Altered length-dependent activation independent of mutant protein in human hypertrophic cardiomyopathy

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(submitted)
ABSTRACT

Background  Hypertrophic cardiomyopathy (HCM), characterized by asymmetric left ventricular hypertrophy, is a major cause of morbidity and mortality. We investigated if altered sarcomeric properties depend on the mutant sarcomeric protein and whether length-dependent activation and responsiveness to β-adrenergic receptor stimulation are altered in human HCM.

Methods and Results  Comparisons were made between cardiac samples from patients with a MYBPC3 mutation (MYBPC3mut; n=17), HCM patients without an identified sarcomere mutation (HCMmn; n=11) and non-failing Donors (n=12). Protein expression of myosin binding protein C (cMyBP-C) was significantly reduced in MYBPC3mut by 33±5% and unaltered in HCMmn compared to Donor. cMyBP-C phosphorylation in MYBPC3mut was similar to Donor, whereas it was significantly lower in HCMmn. Moreover, troponin I phosphorylation was lower in both patient groups compared to Donor. Force measurements in single cardiomyocytes demonstrated comparable sarcomeric dysfunction in both patient groups characterized by reduced maximal force in MYBPC3mut and HCMmn compared to Donor (26.4±2.9, 28.0±3.7 and 37.2±2.3 kN/m², respectively) and higher myofilament Ca²⁺-sensitivity (EC₅₀ = 2.5±0.2, 2.4±0.2 and 3.0±0.2 μmol/L, respectively). The length-dependent increase in Ca²⁺-sensitivity was significantly smaller in both patient groups compared to Donor (ΔEC₅₀: 0.46±0.04, 0.37±0.05 and 0.75±0.07 μmol/L, respectively). Protein kinase A treatment restored myofilament Ca²⁺-sensitivity and length-dependent activation in both patient groups to Donor values.

Conclusions  Depressed maximal force, increased Ca²⁺-sensitivity and perturbed length-dependent activation of sarcomeres likely represent a general feature of HCM and may contribute to impaired cardiac performance in manifest HCM. cMyBP-C haploinsufficiency is characteristic for HCM caused by MYBPC3 mutations and may underlie disease pathogenesis.
INTRODUCTION

Hypertrophic cardiomyopathy (HCM), most often caused by mutations in genes encoding sarcomeric proteins, is a major cause of morbidity and mortality affecting ~1:500 people worldwide at a relative young age.\(^{26,84}\) It is characterized by asymmetric left ventricular (LV) hypertrophy, predominantly involving the interventricular septum, occurring in the absence of other cardiac or systemic disease (such as hypertension or aortic stenosis). Clinical presentation is very heterogeneous in HCM as some patients reach old age with virtually no complaints, while others progress to end-stage heart failure or die at young age from sudden cardiac arrest. A recent longitudinal study (6 years follow-up) showed progressive heart failure (NYHA functional classes III and IV) in 17% of HCM patients. In these patients three diverse clinical profiles were identified based on the predominant pathophysiological alterations: (i) end-stage systolic dysfunction, (ii) LV outflow tract obstruction at rest and (iii) non-obstructive with preserved systolic function (diastolic dysfunction).\(^{17}\) These diverse clinical manifestations of HCM require a targeted treatment strategy. However, currently no specific therapy is available to prevent or reverse cardiac remodeling and dysfunction in HCM.

As a consequence of improved genotyping during past years, many disease causing mutations have been identified, mainly in genes encoding sarcomeric proteins.\(^{6}\) Despite improved genetic testing the cause of HCM remains unidentified in over 40% of the patients.\(^{76}\) Furthermore, the pathophysiological mechanism leading from a genetic defect to cardiac dysfunction is currently largely unknown. Recent studies have shown that even before hypertrophy is overt, carriers of a HCM causing mutation demonstrate signs of cardiac dysfunction.\(^{19;135;136}\) Echocardiographic strain analysis in preclinical HCM patients with mutations in genes encoding β-myosin heavy chain (\textit{MYH7}), cardiac myosin binding protein C (\textit{MYBPC3}), cardiac troponin T (\textit{TNNT2}), cardiac troponin I (\textit{TNNI3}) and α-tropomyosin (\textit{TPM1}) demonstrated a reduced early diastolic (Ea) velocity.\(^{135}\) Systolic strain and strain rates were not different from controls in preclinical HCM patients, while systolic function was diminished in HCM patients with manifest ventricular hypertrophy.\(^{135}\) Cardiovascular magnetic resonance evaluation of carriers with a Dutch founder mutation in \textit{MYBPC3} or a mutation in \textit{TPM1} confirmed diastolic dysfunction...
at the preclinical stage, evident from slower diastolic circumferential strain rate in septal and lateral regions of the LV.\textsuperscript{19} Tissue Doppler imaging in \textit{MYBPC3} mutation carriers without LV hypertrophy also revealed diastolic abnormalities as first feature of preclinical HCM.\textsuperscript{20} Furthermore, structural abnormalities consisting of crypts were discerned in a large percentage of these preclinical patients.\textsuperscript{137} Fibrosis, a hallmark of overt HCM, was not visible in preclinical HCM, but serum level of the profibrotic C-terminal propeptide of type I collagen was already elevated.\textsuperscript{21} These data indicate that sarcomeric mutations initiate modifications in myocardial structure and function early in disease progression, while hypertrophy only evolves later and might reflect an adaptive process.

Recently we have provided evidence for sarcomeric dysfunction in manifest HCM patients with truncating \textit{MYBPC3} founder mutations (c.2373dupG and c.2864_2865delCT) (Chapter 5).\textsuperscript{90} The sarcomeric dysfunction included a reduction in maximal force generating capacity and an increased myofilament Ca\textsuperscript{2+}-sensitivity compared to non-failing human myocardium, which may be the resultant of alterations in sarcomeric protein composition, as we observed haploinsufficiency (i.e. reduced cardiac myosin binding protein C (cMyBP-C) expression) in these patients. Haploinsufficiency was confirmed and extended to HCM caused by missense \textit{MYBPC3} mutations.\textsuperscript{138} Moreover, phosphorylation of the β-adrenergic target proteins cMyBP-C and cardiac troponin I (cTnI) diverged in our patient group, with preserved cMyBP-C and reduced cTnI phosphorylation compared to healthy Donor hearts.\textsuperscript{90} It remains to be investigated if the changes in sarcomeric function and protein composition observed in manifest HCM are specific for the \textit{MYBPC3} mutations or are part of the clinical HCM phenotype.

To gain more insight in the contribution of sarcomere mutations and dysfunction in the pathophysiologic process leading to manifest HCM we investigated whether changes in protein composition and functional properties of the sarcomeres that might underlie cardiomyocyte dysfunction and hypertrophy are unique for HCM patients with mutations in \textit{MYBPC3}, or are a general feature of the clinical HCM phenotype. Sarcomeric protein analysis was combined with force measurements in cells isolated from HCM hearts harboring a \textit{MYBPC3} mutation and in HCM patients in whom no mutation was identified upon screening of 9 genes. Apart from baseline force
measurements, responsiveness to a change in sarcomere length (i.e. Frank-Starling mechanism) and to protein kinase A (PKA) (i.e. β-adrenergic receptor stimulation) were assessed. These two mechanisms are activated during increased cardiac demand (as occurs during exercise) to adapt sarcomere function to increased cardiac stress.

Our study shows unique protein changes in HCM patients with \textit{MYBPC3} mutations, while altered function of the sarcomeres appears to be a general feature of the clinical HCM phenotype.

\textbf{METHODS}

\textbf{Myocardial samples}
Cardiac tissue was obtained from the LV septum of 17 HCM patients with a \textit{MYBPC3} mutation (\textit{MYBPC3}_{mut} group) and 11 mutation negative HCM patients after detailed sequencing of 9 HCM genes (\textit{MYBPC3}, \textit{MYH7}, \textit{TNNT2}, \textit{TNNT3}, \textit{MYL2}, \textit{MYL3}, \textit{ACTC}, \textit{TPM1} and \textit{CSRP3})\textsuperscript{139} (HCM_{mn} group), that underwent myectomy to relieve LV outflow obstruction (Morrow procedure). The \textit{MYBPC3}_{mut} group comprised patients with truncating mutations c.2373dupG (n=9) and c.2864_2865delCT (n=4) and splice site mutations c.927-2A>G (n=2) and c.1458-1G>C (n=1). Echocardiographic and clinical data of the patients are given in table 6.1.

Non-failing cardiac tissue from the free LV wall was obtained from Donor hearts (n=12; 13-65 years of age, mean 42±5 years; 8/4 male/female) when no suitable transplant recipient was found. The Donors had no history of cardiac disease, a normal cardiac examination, normal ECG and normal ventricular function on echocardiography within 24 h of heart explantation.

All samples were immediately frozen and stored in liquid nitrogen. The study protocol was approved by the local ethics committees and written informed consent was obtained.
Table 6.1. Patient characteristics.

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HCM

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M/F indicates male/female; LVOTPG, left ventricular transaortic pressure gradient in mmHg; ST, septal thickness in mm; LVEDD, LV end-diastolic diameter in mm; LVESD, LV end-systolic diameter in mm; LVEF, LV ejection fraction, calculated as (LVEDD-LVESD)/LVEDDx100%; CCB, calcium channel blocker; ATII, angiotensin II receptor antagonist. *P<0.05 HCM vs. MYBPC3 mut.
Protein analysis
Cardiac samples were treated with trichloroacetic acid prior to protein analysis to preserve the endogenous phosphorylation status of the sarcomeric proteins.75

SYPRO Ruby and ProQ Diamond staining of gradient gels
To determine cMyBP-C protein level, proteins were separated on 4-15% pre-cast Tris-HCl gels (BioRad) and stained with SYPRO Ruby. The level of cMyBP-C was expressed relative to α-actinin as described previously.90 The same gels were stained with ProQ Diamond to determine phosphorylation of PKA target proteins (cMyBP-C and cTnI). The phosphorylation status of cMyBP-C and cTnI was expressed relative to SYPRO-stained cMyBP-C and α-actinin, respectively.

Western blot analysis of cMyBP-C and cTnI
To detect truncated or degraded cMyBP-C, samples were separated by one-dimensional gel electrophoresis on a 8% polyacrylamide SDS-gel and subsequently transferred to nitrocellulose paper by wet blotting. Specific antibodies were used directed against the N-terminal part of cMyBP-C (residues 2-14) and phosphorylated sites of cMyBP-C (Ser273, Ser282 and Ser302 mouse MYBPC3 sequence; 275, 284 and 304 in the human sequence)70;140 and against phosphorylated cTnI sites (Ser22/23 and Thr143 in the human sequence) (Cell Signalling, Danvers, MA and Abcam, Cambridge, MA, respectively).

Phos-Tag acrylamide gels
In addition, the recently developed Phos-tag™ acrylamide (FMS Laboratory; Hiroshima University, Japan)141 was used to visualize phosphorylated cTnI species using alkoxide-bridged dinuclear metal (Mn²⁺) complex as phosphate-binding tag (Phos-tag) molecule. Mn²⁺-Phos-tag molecules preferentially capture phosphomonoester dianions bound to Ser, Thr and Tyr residues. Non-phosphorylated and phosphorylated cTnI species were separated in 1D-PAGE with polyacrylamide-bound Mn²⁺-Phos-tag and transferred to Western blots. Phosphorylated cTnI species in the gel are visualized as slower migration bands compared to the corresponding dephosphorylated cTnI form.142;143

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Isometric force measurements
Cardiomyocytes were mechanically isolated from small tissue samples as described previously. Triton-permeabilized cardiomyocytes were glued between a force transducer and a piezoelectric motor. Force measurements were performed at various \([\text{Ca}^{2+}]\) and sarcomere lengths of 1.8 and 2.2 μm. Passive tension (\(F_{\text{pas}}\)) was determined by shortening the cell in relaxation solution by 30%. Maximal calcium activated tension (\(F_{\text{max}}\), i.e. maximal force/cross-sectional area) was calculated by subtracting \(F_{\text{pas}}\) from the total force at saturating \([\text{Ca}^{2+}]\). \(\text{Ca}^{2+}\)-sensitivity is denoted as \(EC_{50}\), i.e. [\(\text{Ca}^{2+}\)] at which 50% of \(F_{\text{max}}\) is reached. Force measurements were repeated after incubation of cells for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U/mL, Sigma).

Data analysis
Data are presented as mean±S.E.M. Cardiomyocyte force values were averaged per sample and mean values for MYBPC3\textsubscript{mut}, HCM\textsubscript{mn} and Donor samples were compared using 1-way ANOVA. Effects of sarcomere length/PKA in single cardiomyocytes were tested with paired t-tests. P<0.05 was considered significant.

RESULTS

Clinical characteristics
Hypertrophic obstructive cardiomyopathy was evident from increased septal thickness (22±1 and 21±1 mm; normal value <13 mm\textsuperscript{115} and high LV transaortic pressure gradient (81±5 and 93±7 mmHg; normal value <30 mmHg\textsuperscript{116} in MYBPC3\textsubscript{mut} and HCM\textsubscript{mn} patients, respectively (Table 6.1). Both LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) were slightly higher in the HCM\textsubscript{mn} (47±2 and 30±2 mm) compared to the MYBPC3\textsubscript{mut} (42±1 and 23±1 mm) patients (both P<0.05 in unpaired Student t-test). LV ejection fraction was moderately depressed in both MYBPC3\textsubscript{mut} (44±2%) and HCM\textsubscript{mn} (38±3%)(Table 6.1).

Haploinsufficiency and altered phosphorylation pattern in MYBPC3\textsubscript{mut}
To investigate if diverse changes are present in protein composition in HCM samples with and without MYBPC3 mutation cardiac samples from all groups
were separated by 1D-gel electrophoresis. Figure 6.1A shows MYBPC3\textsubscript{mut}, HCM\textsubscript{mn} and Donor samples stained with SYPRO and ProQ Diamond to determine cMyBP-C protein level (relative to α-actinin) and sarcomeric protein phosphorylation, respectively. As reported previously (Chapter 5),\textsuperscript{90} a significant reduction of cMyBP-C protein level was found in MYBPC3\textsubscript{mut} hearts compared to Donor myocardium (Figure 6.1C), which was not observed in HCM\textsubscript{mn}. Previous studies indicated ischemia-induced degradation of cMyBP-C at the N-terminus.\textsuperscript{145,146} To exclude that cMyBP-C degradation underlies the reduced expression of full-length cMyBP-C in MYBPC3\textsubscript{mut}, Western blot analysis was performed with a specific antibody directed at the N-terminus of cMyBP-C. No degradation products were observed in any of the samples from the patient (Figure 6.1B) and Donor groups. Furthermore, absence of smaller cMyBP-C products indicates absence of truncated forms of cMyBP-C (encoded by the mutated allele) in the sarcomeres.

Phosphorylation of cMyBP-C did not differ from Donor in MYBPC3\textsubscript{mut}, while it was significantly lower in HCM\textsubscript{mn} (Figure 6.1D). Moreover, phosphorylation of the other PKA target protein, cTnI, was significantly lower in both patient groups compared to Donor (Figure 6.1E). To further unravel the differences in cMyBP-C phosphorylation between MYBPC3\textsubscript{mut} and HCM\textsubscript{mn}, we performed Western blot analysis with phospho-specific antibodies.\textsuperscript{140} cMyBP-C phosphorylation at Ser273 was significantly lower in HCM\textsubscript{mn} compared to Donor, while an intermediate value was observed in MYBPC3\textsubscript{mut} (Figure 6.2A). Phosphorylation of cMyBP-C at Ser282 was also significantly lower in HCM\textsubscript{mn}, but unaltered in MYBPC3\textsubscript{mut} compared to Donor (Figure 6.2B). No significant difference was present in phosphorylation of cMyBP-C at Ser302 among groups (Figure 6.2C).
Figure 6.1. Cardiac myosin binding protein C expression and phosphorylation. A. Cardiac samples separated by 1D-gel electrophoresis and stained with SYPRO for analysis of cardiac myosin binding protein C (cMyBP-C) expression relative to α-actinin, and with ProQ Diamond for analysis of cMyBP-C and cardiac troponin I (cTnI) protein phosphorylation. B. Western blot analysis of cMyBP-C did not reveal truncated or degradation products with a specific antibody. C. Protein level of cMyBP-C was significantly lower in MYBPC3\textsubscript{mut} compared to HCM\textsubscript{mn} and Donor samples, while phosphorylation of cMyBP-C (normalized to its own expression level) was significantly lower in HCM\textsubscript{mn} samples compared to MYBPC3\textsubscript{mut} and Donor tissue (D). E. Phosphorylation of cTnI (normalized to α-actinin) was significantly lower in MYBPC3\textsubscript{mut} and HCM\textsubscript{mn} compared to Donor samples. *P<0.05 versus Donor; #P<0.05 versus patient group.

Figure 6.2. Site-specific phosphorylation of cardiac myosin binding protein C. Western blot with specific antibodies revealed significantly lower phosphorylation at Ser273 (A) and Ser282 (B) in HCM\textsubscript{mn} compared to Donor, while no significant differences in phosphorylation at Ser273 (A), Ser282 (B) and Ser302 (C) was found between MYBPC3\textsubscript{mut} and Donor hearts. *P<0.05 versus Donor.
cTnI was separated on a Phos-tag polyacrylamide gel and subsequently stained on Western blots with a cTnI specific antibody (Figure 6.3A). This analysis yielded the distribution pattern of un- (0P), mono- (1P) and bis- (2P) phosphorylated cTnI. The distribution of the cTnI forms was significantly different among the three groups (Figure 6.3B: tested in 2-way ANOVA; interaction P<0.05). The relative levels of un- and monophosphorylated cTnI were higher in both HCM patient groups compared to Donor, while the proportion of bisphosphorylated cTnI was lower, confirming the low overall cTnI phosphorylation observed in these patients with ProQ analysis (Figure 6.1E). Phosphorylation of cTnI at PKA sites Ser22/23 was significantly lower in both MYBPC3mut and HCMmn compared to Donor, while signals at the protein kinase C (PKC) site Thr143 were extremely low in all groups (data not shown), indicating that the reduction in overall cTnI phosphorylation in patient samples compared to Donor (Figure 6.1E) is mainly due to lower phosphorylation at PKA sites.

Figure 6.3. Phosphorylation forms of cardiac troponin I. A. Cardiac samples were separated on a Phos-tag acrylamide gel to demonstrate the distribution of un- (0P), mono- (1P) and bis- (2P) phosphorylated cardiac troponin I (cTnI). B. Both HCM patient groups had relatively higher levels of un- and monophosphorylated cTnI and a lower level of bisphosphorylated cTnI compared to Donor. C. Western blot with specific antibodies against the phosphorylated PKA sites Ser22/23 on cTnI revealed significantly lower phosphorylation in both HCM patient groups than in Donor. *P<0.05 versus Donor.
Sarcomeric dysfunction in manifest HCM independent of MYBPC3 mutation

Figure 6.4A shows a single cardiomyocyte from a MYBPC3\textsubscript{mut} heart at sarcomere lengths of 1.8 and 2.2 μm. Force development was measured at various (maximal and sub-maximal) calcium concentrations to determine maximal (\(F_{\text{max}}\)) and passive (\(F_{\text{pas}}\)) force and myofilament Ca\(^{2+}\)-sensitivity at sarcomere lengths of 1.8 and 2.2 μm (Figure 6.4B).

At a sarcomere length of 2.2 μm significant reductions in the average \(F_{\text{max}}\) were found in MYBPC3\textsubscript{mut} (26.4±2.9 kN/m\(^2\)) and HCM\textsubscript{mn} samples (28.0±3.7 kN/m\(^2\)) compared to Donor (37.2±2.3 kN/m\(^2\)). Figure 6.5A illustrates that the variety in \(F_{\text{max}}\) was relatively large among individual hearts. Likewise \(F_{\text{pas}}\) varied between hearts (Figure 6.5B) and tended to be somewhat higher in MYBPC3\textsubscript{mut} (3.5±0.6 kN/m\(^2\)) compared to HCM\textsubscript{mn} (2.4±0.3 kN/m\(^2\)) and Donor (2.4±0.3 kN/m\(^2\)), but the difference was not significant.

Figure 6.5C shows force-calcium relations for all groups at a sarcomere length of 2.2 μm: force at submaximal [Ca\(^{2+}\)] was normalized to maximal force at saturating [Ca\(^{2+}\)]. The force-calcium relation in both patient groups was shifted to the left compared to Donor, indicative for an increase in myofilament Ca\(^{2+}\)-sensitivity. The average value for Ca\(^{2+}\)-sensitivity (indicated as EC\textsubscript{50}, which is the [Ca\(^{2+}\)] required to reach 50% of \(F_{\text{max}}\)) was significantly lower in MYBPC3\textsubscript{mut} (2.5±0.2 μmol/L) and HCM\textsubscript{mn} (2.4±0.2 μmol/L) compared to Donor (3.0±0.2 μmol/L) (Figure 6.5D). In addition, the steepness (nH) of the force-calcium relation was significantly reduced in both patient groups (3.1±0.0.2 and 3.0±0.0.2 in MYBPC3\textsubscript{mut} and HCM\textsubscript{mn}, respectively) compared to Donor (3.7±0.1) (Figure 6.5E).
Myofilament length-dependent activation

To assess the length-dependent activation of myofilament contraction, force was measured at a sarcomere length of 1.8 and 2.2 μm in 43 cells from 13 MYBPC3\textsubscript{mut} hearts, 36 cells from 8 HCM\textsubscript{mn} hearts and 32 cardiomyocytes from 9 Donor hearts. Measurements were performed in subsets of patients with clinical characteristics similar to the group averages. In all groups an increase in sarcomere length from 1.8 to 2.2 μm increased maximal force, passive force and Ca\textsuperscript{2+}-sensitivity of the sarcomeres (Figure 6.4B). Noteworthy, the increase in Ca\textsuperscript{2+}-sensitivity upon an increase in sarcomere length from 1.8 to 2.2 μm (i.e. ΔEC\textsubscript{50}) was significantly smaller in both patient groups (0.46±0.04 and 0.37±0.05 μmol/L in MYBPC3\textsubscript{mut} and HCM\textsubscript{mn}, respectively) compared to Donor (0.76±0.06 μmol/L) (Figure 6.6A).

The resultant of reduced F\textsubscript{max} and increased myofilament Ca\textsuperscript{2+}-sensitivity in patient groups compared to Donor is illustrated in figure 6.6B. This figure shows that the altered sarcomeric properties in patient groups (i.e. lower F\textsubscript{max}, increased Ca\textsuperscript{2+}-sensitivity and reduced nH) resulted in depressed force development at [Ca\textsuperscript{2+}] above 3.3 and 2.5 μmol/L at a sarcomere length of 1.8 and 2.2 μm, respectively. In contrast, below this [Ca\textsuperscript{2+}] value force development by the sarcomeres was somewhat higher in MYBPC3\textsubscript{mut} and HCM\textsubscript{mn} compared to Donor cardiomyocytes.
Figure 6.5. Sarcomere force development at a sarcomere length of 2.2 μm.

Force measurements were performed at a sarcomere length of 2.2 μm in single cardiomyocytes from 16 MYBPC3
[mut] (67 cardiomyocytes), 8 HCM
[mn] (38 cardiomyocytes) and 10 Donor samples (45 cardiomyocytes). The maximal force generating capacity (F[\text{max}]) was significantly lower in MYBPC3
[mut] and HCM
[mn] compared to Donor, while passive force (F[\text{pas}]) did not significantly differ among groups. Force was measured at maximal and submaximal [Ca²⁺] to determine Ca²⁺-sensitivity of force. C. Force at submaximal [Ca²⁺] was normalized to the maximal force at saturating [Ca²⁺] which was set to 1 and plotted against [Ca²⁺]. Relative force-calcium relations of patient groups were shifted to the left compared to Donor indicative for an increased Ca²⁺-sensitivity of force. D. The [Ca²⁺] at which 50% of maximal force is reached (i.e. EC[50]) was significantly lower in patient groups compared to Donor. In addition, steepness of the force-calcium relations (nH) was significantly lower in patient groups compared to Donor (C,E). *P<0.05 versus Donor.
Figure 6.6. Myofilament force development at two sarcomere lengths. Force was measured at two sarcomere lengths in cells isolated from 13 MYBPC3 \textsubscript{mut}, 8 HCM \textsubscript{mn} and 9 Donor hearts. A. The length-dependent increase in Ca\textsuperscript{2+}-sensitivity of force was less in MYBPC3 \textsubscript{mut} and HCM \textsubscript{mn} patient groups compared to Donor. B. Absolute force (i.e. tension) plotted against [Ca\textsuperscript{2+}] shows that force development at low [Ca\textsuperscript{2+}] (below 3.3 and 2.5 μmol/L for a sarcomere length of 1.8 and 2.2 μm, respectively) is higher in patient groups compared to Donor, while force development above this [Ca\textsuperscript{2+}] is depressed in patient groups compared to Donor.

Effects of protein kinase A

Force measurements were repeated at a sarcomere length of 2.2 μm in 12 MYBPC3 \textsubscript{mut} (n=32 cells), 8 HCM \textsubscript{mn} hearts (n=19 cells) and 8 Donor samples (n=24 cells) after treatment with exogenous PKA to mimic β-adrenergic receptor stimulation. PKA slightly reduced maximal and passive force in all groups (data not shown), while nH was not altered. Treatment of cells with PKA significantly reduced myofilament Ca\textsuperscript{2+}-sensitivity in all groups. The PKA-induced reduction in Ca\textsuperscript{2+}-sensitivity of force was significantly larger in MYBPC3 \textsubscript{mut} (0.91±0.11 μmol/L) compared to Donor (0.47±0.08 μmol/L) and intermediate in HCM \textsubscript{mn} (0.73±0.12 μmol/L) (Figure 6.7A). PKA abolished the initial difference in Ca\textsuperscript{2+}-sensitivity of force between patient groups and Donor.
PKA pre-treatment of cells significantly enhanced the sarcomere length-dependent change in calcium sensitivity (ΔEC$_{50}$) in MYBPC3$_{mut}$ (4 hearts; 14 cells) and HCM$_{mn}$ (4 hearts; 15 cells), but not in Donor (4 hearts; 12 cells) (Figure 6.7C). The blunted shift in EC$_{50}$ upon an increase in sarcomere length from 1.8 to 2.2 μm in patient groups compared to Donor cells at baseline (Figure 6A) was absent upon PKA pre-treatment (Figure 6.7C). Figure 6.7D illustrates that after PKA force development was depressed in patient cells compared to Donor both at maximal and submaximal [Ca$^{2+}$].

**Figure 6.7. Effects of length and protein kinase A on myofilament function.** A. Treatment of cells with exogenous protein kinase A (PKA) significantly reduced Ca$^{2+}$-sensitivity of force in all groups (12 MYBPC3$_{mut}$, 8 HCM$_{mn}$ and 8 Donor hearts). The change in Ca$^{2+}$-sensitivity of force (i.e. ΔEC$_{50}$) was largest in MYBPC3$_{mut}$ and smallest in Donor hearts. B. The difference in Ca$^{2+}$-sensitivity of force (EC$_{50}$) among groups before PKA (shown in figure 6.5D) was abolished upon PKA treatment. C. Pretreatment of cells with PKA significantly enhanced the length-dependent change in EC$_{50}$ in MYBPC3$_{mut}$ and HCM$_{mn}$ cells and thereby abolished the difference in length-dependent activation among groups. D. After PKA myofilament force development at a sarcomere length of 2.2 μm was lower at maximal and submaximal [Ca$^{2+}$] in MYBPC3$_{mut}$ and HCM$_{mn}$ compared to Donor. n denotes number of cells. *P<0.05 versus Donor. # P<0.05 effect of PKA pre-treatment.
DISCUSSION

This study is the first to compare both protein composition and function of the sarcomeres in well-defined HCM patient groups with \textit{MYBPC3} mutations and without an identified sarcomere mutation. Although unique protein changes were observed in patients with \textit{MYBPC3} mutations (i.e. reduced cMyBP-C protein expression and increased cMyBP-C phosphorylation compared to HCM myocardium without \textit{MYBPC3} mutations), functional properties of the sarcomeres were similarly altered in both patient groups compared to non-failing Donor hearts. Overall, the changes in sarcomere function seem to reflect the clinical HCM phenotype rather than the specific (\textit{MYBPC3}) mutation and likely contribute to impaired systolic and diastolic function in manifest HCM.

\textbf{Cardiac MyBP-C haploinsufficiency and deranged phosphorylation of $\beta$-adrenergic target proteins are unique for HCM with \textit{MYBPC3} mutations}

Our study revealed protein changes unique for MYBPC3\textsubscript{mut} hearts; cMyBP-C haploinsufficiency and relatively high cMyBP-C phosphorylation compared to HCM samples without \textit{MYBPC3} mutation. In contrast, reduced phosphorylation of cTnI compared to Donor hearts was evident in both HCM groups and may rather reflect a phenotype-related protein modification.

Reduced phosphorylation of cTnI, and in the case of the HCM\textsubscript{mn} group also reduced cMyBP-C phosphorylation, compared to non-failing Donor hearts has been observed in previous studies in end-stage failing hearts from patients with idiopathic or ischemic dilated cardiomyopathy,\textsuperscript{48,70,72,92,144} and thus appears to be a general feature of progressive heart failure consistent with down-regulation and desensitization of the $\beta$-adrenergic receptor pathway.\textsuperscript{147} Western blot analysis confirmed reduced phosphorylation at the PKA sites of cTnI downstream of the $\beta$-adrenergic receptor in both MYBPC3\textsubscript{mut} and HCM\textsubscript{mn} samples (Figure 6.3C).

Cardiac MyBP-C has three putative phosphorylation sites (Ser273, Ser282 and Ser302) that are substrates for PKA, PKC and calcium-dependent calmodulin kinase (CaMK2)\textsuperscript{28,126} Analysis of site-specific cMyBP-C phosphorylation using phospho-specific antibodies against these sites (Figure 6.2) confirmed the “high” phosphorylation of cMyBP-C in MYBPC3\textsubscript{mut} samples, as phosphorylation at all
sites did not significantly differ from the Donor group. A significantly lower phosphorylation at Ser282 and Ser273 was observed in the HCM_{mn} samples compared to Donor hearts. The latter observations are in agreement with a recent study by Copeland et al.\textsuperscript{140} who reported a reduction in total cMyBP-C phosphorylation in HCM myectomy samples to 39% of the value found in non-failing myocardium, which was largely attributed to non-phosphorylated Ser273 and Ser282. In the latter study a phosphorylation signal was observed in the failing human samples only with the antibody against Ser302. Recent studies indicated that Ser302 is phosphorylated by CaMK2, which is activated upon an increase in heart rate, rather than by PKA.\textsuperscript{148,149} Our analysis did not reveal a significant difference in Ser302 phosphorylation between MYBPC3_{mut}, HCM_{mn} and Donor samples (Figure 6.2C). Hence, the reduction in total cMyBP-C phosphorylation observed in HCM_{mn} compared to Donor may be the resultant of reduced PKA-mediated phosphorylation compared to non-failing Donor myocardium. The divergent phosphorylation pattern of the PKA targets, cTnI and cMyBP-C in the MYBPC3_{mut} samples may be well-explained by an altered stoichiometry between protein level and PKA expression and/or its activity. This is illustrated in figure 6.8, which shows that the level of phosphorylated cMyBP-C (uncorrected by cMyBP-C expression), which reflects kinase activity, is independent of cMyBP-C expression in the patient groups and significantly lower in both MYBPC3_{mut} and HCM_{mn} compared to Donor.

\textbf{Figure 6.8.} Phosphorylated myosin binding protein C (cMyBP-C), as an indirect measure of kinase activity, plotted against cMyBP-C expression to illustrate that the degree of phosphorylation is independent of cMyBP-C protein expression in the HCM patient groups, and significantly lower compared to non-failing Donor myocardium.
As cMyBP-C exerts a role in myofilament assembly and integrity, the reduced expression of cMyBP-C may underlie disease pathogenesis by disruption of myocardial structure. Cardiac remodeling, including cardiac myocyte disarray and fibrosis has been observed in homozygous cMyBP-C knockout mice, in which cMyBP-C was completely absent. At 11 months of age heterozygous cMyBP-C null mice that only expressed ~75% of normal cMyBP-C content showed asymmetric septal hypertrophy and signs of fibrosis, while no morphological changes were observed at 3-4 months of age. Future longitudinal studies should be performed to answer the question if cMyBP-C haploinsufficiency is cause or consequence of hypertrophic cardiomyopathy in patients with MYBPC3 mutations.

**Depressed force generating capacity and increased Ca\(^{2+}\)-sensitivity: common sarcomere features of HCM**

A reduction in maximal force development may contribute to a reduction in systolic performance of the heart, while an increase in passive force has been associated with diastolic dysfunction. Our data indicate that the reduction in maximal force generating capacity of sarcomeres is a general feature of HCM patients, while passive force is higher only in a few HCM samples with a MYBPC3 mutation (Figure 6.5D). A recent study by Hoskins et al. demonstrated a 40% lower maximal isometric force in 6 HCM hearts with mutations in MYBPC3, MYH7 or no identified mutation, while passive stiffness of the cardiomyocytes was not different from Donor. Thus, the reduction in maximal force generating capacity appears to be characteristics for the clinical HCM phenotype, while perturbed passive stiffness is not a common hallmark of HCM.

Increased Ca\(^{2+}\)-sensitivity of myofilaments has been reported previously in end-stage dilated and ischemic human cardiomyopathy and has been associated with reduced PKA-mediated phosphorylation of cTnI and cMyBP-C. Both cMyBP-C and cTnI have a central role in regulating cardiac performance in response to \(\beta\)-adrenergic receptor stimulation as occurs during exercise. Apart from changes in calcium handling (via phosphorylation of L-type calcium channels, ryanodine receptors and phospholamban) which increase systolic force development, positive lusitropic effects (i.e. increased relaxation) are in part mediated via a reduction in myofilament Ca\(^{2+}\)-sensitivity and increased cross-bridge cycling kinetics. Recent studies have shown that
PKA-induced myofilament desensitization is mediated via phosphorylation of both cTnI and cMyBP-C.\textsuperscript{132,151,152} Thus, reduced phosphorylation of the PKA target proteins may reflect desensitization of the β-adrenergic signalling pathway and may underlie the higher myofilament Ca\textsuperscript{2+}-sensitivity in our HCM patient groups compared to Donor. Indeed, significant correlations are present between EC\textsubscript{50} and phosphorylated cMyBP-C and cTnI (P<0.05; R\textsuperscript{2}=0.34; P<0.05; R\textsuperscript{2}=0.18, respectively; Figure 6.9). Moreover, PKA treatment abolished the initial differences in EC\textsubscript{50} among groups (Figure 6.7B), indicating that enhanced Ca\textsuperscript{2+}-sensitivity might be due to reduced phosphorylation of cMyBP-C and cTnI. Noteworthy, the only (weak) (P=0.07; R\textsuperscript{2}=0.15; Figure 6.10) relation revealed by correlating protein composition and clinical characteristics was observed between LV ejection fraction and myofilament Ca\textsuperscript{2+}-sensitivity, i.e. a low LVEF coincided with a high myofilament Ca\textsuperscript{2+}-sensitivity, which suggests that the enhanced myofilament Ca\textsuperscript{2+}-sensitivity is part of the clinical HCM phenotype and could occur secondary to cardiac remodelling.

**Figure 6.9.** A significant correlation was present between phosphorylated myosin binding protein C (cMyBP-C) and myofilament Ca\textsuperscript{2+}-sensitivity (EC\textsubscript{50}) and between phosphorylation cardiac troponin I (cTnI) and EC\textsubscript{50}.
The combination of reduced maximal force, increased Ca\(^{2+}\)-sensitivity and reduced cooperativity of force development as shown in figure 6.6B indicates that the sarcomere changes contribute to depressed systolic performance at high intracellular [Ca\(^{2+}\)] and may somewhat limit myocardial relaxation at low [Ca\(^{2+}\)] in human HCM. Noteworthy, during increased cardiac stress, i.e. after treatment with exogenous PKA and at a sarcomere length of 2.2 μm, HCM sarcomeres are hypocontractile at all calcium concentrations compared to non-failing Donor hearts, suggesting that perturbed sarcomeric properties of manifest HCM compromise systolic performance of the heart, in particular during increased cardiac load.

As the functional parameters measured in the present study were independent of the mutation, the functional consequences of the mutated protein seem to be minor. However, we cannot exclude a modulating effect of altered cMyBP-C composition in the MYBPC3\(_{\text{mut}}\) patient group that may depend on the level of cMyBP-C expression and/or phosphorylation status.\(^{142,153}\)

**Blunted length-dependent activation in HCM**

In addition to the regulatory effect of the sarcomeres during increased sympathetic activity, the Frank-Starling mechanism, which provides a beat-to-beat regulating mechanism of systolic performance upon enhanced ventricular filling (i.e. increased end-diastolic volume), is caused by an increase in both maximal force generating capacity and Ca\(^{2+}\)-sensitivity of the sarcomeres (reviewed in

![Figure 6.10. Myofilament Ca\(^{2+}\)-sensitivity (EC\(_{50}\)) as a function of left ventricular (LV) ejection fraction in the HCM patient groups.](image)
references\textsuperscript{154,155}). Again, one of the possible mediating proteins involved in the increased force development upon an increase in sarcomere length is cMyBP-C, as the length-dependent activation of force was significantly blunted in muscle strips from cMyBP-C deficient mice.\textsuperscript{152} Interestingly, assessment of regional myocardial function with cardiovascular magnetic resonance imaging indicated blunting of the Frank-Starling mechanism in \textit{MYBPC3} mutation carriers compared to healthy individuals,\textsuperscript{19} which may involve mutant cMyBP-C. However, as similar changes in length-dependent activation of the sarcomeres were observed in patients with and without \textit{MYBPC3} mutations, the perturbed length-dependent increase in myofilament Ca\textsuperscript{2+}-sensitivity is most likely related to the secondary HCM disease phenotype. Konhilas \textit{et al.}\textsuperscript{156} showed reduced length-dependent activation in myocytes from mouse myocardium harbouring skeletal TnI which lacks the PKA phosphorylation sites. Moreover, the length-dependent activation was enhanced upon treatment with PKA in cardiomyocytes from non-transgenic mice.\textsuperscript{156} Likewise, in the present study incubation with PKA restored the length-dependent increase in Ca\textsuperscript{2+}-sensitivity in both HCM groups to the response observed in Donor (Figure 6.7C). As PKA incubation lowered baseline Ca\textsuperscript{2+}-sensitivity, likely via phosphorylation of cTnI, this might restore the margin of Ca\textsuperscript{2+}-sensitivity response and allow for an increase upon increased sarcomere length.

\textbf{Clinical implications}

Medication aimed at restoring the \(\beta\)-adrenergic signalling pathway may help to correct altered sarcomeric properties in HCM. In a recent study we have shown that \(\beta\)-blocker therapy (bisoprolol) in pigs with a myocardial infarction corrected sarcomeric dysfunction (i.e. reduced maximal force and increased Ca\textsuperscript{2+}-sensitivity) in infarct animals to levels found in sham animals.\textsuperscript{157} Moreover, maximal force development of single cardiomyocytes was significantly higher in heart failure patients who received chronic \(\beta\)-blocker treatment compared to patients who did not receive \(\beta\)-blockers.\textsuperscript{158} It should be noted that most of the patients included in the present study already received \(\beta\)-blockers or a Ca\textsuperscript{2+}-antagonist aimed to improve relaxation and hemodynamics. Thus part of the variability in myofilament functional properties may be due to effects of drug therapy.

In conclusion, investigation of protein composition and function of the sarcomeres in a large well-characterized HCM patient group with and without
MYBPC3 mutations revealed cMyBP-C haploinsufficiency to be unique for HCM patients with MYBPC3 mutations, while reduced protein phosphorylation appears common for the clinical HCM phenotype. Reduced PKA-mediated protein phosphorylation may largely underlie altered sarcomere function in human HCM, yielding an increased Ca\(^{2+}\)-sensitivity and a bunted length-dependent sarcomere responsiveness, which may explain defects in activation and relaxation of the heart muscle in hypertrophic cardiomyopathy.

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