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

Donaldson et al: Cross-Bridge Dynamics in LV Remodeling

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Abstract

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

**Methods and Results**—Intra-operative myocardial biopsies were obtained in 15 male patients undergoing coronary bypass grafting (CBG), all with normal LV EF 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 8–4.5. Sinusoidal analysis provides estimates of cross-bridge dynamics, including rate constants of attachment and detachment and cross-bridge attachment time (t_{on}). The normalized isometric tension-pCa relation was similar in HTN and controls. However, t_{on} was significantly prolonged at submaximal [Ca^{2+}] (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 t_{on} at submaximal [Ca^{2+}] without a change in the tension-pCa relation. Prolonged t_{on} 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.

**Key Words**: diastole, heart failure, hypertrophy, myocardium, remodeling
Hypertension (HTN) causes concentric left ventricular (LV) remodeling, defined as either increased relative wall thickness or overt LV hypertrophy (LVH) (1,2), and diastolic dysfunction. HTN is also the most common risk factor for heart failure with preserved ejection fraction (HFpEF) (3-9).

The myocardial basis of diastolic dysfunction in human HTN with concentric remodeling is incompletely understood. In patients, ecollagen volume fraction is increased (10,11). 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 EF and LVH due to pressure overload (12). This finding appears to be related to abnormalities in $\text{Ca}^{2+}$ 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/or 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/or completeness of relaxation is thin filament sensitivity to calcium activation, i.e., $\text{pCa}_{50}$ 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 (13-15).
The present study was performed in myocardium obtained from patients undergoing coronary bypass grafting (CBG) with HTN, normal LV EF and concentric remodeling to determine if 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 employed 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 intra-operative myocardial biopsy from amongst those scheduled for CBG at Fletcher Allen Health Care in Burlington, VT, the clinical facility of the University of Vermont College of Medicine (UVM), and the VA Medical Center and Medical University of South Carolina (MUSC) Hospital in Charleston, SC between October 1, 2008 and May 31, 2010 who satisfied the inclusion and exclusion criteria specified below. All patients signed consent forms approved by their respective IRBs. Some potentially eligible patients were not screened due to vacations, illnesses, or periods of time when there was inadequate capacity to perform studies or equipment maintenance. Patients over 21 years of age with normal LVEF, wall motion and end-diastolic volume index (EDVI) based on an echocardiogram obtained less than two weeks before surgery were eligible for inclusion. If a pre-operative echocardiogram could not be obtained within the two week time frame a pre-operative contrast left ventriculogram demonstrating normal LVEF and wall motion was accepted for inclusion. In patients who did not undergo pre-operative echocardiography, an echocardiogram was performed 2-4 weeks after surgery. Patients were categorized as having
HTN if this was documented in their medical records and/or they had been told of this diagnosis by a physician and, in addition, were receiving anti-hypertensive medications. Based on echocardiographic results, we excluded HTN patients without evidence of concentric LV remodeling [mass index < 115 gm/m² or relative wall thickness (RWT) < 0.42 (3)]. Patients without HTN were classified as controls. A total of eight patients comprised the HTN group and seven comprised the control group.

Exclusion criteria included diabetes mellitus, acute myocardial infarction within 4 weeks before CBG, EF < 0.50, significant valvular or other non-coronary heart disease, obstructive sleep apnea and/or chronic pulmonary disease requiring home O₂, any non-cardiac disease or condition known to affect myocardial function, anemia (Hgb < 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 end-diastolic pressure if measured) were tabulated. The angiographic severity of coronary artery disease (CAD) was graded based on the number of major vessels (left anterior descending, left circumflex, right coronary arteries) with a stenosis >70% with left main coronary artery considered as two vessels.

Myocardial Biopsy Procedure

Anterior LV free wall sub-epicardial biopsies weighing ~25 to 50 mg were obtained during CBG soon after the patient was placed on cardiopulmonary bypass, as previously described (17,18).
All patients were followed post-operatively until discharge. No adverse effects or post-operative 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 below). The tissue was then cut into pieces < 2 mm in length and placed in skinning solution containing Triton-X100 at 4°C. For samples obtained at MUSC, the skinning period coincided with overnight transit to UVM at 4°C. After 18-24 hour skinning, strips were dissected to 150-200 µm diameter and 800-1200 µm length, stored at -20°C in storage solution and studied within one week.

Solutions

Reagents were purchased from Sigma (St. Louis, MO). Solutions were formulated by solving ionic equilibria equations (19). 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 BES, 240 U/mL creatine kinase (CK), 40 phosphocreatine (PCr), 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, CK or PCr. 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 if 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 (cTnI) and tropomyosin (Tm). For positive controls, ~3 mg samples of human myocardium were skinned with the addition of 400 mM KCl, which separated proteins without cleavage. Protein samples were loaded and separated on 10% Novex bis-tris gel with MES SDS running buffer (Invitrogen, WG1201, NP0002). Protein was semi-dry transferred onto nitrocellulose (Trans-Blot SD, BioRad, Hercules, CA). Nitrocellulose was stained with Ponceau-S solution (Sigma, P7170) to confirm successful transfer and then washed with TBS-T and blocked with 5% non-fat dry milk. Blots were probed with either anti-cTnI (Abcam, ab10231) (1:2000) or anti-Tm (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 (T_{max}-T_{pCa8}) and fit to the Hill equation:

\[
\frac{\text{T}-\text{T}_{\text{pCa8}}}{\text{T}_{\text{max}}-\text{T}_{\text{pCa8}}} = \frac{[\text{Ca}^{2+}]^{n_{\text{Hill}}}}{([\text{Ca}^{2+}]_{50}^{n_{\text{Hill}}} + [\text{Ca}^{2+}]^{n_{\text{Hill}}})},
\]  

(Equation 1)
where [Ca$^{2+}$]$_{50}$ = calcium concentration at half activation, pCa$_{50}$ = -log [Ca$^{2+}$]$_{50}$, and n$^{\text{Hill}}$ = Hill coefficient using a nonlinear least squares algorithm (Sigma Plot 8.0, SPSS, Chicago, IL).

The complex modulus ($Y(\omega)$), 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 (20). 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, since 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 were fit to a multi-parameter model in the frequency domain (20):

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

(Equation 2)

where $A$, $B$ and $C$ are magnitudes expressed in mN/mm$^2$, $k$ = a unitless exponent, $2\pi b$ and $2\pi c$ are characteristic rates expressed in $s^{-1}$, $\omega$ = angular frequency in units of $s^{-1}$ of the length perturbation equal to $2\pi \times$ frequency of perturbation, and $i = -1^{1/2}$ (21). Parameters $A$ and $k$ reflect the viscoelastic properties of structural elements of the muscle strip, including collagen, titin and a Ca$^{2+}$-dependent portion ascribed to myosin heads attached to actin at any given time (22,23). 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) (20). The apparent rate constants $2\pi b$ and $2\pi c$ reflect the rates of myosin cross-bridge attachment and detachment, where $2\pi b$ is the rate force develops in response to step strain and is akin to $k_{\text{Phase3}}$ measured in stretch activation experiments (20,21,23,24). $2\pi c$ represents the cross-bridge detachment rate and its reciprocal, $1/2\pi c$, is the average time the myosin cross-bridge is attached to actin ($t_{on}$) (20). A non-linear 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 six controls and five HTN patients. 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/pis/mp33300.pdf).

**Echocardiographic-Doppler Studies**

Studies performed at UVM and MUSC were interpreted at a Core Laboratory at MUSC. DVDs were de-identified and interpreted by Dr. Zile in a blinded fashion. LVEF, EDVI, RWT and mass index, and left atrial (LA) volume and E/E’ were calculated (3).

**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, two-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 $t_{on}$ as a function of pCa. An unpaired t-test was then used to detect differences in $t_{on}$ between the groups at each pCa condition. Linear correlations were tested between $t_{on}$ 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. 14 patients were Caucasian; one HTN patient was African-American. Control and HTN patients were similar with respect to age, serum creatinine and number of vessels with $> 70\%$ stenosis. LVEDP was slightly higher in HTN patients, but this difference was not significant. Only two of the control patients were receiving beta-blockers and none were receiving angiotensin converting enzyme inhibitors or receptor blocking drugs. HTN patients were receiving a variety of blood-pressure lowering drugs.

Echocardiographic data are shown in Table 2. LVEF and EDVI 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), four of the HTN patients had LVH and four 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 HTN compared with controls. This result is consistent with higher passive stiffness, i.e., 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, T_dev, 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_{50} 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 pCa8 were not significantly different between HTN and controls (Figures 2A,B). 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 to 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 cTnI and Tn, respectively.
Analysis $A$, $B$ and $C$, which reflect myosin cross-bridge number \times crossbridge stiffness \times duty ratio (20), 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 [Ca$^{2+}$] conditions but indistinguishable at low [Ca$^{2+}$], with the differences becoming evident at pCa < 5.75-6.0. The magnitude of $B$ trended in the same direction, but was not significant at $P < 0.05$. These changes $A$, $B$ and $C$ are similar to those for $T_{dev}$ 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,E). Myosin cross-bridge detachment rate, $2\pi c$, was significantly lower in HTN compared with controls at low [Ca$^{2+}$] conditions, with the difference evident at pCa$_{50} \geq \sim 6.50$ (Figure 3F).

Results for myosin cross-bridge $t_{on}$ are shown in Figure 4A. ANOVA revealed a significant within subject pCa main effect ($p < .001$) and a significant within subject pCa versus HTN interaction ($p = .001$). There was also a significant between subject HTN main effect ($p = .001$). Mirroring changes in $2\pi c$ (Figure 3F), $t_{on}(2\pi c^{-1})$ was substantially prolonged at pCa $\geq \sim 6.5$ in the HTN group. The lower $2\pi c$ and higher $t_{on}$ at submaximal [Ca$^{2+}$] 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 $C$.

In order to examine relationships between $t_{on}$ and variables of interest, we tested for linear correlations by combining the control and HTN patients. There were no significant correlations with age ($r = .14, p = .72$), LVEDP ($r = .40, p = .27$), relative wall thickness ($r = -.44, p = .16$), LV mass index ($r = -.47, p = .13$), LA volume ($r = .20, p = .54$), or E/E’ ($r = -.55, p = .08$).

**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 data supplement.

**Discussion**

HTN is the most important substrate for HFpEF, which currently accounts for at least 50% of cases of HF (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 parallels the progression to HFpEF (8,9). Thus, elucidating the mechanism(s) of diastolic dysfunction in patients with HTN and concentric remodeling is likely very important in understanding the underlying pathophysiology that leads to HFpEF. As a result of ready availability of myocardial tissue at the time of cardiac transplantation, there is an extensive body of knowledge in regard to myocardial abnormalities in end-stage HF with reduced EF (HFrEF).
Viable tissue from patients with HTN-associated concentric remodeling is less commonly available. As a result, much less is known about myocardial properties in these patients.

Echocardiographic-Doppler indices are commonly used to evaluate diastolic function. However, these measurements cannot determine if 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 $[Ca^{2+}]_{in}$ to normal diastolic levels (SERCA-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 one or more of these myocardial determinants.

Prior studies in patients indicate that increased collagen volume fraction (10,11) and possibly post-translational modifications of collagen such as glycation (25) contribute to elevated passive myocardial stiffness LVH. Recently, we reported abnormal rate-dependent incomplete relaxation as well as increased resting tension in excitable strips from LVH patients (12), 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, i.e., $pCa_{50}$, is increased and is thought to contribute to slowed relaxation (26,27). This change has been ascribed to reduced phosphorylation of Ser 23,24 on TnI, sites phosphorylated by protein kinases A (PKA) and G (PKG). In studies in rodents with pressure overload LVH,
myofilament calcium sensitivity has been reported to be increased (14), decreased (15) and unchanged (13). 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 (12), diabetes mellitus (18) and mitral regurgitation (23). However, at low $[\text{Ca}^{2+}]$ (pCa $> 6.5$) $t_{on}$ was substantially prolonged in HTN. Since $t_{on}$ determines and mathematically is simply the inverse of cross-bridge detachment rate, prolongation at low $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$ below the pCa50 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 $[\text{Ca}^{2+}]$ 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 \( t_{on} \) with clinical data, echocardiographic-Doppler measures of LV remodeling (LV mass index) or diastolic function (left atrial 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 \( t_{on} \) at low \([Ca^{2+}]\) suggested that hypo-phosphorylation of PKA/PKG sites on TnI and/or MyBPC could be responsible. Phosphorylation of these TnI sites accelerates relaxation (28-31) and, as discussed previously, hypo-phosphorylation 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 \( t_{on} \) (38), arguing strongly against a role for a decrease in MyBPC phosphorylation as a factor in prolonging \( t_{on} \) in HTN. Thus, reduced phosphorylation of Ser 23,24 on TnI remains as a potential mechanism of our \( t_{on} \) findings in HTN. In HFrEF, hypo-phosphorylation of Ser 23,24 is quite marked (39), 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 $t_{on}$ was prolonged only at low $[\text{Ca}^{2+}]$ without a change in $p\text{Ca}_{50}$.

The ProQ Diamond method is not site-specific and we cannot state which TnI sites were in fact hypo-phosphorylated. Thus, for example, Ser 43,44 are important targets of PKC. However, most PKC isoforms are up-regulated in pressure overload hypertrophy in both animals and patients (40,41). Therefore, it seems unlikely that these sites would be hypo-phosphorylated 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 $t_{on}$.

Alterations in myosin heavy chain isoforms (MHCs) represent another potential mechanism for the changes in $t_{on}$ 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 93-95%, with increases to 98-100% in HFrEF (43,44). We have shown that changes in MHC isoforms of this magnitude have minimal functional consequences at best (43,44). 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, i.e., 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 $T_{dev}$, 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 HTN patients. All of these results could be explained by a decrease in the number of cross-bridges/cross-sectional area and/or 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 $t_{on}$. It was also comprised exclusively of males who were largely Caucasian. 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 submit, 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 (46,47) attests to the likelihood that sub-clinical 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 believe 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.

**Summary and 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 sub-maximal [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 employed to infer a myofilament abnormality that slows relaxation. We also detected reduced phosphorylation of TnI, which suggests the possibility that hypo-phosphorylation 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.

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**Disclosures**

None.

**References**


failure. 


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Table 1. Demographic and clinical characteristics (mean ± SE).

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<tr>
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<th>Controls (n=7)</th>
<th>HTN (n=8)</th>
<th>p value</th>
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<tr>
<td>Age (yrs)</td>
<td>67±2</td>
<td>63±4</td>
<td>.44</td>
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<tr>
<td>Serum creatinine</td>
<td>1.02±0.09</td>
<td>1.00±0.13</td>
<td>.93</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>12±2 (6)</td>
<td>15±2 (6)</td>
<td>.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 = hypertension; LVEDP = left ventricular end-diastolic pressure (# patients measured); BB = beta-blocker; ACEI = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker.
Table 2. Echocardiographic-Doppler data (mean ± SE)

<table>
<thead>
<tr>
<th></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>
Table 3. Myofilament activation characteristics.

* = different from CAD at \( P<0.05 \), †\( P<0.01 \).

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=7)</th>
<th>HTN (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{BDM} ) (mN.mm(^{-2}))</td>
<td>2.15±0.24</td>
<td>3.01±0.40 *</td>
</tr>
<tr>
<td>( T_{pCa8} ) (mN.mm(^{-2}))</td>
<td>4.26±0.52</td>
<td>4.30±0.51</td>
</tr>
<tr>
<td>( T_{dev} ) (mN.mm(^{-2}))</td>
<td>20.64±3.29</td>
<td>10.67±1.38 †</td>
</tr>
<tr>
<td>([Ca^{2+}]_{50}) (nM)</td>
<td>940±163</td>
<td>914±75</td>
</tr>
<tr>
<td>( pCa_{50} )</td>
<td>6.07±0.07</td>
<td>6.06±0.04</td>
</tr>
<tr>
<td>( nHill )</td>
<td>2.91±0.11</td>
<td>2.91±0.24</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Tension-pCa relationships.
A. Passive tension under BDM conditions at 2.2 μm sarcomere length was greater in HTN compared with controls. Calcium-dependent tension development was reduced in the HTN compared to controls at calcium concentrations at pCa ≤ 6.
B. Thin filament sensitivity to calcium activation, presented here using normalized tension, was indistinguishable between the two groups. * P < 0.05

Figure 2. A,B. Viscoelastic mechanics under relaxed conditions.
Under conditions of pCa 8 and 30 mM BDM, the elastic and viscous moduli were similar between CAD (Panel A) and HTN (Panel B). Upon removal of BDM, viscoelastic characteristics demonstrated the signature mechanical characteristics (i.e., dips and shoulders) due to myosin crossbridge formation in both groups.
C,D. Skinning and storage procedures did not result in detectable levels of cTnI (Panel C) or cTm (Panel D) by Western blot analysis, which verifies that these procedures do not result in significant loss of thin filament regulation. The skinning solution with 400 mM KCl provided a positive control.

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 HTN compared with controls. *P < 0.05; #P < 0.01
D. Parameter $k$ reflects the degree of elastic ($k \to 0$) vs viscous ($k \to 1$) 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, i.e., pCa $> 6.25$. *$P < 0.05$

Figure 4. Myosin cross-bridge attachment time ($t_{on}$) and stiffness.

A. Mean $t_{on}$ was calculated as the reciprocal of $2\pi c$ and was significantly prolonged in 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.

Figure 5. Myosin cross-bridge viscoelastic stiffness measured under rigor conditions, i.e., with all myosin crossbridges formed and without MgATP to facilitate detachment, was significantly lower in HTN compared with controls. The moduli under BDM conditions were subtracted to demonstrate crossbridge-dependent viscoelastic properties.

Figure 6. Relative phosphorylation of MyBP-C and TnI.

A. Phosphorylation of MyBPC and TnI was detected by Pro-Q diamond phosphostain compared with Coomassie Blue stain for protein density.
B. Relative phosphorylation of MyBP-C and TnI was reduced in myocardium of HTN patients (n = 6) compared with controls (n = 5). *$P < 0.05$. 
Figure 1
Figure 2

(A) Elastic Modulus (B) Viscous Modulus (mN/mm²)

Control

- pCa 8+BDM
- pCa 8

HTN

- pCa 8+BDM
- pCa 8

Frequency (Hz)

MW kD

Skin

+KCl

Stnd

Skin

+KCl

Stnd

C

Western

Ponceau

30

20

D

Western

Ponceau

30

20

MW kD

Skin

+KCl

Stnd

Skin

+KCl

Stnd
Figure 5
Figure 6

A

ProQ Diamond

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HTN</th>
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<tr>
<td>MyBPC</td>
<td><img src="MyBPC_Control.png" alt="Image" /></td>
<td><img src="MyBPC-HTN.png" alt="Image" /></td>
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<tr>
<td>TnI</td>
<td><img src="TnI_Control.png" alt="Image" /></td>
<td><img src="TnI-HTN.png" alt="Image" /></td>
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</tbody>
</table>

Coomassie Blue

<table>
<thead>
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<th>Control</th>
<th>HTN</th>
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</thead>
<tbody>
<tr>
<td>MyBPC</td>
<td><img src="MyBPC_Control.png" alt="Image" /></td>
<td><img src="MyBPC-HTN.png" alt="Image" /></td>
</tr>
<tr>
<td>TnI</td>
<td><img src="TnI_Control.png" alt="Image" /></td>
<td><img src="TnI-HTN.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

Relative Phosphorylation

- Control
- HTN

MyBP-C

- Control: ![Image](Control_MyBP-C.png)
- HTN: ![Image](HTN_MyBP-C.png)

Troponin I

- Control: ![Image](Control_Troponin.png)
- HTN: ![Image](HTN_Troponin.png)
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

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