</td>
<td>3.7(2.7,4.1)*</td>
<td>0.005</td>
</tr>
<tr>
<td>Stroke Volume, ml (IQR)</td>
<td>30.6(21.2,35.8)</td>
<td>33.5(21.7,38.3)</td>
<td>35.9(25.0,41.9)*</td>
<td>0.005</td>
</tr>
<tr>
<td>Arterial Elastance (Em), mmHg/ml (IQR)</td>
<td>3.2(2.5,5.9)</td>
<td>2.9(2.2,5.5)</td>
<td>2.7(1.7,4.0)*</td>
<td>0.009</td>
</tr>
<tr>
<td>Ejection Fraction, % (IQR)</td>
<td>48.2(35.1,58.2)</td>
<td>50.3(36.3,63.4)</td>
<td>49.9(38.2,64.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>1564(1449,2277)</td>
<td>1723(1558,2213)</td>
<td>1733(1486,2162)</td>
<td>0.92</td>
</tr>
</tbody>
</table>

ESPVR analysis

End Systolic Elastance (Ees), mmHg/ml (IQR) | 1.88(1.59,2.57) | 2.12(1.43,3.28) | 2.08(1.49,2.81) | 0.558
Ees V0, ml (IQR) | -25.1(-35.9,-6.0) | -16.8(-36.4,-1.0)* | -9.4(-26.6,0.0)* | 0.046
Preload Recruitable Stroke Work (PRSW), mmHg (IQR) | 58.0(41.1,69.7) | 54.4(44.5,71.2) | 59.5(53.1,65.6) | 0.368
PRSW, Vo, ml (IQR) | 7.6(-5.9,18.5) | 8.3(-1.3,20.0)* | 16.9(11.3,23.1)* | 0.001
Systolic Capacitance, ml | 19.2(13.0,26.7) | 19.3(15.2,27.1)* | 25.0(21.6,32.8)* | 0.001

EDPVR analysis

Diastolic Capacitance, ml | 47.0(42.8,58.2) | 48.2(43.7,61.6)* | 54.4(44.9,68.6)* | <0.001
Diastolic stiffness coefficient (β, mmHg/ml) | 0.04(0.02,0.09) | 0.05(0.03,0.11) | 0.05(0.03,0.09) | 0.071
Curve fitting constant (α) | 0.57(0.07,2.81) | 0.42(0.02,1.17) | 0.31(0.02,0.79) | 0.132

Plasma hormones

cGMP, pmol/ml | 6.8(4.9,9.9) | 28.8(19.4,31.2)* | 73.9(61.8,77.2)* | <0.001
cAMP, pmol/ml | 52.7(26.8,56.5) | 47.2(23.9,58.4) | 50.7(33.8,61.6) | 0.614

‡ Friedman's test; * P-value <0.05 Vs Baseline and † P-value <0.05 Vs sildenafil by Wilcoxon signed-rank test
### Supplementary Table 3: Hemodynamics and plasma hormones in young normal and old hypertensive dogs, n=12

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Sildenafil</th>
<th>BNP + Sildenafil</th>
<th>p-value, ANOVA*</th>
<th>Baseline</th>
<th>Sildenafil</th>
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</tr>
<tr>
<td>Heart Rate, bpm</td>
<td>99.9±14.3</td>
<td>99.7±14.1</td>
<td>102.2±17.7</td>
<td>0.431</td>
<td>107.1±8.3</td>
<td>106.9±8.1</td>
<td>106.9±8.0</td>
<td>0.555</td>
</tr>
<tr>
<td>Mean Aortic Pressure, mmHg</td>
<td>76.3±9.6</td>
<td>74.6±11.0</td>
<td>70.7±15.7</td>
<td>0.498</td>
<td>107.3±22.5</td>
<td>101.3±22.3†</td>
<td>96.4±21.2†</td>
<td>0.003</td>
</tr>
<tr>
<td>Mean Left Atrial Pressure, mmHg</td>
<td>6.68±3.89</td>
<td>4.52±0.87</td>
<td>4.02±1.75</td>
<td>0.406</td>
<td>9.58±3.01</td>
<td>6.99±2.05†</td>
<td>6.44±4.52</td>
<td>0.07</td>
</tr>
<tr>
<td>LV End Systolic Pressure, mmHg</td>
<td>86.3±8.6</td>
<td>86.3±10.6</td>
<td>79.0±13.9</td>
<td>0.229</td>
<td>112.2±23.3</td>
<td>106.7±23.5</td>
<td>98.1±22.2†</td>
<td>0.002</td>
</tr>
<tr>
<td>LV End Diastolic Pressure, mmHg</td>
<td>8.90±3.78</td>
<td>9.22±4.60</td>
<td>8.70±5.10</td>
<td>0.667</td>
<td>8.34±2.81</td>
<td>7.47±2.89</td>
<td>7.79±2.98</td>
<td>0.144</td>
</tr>
<tr>
<td>LV End Systolic Volume, ml</td>
<td>28.4±7.9</td>
<td>28.2±10.1</td>
<td>29.2±8.4</td>
<td>0.727</td>
<td>41.0±19.2</td>
<td>39.0±17.9</td>
<td>42.5±20.6</td>
<td>0.45</td>
</tr>
<tr>
<td>LV End Diastolic Volume, ml</td>
<td>59.6±7.5</td>
<td>62.1±10.5</td>
<td>68.2±7.1</td>
<td>0.055</td>
<td>70.1±19.4</td>
<td>68.5±16.5</td>
<td>74.2±21.9</td>
<td>0.073</td>
</tr>
<tr>
<td>Cardiac Output, L/min</td>
<td>3.09±0.24</td>
<td>3.33±0.29</td>
<td>3.97±0.85</td>
<td>0.095</td>
<td>3.11±1.06</td>
<td>3.15±1.06</td>
<td>3.36±0.82</td>
<td>0.137</td>
</tr>
<tr>
<td>Stroke Volume, ml</td>
<td>31.3±3.6</td>
<td>33.8±5.5</td>
<td>39.0±5.8</td>
<td>0.053</td>
<td>29.1±10.2</td>
<td>29.5±9.9</td>
<td>31.6±8.3</td>
<td>0.122</td>
</tr>
<tr>
<td>Arterial Elastance (Ea), mmHg/ml</td>
<td>2.81±0.56</td>
<td>2.64±0.77</td>
<td>2.11±0.75</td>
<td>0.117</td>
<td>4.46±2.20</td>
<td>4.16±2.05†</td>
<td>3.40±1.49†</td>
<td>0.006</td>
</tr>
<tr>
<td>Ejection Fraction, %</td>
<td>52.0±8.3</td>
<td>55.4±10.8</td>
<td>57.6±9.8</td>
<td>0.214</td>
<td>43.3±15.3</td>
<td>44.8±16.4</td>
<td>45.0±14.5</td>
<td>0.692</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>1532±142</td>
<td>1644±190</td>
<td>1732±171</td>
<td>0.099</td>
<td>1927±513</td>
<td>1940±429</td>
<td>1886±535</td>
<td>0.649</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>36.6±2.9</td>
<td>35.9±3.7</td>
<td>35.2±2.9†</td>
<td>0.021</td>
<td>43.7±9.7</td>
<td>45.0±11.1</td>
<td>48.3±13.5††</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>ESPVR analysis</strong></td>
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<tr>
<td>End Systolic Elastance (Ees), mmHg/ml</td>
<td>1.54±0.32</td>
<td>1.38±0.26</td>
<td>1.62±0.44</td>
<td>0.492</td>
<td>2.47±0.76</td>
<td>2.96±1.05†</td>
<td>2.52±0.87†</td>
<td>0.004</td>
</tr>
<tr>
<td>Ees V₀, ml</td>
<td>-36.8±15.6</td>
<td>-38.0±9.2</td>
<td>-27.3±16.5</td>
<td>0.134</td>
<td>-15.0±18.3</td>
<td>-7.0±20.8†</td>
<td>-7.7±20.3†</td>
<td>0.007</td>
</tr>
<tr>
<td>Preload Recruitable Stroke Work (PRSW), mmHg</td>
<td>43.6±5.5</td>
<td>45.1±7.9</td>
<td>53.8±10.9†</td>
<td>0.012</td>
<td>65.1±17.4</td>
<td>63.7±14.4</td>
<td>64.2±12.4</td>
<td>0.929</td>
</tr>
<tr>
<td>PRSW, V₀, ml</td>
<td>-0.6±9.0</td>
<td>0.9±7.5</td>
<td>10.7±10.1††</td>
<td>0.009</td>
<td>15.3±25.0</td>
<td>19.6±18.8</td>
<td>23.5±20.9††</td>
<td>0.025</td>
</tr>
<tr>
<td>Systolic Capacitance, ml</td>
<td>14.6±7.8</td>
<td>17.1±6.9</td>
<td>22.7±2.8</td>
<td>0.119</td>
<td>27.0±16.9</td>
<td>29.1±18.3††</td>
<td>35.1±19.4††</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>EDPVR analysis</strong></td>
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<td></td>
</tr>
<tr>
<td>Diastolic Capacitance, ml</td>
<td>44.0±8.5</td>
<td>48.5±9.8</td>
<td>56.0±8.6††</td>
<td>0.01</td>
<td>55.1±19.2</td>
<td>56.4±19.4</td>
<td>62.0±23.4†</td>
<td>0.018</td>
</tr>
<tr>
<td>Diastolic Stiffness Coefficient (β, mmHg/ml)</td>
<td>0.048±0.042</td>
<td>0.052±0.039</td>
<td>0.050±0.029</td>
<td>0.784</td>
<td>0.056±0.038</td>
<td>0.079±0.064</td>
<td>0.063±0.042</td>
<td>0.064</td>
</tr>
<tr>
<td>Curve Fitting Constant (α)</td>
<td>1.88±2.47</td>
<td>1.72±2.53</td>
<td>1.34±2.30</td>
<td>0.11</td>
<td>1.14±1.58</td>
<td>0.70±1.08</td>
<td>0.65±1.02</td>
<td>0.346</td>
</tr>
<tr>
<td><strong>Plasma cyclic nucleotides</strong></td>
<td></td>
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<tr>
<td>cGMP, pmol/ml</td>
<td>7.1±2.7</td>
<td>19.4±11.9</td>
<td>71.6±4.1††</td>
<td>&lt;0.001</td>
<td>7.4±2.4</td>
<td>29.7±8.8†</td>
<td>70.0±9.4††</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cAMP, pmol/ml</td>
<td>24.0±3.1</td>
<td>17.6±9.2</td>
<td>82.1±100.4</td>
<td>0.431</td>
<td>56.4±8.5</td>
<td>53.2±10.3</td>
<td>53.2±10.9</td>
<td>0.666</td>
</tr>
</tbody>
</table>

*Repeated measures ANOVA, † P-value <0.05 Vs Baseline, ‡ P-value <0.05 Vs Sildenafil
Supplementary Figure Legend

Supplemental Figure 1: Preload Recruitable Stroke Work (PRSW). Top panel shows data from a pressure transducer and volume conductance catheter in the left ventricle of an old hypertensive dog during IVC occlusion. Stroke work for each cycle is the area of the individual loop of cardiac cycle as illustrated by the gray area. The bottom panel shows the PRSW relationship which is a linear regression of the stroke work vs the end diastolic volume for each loop. PRSW is the slope for this relationship. PRSW-Vo is the volume axis intercept for this relationship.
Supplemental References
비아그라와 BNP는 심장세포의 Titin 인산화를 촉진하여 심실의 확장기 이완능을 개선시킨다

최 동 주 교수 분당서울대학교병원 순환기내과

Summary

배경

최근 연구들에 의하면 titin의 인산화는 심근세포의 경직도를 감소시킬 수 있다고 한다. Titin의 인산화는 cGMP를 활성화시킴으로써 촉진될 수 있다. 세포 내의 cGMP는 B-type natriuretic peptide(BNP)에 의해 증가하는 반면, phosphodiesterase-5A 등의 phosphodiesterase(PDE)에 의해 분해되어 감소된다. 본 연구는 phosphodiesterase-5A(PDE-5A) 억제제인 sildenafil(비아그라)이 단독 혹은 BNP와 병용하여 titin을 인산화 시킴으로써 좌심실 확장능을 개선할 수 있을 것이라는 가설에서 시행되었다.

방법 및 결과

실험적 고혈압을 동반한 8마리의 개와 정상 4마리에서 압-용적(pressure-volume) 곡선을 sildenafil과 BNP 주입 전후로 구하였다. 별개의 그룹으로 각각 7마리의 고령 고혈압 개와 어린 정상 개에서도 동일한 약물주입 후 좌심실을 일정한 간격의 조직을 얻어 심근섬유 단백의 인산화를 측정하였다. 혈액의 cGMP 농도는 sildenafil과 BNP 주입 후 증가하였다(7.31±2.37 to 26.9±10.3 to 70.3±8.1 pmol/L; P<0.001). 좌심실의 이완기 용적능(diastolic capacitance)도 sildenafil 투여 후 증가하였으며, BNP 투여 후 더욱 증가했다(51.4±16.9 to 53.7±16.8 to 60.0±19.4mL; P<0.001). 고령 고혈압 군과 어린 정상군에서도 유사한 결과를 보였다. Troponon I, troponin T, phospholamban, myosin light chain(MLC)-1, 혹은 -2 에서는 인산화 효과가 없었다. 반면, titin은 sildenafil과 BNP에 의해 인산화가 증가하며, titin에 의한 좌심실 경직도 역시 감소하였다.

결론

단기적 sildenafil과 BNP 투여에 의한 cGMP 증가 치료는 좌심실 확장기 이완능을 개선하였으며, 이는 일부 titin의 인산화에 의한 영향으로 사료된다.
심부전 환자의 절반은 수축능이 정상으로 유지되는 확장기심부전 혹은 정상수축기능 심부전(heart failure with preserved ejection fraction, HFpEF)이다. HFpEF의 원인이나 병태생리는 정확히 밝혀지지 않았으나, 좌심실의 확장성(distensibility) 감소와 연관되며, 이는 고령에서의 고혈압, 좌심실비후, 심근세포 간질의 증가 등이 연관되는 것으로 알려져 있다.

Titin은 sarcomere의 구성요소로 myosin을 Z-disc에 부착시켜 심근의 수축과 이완에 중요한 역할을 한다. Titin의 인산화는 cAMP- 혹은 cGMP-dependent kinase인 PKA(protein kinase A) 혹은 PKG(protein kinase G)에 의해 촉진되어 심근세포의 긴장도를 완화시킨다. 즉, titin의 인산화는 심부전에서 확장기능 장애와 연관된다. 세포내에서 cGMP는 NO(nitric oxide)와 BNP에 의해 생성이 촉진되고 PDE에 의해 분해되는데, sildenafil은 선택적으로 PDE-5A를 억제하여 cGMP의 분해를 막는다.

좌심실 확장기 이완능의 감소는 HFpEF의 중요한 병태생리로 이해되고 있으나, 아직 이에 대한 효과적인 치료법은 없다. 실험 연구에 의하면 cGMP는 좌심실 확장기능 개선에 효과적일 수 있으며, 이는 cGMP-dependent kinase의 활성화와 titin 인산화와 연관된다고 알려져 있다. 본 연구에서 PDE-5A 억제제인 sildenafil과 BNP가 HFpEF 모델과 정상 개에서 압-용적 곡선에 의한 좌심실확장기능 개선, 심근세포에서 수동적 긴장능 완화, 조직검사로 얻은 심근섬유의 인산화에 효과가 있음을 보였다. 특히, 혈액의 cGMP 변화는 심근 조직에서의 변화와 일치하였으며, troponin I, troponin T, phospholamban, MLC, MBP-C 등의 다른 심근섬유 단백 물질의 인산화에는 영향을 주지 않았다.

본 연구의 결과로 향후 HFpEF의 치료로 cGMP를 상승시키는 것이 효과적일 것으로 기대되며, 특히 단기적 효과뿐 아니라 장기적 효과에 대한 연구가 기대된다.

**Commentary**

Figure 1. Arrangement of titin in the sarcomere
Sildenafil and B-Type Natriuretic Peptide Acutely Phosphorylate Titin and Improve Diastolic Distensibility In Vivo

Kalkidan Bishu, MD*; Nazha Hamdani, PhD*; Selma F. Mohammed, MBBS; Martina Kruger, PhD; Tomohito Ohtani, MD, PhD; Ozgur Ogut, PhD; Frank V. Brozovich, MD, PhD; John C. Burnett, Jr, MD; Wolfgang A. Linke, PhD; Margaret M. Redfield, MD

Background—In vitro studies suggest that phosphorylation of titin reduces myocyte/myofiber stiffness. Titin can be phosphorylated by cGMP-activated protein kinase. Intracellular cGMP production is stimulated by B-type natriuretic peptide (BNP) and degraded by phosphodiesterases, including phosphodiesterase-5A. We hypothesized that a phosphodiesterase-5A inhibitor (sildenafil) alone or in combination with BNP would increase left ventricular diastolic distensibility by phosphorylating titin.

Methods and Results—Eight elderly dogs with experimental hypertension and 4 young normal dogs underwent measurement of the end-diastolic pressure-volume relationship during caval occlusion at baseline, after sildenafil, and BNP infusion. To assess diastolic distensibility independently of load/extrinsic forces, the end-diastolic volume at a common end-diastolic pressure on the sequential end-diastolic pressure-volume relationships was measured (left ventricular capacitance). In a separate group of dogs (n=7 old hypertensive and 7 young normal), serial full-thickness left ventricular biopsies were harvested from the beating heart during identical infusions to measure myofilament protein phosphorylation. Plasma cGMP increased with sildenafil and further with BNP (7.31 ± 2.37 to 26.9 ± 10.3 to 70.3 ± 8.1 pmol/mL; P < 0.001). Left ventricular diastolic capacitance increased with sildenafil and further with BNP (51.4 ± 16.9 to 53.7 ± 16.8 to 60.0 ± 19.4 mL; P < 0.001). Changes were similar in old hypertensive and young normal dogs. There were no effects on phosphorylation of troponin I, troponin T, phospholamban, or myosin light chain-1 or -2. Titin phosphorylation increased with sildenafil and BNP, whereas titin-based cardiomyocyte stiffness decreased.

Conclusion—Short-term cGMP-enhancing treatment with sildenafil and BNP improves left ventricular diastolic distensibility in vivo, in part by phosphorylating titin. (Circulation. 2011;124:2882-2891.)

Key Words: cyclic GMP-dependent protein kinases ■ heart failure, diastolic ■ natriuretic peptide, brain ■ sildenafil

Half of heart failure (HF) patients have preserved ejection fraction (HFpEF), and age and hypertension are potent risk factors for HFpEF. Although the pathophysiology of HFpEF is complex, reduced left ventricular (LV) diastolic compliance or distensibility is often present in HFpEF and elderly hypertensive patients and may be due to hypertrophy, increased extracellular matrix, or changes in cardiomyocyte function. Transcriptional (isoform distribution) and post-translational (phosphorylation state) modifications of titin alter its tensile properties and, in turn, passive myofiber stiffness. Titin exists in 2 isoforms, the longer and more compliant isoform N2BA and the shorter, stiffer isoform N2B, both of which contain a common N2-B unique sequence (N2-Bus). Phosphorylation of the N2-Bus by cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) decreases passive tension in skinned cardiac strips and isolated cardiomyocytes in vitro. PKG-mediated reductions in passive tension were linked to phosphorylation and increased the compliance of the N2-Bus region of titin. Furthermore, hypophosphorylation of titin in myocardium harvested from humans with dilated cardiomyopathy has been reported. Thus, titin hypophosphorylation may contribute to diastolic dysfunction in HF, and cGMP-activating therapies may phosphorylate titin and improve diastolic function.

Clinical Perspective on p131

Nitric oxide (NO) activates soluble guanylyl cyclase and natriuretic peptides activate particulate guanylyl cyclase to...
produce cGMP.\textsuperscript{10} cGMP signaling is terminated by cGMP-
hydrolyzing phosphodiesterase (PDE) enzymes.\textsuperscript{10} PDE-5A,
which is inhibited by sildenafil, selectively hydrolyzes cGMP. The potential for cGMP-activating therapies to modu-
late diastolic function in vivo is also supported by earlier studies in variable patient populations in which agents that
increase myocardial NO resulted in downward shifts in the end-diastolic pressure (EDP)-volume (EDV) relation-
ship (EDPVR; increased distensibility) represented by increases in
EDV at matched EDP (increased capacitance).\textsuperscript{11,12} However,
in these elegant human studies, concomitant effects on load or
extrinsic forces were difficult to exclude, and myofilament
protein phosphorylation was not investigated.

We hypothesized that agents that increase cGMP may en-
hance titin compliance by phosphorylation, leading to improved
LV diastolic stiffness or distensibility in vivo. Thus, we tested
the effect of a PDE-5A inhibitor (sildenafil) alone or in combi-
nation with a natriuretic peptide (B-type natriuretic peptide
[BNP]) on LV diastolic function in elderly canines with exper-
imental hypertension (OH dogs) and normal young adult canines
(YN dogs). Furthermore, we defined associated alterations in
phosphorylation of titin and other sarcomeric proteins.

\section*{Methods}

\subsection*{Study Design}

The study included 11 YN and 15 OH dogs. All experimental pro-
ductures were designed in accordance with National Institutes of Health
guidelines and approved by the Mayo Institutional Animal Care and Use
Committee. Dogs were euthanized by intravenous potassium chloride
under deep anesthesia, consistent with guidelines of the Panel on
Euthanasia of the American Veterinary Medical Association.

\subsection*{Animal Model}

Elderly (age, \(\geq 8-13\) months) mongrel dogs were made hypertensive
(OH) by bilateral renal wrapping with implantation of an aortic
catheter for blood pressure measurement as previously described.\textsuperscript{14} YN (age \(\leq 1\) year) dogs underwent sham surgery and aortic catheter
placement. YN dogs were used as controls to determine whether any
actions of sildenafil and BNP as putative PKG activators are blunted
or heightened in conditions associated with the HFpEF phenotype
(age and hypertensive remodeling). All dogs underwent echocardi-
ography in the conscious state before and 8 weeks after surgery.

\subsection*{Hemodynamic Study}

Short-term hemodynamic studies were performed in 4 YN and 8 OH
dogs 8 weeks after renal wrapping or sham surgery. Animals were
anesthetized (fentanyl 0.25 mg/kg followed by 0.18 mg · kg\(^{-1}\) · h\(^{-1}\),
and midazolam 0.75 mg/kg followed by 0.59 mg · kg\(^{-1}\) · h\(^{-1}\))
intubated, ventilated, and given maintenance saline infusion (3
mL · kg\(^{-1}\) · min\(^{-1}\)). Thoracotomy and pericardiectomy were per-
formed. Under fluoroscopic guidance, animals were instrumented
with an ECG, a pulmonary artery catheter, an LV integrated
pressure-conductance catheter (Millar), a left atrial and central aortic
high-fidelity pressure transducer (Millar), a pulmonary occluding
device around the thoracic inferior vena cava, and an atrial lead for
pacing at 10 to 20 bpm above sinus rate. The conductance catheter
was calibrated with the measurement of blood conductance \(\rho\),
thermodilution stroke volume, and parallel conductance (hypertonic
saline method) as previously described.\textsuperscript{14} All dogs received auto-
nomic blockade with atropine (1 mg) and propranolol (2 mg/kg).
Steady-state and inferior vena cava occlusion (in triplicate) data were
collected at suspended end expiration at baseline (after autonomic
blockade). Sildenafil (2 mg/kg intravenous bolus) was then admin-
istered and data collection was repeated 30 minutes later. Next, BNP
was administered (2-\(\mu\)g/kg bolus and 0.01-\(\mu\)g · kg\(^{-1}\) · min\(^{-1}\)
infu-
sion) with data collection 30 minutes later. Blood for plasma cAMP
and cGMP concentrations by radioimmunoassay was collected at
each experimental period. After each stage of drug treatment,
hemodynamic data collection or plasmatic/issue biopsy specimen
collection was completed in 5 to 10 minutes; then the next treatment
was started.

\subsection*{Pressure-Volume Analysis}

Data were collected and analyzed with Sonoview and Cardiosoft
software (Sonometrics Corp.). The end-systolic pressure (ESP)-volume
(ESV) relationship (ESPVR) was defined as ESP = \(Ees \cdot ESV \cdot V_0\), where
Ees (end-systolic elastance) is the slope of ESPVR and \(V_0\) is its volume
axis intercept. The ESPVR data points were fit to the monoequponential
equation \(E_{DP} = cE^{k \cdot EDV}\) using least-squares nonlinear regression.\textsuperscript{15} To
assess LV diastolic distensibility independently of load/extrinsic
pericardium-mediated forces, the EDV at a common EDP on the sequential
ESPVRs was measured (LV diastolic capacitance) with the
EDP midway down the baseline ESPVR used as the comparator (Figure
1). Similarly, we measured systolic capacitance on the sequential
ESPVRs measuring ESV at a common ESP defined at the midpoint of
the baseline ESPVR.

\subsection*{Ventricular Biopsy and Biochemical and
Mechanical Measurements}

Although sarcomeric protein phosphorylation status may display
transmural variation,\textsuperscript{16} we assessed average phosphorylation on
full-thickness LV biopsies. Because the biopsy and hemostatic
sutures would alter chamber diastolic properties, serial samples were
harvested from different regions of the anterior or anterior lateral
wall from 7 YN and 7 OH dogs subjected to an identical experimen-
tal protocol without collection of hemodynamic data. Biopsy
samples were frozen in liquid nitrogen within seconds and stored at
\(-80^\circ\)C until use. Protein phosphorylation states were analyzed by
1-dimensional and, in a subset of dogs, 2-dimensional SDS-PAGE
with Western blotting, phosphoprotein staining, or autoradiography
as previously described.\textsuperscript{4,17} Passive force measurements were performed in mechanically isolated, skinned, single cardiomyocytes as
described previously.\textsuperscript{10} Single cardiomyocytes (2-5 per dog) from
3 OH and 3 YN dogs obtained at baseline and after each serial
treatment were attached between a force transducer and a motor.
Their sarcomere length was adjusted to 2.2 \(\mu\)m, and passive force
\(F_{pass}\) was measured in relaxing buffer. See the online-only Data
Supplement for details.

\subsection*{Statistics}

All continuous variables are presented as mean\(\pm\)SD. The Student t
test was used to compare echocardiographic characteristics between
OH and YN dogs at baseline and to assess change (paired t-test) from
baseline to 8 weeks after surgery. Two-way ANOVA with repeated
measures was used to test for significant changes during serial
treatment and to determine whether changes with sildenafil and BNP
differed between YN and OH dogs. Normal distribution is difficult to
confirm in the relatively small experimental groups used in these
types of translational studies. Thus, data are also reported as median
(25th and 75th percentiles), and significance of changes across and
between experimental periods was confirmed by the Friedman test,
followed by Wilcoxon signed-rank test for individual group compar-
tions (Tables 1 and II in the online-only Data Supplement). To test for
interaction between serial treatment and OH versus YN status
with a nonparametric method, we performed the Scheirer-Ray-Hare
extension of the Kruskal-Wallis test.\textsuperscript{18} Relationships between vari-
bles were tested with the Spearman rank correlation. Statistical
significance was set at \(P<0.05\) without adjustment for multiple
comparisons. Statistical analyses use SPSS (version 19.0, SPSS Inc,
Chicago, IL).

The authors had full access to and take full responsibility for the
integrity of the data. All authors have read and agree to the manuscript
as written.
Results

Before surgery, OH dogs had similar body weight and EF but higher LV mass and LV/body weight ratio than YN dogs (Table 1 and Table I in the online-only Data Supplement). At 8 weeks after surgery, systolic, diastolic, mean, and pulse pressures were higher in OH than in YN dogs. Furthermore, LV mass had increased by ~25% in OH but remained stable in YN dogs. There was no significant change in EF in either group.

Effects of Sildenafil and BNP on Ventricular Function

Plasma cAMP and cGMP levels were similar in OH and YN dogs at baseline. Plasma cAMP did not change significantly with sildenafil or BNP treatment (Table 2 and Table II in the online-only Data Supplement). Plasma cGMP levels increased serially in both OH and YN dogs during treatment with sildenafil and BNP.

In all dogs, diastolic capacitance increased with sildenafil and increased further with BNP but did so similarly ($P = 0.37$ for interaction) in OH and YN dogs (Figures 1 and 2, Table 2, and Table II in the online-only Data Supplement). There was a rightward shift in the PVRs without consistent changes in the shape of the curve ($\beta$ and $\alpha$), indicative of an increase in distensibility.

Table 1. Echocardiography at Baseline (Before Surgery) and at 8 Weeks

<table>
<thead>
<tr>
<th>Variables</th>
<th>YN Dogs (n=4)</th>
<th>OH Dogs (n=8)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>25.40±1.34</td>
<td>26.83±3.78</td>
<td>0.43</td>
</tr>
<tr>
<td>Baseline LV mass, g</td>
<td>104±5</td>
<td>147±25</td>
<td>0.002</td>
</tr>
<tr>
<td>LV mass at 8 wk, g</td>
<td>106±5</td>
<td>188±46†</td>
<td>0.0008</td>
</tr>
<tr>
<td>LV/BW at 8 wk, g/kg</td>
<td>4.11±0.24</td>
<td>5.52±0.85</td>
<td>0.003</td>
</tr>
<tr>
<td>LV mass change, %</td>
<td>0.01±0.02</td>
<td>0.27±0.16</td>
<td>0.004</td>
</tr>
<tr>
<td>Systolic blood pressure at 8 wk, mm Hg</td>
<td>167±12</td>
<td>220±25</td>
<td>0.0005</td>
</tr>
<tr>
<td>Diastolic blood pressure at 8 wk, mm Hg</td>
<td>94±6</td>
<td>126±21</td>
<td>0.005</td>
</tr>
<tr>
<td>Mean arterial pressure at 8 wk, mm Hg</td>
<td>122±7</td>
<td>159±20</td>
<td>0.002</td>
</tr>
<tr>
<td>Pulse pressure at 8 wk, mm Hg</td>
<td>73±7</td>
<td>94±15</td>
<td>0.01</td>
</tr>
<tr>
<td>Baseline ejection fraction, %</td>
<td>74±3</td>
<td>70±3</td>
<td>0.1</td>
</tr>
<tr>
<td>Ejection fraction at 8 wk, %</td>
<td>74±2</td>
<td>68±11</td>
<td>0.25</td>
</tr>
</tbody>
</table>

YN indicates young normal; OH, old hypertensive; LV, left ventricular; and BW, body weight.

*Student $t$ test.
†$P<0.05$ versus baseline.
was a rightward shift in the ESPVR without consistent changes in the slope of the relationship (no change in Ees but an increase in V0). Similarly, the stroke work–EDV relationship (preload-recruitable stroke work) was shifted rightward (increased volume intercept) without a change in slope. However, there was no change in load-dependent indexes of contractility (EF and peak dP/dt). Despite the rightward shift in the ESPVR, the steady-state ESV did not increase as a result of the concomitant decrease in steady-state ESP.

There was no correlation between changes in afterload (Ea) and changes in systolic (P=0.36) and diastolic (P=0.71) capacitance during sequential therapy.

**Passive Force of Isolated Skinned Cardiomyocytes**

At baseline, Fpassive was higher in cardiomyocytes isolated from OH dogs than in those from YN dogs (Figure 3). In both OH and YN dogs, Fpassive was lower in cardiomyocytes obtained from

**Figure 2.** Diastolic and systolic capacitance. Left, Group data for diastolic capacitance during sequential therapy indexed to baseline (BL) for 8 old hypertensive and 4 young normal dogs. Right, Group data for systolic capacitance during sequential therapy indexed to baseline. P<0.05 (ANOVA) for the effect of serial therapy on diastolic and systolic capacitance. BNP indicates B-type natriuretic peptide. †P<0.05 vs baseline; ‡P<0.05 vs sildenafil.

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**Table 2. Hemodynamic and Plasma Evaluation (n=12)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Sildenafil</th>
<th>BNP + Sildenafil</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state hemodynamics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>105±11</td>
<td>105±10</td>
<td>105±12</td>
<td>0.16</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>97.0±24.1</td>
<td>92.4±22.8†</td>
<td>87.8±22.6†</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean left atrial pressure, mm Hg</td>
<td>8.6±3.5</td>
<td>6.2±2.1†</td>
<td>5.6±3.9†</td>
<td>0.048</td>
</tr>
<tr>
<td>LV end-systolic pressure, mm Hg</td>
<td>103.5±23.0</td>
<td>99.9±21.9</td>
<td>91.8±21.3†‡</td>
<td>0.002</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>8.5±3.0</td>
<td>8.1±3.4</td>
<td>8.1±3.6</td>
<td>0.57</td>
</tr>
<tr>
<td>LV end-systolic volume, mL</td>
<td>36.8±17.0</td>
<td>35.4±16.1</td>
<td>38.1±18.2</td>
<td>0.55</td>
</tr>
<tr>
<td>LV end-diastolic volume, mL</td>
<td>66.6±16.8</td>
<td>66.4±14.6</td>
<td>72.2±18.1†‡</td>
<td>0.006</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>3.1±0.9</td>
<td>3.2±0.9</td>
<td>3.6±0.8†</td>
<td>0.003</td>
</tr>
<tr>
<td>Stroke volume, mL</td>
<td>29.9±8.4</td>
<td>31.0±8.7</td>
<td>34.1±8.1†‡</td>
<td>0.001</td>
</tr>
<tr>
<td>Ea, mm Hg/mL</td>
<td>3.9±2.0</td>
<td>3.7±1.8</td>
<td>3.0±1.4†‡</td>
<td>0.003</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>46.5±13.8</td>
<td>48.3±15.2</td>
<td>49.2±14.1</td>
<td>0.22</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg/s</td>
<td>1795±459</td>
<td>1841±385</td>
<td>1834±442</td>
<td>0.28</td>
</tr>
<tr>
<td>r, ms</td>
<td>41.3±8.6</td>
<td>42.0±10.1</td>
<td>43.9±12.7</td>
<td>0.13</td>
</tr>
<tr>
<td>ESPVR analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ees, mm Hg/mL</td>
<td>2.16±0.78</td>
<td>2.43±1.15</td>
<td>2.22±0.85</td>
<td>0.37</td>
</tr>
<tr>
<td>Ees V0, mL</td>
<td>−22.3±19.9</td>
<td>−17.3±23.0</td>
<td>−14.2±20.8§</td>
<td>0.007</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>57.9±17.7</td>
<td>57.5±15.2</td>
<td>60.7±12.5</td>
<td>0.2</td>
</tr>
<tr>
<td>PRSW, V0, mL</td>
<td>10.0±21.9</td>
<td>13.4±18.1</td>
<td>19.3±18.6§‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic capacitance, mL</td>
<td>22.9±15.3</td>
<td>25.1±16.2†</td>
<td>31.0±16.7†‡</td>
<td>0.001</td>
</tr>
<tr>
<td>EDPVR analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic capacitance, mL</td>
<td>51.4±16.9</td>
<td>53.7±16.8†</td>
<td>60.0±19.4†‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic stiffness coefficient, β, mm Hg/mL</td>
<td>0.053±0.04</td>
<td>0.069±0.06</td>
<td>0.059±0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Curve-fitting constant, α</td>
<td>1.39±1.84</td>
<td>1.04±1.65</td>
<td>0.88±1.50</td>
<td>0.18</td>
</tr>
<tr>
<td>Plasma cyclic nucleotides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP, pmol/mL</td>
<td>7.31±2.37</td>
<td>26.9±10.3†</td>
<td>70.3±8.1†‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cAMP, pmol/mL</td>
<td>47.6±16.8</td>
<td>43.5±19.2</td>
<td>61.1±47.7</td>
<td>0.07</td>
</tr>
</tbody>
</table>

BNP indicates B-type natriuretic peptide; LV, left ventricular; Ea, arterial elastance; ESPVR, end-systolic pressure–volume relationship; Ees, end-systolic elastance; PRSW, preload-recruitable stroke work; EDPVR, end-diastolic pressure–volume relationship.

*Repeated measures ANOVA.

†P<0.05 versus baseline; ‡P<0.05 vs sildenafil.
LV tissue after sildenafil and BNP treatment compared with cardiomyocytes obtained from LV tissue at baseline.

**Sarcomeric Protein Phosphorylation**

In the LV tissue samples obtained after autonomic blockade, serine 23/24 phosphorylation of troponin I (TnI) did not change with treatment, and the proportion of monophosphorylated and diphosphorylated TnI isoelectric variants remained unaltered (Figure 4). Troponin T (TnT) was predominantly phosphorylated, but there was no change in phosphorylation status after therapy (Figure 5). Myosin light chain (MLC)-1 was primarily nonphosphorylated and did not change after therapy (Figure 5). MLC-2 was ~40% monophosphorylated but did not change during treatment (Figure 5). There was nearly complete dephosphorylation of the serine 16 site of phospholamban (after autonomic blockade), and this did not change during treatment (Figure 6).

The ratio of phosphorylated N2B and N2BA isoforms (ProQ Diamond stain) to total titin (Sypro Ruby Stain) increased ~100% with sildenafil compared with baseline, and the higher level of phosphorylation was maintained with BNP (Figure 7A through 7C). The ratio of the phosphorylated N2B isoform to the phosphorylated N2BA isoform increased with sildenafil and remained elevated after BNP treatment (Figure 7A and 7C). To confirm the findings with the ProQ phosphostain, we also performed back-phosphorylation assays in which skinned fibers were either phosphorylated by adding PKG or first dephosphorylated with a phosphatase and then treated with PKG to assess inherent phosphorylation as the difference in the phosphorylation signal between PKG and phosphatase plus PKG (Figure 7D through 7F). Inherent phosphorylation was found to be higher with sildenafil and remained elevated after treatment with BNP in the N2B titin isoform (Figure 7D and 7E). In contrast, inherent phosphorylation levels remained unaltered in the T2 titin species, which is considered a degraded form containing the A-band region but little of the elastic I-band region (Figure 7D and 7F). Hence, the effect of sildenafil/BNP on PKG-mediated titin phosphorylation is mainly an effect on I-band titin.

**Discussion**

Our primary finding is that sildenafil and sildenafil plus BNP increased diastolic and systolic capacitance in association with increases in total titin phosphorylation and decreases in titin-based myocyte passive stiffness. In these autonomically blocked canines, sildenafil or sildenafil plus BNP did not alter phosphorylation of other sarcomeric proteins. These findings confirm and extend previous in vitro studies in which incubation of myocardial preparations with cGMP-PKG increased phosphorylation of titin and reduced passive diastolic myofiber stiffness. These data suggest that therapies elevating cGMP may provide benefit in the short- or long-term treatment of HFpEF.

**Figure 3.** Passive force measurements in isolated skinned cardiomyocytes from old hypertensive (OH) and young normal (YN) dogs. Passive force ($F_{\text{passive}}$) was higher in cardiomyocytes from OH dogs, but in both YN and OH dogs, $F_{\text{passive}}$ was lower in cardiomyocytes obtained after sildenafil and B-type natriuretic peptide (BNP) treatment compared with cardiomyocytes obtained from left ventricular tissue at baseline. †$P<0.05$ vs baseline.

**Figure 4.** Phosphorylation of troponin I (TnI) by Western blotting and 2-dimensional SDS-PAGE. A, Multiplex Western blots using a serine 23/24 phosphospecific antibody and a total TnI antibody in old hypertensive and young normal dogs. A common standard (STD) was loaded alongside the baseline (BL), sildenafil (SIL), and B-type natriuretic peptide (BNP) biopsy samples to allow comparisons across gels. B, Group data for the phosphorylation ratio of serine 23/24 TnI to total TnI indexed to baseline. $P=\text{NS}$. C, Total protein stain of a sample homogenate resolved by 2-dimensional SDS-PAGE. Two phosphorylated isoelectric variants, 1-P and 2-P, of TnI are observed. D, Proportion of the 1-P and 2-P isoelectric variants of TnI with serial therapy. $P=\text{NS}$. 

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Effect of Sildenafil and BNP on Diastolic Distensibility

Improvement in passive LV diastolic properties may include decreases in diastolic stiffness (decreased $\beta$, slope of EDPVR) or increased distensibility in which the EDPVR shifts downward without changes in shape. In vivo, sildenafil and BNP resulted in acute increases in LV diastolic capacitance unassociated with consistent changes in $\alpha$ or $\beta$, suggesting an acute effect on LV distensibility. Short-term increases in LV distensibility may be due to release of extrinsic forces, altered extracellular matrix, or changes in the cardiomyocyte. Improved diastolic function. The shift indicates that, for a particular LV volume, the LV operates at lower LV pressure, which is reflected in lower left atrial pressure, suggesting potential relief of symptoms and other manifestations of pulmonary congestion.

In these anesthetized, instrumented, open-chest, autonomically blocked canines, EDP was normal at baseline. Thus, we cannot exclude the possibility that changes in the shape of the EDPVR (altered diastolic chamber stiffness constant) would have been evident if data had been collected over a larger range of EDV, providing a more accurate (less extrapolation) characterization of the monoexponential EDPVR. Furthermore, detection of changes in shape of the EDPVR may be obscured by concomitant and reciprocal changes in the stiffness and curve-fitting constants. Improvement in diastolic distensibility has similarly been reported during intracoronary nitroprusside, NO, and substance P infusion in humans. Although the presence of the pericardium in these human studies increased the potential for altered right ventricular loading and ventricular interdependence to mediate shifts in the EDPVR, it is notable that our findings (in the absence of the pericardium and with measurements in the midportion of the EDPVR) are similar. In human subjects with dilated cardiomyopathy, $N^\gamma$-monomethyl-L-

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Figure 5. Phosphorylation of troponin T (TnT), myosin light chain (MLC)-1, and MLC-2. A, Representative total protein stain of a left ventricular homogenate resolved by 2-dimensional SDS-PAGE. Three prominent TnT isoforms are identified. Isoform 1 demonstrated 3 isoelectric variants indicating 3 (bi-, mono- and non-) phosphorylation states, whereas the less abundant isoforms 2 and 3 were each present in 2 (non- and mono-) phosphorylated forms. MLC-1 is present as 2 (non- and mono-) phosphorylated forms, whereas MLC-2 is present as 3 (non-, mono-, and a rarely detected bi-) phosphorylated form. B, The distribution of isoelectric variants of the abundant TnT isoform (isoform 1) is plotted at baseline and in response to sildenafil and B-type natriuretic peptide (BNP) treatment. There was no change in the less abundant isoforms of TnT (isoforms 2 and 3; not shown). C, The percentages of nonphosphorylated and monophosphorylated MLC-1 and MLC-2 at baseline and after sequential treatment with sildenafil and BNP are shown. $P=NS$.

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Figure 6. Phosphorylation of phospholamban. Representative Western blot for serine 16 phosphorylated (A) and total (B) phospholamban pentamer and monomer. Phospholamban was dephosphorylated after baseline autonomic blockade (BL) and did not change with sequential sildenafil (Sil) or B-type natriuretic peptide (BNP) therapy. Phosphorylation of phospholamban in the standard (STD) is evident. OH indicates old hypertensive; YN, young normal.
arginine, an NO synthase inhibitor, impaired diastolic compliance.22 The present findings with sildenafil and BNP are consistent with these previous studies in which cGMP was increased via NO and soluble guanylyl cyclase.

**Effect of Sildenafil and BNP on Systolic Capacitance**

Sildenafil and BNP increased systolic capacitance, as exhibited by the rightward shift in the ESP VRs with increased ESV at a common ESP and an increase in the volume axis intercept of the ESP VR (V0) without a change in the slope of the ESP VR (Ees). Similarly, the stroke work–EDV relationship (preload-recruitable stroke work) was shifted rightward without a change in slope. Although these changes are consistent with a negative inotropic effect, more load-dependent indexes of systolic performance (EF, peak dP/dt) did not change with sequential treatment, likely owing to the concomitant decrease in afterload.

It is now well understood that the ESP VR is not linear, that the degree and nature of nonlinearity vary with inotropic state, and that the volume intercept (V0) and slope (Ees) may change with changes in inotropic state (reviewed by Burkhoff et al15). Furthermore, the ESP VR is not truly load independent because the inotropic state varies directly with afterload impedance, an effect attributed to load-dependent changes in actin-myosin interactions and calcium sensitivity of the troponin regulatory complex.15 Thus, sildenafil- and sildenafil plus BNP–mediated decreases in afterload could contribute to the rightward shift in the ESP VR,14,23 although the changes in afterload were subtle (≈10-mm Hg change in ESP) and there was no correlation between change in afterload and changes in systolic capacitance. Although we believe that the decrease in the passive tensile properties of cardiomyocytes after sildenafil and BNP infusion explains the increased systolic capacitance, a contribution of afterload reduction, particularly seen after BNP infusion, cannot be excluded.

Some24,25 but not all26,27 in vivo studies have reported a subtle but detectable negative inotropic effect of cGMP-activating compounds, although subject characteristics, methodology, and experimental conditions in these studies varied and none investigated the combination of PDE-5A inhibition and natriuretic peptides.

Although still controversial, several recent reports have supported a contribution from titin-based passive tension to length-dependent activation (Frank-Starling relationship) in the cardiac sarcomere.28,29 However, despite decreased passive tension in isolated cardiomyocytes, we have not detected a clear change in preload-recruitable stroke work or Ees with sildenafil or the addition of BNP.

**Figure 7. Phosphorylation of titin. A.** Representative phosphoprotein stain (ProQ diamond) and total protein stain (Sypro Ruby) of a titin gel at baseline and after sequential treatment with sildenafil and B-type natriuretic peptide (BNP). **B.** Group data (n=7 per group) for the ratio of phosphorylated titin (ProQ diamond) to total titin (Sypro Ruby) indexed to baseline. **C.** Group data (n=7 per group) for the ratio of the phosphorylated N2B isoform to the N2BA isoform (ProQ diamond). **D.** Back-phosphorylation assay by autoradiography to assess titin phosphorylation. Skinned fibers were either phosphorylated by adding cGMP-activated protein kinase (PKG) or first dephosphorylated with a phosphatase and then treated with PKG (PP+PKG) to assess inherent phosphorylation (difference in the phosphorylation signal between PKG and PP+PKG). The control sample was not treated with PKG. Back-phosphorylation assays were assessed for N2B isoform and T2 degradation product. The signal from the N2BA band on the autoradiograms was too faint to allow a meaningful analysis. **E.** Group data (n=5) for inherent phosphorylation of the N2B isoform. **F.** Group data (n=5) for the inherent phosphorylation of the T2 degradation product. †P<0.05 vs baseline.
modulating cyclic nucleotide signaling through cGMP-stimulated (PDE-2 and -5) or cGMP-inhibited (PDE-3) PDEs. Ex vivo studies of myocytes or multicellular preparations have shown that cGMP stimulators or analogs produce an acceleration of relaxation, an increase in resting cell length, and reduced inotropy and that these effects occur in the absence of changes in calcium transient amplitude or kinetics. Although NO-mediated increases in cGMP blunt the inotropic response to β-adrenergic stimulation,32,33 the negative inotropic effect of cGMP is PKG-dependent and can occur independently of PKA signaling.44 The mechanisms whereby PKG alters calcium sensitivity are unclear, although PKG-mediated phosphorylation of TnI has been implicated in vitro studies.30,31,35

Phospholamban is phosphorylated by PKA and PKG, and modulation of phospholamban phosphorylation alters relaxation and potentially inotropic function, although the circumstances in which PKG versus PKA-mediated phosphorylation affects phospholamban phosphorylation status in vivo are poorly understood.36,37 TnT, MLC-1, and MLC-2 are not known to be phosphorylated by PKG, but this has not been widely studied. Importantly, in our autonomically blocked dogs, neither sildenafil nor sildenafil plus BNP altered the phosphorylation status of TnI, phospholamban, TnT, MLC-1, or MLC-2. Of note, TnI and particularly phospholamban were highly dephosphorylated, likely owing to the presence of autonomic blockade. Consistent with the lack of change in TnI or phospholamban phosphorylation status, there was no consistent effect on the speed of relaxation with sildenafil or sildenafil plus BNP.

Although sildenafil and sildenafil plus BNP had no effect on the regulatory proteins noted above, titin phosphorylation was increased with sildenafil and sildenafil plus BNP with preferential phosphorylation of the springy I-band region. In vitro studies show that passive tension in the physiological range of sarcomere length is critically influenced by titin.38 Relative expression of the N2BA titin isoform increases in proportion to body size in mammals and allows longer operating sarcomere lengths without increases in passive tension.39,40

Phosphorylation of titin by incubation with PKA and PKG acutely decreases passive tension in cardiomyocytes and myofibers in vitro, most dramatically after initial dephosphorylation.5,7,9 Both kinases phosphorylate titin at the N2-Bus segment, and this posttranslational event enhances titin compliance and thus reduces myofiber passive stiffness.6 We observed that titin phosphorylation did not increase further after BNP was added to sildenafil despite a substantial increase in plasma cGMP. However, the 80% inherent phosphorylation after sildenafil administration, as measured with back-phosphorylation assays, suggests potential saturation.

We used sildenafil, a PDE-5A inhibitor, and BNP to enhance cGMP signaling. Recently, there has been uncertainty regarding the expression of PDE-5A in cardiomyocytes.41,42 However, the presence of sildenafil-induced physiological effects in cardiomyocytes is supported by studies demonstrating sildenafil stimulation of cGMP-associated electric currents in isolated rat cardiomyocytes transfected with cGMP-gated ion channels.43 Sildenafil may also have nonselective inhibitory effects on a different PDE known to be abundantly expressed in the heart, PDE-1.44,45 Regardless of the mechanism for cGMP enhancement by sildenafil in the heart, the short-term hemodynamic and titin phosphorylation effects of sildenafil in our experiments are unequivocal.

The ratio of phosphorylated titin to total titin increased after sildenafil therapy with no further increase with BNP treatment. Most changes in titin phosphorylation appeared to occur within the I-band region. Moreover, the ratio of phosphorylated N2B to N2BA titin isoforms increased after sildenafil treatment with no further change after the addition of BNP. Fukuda et al5 showed that the extent of reduction in passive tension during phosphorylation of titin by PKA is highest in myocardial tissue with more of the N2B isoform. They postulated that passive force reduction is due to a reduction in the ratio of the length of the phosphorylated segment to the length of the titin isoform. Thus, phosphorylation of the shorter N2B isoform will be associated with a larger passive force reduction. Although phosphorylation of the N2-Bus segment is linked to changes in titin elasticity, there are other PKG-sensitive phosphorylation sites on titin.6 Thus, correlation between overall titin phosphorylation and changes in diastolic function is not likely to be linear.

We measured Fpassive in isolated skinned cardiomyocytes ex vivo, which was lower after treatment with sildenafil and maintained after the addition of BNP. Fpassive has a strong correlation with LVEDP and other indices of diastolic function, including radial myocardial stiffness modulus (E) and circumferential LV end-diastolic wall stress (σ).46 Human subjects with diastolic HF have higher Fpassive compared with normal subjects.46 Because the cardiomyocytes were treated in a manner that disrupts the sarcolemma and other membranes and with experiments performed in nonactivating (Ca2+-free) buffer, altered calcium handling is not responsible for the observed changes in Fpassive. Because we excluded posttranslational changes in myofilament-associated proteins (TnI, TnT, MLC-1, and MLC-2), titin phosphorylation changes are most likely to explain the change in Fpassive. The lack of improvement in Fpassive after the addition of BNP to sildenafil despite an increase in plasma cGMP may be secondary to saturation of titin phosphorylation.

The EDPVR represents a summary of the PVR at the end of diastole in each cardiac cycle and is affected by any intervention or physiological process that contributes to active relaxation and passive stiffness properties of the ventricular chamber. Impairment or incomplete relaxation (at higher heart rates) and increased passive diastolic tone will contribute to changes in the EDPVR. Impaired relaxation may be due to posttranslational changes in myofilament-associated proteins (TnI, TnT, MLC-1, and MLC-2) regulating calcium sensitivity or calcium transients (phospholamban), which were not affected in our experiments with sildenafil or sildenafil plus BNP.

We found a short-term increase in LV distensibility reflected in diastolic LV capacitance and the rightward shift in the EDPVR during treatment with sildenafil that was further enhanced with the addition of BNP. However, titin phosphorylation and cardiomyocyte Fpassive did not change further after the addition of BNP. There are several potential explanations
for the improved diastolic distensibility with BNP other than posttranslational changes in titin. Changes in systemic afterload may influence systolic and diastolic stiffness or distensibility.22,32 Ludbrook et al.47 observed a downward shift of the EDPVR with no appreciable alteration in the stiffness constant with a vasodilator (16% decrease in mean arterial pressure) in human HF. Although PDE-5 is thought to be specific for NO-soluble guanylyl cyclase–derived cGMP,43 pulmonary arterial vasodilation in response to BNP is markedly accentuated in the presence of sildenafil.48 Thus, despite pericardiotomy, we cannot exclude that an effect of BNP is to reduce ventricular interdependence via effects on right ventricular load. Finally, sildenafil- and BNP-mediated reduction in coronary perfusion pressure may enhance diastolic distensibility.49 Unfortunately, we did not measure right ventricular or coronary perfusion pressure.

Limitations
The inability to collect myocardial samples and hemodynamics in the same animals limits the ability to correlate changes in phosphorylation with changes in LV function, and differential dose-dependent and time course effects were not explored. The order of infusions was not altered, so we are unable to determine whether BNP alone phosphorylates titin or alters distensibility. Studies were conducted in the presence of autonomic blockade, and this may influence the interaction of PKG- and PKA-mediated phosphorylation. However, the use of β-blockers and age-related decrements in adrenergic responsiveness are ubiquitous in HFP EF and hypertension. A study of the effect of alternate particulate or soluble guanylyl cyclase activators alone and in combination with PDE inhibitors would be of interest.

Conclusions
Diastolic dysfunction is thought to contribute to the pathophysiology of HFP EF, but to date, no therapy has been demonstrated to improve diastolic dysfunction, symptoms, or outcomes in HFP EF. In vitro studies suggest dramatic effects of cGMP-PKG titin phosphorylation on diastolic function. We show here for the first time that therapies known to enhance cGMP enhance diastolic distensibility in association with enhanced titin phosphorylation and reduced titin-based passive stiffness in vivo in a clinically relevant large mammalian model. Although evidence of a negative inotropic effect was also apparent, this was subtle and not associated with decreases in EF or cardiac output. These data provide support for ongoing trials of sildenafil in HFP EF (Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure [RELAX] trial; www.clinicaltrials.gov, NCT00763867) and for future investigation of combining sildenafil and natriuretic peptide therapy to treat HFP EF.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

Reduced left ventricular diastolic compliance contributes to the pathophysiology of heart failure with preserved ejection fraction, a disease entity with no effective therapy. In vitro studies suggest that cGMP may have favorable effects on diastolic function, in part via activation of cGMP-dependent protein kinase and titin phosphorylation. Phosphodiesterase-5A degrades cGMP, whereas nitric oxide and natriuretic peptides enhance cGMP production. We studied the effects of short-term treatment with sildenafil (a phosphodiesterase-5A inhibitor) and brain natriuretic peptide on diastolic function in vivo (pressure-volume analysis) and on skinned cardiomyocyte passive stiffness and myofilament protein phosphorylation (serial myocardial biopsies) in a canine model of heart failure with preserved ejection fraction (elderly dogs with experimental hypertension) and young normal controls. In vivo, plasma cGMP levels and left ventricular diastolic and systolic capacitance increased and ex vivo measurements of cardiomyocyte passive stiffness decreased during serial treatment with sildenafil and with brain natriuretic peptide. These functional changes were associated with increases in titin phosphorylation without effects on troponin I, troponin T, phospholamban, myosin light chain, or myosin binding protein C phosphorylation. These data suggest that therapies elevating cGMP may provide benefit in the treatment of heart failure with preserved ejection fraction and support further investigation of short- or long-term administration of cGMP-enhancing therapies in this syndrome.