Myosin Cross-Bridge Dynamics in Patients With Hypertension and Concentric Left Ventricular Remodeling

Cameron Donaldson, MD; Bradley M. Palmer, PhD; Michael Zile, MD; David W. Maughan, PhD; John S. Ikonomidis, MD, PhD; Henk Granzier, PhD; Markus Meyer, MD, PhD; Peter VanBuren, MD; Martin M. LeWinter, MD

Background—Hypertension (HTN) causes concentric left ventricular remodeling, defined as an increased relative wall thickness or overt left ventricular hypertrophy, and associated diastolic dysfunction. HTN and concentric remodeling are also common precursors to heart failure with a preserved ejection fraction. It is not known whether the myofilament contributes to diastolic dysfunction in patients with concentric remodeling.

Methods and Results—Intraoperative myocardial biopsies were obtained in 15 male patients undergoing coronary bypass grafting, all with normal left ventricular ejection fraction and wall motion. Eight patients had a history of HTN and concentric remodeling. Seven without HTN or remodeling served as controls. Myocardial strips were dissected and demembranated with detergent. Isometric tension was measured and sinusoidal length perturbation analysis performed at sarcomere length 2.2 μm and pCa 8 to 4.5. Sinusoidal analysis provides estimates of cross-bridge dynamics, including rate constants of attachment and detachment and cross-bridge attachment time. The normalized isometric tension–pCa relation was similar in HTN and controls. However, cross-bridge attachment time was significantly prolonged at submaximal [Ca²⁺] (pCa ≥6.5) in HTN patients. Analysis of protein phosphorylation revealed ≥25% reduction in phosphorylation of troponin I in HTN patients (P<0.05).

Conclusions—Compared with controls, patients with HTN and concentric remodeling display prolonged cross-bridge attachment time at submaximal [Ca²⁺] without a change in the tension–pCa relation. Prolonged cross-bridge attachment time implicates altered cross-bridge dynamics as a cause of slowed relaxation in these patients. This finding was associated with reduced phosphorylation of troponin I, suggesting decreased phosphorylation of protein kinase A/G sites as a mechanism. (Circ Heart Fail. 2012;5:803-811.)

Key Words: diastole ■ heart failure ■ hypertrophy ■ myocardium ■ remodeling

Hypertension (HTN) causes concentric left ventricular (LV) remodeling, defined as either increased relative wall thickness (RWT) or overt LV hypertrophy (LVH), and diastolic dysfunction. HTN is also the most common risk factor for heart failure with preserved ejection fraction (HFrEF).

Clinical Perspective on p 811

The myocardial basis of diastolic dysfunction in human HTN with concentric remodeling is incompletely understood. In patients, collagen volume fraction is increased. Although direct measurements have never been reported, such changes in collagen would be expected to increase passive myocardial tension and stiffness. We recently reported abnormally high resting tone and incomplete relaxation at abnormally low stimulation frequencies in excitable myocardial strips from patients with normal ejection fraction and LVH because of pressure overload. This finding seems to be related to abnormalities in Ca²⁺ homeostasis and is the first time ion handling has been implicated as a mechanism of diastolic dysfunction in patients with LVH.

To date, there have been no reports of abnormal myofilament function and cross-bridge dynamics that could contribute to impaired relaxation and diastolic dysfunction in myocardium from patients with HTN and concentric remodeling. The myofilament property most commonly used to infer an effect on the speed and completeness of relaxation is thin filament sensitivity to calcium activation, that is, pCa₉₀ of the isometric tension–pCa relation, usually determined in demembranated (skinned) strips of muscle. In a few studies in rodent models of pressure overload LVH, myofilament functional changes have been inconsistent.
The present study was performed in myocardium obtained from patients undergoing coronary bypass grafting (CBG) with HTN, normal LV ejection fraction, and concentric remodeling to determine whether there are changes in myosin cross-bridge dynamics that influence the rate and completion of relaxation compared with control CBG patients. In addition to measuring the isometric tension–pCa relation, we used sinusoidal length perturbation analysis to characterize features of myosin cross-bridge dynamics that cannot be derived from the tension–pCa relation. The results are confined to males; studies in females are ongoing.

Methods

The study cohort consisted of 15 consecutive male patients recruited to undergo intraoperative myocardial biopsy from among those scheduled for CBG at Fletcher Allen Health Care in Burlington, VT, the clinical facility of the University of Vermont College of Medicine, and the VA Medical Center and Medical University of South Carolina Hospital in Charleston, SC, between October 1, 2008 and May 31, 2010, who satisfied the inclusion and exclusion criteria specified later. All patients signed consent forms approved by their respective Institutional Review Boards. Some potentially eligible patients were not screened because of vacations, illnesses, or periods of time when there was inadequate capacity to perform studies or equipment maintenance.

Patients >21 years of age with normal left ventricular ejection fraction, wall motion, and end-diastolic volume index based on an echocardiogram obtained <2 weeks before surgery were eligible for inclusion. If a preoperative echocardiogram could not be obtained within the 2-week time frame a preoperative contrast left ventriculogram demonstrating normal left ventricular ejection fraction and wall motion was accepted for inclusion. In patients who did not undergo preoperative echocardiography, an echocardiogram was performed 2 to 4 weeks after surgery. Patients were categorized as having HTN if this was documented in their medical records and they had been told of this diagnosis by a physician and, in addition, were receiving antihypertensive medications. On the basis of echocardiographic results, we excluded HTN patients without evidence of concentric LV remodeling (mass index <115 gm/m² or RWT <0.42). Patients without HTN were classified as controls. A total of 8 patients comprised the HTN group and 7 comprised the control group.

Exclusion criteria included diabetes mellitus, acute myocardial infarction within 4 weeks before CBG, ejection fraction <50%, significant valvular or other noncoronary heart disease, obstructive sleep apnea and chronic pulmonary disease requiring home O2, any noncardiac disease or condition known to affect myocardial function, anemia (hemoglobin <13.0 g/dL), serum creatinine >2.0, poorly controlled HTN by JNC 7 guidelines, 16 off-pump or emergency CBG, any noncoronary bypass grafting inclusion. If a preoperative echocardiogram could not be obtained within 2 weeks before surgery. Patients were categorized as having HTN if this was documented in their medical records and they had been told of this diagnosis by a physician and, in addition, were receiving antihypertensive medications. On the basis of echocardiographic results, we excluded HTN patients without evidence of concentric LV remodeling (mass index <115 gm/m² or RWT <0.42). Patients without HTN were classified as controls. A total of 8 patients comprised the HTN group and 7 comprised the control group.

Exclusion criteria included diabetes mellitus, acute myocardial infarction within 4 weeks before CBG, ejection fraction <50%, significant valvular or other noncoronary heart disease, obstructive sleep apnea and chronic pulmonary disease requiring home O2, any noncardiac disease or condition known to affect myocardial function, anemia (hemoglobin <13.0 g/dL), serum creatinine >2.0, poorly controlled HTN by JNC 7 guidelines, 16 off-pump or emergency CBG, morbid obesity, history of significant substance abuse, and inability to provide informed consent.

Routine demographic, historical and laboratory data, medications and cardiac catheterization results (coronary anatomy and LV enddiastolic pressure if measured) were tabulated. The angiographic section containing 30 mmol/L 2,3-butanedione monoxime (BDM) at room temperature,17 followed postoperatively until discharge. No adverse effects or postoperative complications ascribable to the biopsy were detected and all patients were discharged alive.

The biopsy was placed in oxygenated HEPES-based Krebs solution containing 30 mmol/L 2,3-butanedione monoxime (BDM) at room temperature,17 with small samples (<5 mg) removed and frozen for phosphorylation studies (see Protein Phosphorylation Studies section). The tissue was then cut into pieces <2 mm in length and placed in skinnng solution containing Triton-X100 at 4°C. For samples obtained at Medical University of South Carolina, the skinnning period coincided with overnight transit to University of Vermont College of Medicine at 4°C. After 18- to 24-hour skinnning, strips were dissected to 150 to 200 μm diameter and 800 to 1200 μm length, stored at −20°C in storage solution and studied within 1 week.

Solutions

Reagents were purchased from Sigma (St. Louis, MO). Solutions were formulated by solving ionic equilibria equations.18 Concentrations are provided in mmol/L unless otherwise noted. Relaxing solution, pH 8.0, consisted of pCa 8, 5 EGTA, 5 ATP 1 Mg2+, 30 N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 240 μM creatine kinase, 40 phosphocreatine, and 190 ionic strength. Fully activating solution was the same as relaxing solution but pCa was 4.5. During the experimental protocol, pCa was varied between 8 and 4.5. Rigor solution was the same as activating solution but without ATP, creatine kinase, or phosphocreatine. Storage solution was the same as relaxing solution but also contained 10 μg/mL leupeptin and 50% wt/vol glycerol. Skinning solution was the same as relaxing solution but also contained 10 μg/mL leupeptin, 1% wt/vol Triton-X100, and 50% wt/vol glycerol.

To determine whether our procedures lead to proteolysis, samples exposed to skinning solution for 24 hours (4°C) and then to storage solution for 48 hours (−20°C) were prepared for Western blot analysis of cardiac troponin I (TnI) and tropomyosin. For positive controls, ±3 mg samples of human myocardium were skinned with the addition of 400 mmol/L KCl, which separated proteins without cleavage. Protein samples were loaded and separated on 10% Novex bis-tris gel with MES SDS running buffer (Invitrogen, W1, 100). Protein was semidry transferred to nitrocellulose (Trans-Blot SD, BioRad, Hercules, CA). Nitrocellulose was stained with Ponceau-S solution (Sigma, P1701) to confirm successful transfer and then washed with TBS-T and blocked with 5% nonfat dry milk. Blots were probed with either anti-cardiac TnI (Abcam, ab102351) (1:2000) or anti-tropomyosin (Santa Cruz, sc-28543) (1:200), and incubated with either SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34077) or West Femto (Thermo Scientific, 34095).

Isometric Tension and Sinusoidal Length Perturbation Measurements

At time of study, aluminum T-clips were attached to the ends of each strip ±300 μm apart. The strip was mounted between a piezoelectric motor (Physik Instrumente, Auburn, MA) and a strain gauge (SensorNor, Horten, Norway) and lowered into a 30 μL droplet of relaxing solution plus 30 mmol/L BDM maintained at 37°C. Sarcomere length was set at 2.2 μm by Fourier Transform of digital images (IonOptix Corp, Milton, MA). Both steady-state isometric force and dynamic stiffness were measured at the initial pCa 8 plus BDM condition and as pCa was varied from 8.0 to 4.5 in the absence of BDM. A rigor condition was then created by multiple exchanges of rigor solution. Tension (T) was calculated as force per cross-sectional area. Individual recordings of T minus relaxed tension at pCa 8 (T(pCa8)) were normalized to maximum developed tension (Tmax–T(pCa8)) and fit to the Hill equation: (T−T(pCa8))(Tmax−T(pCa8)) = [Ca2+]i half(κH ([Ca2+]i)50 + [Ca2+]i half) (1)

where [Ca2+]i half = calcium concentration at half activation, pCa50 =−log [Ca2+]i 50% and nHill = Hill coefficient using a nonlinear least squares algorithm (Sigma Plot 8.0, SPSS, Chicago, IL).

The complex modulus (κH), which refers to the frequency dependence of fiber viscoelastic stiffness, was measured at each pCa by sinusoidal length perturbations of 0.125% clip-to-clip strip length over a frequency range of 0.125 to 250 Hz. For each condition, the complex modulus was measured as change in tension divided by change in muscle length in phase with the length change (elastic modulus) and out of phase (viscous modulus). Cross-bridge-dependent complex modulus
was calculated as dynamic stiffness at any pCa condition minus that under BDM conditions, as BDM prevents cross-bridge formation.

Characteristics of the elastic and viscous moduli over the frequency range tested provide a signature of cross-bridge kinetics. Elastic and viscous moduli of cross-bridge-dependent dynamic stiffness fitted a multiparameter model in the frequency domain:

$$Y(\omega) = A(\omega)^2 - B\left(\frac{\omega}{2\pi b + \omega}\right) + C\left(\frac{\omega}{2\pi c + \omega}\right)$$  

(2)

where A, B, and C are magnitudes expressed in mN/m², k = a unitless exponent, 2πb and 2πc are characteristic rates expressed in s⁻¹, ωₚ is angular frequency in units of s⁻¹ of the length perturbation equal to 2πxfrequency of perturbation, and i = -1. Parameters A and k reflect the viscoelastic properties of structural elements of the muscle strip, including collagen, titin, and a Ca²⁺-dependent portion ascribed to myosin heads attached to actin at any given time. Coefficients B and C are proportional to the unitary stiffness of the cross-bridge, the total number of force-generating myosin heads and the duty ratio of cycling cross-bridges (the average proportion of the cycle that cross-bridges are attached and generating force). The apparent rate constants 2πb and 2πc reflect the rates of myosin cross-bridge attachment and detachment, where 2πb is the rate at which force develops in response to step strain and is akin to 1/kₚmax measured in stretch activation experiments. 2πc represents the cross-bridge detachment rate and its reciprocal, 1/2πc, is the average time the myosin cross-bridge is attached to actin (tₐ). A nonlinear least squares curve-fitting algorithm was used to estimate these parameters from the recorded elastic and viscous moduli.

### Protein Phosphorylation Studies

Studies were performed in frozen tissue samples from 6 controls and 5 patients with HTN. Phosphorylation of TnI, myosin binding protein C (MyBPC), troponin T, and myosin light chain 2 was estimated using the ProQ Diamond method (Invitrogen; http://probes.invitrogen.com/media/its/mp33300.pdf).

### Echocardiographic-Doppler Studies

Studies were performed at University of Vermont College of Medicine and Medical University of South Carolina were interpreted at a Core Laboratories at Medical University of South Carolina. DVDs were de-identified and interpreted by Dr Zile in a blinded fashion. Left ventricular ejection fraction, end-diastolic volume index, RWT, and mass index, and left atrial (LA) volume and E/E' (ratio of mitral inflow peak early diastolic filling velocity to lateral mitral annular tissue peak early diastolic lengthening rate) were calculated.

### Statistics

Data are reported as mean±SEM. Two activated skinned strips were examined from each heart, and mechanical and kinetic characteristics averaged to provide one measure for each heart. An unpaired, 2-tailed t test was used to test for group differences in clinical variables, echocardiographic-Doppler measurements, and parameters of tension development. Repeated measures ANOVA with an assumption of compound symmetry was employed to test for differences in tₐ as a function of pCa. An unpaired t test was then used to detect differences in tₐ between the groups at each pCa condition. Linear correlations were tested between tₐ and variables of interest. P<0.05 was considered statistically significant. The study was not formally powered to a specific end point.

### Results

Clinical and demographic data are shown in Table 1. Fourteen patients were white; 1 patient with HTN was African-American. Control and patients with HTN were similar with respect to age, serum creatinine, and number of vessels with >70% stenosis. LVEDP was slightly higher in patients with HTN, but this difference was not significant. Only 2 of the control patients were receiving β-blockers and none were receiving angiotensin converting enzyme inhibitors or receptor blocking drugs. Patients with HTN were receiving a variety of blood-pressure lowering drugs.

Echocardiographic data are shown in Table 2. Left ventricular ejection fraction and end-diastolic volume index were not significantly different in control and HTN groups. RWT was greater in the HTN group, but this did not reach significance (P=0.057). LV mass index was significantly greater in the HTN group (P=0.011). There were trends toward an increased LA volume and E/E' in the HTN group (P=0.08, 0.09, respectively). Using the aforementioned criteria, 3 of the patients with HTN had LVH and 4 had increased RWT. None of the controls had concentric remodeling.

### Activated Skinned Strip Studies

Table 3 presents isometric tension results. Tension under BDM conditions at 2.2 μm sarcomere length was higher in

### Table 1. Demographic and Clinical Characteristics (mean±SE)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n=7)</th>
<th>HTN (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>67 ± 2</td>
<td>63 ± 4</td>
<td>0.44</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>1.02 ± 0.09</td>
<td>1.00 ± 0.13</td>
<td>0.93</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>12 ± 2 (6)</td>
<td>15 ± 2 (6)</td>
<td>0.70</td>
</tr>
<tr>
<td>No. vessels &gt;70% stenosis</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>1.00</td>
</tr>
<tr>
<td>No. receiving BB</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>No. receiving ACEI/ARB</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>No. receiving other BP lowering drug</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

HTN indicates hypertension; LVEDP, left ventricular end-diastolic pressure (No. of patients measured); BB, β-blocker; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

### Table 2. Echocardiographic-Doppler Data (mean±SE)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n=7)</th>
<th>HTN (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEF</td>
<td>64 ± 3</td>
<td>59 ± 6</td>
<td>0.49</td>
</tr>
<tr>
<td>LVEDVI, mL/m²</td>
<td>56 ± 6</td>
<td>58 ± 5</td>
<td>0.84</td>
</tr>
<tr>
<td>Relative WT</td>
<td>3.7 ± 0.1</td>
<td>4.5 ± 0.4</td>
<td>0.06</td>
</tr>
<tr>
<td>LV mass index, g/m²</td>
<td>84 ± 7</td>
<td>111 ± 8</td>
<td>0.01</td>
</tr>
<tr>
<td>LA volume, mL</td>
<td>22 ± 1.3</td>
<td>42 ± 9.1</td>
<td>0.08</td>
</tr>
<tr>
<td>E/E'</td>
<td>7.2 ± 1.3</td>
<td>11.1 ± 1.6</td>
<td>0.09</td>
</tr>
</tbody>
</table>

LVEF indicates left ventricular ejection fraction; LVEDVI, left ventricular end-diastolic volume index; WT, wall thickness; LV, left ventricular; LA, left atrial; E, E'.

### Table 3. Myofilament Activation Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n=7)</th>
<th>HTN (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>tₐ [s]</td>
<td>2.15 ± 0.24</td>
<td>3.01 ± 0.40*</td>
<td>0.05</td>
</tr>
<tr>
<td>Tₛₜₜ [mm²]</td>
<td>4.26 ± 0.52</td>
<td>4.30 ± 0.51</td>
<td>0.16</td>
</tr>
<tr>
<td>Tₛₜₜ [nm²]</td>
<td>20.64 ± 3.29</td>
<td>10.67 ± 1.38†</td>
<td>0.01</td>
</tr>
<tr>
<td>[Ca²⁺]ₚₜₜ [nmol/L]</td>
<td>940 ± 163</td>
<td>914 ± 75</td>
<td></td>
</tr>
<tr>
<td>pCaₚₜₜ</td>
<td>6.07 ± 0.07</td>
<td>6.06 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>&amp;nₚₜₜ</td>
<td>2.91 ± 0.11</td>
<td>2.91 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

HTN indicates hypertension; T, tension; BDM, butanedione monoxime; pCa, calcium concentration; Tₛₜₜ, maximum developed tension; nₚₜₜ, Hill coefficient. *Different from coronary artery disease at P<0.05, †P<0.01.
HTN compared with controls. This result is consistent with higher passive stiffness, that is, increased stiffness independent of myosin cross-bridges, in HTN patients. At pCa 8 tension was greater relative to BDM conditions in both groups, and a group difference in tension was no longer evident. Maximum developed tension was markedly lower in HTN compared with controls (P < 0.01). The absolute isometric tension–pCa relationship (Figure 1A) reveals differences in tension under BDM conditions (HTN>controls) and at pCa <6 (controls>HTN). The normalized isometric tension–pCa relation, however, was indistinguishable between control and HTN (Figure 1B). Correspondingly, pCa_in and the Hill coefficient of activation cooperativity, n_Hill were virtually identical in HTN and controls (Table 3).

The complex moduli detected under BDM conditions at pCa 8 were not significantly different between HTN and controls (Figures 2A and 2B). Upon removal of BDM we detected cross-bridge formation under pCa 8 conditions in both HTN and control groups. As illustrated in Figures 2A and 2B, the dips and rises in the elastic and viscous moduli reflect the mechanical consequences of cycling cross-bridges at pCa 8. The pCa 8 condition is normally assumed to not elicit activation of the thin filament. Our measures of tension (Table 3) and the complex moduli at pCa 8 compared with BDM conditions suggest that the thin filament is activated at a nominal yet detectable amount under otherwise relaxed conditions in both HTN and control patients. Figures 2C and 2D are Western blots demonstrating lack of proteolysis of cardiac TnI and Tn, respectively.

Analysis of A, B, and C, which reflect myosin cross-bridge number × cross-bridge stiffness × duty ratio, revealed that their magnitudes rose with thin filament activation (Figures 3A–3C). The magnitudes of A and C were lower in HTN compared with controls under high [Ca2+] conditions but indistinguishable at low [Ca2+], with the differences becoming evident at pCa <5.75 to 6.0. The magnitude of B trended in the same direction, but was not significant at P<0.05. These
changes in A, B, and C are similar to those for maximum developed tension and consistent with the fact that these parameters are all proportional to cross-bridge number, duty ratio, and stiffness. Parameters k and $2\pi b$ were not significantly different between controls and HTN (Figures 3D and 3E). Myosin cross-bridge detachment rate, $2\pi c$, was significantly lower in HTN compared with controls at low [Ca2+] conditions, with the difference evident at pCa $\geq 6.50$ (Figure 3F).

Results for myosin cross-bridge $t_{im}$ are shown in Figure 4A. ANOVA revealed a significant within-subject pCa main effect ($P<0.001$) and a significant within-subject pCa versus HTN interaction ($P=0.001$). There was also a significant between-subject HTN main effect ($P=0.001$). Mirroring changes in $2\pi c$ (Figure 3F), $t_{im}$ ($2\pi c^{-1}$) was substantially prolonged at pCa $\geq 6.5$ in the HTN group. The lower $2\pi c$ and higher $t_{im}$ at submaximal [Ca2+] in HTN can be visualized in Figure 4B, where the dips and peaks of the moduli in HTN occur at lower frequencies compared with controls.

Dynamic stiffness of myosin cross-bridges was also measured under rigor conditions (Figure 5). We found that cross-bridges from HTN patients demonstrated half the elastic and viscous moduli than controls. These results indicate that in HTN there are either a smaller number of cross-bridges per cross-sectional area or less stiff cross-bridges, or some combination of the two. This result detected under rigor conditions offers a partial explanation for the reduced developed tension and magnitudes of A, B, and C.

To examine relationships between $t_{im}$ and variables of interest, we tested for linear correlations by combining the control and HTN patients. There were no significant correlations with age ($r=0.14, P=0.72$), LVEDP ($r=0.40, P=0.27$), RWT ($r=-0.44, P=0.16$), LV mass index ($r=-0.47, P=0.13$), LA volume ($r=0.20, P=0.54$), or E/E' ($r=-0.55, P=0.08$).

**Protein Phosphorylation**

Phosphorylation of both TnI and MyBPC was significantly reduced ($P<0.05$) in HTN compared with controls (Figure 6). There were no significant differences in the levels of TnT or myosin light chain 2 phosphorylation (data not shown). Full gels are shown in the online-only Data Supplement.

**Discussion**

HTN is the most important substrate for HFpEF, which currently accounts for >50% of cases of heart failure.$^3$-$^9$ Most patients with HTN and HFpEF have concentric remodeling and virtually all have evidence of diastolic dysfunction. In population studies of HTN, worsening of diastolic function...

![Figure 3. Model parameters of cross-bridge-dependent viscoelasticity. A-C. Magnitudes of processes (A, B, and C) in mN/mm² are proportional to myosin cross-bridge number, duty ratio, and stiffness. These magnitudes rise with calcium activation, less so in hypertension (HTN) compared with controls. P<0.05; #P<0.01. D. Parameter k reflects the degree of elastic (k→0) vs viscous (k→1) response and was not significantly different between the groups. E. $2\pi b$, representing the forward and reverse rate of myosin isomerization, was not different between the groups. F. The myosin detachment rate, $2\pi c$, was significantly lower in HTN compared with controls at low calcium concentrations, that is, pCa $>6.25$. P<0.05.](http://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.117.030503)
properties.

subtracted to demonstrate cross-bridge-dependent viscoelastic moduli under butanedione monoxime (BDM) conditions were significantly lower in hypertension (HTN) compared with controls. The formed and without MgATP to facilitate detachment, was significantly prolonged in hypertension (HTN) compared with controls at lower calcium concentrations. *P<0.05

Figure 4. Myosin cross-bridge attachment time (t_on) and stiffness. A, Mean t_on was calculated as the reciprocal of 2πc and was significantly prolonged in hypertension (HTN) compared with controls at lower calcium concentrations. *P<0.05 B, Prolonged t_on in HTN at pCa 8 underlies the lower frequencies at which the dips and shoulders of the viscoelastic characteristics occur. BDM indicates butanedi-one monoxime.

Echocardiographic-Doppler indices are commonly used to evaluate diastolic function. However, these measurements cannot determine whether there is a myocardial basis for diastolic dysfunction, as opposed to a change in chamber geometry or arterial load. At the myocardial level, the determinants of diastolic function can be divided into those responsible for deactivation (relaxation) of the myofilament, specifically, acto-myosin cross-bridge dynamics and restoration of [Ca^{2+}]i to normal diastolic levels (sarcoplasmic reticulum calcium ATPase-2/phospholamban and the Na^{+}-Ca^{2+} exchanger), and those responsible for passive stiffness, specifically, extracellular matrix collagen and titin, the myofilament protein that functions as a molecular spring. A decrease in chamber compliance can result from abnormalities of ≥1 of these myocardial determinants.

Prior studies in patients indicate that increased collagen volume fraction and possibly posttranslational modifications of collagen, such as glycation, contribute to elevated passive myocardial stiffness in LVH. Recently, we reported abnormal rate-dependent incomplete relaxation as well as increased resting tension in excitable strips from patients with HTN, findings which are likely due to alterations in Ca^{2+} handling. There have been no previous reports implicating involvement of the myofilament as a cause of slowed relaxation in patients with HTN and associated concentric remodeling. In end-stage HFrEF, myofilament Ca^{2+} sensitivity, that is, pCa_{50}, is increased and is thought to contribute to slowed relaxation. This change has been ascribed to reduced phosphorylation of Serine 23,24 on TnI, sites phosphorylated by protein kinases A (PKA) and G. In studies in rodents with pressure overload LVH, myofilament calcium sensitivity has been reported to be increased, decreased, and unchanged. These inconsistent results underscore the importance of elucidating myofilament involvement in slowed relaxation in patients with HTN and concentric remodeling.

Our analysis of myofilament properties revealed that the normalized isometric tension–pCa relation was indistinguishable in controls and HTN, identical to what we reported in LVH, diabetes mellitus, and mitral regurgitation. However, at low [Ca^{2+}] (pCa >6.5) t_on was substantially prolonged in HTN. Because t_on determines and mathematically is simply the inverse of cross-bridge detachment rate, prolongation at low [Ca^{2+}] would be expected to slow LV relaxation, especially during its later phases and including the period after the mitral valve opens. The fact that this effect was only evident at [Ca^{2+}] below the pCa_{50} level accounts at least in part for the lack of change in the normalized isometric tension–pCa relation. Moreover, changes in t_on need not be associated with changes in active tension development. As for processes B and C, active tension is a function of the duty ratio, the unitary force produced by each cross-bridge and the number of cycling cross-bridges.

As expected, LV diastolic function was impaired in HTN patients based on increased LA volume and E/E', although these changes did not quite achieve significance at P<0.05. The fact that myofilament effects on relaxation in HTN were confined to t_on and only evident at low [Ca^{2+}] might make them difficult to detect using either echocardiographic-Doppler measures of LV function or invasively determined rate of
pressure decline, all of which are load-sensitive. Nonetheless, these myofilament changes could have significant functional consequences early in diastole, when ventricular inflow rate is high. Our results underscore the fact that in evaluating the role of the myofilament in relaxation, it is important to consider cross-bridge dynamic properties as well as isometric tension.

We did not detect any significant correlations of \( t_\text{on} \) with clinical data, echocardiographic-Doppler measures of LV remodeling (LV mass index) or diastolic function (LA volume and \( E/E' \)). However, the number of HTN patients was much too small to conclude that such correlations do not exist.

The increase in \( t_\text{on} \) at low \([Ca^{2+}]\) suggested that hypophosphorylation of protein kinases A/G sites on TnI and MyBPC could be responsible. Phosphorylation of these TnI sites accelerates relaxation\(^{38-37}\) and, as discussed previously, hypophosphorylation has been implicated as a mechanism of increased myofilament \( Ca \) sensitivity in HFrEF. The effect of phosphorylation of PKA sites on MyBPC is less well established. Some studies suggest that phosphorylation results in effects on relaxation that are indistinguishable from those for TnI.\(^{32-35}\) Others suggest that the effects of phosphorylation are mainly manifest as changes in the kinetics of force generation rather than force decline or that effects of adrenergic stimulation on relaxation are mediated exclusively by PKA phosphorylation of TnI.\(^{36,37}\) Our phosphorylation studies demonstrated significantly reduced phosphorylation of both proteins, with the change being more prominent for TnI than MyBPC. Recently, we showed that dephosphorylation of PKA sites on MyBPC actually shortens \( t_\text{on} \), arguing strongly against a role for a decrease in MyBPC phosphorylation as a factor in prolonging \( t_\text{on} \) in HTN. Thus, reduced phosphorylation of Ser 23,24 on TnI remains as a potential mechanism of our \( t_\text{on} \) findings in HTN. In HFrEF, hypophosphorylation of Ser 23,24 is quite marked,\(^{38}\) whereas the decrease in TnI phosphorylation we observed in HTN was relatively modest, amounting to about 25% (Figure 6). This could also potentially contribute to the fact that \( t_\text{on} \) was prolonged only at low \([Ca^{2+}]\) without a change in \( pCa_{50} \).

The ProQ Diamond method is not site-specific and we cannot state which TnI sites were in fact hypophosphorylated. Thus, for example, Ser 43,44 are important targets of protein kinase C. However, most protein kinase C isoforms are upregulated in pressure overload hypertrophy in both animals and patients.\(^{40,41}\) Therefore, it seems unlikely that these sites would be hypophosphorylated in patients with HTN and concentric remodeling. Phosphorylation of TnT also modulates cross-bridge dynamics,\(^{42}\) but we did not detect a change in TnT phosphorylation. It is important to emphasize the preliminary nature of our phosphorylation results. Thus, complex changes in phosphorylation of the proteins that modulate cross-bridge dynamics could have been missed. More detailed studies of the phosphorylation of these proteins will be required to definitively establish a mechanistic link with changes in \( t_\text{on} \).

Alterations in myosin heavy chain isoforms (MHCs) represent another potential mechanism for the changes in \( t_\text{on} \) we observed. Shifts toward a higher percentage of the \( \beta \)-MHC isoform, which has slower ATPase activity, are observed under conditions of hemodynamic stress and end-stage HFrEF. However, in normal humans the proportion of \( \beta \)-MHC is high, generally 93% to 95%, with increases to 98% to 100% in HFrEF.\(^{43,44}\) We have shown that changes in MHC isoforms of this magnitude have minimal functional consequences at best.\(^{45,44}\) Given the normal MHC distribution and the magnitude of increase in HFrEF, it is not possible to have a significantly larger change in patients with concentric remodeling.

Two other of our findings merit discussion. Our BDM results in both HTN and controls reveal cross-bridge activation at \( pCa \) 8, that is, cross-bridge-dependent tension is present under conditions usually considered to be fully relaxed. This observation is consistent with single particle analyses of variation in tropomyosin position,\(^{45}\) which reveal that a \( Ca^{2+} \) induced position is normally present in a small proportion of cross-bridges at low \([Ca^{2+}]\), indicating the possibility of low-level activation. It is also possible that the presence of CAD in both HTN patients and controls could in some way have accounted for this finding. Second, we observed major decreases in maximum developed...
tension, the magnitudes of processes A and C (with a trend toward the same result in process B) at activating [Ca\(^{2+}\)] and a large decrease in dynamic stiffness under rigor conditions in patients with HTN. All of these results could be explained by a decrease in the number of cross-bridges/cross-sectional area and a decrease in the stiffness of the cross-bridges. Our studies were not designed to examine the mechanism of these changes. For example, we did not measure myofilament cross-sectional fraction. Nonetheless, these findings suggest important changes in the properties of the fully activated myofilament in HTN with concentric remodeling that merit future investigation.

**Study Limitations**

As noted earlier, this was a relatively small study and was not powered to detect correlations of various clinical parameters and echocardiographic-Doppler indexes with \( t_{1/2} \). It was also comprised exclusively of males who were largely white. Unfortunately, we were constrained by the racial-ethnic make-up of eligible patients referred for CBG at the participating institutions.

The fact that all of our patients had concomitant CAD requiring CBG might be considered a confounding feature. The angiographic severity of CAD was similar in both groups and all had normal LV wall motion, eliminating any effects of large areas of chronic infarction. Nonetheless, it is possible that the presence of CAD could itself have influenced the myocardial properties we studied in some as yet undefined way. We would suggest, however, that studying these properties in patients with CAD is in fact clinically appropriate. A population of middle-aged to older males with HTN inevitably has a relatively high incidence of underlying CAD. The fact that endothelial function is so often abnormal in HTN patients\(^{36,47} \) attests to the likelihood that subclinical CAD is common and may be the rule. Thus, while it certainly would be of great interest to perform similar measurements in a cohort without CAD, we think that age- and sex-matched control and HTN/concentric remodeling groups with a common background of CAD constitute a real-world patient cohort that is also quite representative of those at risk for the development of HFpEF. Finally, it is possible that storage in glycerol could have modified the behavior of our skinned strips in some way, although this would not explain differences between controls and HTN patients.

**Conclusions**

Our results show that CBG patients with a history of HTN and concentric LV remodeling display substantial prolongation of cross-bridge attachment time estimated at submaximal [Ca\(^{2+}\)] in skinned myocardial strips compared with controls. This change in attachment time is the first report of an alteration in myofilament properties that could contribute to the slowing of LV relaxation observed in patients with LVH. In contrast, there were no differences in pCa\(_{50}\) of the isometric tension–pCa relation, the usual measurement used to infer a myofilament abnormality that slows relaxation. We also detected reduced phosphorylation of TnI, which suggests the possibility that hypophosphorylation of Ser 23,24 might be responsible for prolonged attachment time. Additional studies directed at more specifically delineating TnI phosphorylation and its functional consequences will be required to test this hypothesis.

**Sources of Funding**

This research was supported by National Institute of Health grants R01 HL089944 (M.M.L., B.M.P., P.V.B., D.W.M., M.Z.), T32 HL07647 (C.M., M.M.L.), R01 AG036954 (J.I.), R01 HL102121 (J.J.), and the Research Service of the Department of Veterans Affairs (M.Z.).

**Disclosures**

None.

**References**

reduced phosphorylation of troponin I as a mechanism of this alteration. The implication of these findings is that abnormal increase in cross-bridge attachment time (t on) and a corresponding reduction in its inverse, cross-bridge detachment rate, diastolic dysfunction in patients with concentric left ventricular remodeling due to hypertension. We found that compared but underlying mechanisms are not fully understood. This study, performed in human left ventricular myocardial tissue at submaximal [Ca2+]. These parameters are key determinants of the rate of tension decline. Preliminary results implicate nants of matrix composition identify the presence of left ventricular hy -

cardiac myosin binding protein C modulates cardiac function. 

811

CLINICAL PERSPECTIVE

Hypertension is the most common substrate for the syndrome of heart failure with a preserved ejection fraction. Abnormal diastolic function is thought to represent a major pathophysiologic feature of heart failure with a preserved ejection fraction, but underlying mechanisms are not fully understood. This study, performed in human left ventricular myocardial tissue obtained at time of coronary bypass surgery, was designed to determine if abnormal myofilament properties contribute to diastolic dysfunction in patients with concentric left ventricular remodeling due to hypertension. We found that compared with controls, patients with hypertension have abnormal myosin cross.bridge dynamics, consisting of a substantial increase in cross-bridge attachment time (t on) and a corresponding reduction in its inverse, cross-bridge detachment rate, at submaximal [Ca2+]. These parameters are key determinants of the rate of tension decline. Preliminary results implicate reduced phosphorylation of troponin I as a mechanism of this alteration. The implication of these findings is that abnormal myofilament cross.bridge dynamics contribute to slowed relaxation in patients with hypertension and concentric remodeling and, potentially by extension, in patients with hypertension associated heart failure with a preserved ejection fraction.
Myosin Cross-Bridge Dynamics in Patients With Hypertension and Concentric Left Ventricular Remodeling

Cameron Donaldson, Bradley M. Palmer, Michael Zile, David W. Maughan, John S. Ikonomidis, Henk Granzier, Markus Meyer, Peter VanBuren and Martin M. LeWinter

_Circ Heart Fail._ 2012;5:803-811; originally published online September 26, 2012; doi: 10.1161/CIRCHEARTFAILURE.112.968925

_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/5/6/803

Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2012/09/26/CIRCHEARTFAILURE.112.968925.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Heart Failure_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation: Heart Failure_ is online at:
http://circheartfailure.ahajournals.org//subscriptions/
Supplemental Material
Supplemental Figure. Complete ProQ Diamond and total protein stains.