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Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction

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Aims Heart failure (HF) with preserved ejection fraction (HFpEF) is a major cause of morbidity and mortality. Key alterations in HFpEF include increased left ventricular (LV) stiffness and abnormal relaxation. We hypothesized that myofilament protein phosphorylation and function are deranged in experimental HFpEF vs. normal myocardium. Such alterations may involve the giant elastic protein titin, which contributes decisively to LV stiffness.

Methods and results LV tissue samples were procured from normal dogs (CTRL) and old dogs with hypertension-induced LV hypertrophy and diastolic dysfunction (OHT/HFpEF). We quantified the expression and phosphorylation of myofilament proteins, including all-titin and site-specific titin phosphorylation, and assessed the expression/activity of major protein kinases (PKs) and phosphatases (PPs), myofilament calcium sensitivity (pCa_{50}), and passive tension ($F_{passive}$) of isolated permeabilized cardiomyocytes. In OHT vs. CTRL hearts, protein kinase-G (PKG) activity was decreased, whereas PKCα activity and PP1/PP2a expression were increased. Cardiac MyBPC, TnT, TnI and MLC2 were less phosphorylated and pCa_{50} was increased in OHT vs. CTRL. The titin N2BA (compliant) to N2B (stiff) isoform-expression ratio was lowered in OHT. Hypophosphorylation in OHT was detected for all-titin and at serines S4010/S4099 within titin-N2Bus, whereas S11878 within proline, glutamate, valine, and lysine (PEVK)-titin was hyperphosphorylated. Cardiomyocyte $F_{passive}$ was elevated in OHT, but could be normalized by PKG or PKA, but not PKCα, treatment.

Conclusions This patient-mimicking HFpEF model is characterized by titin stiffening through altered isoform composition and phosphorylation, both contributing to increased LV stiffness. Hypophosphorylation of myofilament proteins and increased calcium sensitivity suggest that functional impairment at the sarcomere level may be an early event in HFpEF.

Keywords Diastolic heart failure • Hypertrophy • Titin • Passive stiffness

1. Introduction

Heart failure (HF) is a major cause of mortality and morbidity and a frequent reason for hospital admission in the USA and Europe.[1] More than 50% of HF patients have a left ventricular (LV) ejection fraction (EF) >50% and are referred to as patients with HF with a preserved EF (HFpEF). Typically, HFpEF patients show impaired LV filling resulting from abnormal relaxation and increased LV diastolic stiffness.[2,3] The factors contributing to the increased LV passive stiffness include cardiac hypertrophy, fibrosis,[4] abnormal Ca^{2+}-handling,[3] or deranged expression/phosphorylation of the elastic sarcomere protein titin.[5–7]

Cardiac titin is expressed as stiff N2B isoform (3000 kDa) and more compliant N2BA isoform (3200–3700 kDa),[8] and the N2BA:N2B expression ratio partly defines myofibrillar passive stiffness.[9–11] Pathologically increased N2BA:N2B ratios have been found in end-stage human HF with reduced EF (HFrEF).[9,11] In human HFpEF, the proportion of compliant N2BA titin is also increased, but cardiomyocytes have been found to be stiffer than normal.[7] The increased cardiomyocyte passive tension ($F_{passive}$) may be due to deranged titin

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phosphorylation. A cardiac-specific segment in titin, the N2Bus, is phosphorylated by protein kinase (PK)A[12,13] and cGMP-activated protein kinase-G (PKG),[14] an effect augmented by PDE5A-inhibitor sildenafil, *in vivo*.[15] This titin modification reduces cardiomyocyte $F_{passive}$,[6,7,12–15] whereas a deficit in phosphorylation at the N2Bus-titin site would increase $F_{passive}$.[7,14] However, if the elastic titin segment is phosphorylated at a different site, the proline, glutamate, valine, and lysine-(PEVK) domain, by PKCα, titin-based stiffness increases.[16] PKCα is elevated in HF[17] and hyperphosphorylation of the PEVK domain could increase $F_{passive}$ in failing hearts.[18] Thus, while titin phosphorylation changes most probably alter $F_{passive}$ in HF, we are only beginning to understand which sites on titin become more or less phosphorylated in failing hearts and how these modifications alter diastolic stiffness, particularly in HFpEF.

Cardiac remodelling in HF also involves phosphorylation changes in other myofilament proteins, notably the regulatory proteins.[19–21] Little is known about alterations in regulatory protein phosphorylation in HFpEF, whereas HFrEF has been well studied for such alterations.[19–21] A prominent example is cardiac troponin I (cTnI), which is largely responsible for myofilament calcium sensitivity. TnI has been found to be hyperphosphorylated in HFrEF in some studies, but hypophosphorylated in others, and similar differences have been reported for other regulatory proteins.[22–27] A firm cause for these discrepancies is not known.

We speculated that derangements in phosphorylation and function of cardiac myofilament proteins may occur in experimental dogs with advanced age and hypertension-induced LV hypertrophy,[15,28] in comparison to normal dog hearts. Whereas the dog model has been shown to mimic some forms of human HFpEF,[15,28] these dogs have not been studied yet for biochemical and functional properties at the level of the sarcomere proteins. We find hypophosphorylation of regulatory proteins and increased Ca^{2+} sensitivity of the contractile apparatus in the experimental HFpEF model. We also detect elevated $F_{passive}$ in HFpEF dog hearts owing to both titin-isoform switching and altered titin phosphorylation, including site-specific phosphorylation revealed by novel phospho-specific antibodies. While these alterations are already apparent in the HFpEF model, *in vivo* cardiac mechanical function is still maintained. Our findings provide a novel mechanistic insight into the remodelling processes during HFpEF development and suggest new possibilities for therapeutic interventions in this syndrome.

2. Methods

A detailed methods and additional data section is provided in the Supplementary material online.

2.1 Animal model, *in vivo* mechanical analysis, and tissue sampling

The study employed mongrel dogs ($n = 39$; Supplementary material online, *Table S1*) that were divided into controls [CTRL; aged one year ('young') or 8–12 years ('old')] and old dogs made hypertensive by bilateral renal wrapping (OHT; aged 8–13 years).[28] All dogs underwent echocardiography in the conscious unsedated state. Short-term haemodynamic studies were performed in CTRL and OHT dogs 8 weeks after renal wrapping or sham surgery.[28] Animals were anaesthetized using fentanyl (0.25 mg kg^{-1} intravenous bolus followed by 0.18 mg kg^{-1} h^{-1}) and midazolam (0.75 mg kg^{-1} intravenous bolus followed by 0.59 mg kg^{-1} h^{-1}). Adequacy of anaesthesia was monitored from the disappearance of the corneal reflex and jaw tone. Dogs were intubated, ventilated, and given maintenance saline infusion (3 mL kg^{-1} min^{-1}), and they received autonomic blockade with atropine (1 mg) and propranolol (2 mg kg^{-1}). Thoracotomy and pericardiotomy were performed. Under fluoroscopic guidance, animals were instrumented with a pulmonary artery catheter, an LV integrated pressure-conductance catheter (Millar), a left atrial and central aortic high-fidelity pressure transducer (Millar), a pneumatic occluding device around the thoracic inferior vena cava, and an atrial lead for pacing at 10–20 bpm above the sinus rate. LV tissue samples were procured from CTRL and OHT (8 weeks after renal wrapping), either by taking full-thickness LV biopsies from the beating heart ($n = 7$ per group) or by excising tissue post-mortem ($n = 7–9$ per group).

In the beating-heart biopsy group, serial samples were harvested from different regions of the anterior or anterior lateral wall from seven CTRL and seven OHT dogs subjected to an identical experimental protocol without collection of haemodynamic data, because the biopsy and haemostatic sutures would alter chamber diastolic properties. Biopsy samples were frozen in liquid nitrogen within seconds and stored at −80°C until use. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and was approved by the Mayo Institutional Animal Care and Use Committee. Dogs were euthanized by intravenous KCl under deep anaesthesia, consistent with the Panel on Euthanasia guidelines for the American Veterinary Medical Association. Hearts were removed post-mortem, weighed, and the LV sectioned for samples that were flash frozen in liquid nitrogen. This procedure occurred as quickly as possible but could take up to 60 min. Samples were stored at −80°C until use. All data shown for the CTRL post-mortem group were obtained from old (aged) animals; however, young CTRL hearts procured post-mortem revealed a similar myofilament protein expression and phosphorylation and cardiomyocyte mechanical properties compared with old CTRL post-mortem hearts (data not shown).

2.2 Protein analysis

Titin isoform separation. Homogenized myocardial samples were analysed by 2% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).[9] Protein bands were visualized using Coomassie blue or SYPRO Ruby, scanned, and analysed densitometrically.

Total protein phosphorylation assays. Tissue samples (20 μg dry weight/lane) were separated on 2% SDS–PAGE gels for titin, or on gradient gels for other myofilament proteins. Gels were stained with Pro-Q Diamond for 1 h and subsequently with SYPRO Ruby overnight. Phosphorylation signals for myofilament proteins on Pro-Q Diamond-stained gels were normalized to SYPRO Ruby-stained total protein signals.

Immunoblotting. Expression of cMyBPC, phospho-cMyBPC (S282), cTnI, phospho-cTnI (S23/S24), cMLC2, phospho-cMLC2 (S19), PKCα, phospho-PKCα, PP1, and PP2a was measured by 15% SDS–PAGE and western blot, expression of titin phospho-N2Bus (S4010; S4099) and phospho-PEVK (S11878) by 2% SDS–PAGE and western blot. The position of titin phosphosites is according to full-length human titin, UniProtKB identifier, Q8WZ42. Affinity-purified phosphospecific and sequence-specific anti-titin antibodies were custom-made by Eurogentec (Belgium).

2.3 Myocardial protein kinase G and A activity tests

PKG activity (pmol/min/mg protein) was measured using radiolabeled ATP. PKA activity (ng μL^{-1}) was measured using a nonradioactive PKA kinase activity-assay kit.

2.4 Force measurements on isolated skinned cardiomyocytes

Cardiomyocytes were demembranated and isolated cells were attached between a force transducer and a motor.[29] $F_{passive}$ was recorded between 1.8 and 2.4 μm sarcomere length (SL). Ca^{2+} sensitivity of the contractile apparatus (pCa_{50}) was determined at 2.2 μm SL.

2.5. Statistics

The values are given as mean ± SEM in each group. Data were tested for statistically significant differences using the Bonferroni-adjusted *t*-test, apart from *Figure 6* and Supplementary material online, *Figure S10*, where the paired Student's *t*-test was used. A *P*-value of <0.05 was considered significant.

3. Results

Clinical, haemodynamic (anaesthesia), and conscious echocardiography data were available for all groups of dogs (Supplementary material online, *Table S1*). OHT dogs had chronic hypertension as described previously.[28,30] Both systolic and diastolic blood pressure were significantly increased in OHT vs. CTRL, as were the LV end-systolic pressure, the relaxation constant Tau, and the LV weight/body weight ratio. Hypertrophy was present in OHT, as indicated by an increased mean cardiomyocyte diameter in this group compared with CTRL (Supplementary material online, *Figure S1*). LV EF was unaltered in OHT vs. CTRL dogs. Thus, the OHT dogs showed typical signs of early HFpEF.

3.1. Hypophosphorylation of myofilament regulatory proteins in OHT

Myofilament regulatory proteins cMyBPC, cTnI, cTnT, and cMLC2 showed reduced phosphorylation in biopsies of OHT vs. CTRL hearts, as detected by Pro-Q Diamond/SYPRO Ruby staining (*Figure 1*). In post-mortem OHT hearts, cMyBPC, cTnI, and cTnT were also hypophosphorylated compared with CTRL, whereas cMLC2 phosphorylation was unaltered (Supplementary material online, *Figure S2*). Using western blots for the detection of myofilament protein expression and phosphorylation, we found cTnI phosphorylation at S23/S24 to be significantly reduced by >50% in OHT vs. CTRL, in beating-heart biopsies (*Figure 2B*) and post-mortem tissues (Supplementary material online, *Figure S3*). Furthermore, cMyBPC phosphorylation at S282 was reduced by ~80% in biopsied OHT vs. CTRL samples and cMLC2 phosphorylation at S19 was reduced by ~70% (*Figure 2A* and *C*). Total cMyBPC, cTnI, and cMLC2 expression remained unaltered in OHT (*Figure 2*).

3.2. Alterations in expression/activity of major protein kinases and phosphatases in OHT

The activity of PKG was reduced in OHT vs. CTRL (Supplementary material online, *Figure S4A* and *B*). PKCα expression was similar in CTRL and OHT (Supplementary material online, *Figure S4C* and *D*), but phosphorylation of PKCα (a measure of kinase activity) was higher in OHT vs. CTRL (Supplementary material online, *Figure S4E* and *F*). PKA activity was not different between sample groups (Supplementary material online, *Figure S5*). Expression of PP1 (Supplementary material online, *Figure S6A* and *B*) and PP2a (Supplementary material online, *Figure S6C* and *D*) was increased in OHT vs. CTRL.

3.3. Altered calcium sensitivity of cardiomyocytes in OHT and effect of PKA treatment

The force–pCa relationship of skinned single cardiomyocytes from beating-heart biopsies (*n*, 10–16 cells per group; from 2 to 3 hearts per group) revealed significantly higher myofilament calcium sensitivity (pCa_{50}) in OHT (5.77 ± 0.01) vs. CTRL (5.64 ± 0.02) (*Figure 3A*). Maximum Ca^{2+}-activated tension was reduced in OHT (*Figure 3A*, inset). In cardiomyocytes from post-mortem hearts, pCa_{50} was significantly lower in OHT (5.52 ± 0.01) than in CTRL (5.61 ± 0.01), while maximum Ca^{2+}-activated tension was also reduced (Supplementary material online, *Figure S7*). The Hill coefficient indexing the steepness of the force–Ca^{2+} curve was decreased in OHT (1.9 ± 0.2) vs. CTRL (2.5 ± 0.2) biopsy samples (*Figure 3A*), but increased in OHT (3.9 ± 0.5) vs. CTRL (3.0 ± 0.8) post-mortem samples (Supplementary material online, *Figure S7*). The Ca^{2+} sensitivity of the contractile apparatus was significantly reduced ($P = 0.012$) in OHT cardiomyocytes upon incubation with a PKA catalytic subunit (pCa_{50} shift, 0.16 ± 0.02 units), whereas in CTRL myocytes this effect was not significant (pCa_{50} shift, 0.06 ± 0.01 units) (*Figure 3B*).

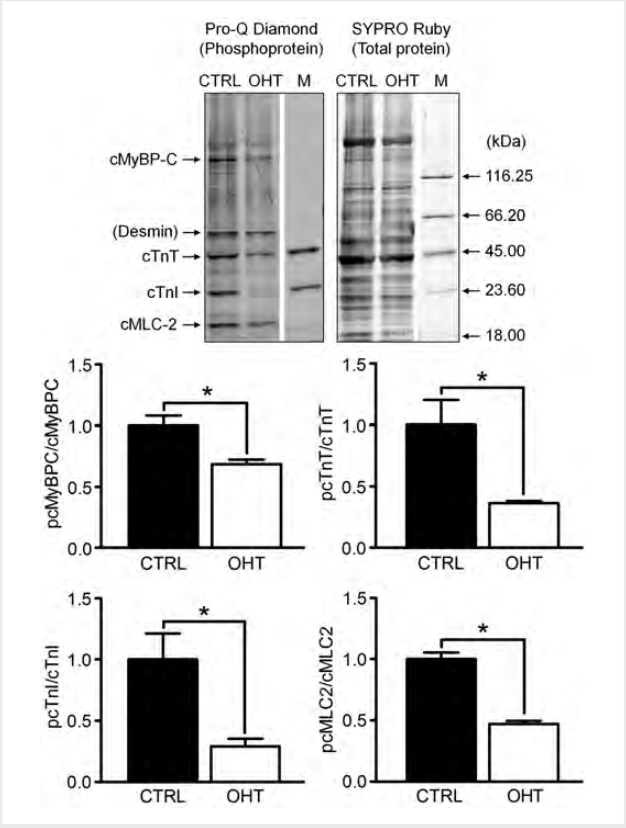

Figure 1 Phosphorylation of regulatory myofilament proteins in biopsy samples from OHT and CTRL dog hearts, by Pro-Q Diamond/SYPRO Ruby staining. Top panels show representative SDS–PAGE gels. M, peppermint-stick marker. Graphs show mean phospholevels of cMyBPC, cTnI, cTnT, and cMLC2, normalized to their respective total protein level. Heart samples ($n = 7–9$) were analysed in duplicate. *$P < 0.05$.

3.4. Titin isoform shift towards N2B in OHT

By measuring the titin N2BA/N2B isoform composition (*Figure 4A*; Supplementary material online, *Figure S8A*), we found that the mean N2B proportion in biopsied samples increased from 63.3 ± 3.3% in CTRL to 72.6 ± 3.3% in OHT (*Figure 4B*), and in post-mortem

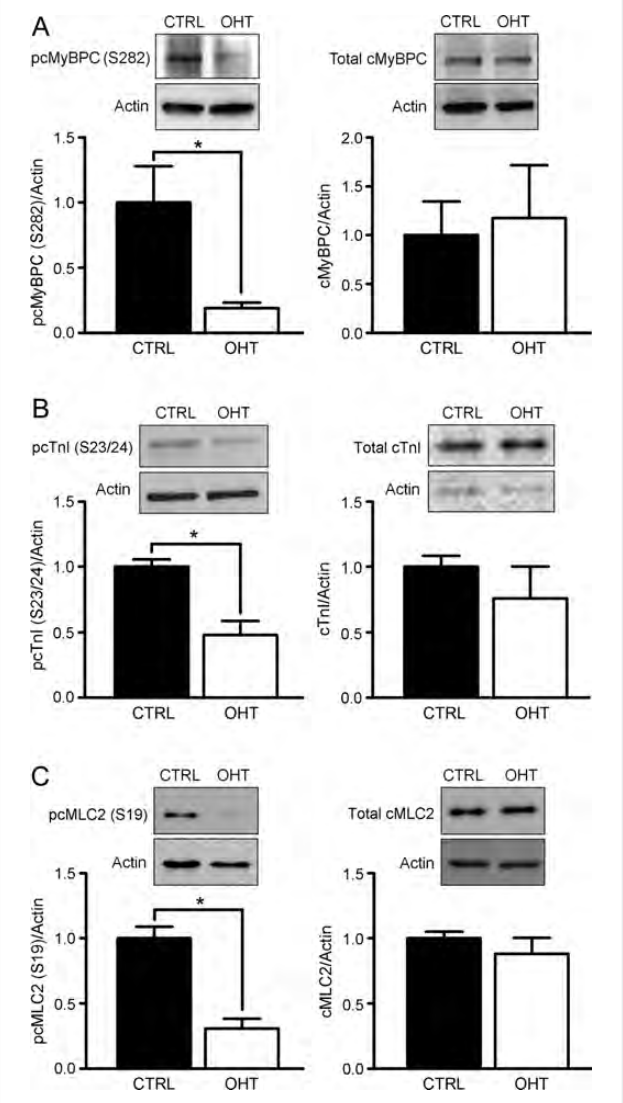

Figure 2 Expression and phosphorylation of cMyBPC, cTnI, and cMLC2, in CTRL and OHT biopsy samples by western blot. Left panels show phosphorylated, right panels total protein levels; representative immunoblots above graphs, which indicate mean phosphorylation/expression. (*A*), cMyBPC (S282) phosphorylation and cMyBPC expression; (*B*), cTnI (S23/S24) phosphorylation and cTnI expression; (*C*), cMLC2 (S19) phosphorylation and cMLC2 expression. Data in graphs are normalized to beta-actin signals. Heart samples ($n = 7–9$) were analysed in duplicate. $^*P < 0.05$.

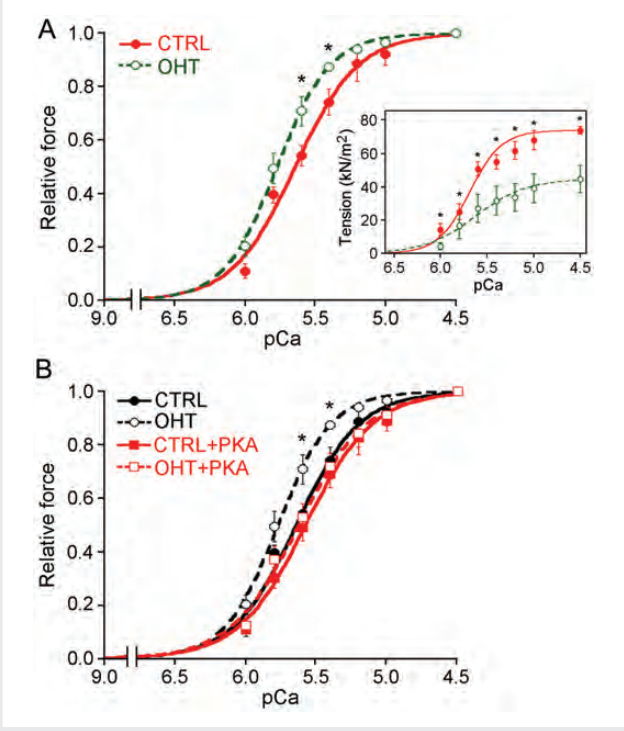

Figure 3 Myofilament calcium sensitivity of skinned isolated cardiomyocytes from biopsy samples. (*A*), Relative force vs. pCa relationship at 2.2 μm SL of OHT compared with CTRL cardiomyocytes. Inset, absolute values for actively developed tension vs. pCa. (*B*), Relative force vs. pCa relationship for CTRL and OHT cardiomyocytes at 2.2 μm SL, before (black symbols and curves; data taken from panel A) and after incubation with a PKA catalytic subunit (red symbols and curves). $n = 10–16$ myocytes per group, from three different hearts per group. $^*P < 0.05$.

tissues from 57.5 ± 6.1% in CTRL to 65.9 ± 5.1% in OHT (Supplementary material online, *Figure S8B*). A 'T2' titin degradation band was barely detectable in biopsy samples, but was more frequent and more intense in post-mortem tissues.

3.5. Phosphorylation deficit of all-titin in OHT and rescue by PKG

All-titin phosphorylation measured by Pro-Q Diamond/SYPRO Ruby staining decreased by ~30% in OHT vs. CTRL biopsies, and by ~60% in post-mortem tissue (*Figure 4C*; Supplementary material online, *Figure S8C*). Both N2BA and N2B titin isoforms were hypophosphorylated in OHT. Importantly, *ex vivo* phosphorylation by cGMP-dependent PKG significantly increased all-titin phosphorylation in OHT, up to the level measured in CTRL (*Figure 4C*; Supplementary material online, *Figure S8C*). This increase was larger in N2B than in N2BA titin.

3.6. Site-specific phosphorylation at titin-N2Bus (S4010/S4099) and titin-PEVK (S11878)

Alterations in all-titin phosphorylation reflect modifications at potentially hundreds of amino acids within titin. Furthermore, the Pro-Q Diamond stain reportedly fails to detect phosphosites within titin's PEVK domain.[18] Using custom-made phosphospecific titin antibodies, we measured changes in phosphorylation at two conserved serines within the N2Bus (S4010 and S4099 of full-length human titin) and at a conserved serine of the PEVK segment (S11878) by western blot (*Figure 5*; Supplementary material online, *Figure S9*). The mean proportions of titin N2Bus phosphorylation at S4010 (*Figure 5A*; Supplementary material online, *Figure S9A*) and S4099 (*Figure 5B*; Supplementary material online, *Figure S9B*) were significantly lower in OHT vs. CTRL. In contrast, the mean proportion of phospho-PEVK (S11878) was significantly higher in OHT than in CTRL (*Figure 5C*; Supplementary material online, *Figure S9C*). These phosphorylation changes occurred in both titin isoforms.

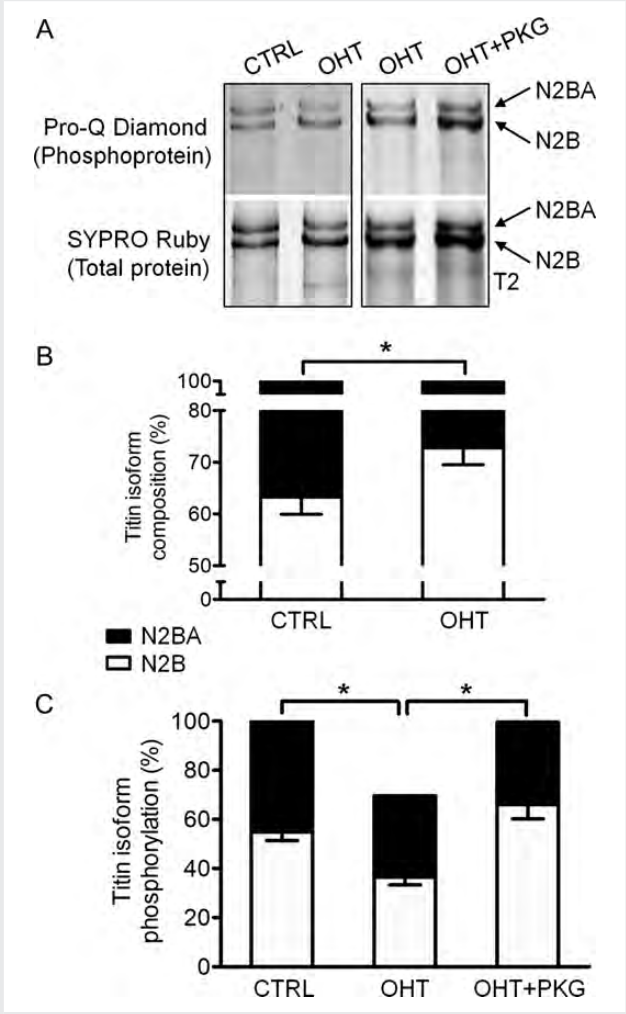

Figure 4 Titin-isoform composition and all-titin phosphorylation in biopsy samples, by Pro-Q Diamond/SYPRO Ruby staining. (*A*), Representative titin gels comparing CTRL and OHT samples, and OHT samples before and after incubation with cGMP-dependent PKG. Indicated are the N2BA and N2B titin isoforms and titin proteolytic fragment, T2. (*B*), Mean titin-isoform composition in OHT and CTRL hearts, measured on SYPRO Ruby-stained gels. Combined N2BA + N2B signal intensities are 100%. (*C*) Mean all-titin phosphorylation in CTRL and OHT, and in OHT samples incubated *ex vivo* with cGMP-dependent PKG. Combined N2BA + N2B phosphorylation of CTRL set to 100%. $n = 7–9$ per group; heart samples analysed in triplicate. $^*P < 0.05$.

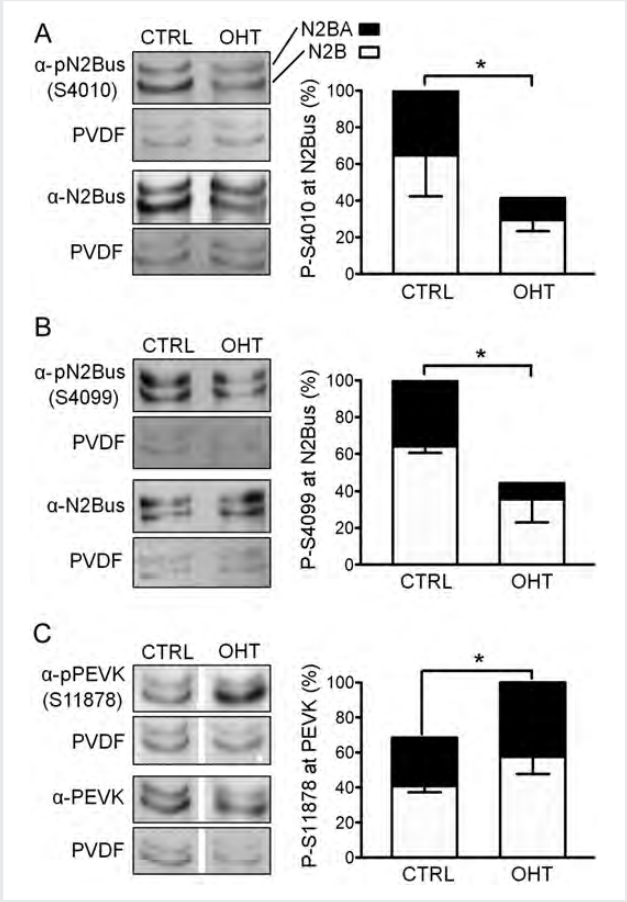

Figure 5 Site-specific phosphorylation of titin in biopsy samples by western blot. Images on left panels show representative immunoblots with OHT and CTRL heart tissue using phosphosite-specific (top) and corresponding sequence-specific (bottom) anti-titin antibodies. PVDF stains are included for comparison. Graphs on right show mean phosphorylation levels for N2BA and N2B isoforms in OHT and CTRL, at position S4010 (N2Bus) (*A*), S4099 (N2Bus) (*B*), and S11878 (PEVK domain) (*C*). (*A*) and (*B*) combined N2BA + N2B signal intensities of the CTRL set to 100%; (*C*) combined N2BA + N2B signal intensities of OHT set to 100%. Heart samples ($n = 7–9$) were analysed in triplicate. $^*P < 0.05$.

3.7. Cardiomyocyte $F_{passive}$ is increased in OHT but lowered by administration of PKA or PKG

The passive SL–tension relationship of isolated skinned cardiomyocytes (*n*, 10–16 per group, from 2 to 3 hearts per group) was generally steeper in OHT than in CTRL (*Figure 6*; Supplementary material online, *Figure S10*). Administration of PKA significantly reduced $F_{passive}$ of OHT cells at a SL of 2.2–2.4 μm, sometimes already at shorter SLs (*Figure 6A*; Supplementary material online, *Figure S10A*). Even in cardiomyocytes from beating-heart biopsies, PKA significantly lowered $F_{passive}$ also in CTRL (*Figure 6A*). Additional administration of cGMP-dependent PKG had no obvious mechanical effect in all groups (*Figure 6A*; Supplementary material online, *Figure S10A*). If PKG was administered first, $F_{passive}$ dropped as seen with PKA first and additional administration of PKA caused no further $F_{passive}$ change in the myocytes (*Figure 6B*; Supplementary material online, *Figure S10B*). We also tested whether administration of PKCα alters $F_{passive}$, but found no significant effect in both CTRL and OHT (*Figure 6C*). These results demonstrate that $F_{passive}$ is increased in OHT, but can be corrected by PKG- or PKA-mediated protein phosphorylation.

4. Discussion

The number of patients hospitalized for HFpEF grows steadily, but neither is the aetiology of the disease understood well, nor are effective treatment strategies available.[1–3] Mechanistic studies on human

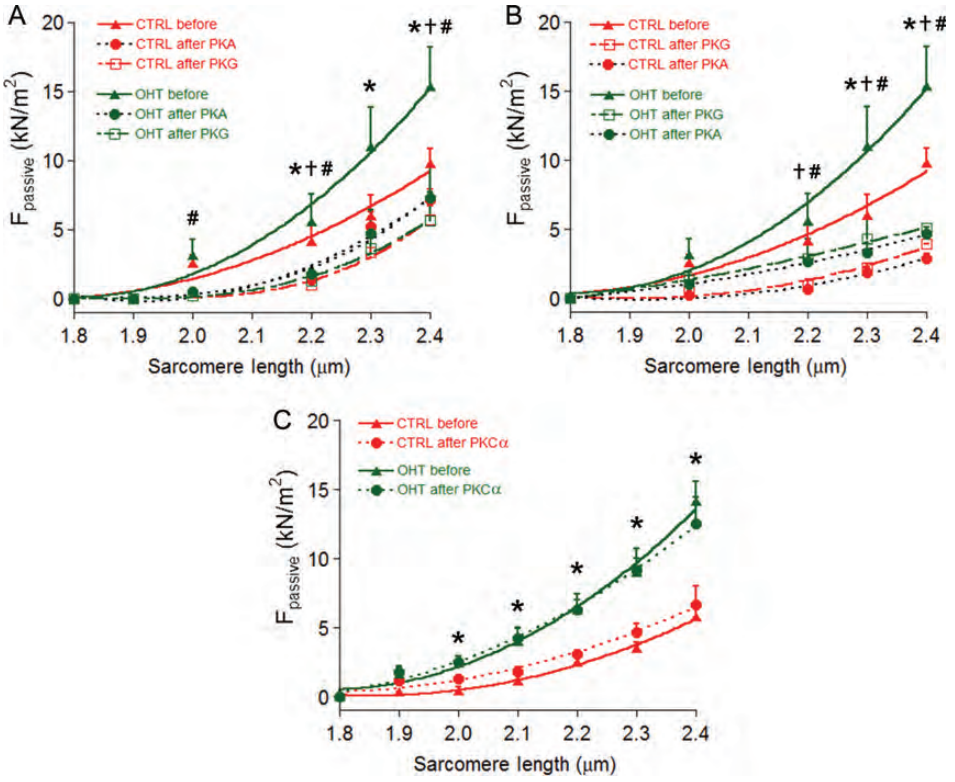

Figure 6 Passive tension of skinned cardiomyocytes isolated from biopsy samples, and effect of incubation with PKA, PKG, or PKCα. (*A*) and (*B*), $F_{passive}$ at SL 1.8–2.4 μm recorded on CTRL (red symbols and curves) or OHT myocytes (green symbols and curves) in non-activating buffer (solid curves; 'before'), following incubation with a PKA catalytic subunit (dotted curves; 'after PKA'), and following incubation with PKG and activator cGMP (dashed curves; 'after PKG'). (*A*) PKA administered first, followed by PKG; (*B*) PKG administered first, followed by PKA. (*C*), $F_{passive}$ at SL 1.8–2.4 μm recorded with CTRL or OHT myocytes in non-activating buffer (solid curves; 'before') and following incubation with PKCα (dotted curves; 'after PKCα'). $n = 10–16$ cardiomyocytes per group, from 2 to 3 hearts per group. Curves are three-order regressions. $^{*}P < 0.05$ CTRL'before' vs. OHT'before'; $^{\#}P < 0.05$ OHT'before' vs. OHT'after PKA' (in *A*) and OHT'before' vs. OHT'after PKG' (in *B*); $^{\dagger}P < 0.05$ CTRL'before' vs. CTRL'after PKA' in (*A*) and CTRL'before' vs. CTRL'after PKG' in (*B*).

HFpEF are limited by the low availability of tissue samples from diseased and healthy control hearts. Unlike hearts in end-stage failure, which after transplantation can be used for research purposes, HFpEF hearts usually do not get explanted. Biopsy samples are sometimes obtained from human HFpEF myocardium, but obviously not from healthy control hearts. Non-transplanted non-failing donor hearts occasionally become available for research, but their preservation is highly variable. These limitations with human cardiac tissue warrant the study of well-defined animal models of HFpEF, which can be controlled in terms of age, genetic background, or pharmacological treatment.

In this study, we used an old dog model of hypertrophy-associated early HFpEF, which shows signs of diastolic dysfunction resembling those frequently seen in elderly HFpEF patients: impaired LV relaxation, unaltered coefficient of LV diastolic stiffness but reduced diastolic capacitance, along with elevated natriuretic peptides, normal LV volume but increased LV mass and myocardial fibrosis.[28,30] We detected profound alterations in cardiac myofilament phosphorylation and function in dog HFpEF compared with normal dog hearts. Hypophosphorylation of sarcomeric proteins in experimental HFpEF presumably resulted, at least in part, from the increased PP1 and PP2a expression and the reduced PKG activity. The deranged phosphorylation in dog HFpEF altered the calcium sensitivity of the contractile apparatus and increased cardiomyocyte $F_{passive}$. By focusing on the elastic protein titin, we found that both isoform switching and altered phosphorylation increased cardiomyocyte $F_{passive}$ in HFpEF. Since hypophosphorylation of sarcomeric proteins persists in end-stage human HF,[20] a deficit in phosphorylation of these proteins could be a general property in the transition to HF. *In vivo* heart function was modestly impaired in the dog HFpEF model at rest, but reserve function, including catecholamine responsiveness (not studied here), could be more strongly affected. In summary, contributing factors that drive the HFpEF hearts into diastolic dysfunction likely include biochemical changes at myofilament proteins leading to increased calcium responsiveness of force generation and elevated titin-based passive stiffness.

4.1 Hypophosphorylation of regulatory myofilament proteins and increased pCa_{50} in dog HFpEF

Myofilament Ca^{2+} sensitivity is decreased after beta-adrenergic stimulation of cardiac muscle, an effect largely mediated by increased PKA-dependent phosphorylation of cTnI.[20,21,24,29,31] Because the

beta-adrenoceptor density and adenylate cyclase activity are reduced in HF,[32] hypophosphorylation of cTnI is a usual consequence, particularly at the PKA[24] (and PKG[33])-dependent phosphosites, S23/S24. In dog HFpEF, we found decreased cTnI phosphorylation (including S23/S24 phosphorylation) compared with normal dog hearts, presumably explaining the increased Ca^{2+} sensitivity of skinned cardiomyocytes from biopsied HFpEF hearts. In line with this interpretation, the increased Ca^{2+} sensitivity of HFpEF (biopsy) cardiomyocytes could be normalized by *ex-vivo* PKA treatment. Thus, the dog HFpEF hearts show alterations in cTnI phosphorylation and myofilament calcium sensitivity resembling those frequently (but not consistently[23,24]) reported in human HF vs. donor hearts and in animal models of HF.[20,21,29,31] Along this line, β-blockade in HFpEF patients has been associated with increased cardiomyocyte pCa_{50} compared with HFpEF patients not treated with β-blockers.[29] Furthermore, myofilament pCa_{50} changes are unlikely to be due only to cTnI phosphorylation, but also due to phosphorylation of cMyBPC and cMLC2. We found both these myofilament proteins, as well as cTnT, to be hypophosphorylated in biopsy samples of HFpEF dogs. In summary, hypophosphorylation of regulatory myofilament proteins and increased calcium sensitivity in this model suggest that functional impairment at the sarcomere level may be an early event in the development of HFpEF.

Surprisingly, reduced Ca^{2+} sensitivity was found in skinned cardiomyocytes from HFpEF hearts procured post-mortem, although phosphorylation of cTnI, cMyBPC, and cTnT was also lowered in the post-mortem HFpEF group. PKA activity was similar in biopsy and post-mortem samples, perhaps because all dogs were under autonomic blockade. The different direction of Ca^{2+}-sensitivity shift in the post-mortem compared with the beating-heart biopsy group likely originates in events associated with death, such as a catecholamine surge, enzymatic dysfunction, or activation of proteases. Unlike in biopsy samples, cMLC2 phosphorylation was unaltered in post-mortem HFpEF vs. CTRL, which might contribute to the differences in Ca^{2+}-sensitivity shift. Additionally, phosphosites in myofilament proteins not tested by us could be modified differently in the post-mortem and beating-heart groups. Cardiac TnI contains sites other than S23/S24 which can be phosphorylated and possibly be important for the Ca^{2+} sensitivity,[26] and some functionally relevant cTnI phosphosites may still be unknown. In conclusion, since we consider the beating-heart biopsies as the gold standard, the dog HFpEF hearts have increased Ca^{2+} sensitivity. Our results confirm that degradation processes or other modifications at the time of death can impact protein phosphorylation and function, which has implications for the interpretation of data from the samples obtained post-mortem.

4.2. Titin-isoform switch in HFpEF vs. HFrEF

The pattern of titin-isoform expression correlates with systolic and diastolic functional parameters in patients, including LV end-diastolic wall stiffness,[6] EF, EDV, and ESV.[10] Titin-isoform shift towards the more compliant N2BA variants occurs in end-stage failing hearts of patients with ischaemic cardiomyopathy[9] or non-ischaemic dilated cardiomyopathy (DCM).[10,11] In contrast, we found a modest decrease in the proportion of N2BA titin in HFpEF dogs. Similarly, a decreased N2BA proportion has been reported in rapid pacing canine models of DCM.[34,35] Possibly, then, dog hearts are unique in their remodelling response to mechanical stress. However, in aortic stenosis patients, the N2BA proportion was also lowered compared with donor hearts[36]—although the opposite result was found elsewhere.[7] In human hypertrophic cardiomyopathy, the cardiac titin-isoform pattern did not change compared with donor hearts,[37] whereas spontaneously hypertensive rats expressed slightly less N2BA proportions than normotensive rat hearts.[38] In summary, a decreased N2BA:N2B titin expression ratio may be a frequent (albeit not general) feature of hypertrophied hearts, including those developing HFpEF. Titin switching towards the N2B isoform increases cardiomyocyte $F_{passive}$ in HFpEF (this study), switching towards the N2BA isoform decreases $F_{passive}$ in HFrEF.[9–11]

4.3. Titin phosphorylation and $F_{passive}$ in HFpEF

Like human end-stage failing hearts,[7,14] dog HFpEF hearts showed a deficit in phosphorylation of all-titin and at S4010 and S4099 within titin's cardiac-only N2Bus. Importantly, *ex-vivo* administration of cGMP-dependent PKG corrected the all-titin phosphorylation deficit in HFpEF heart tissue and administration of PKA or cGMP-dependent PKG reduced the pathologically increased $F_{passive}$ of skinned OHT cardiomyocytes to CTRL levels. Since PKA activity was unaltered among the dog groups, the deficit in all-titin phosphorylation in HFpEF hearts may, at least partly, be a deficit in PKG-mediated phosphorylation, causing the higher-than-normal $F_{passive}$. Lowering $F_{passive}$ via increased PKG-mediated phosphorylation at the titin N2Bus, which improves diastolic function in these dog hearts,[15] could thus be a useful therapeutic approach in HFpEF.

Against the reduced phosphorylation of all-titin, the PKCα-dependent phosphosite at S11878 within the PEVK-titin segment was hyperphosphorylated. Active PKCα can be increased in HF[17] and an elevated PKCα activity was also apparent in the HFpEF dog hearts. Because PKCα-dependent phosphorylation at titin-S11878 (PEVK) increases cardiomyocyte $F_{passive}$,[16] hyperphosphorylation at this site presumably added to the higher-than-normal $F_{passive}$ in HFpEF.

The increased cardiomyocyte $F_{passive}$ in dog HFpEF is consistent with previous reports of elevated $F_{passive}$ in HFpEF patients[39] or those with diabetes[40] or under β-blockade.[29] Regarding HFrEF, either reduced[9,11] or elevated[7] $F_{passive}$ has been observed compared with donor hearts. The decreased $F_{passive}$ in human HFrEF was explained by a titin-isoform shift towards the compliant N2BA variants,[9,11] the increased $F_{passive}$ by depressed titin phosphorylation, because administration of PKA lowered $F_{passive}$ to control levels.[7] These findings underscore the importance of both titin-isoform transitions and titin phosphorylation changes for cardiomyocyte $F_{passive}$ in chronic HF. In dog HFpEF, the changes in titin phosphorylation may be more important for altering LV passive stiffness than the relatively small transitions in titin isoforms. In any case, we conclude that titin-based $F_{passive}$ is increased in dog experimental HFpEF and contributes to elevated LV passive stiffness, the hallmark of HFpEF.

4.4. Conclusions

This clinically relevant large-animal model of HFpEF is characterized by cardiac titin-isoform switch towards the stiffer N2B variant, a deficit in phosphorylation of all-titin and at specific serines within the N2Bus-titin domain, but hyperphosphorylation at titin's PEVK domain. These alterations act synergistically to elevate cardiomyocyte $F_{passive}$. A stiffer titin may be a key determinant of diastolic dysfunction

resulting from increased LV passive stiffness in HFpEF. Regulatory myofilament proteins are hypophosphorylated in dog HFpEF, causing increased Ca^{2+} sensitivity. A phosphorylation deficit for myofilament proteins could be an early and general event in the transition to HF, thus unbalancing cardiac mechanical function. Reversing the phosphorylation deficit by pharmacological manipulation of PK or phosphatase (PP) signalling pathways may be a useful therapeutic strategy in HFpEF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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