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Interventricular differences in myofilament function in experimental congestive heart failure

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Abstract

This study was conducted to identify molecular mechanisms which explain interventricular differences in myofilament function in experimental congestive heart failure (CHF). CHF was induced in rats by chronic aortic banding or myocardial infarction for 32–36 weeks. Right and left ventricular (RV, LV) myocytes were mechanically isolated, triton-skinned, and attached to a force transducer and motor arm. Myofilament force– $[Ca^{2+}]$ relations assessed maximal Ca^{2+} -saturated force (F_{max}) and the $[Ca^{2+}]$ at 50% of F_{max} (EC_{50}). Myofilament protein phosphorylation was determined via ProQ diamond phospho-staining. Protein kinase C (PKC)- α expression/activation and site-specific phosphorylation of cardiac troponin I (cTnI) and cardiac troponin T (cTnT) were measured via immunoblotting. Relative to controls, failing RV myocytes displayed a ~45% decrease in F_{max} with no change in EC_{50} , whereas failing LV myocytes displayed a ~45% decrease in F_{max} and ~50% increase in EC_{50} . Failing LV myofilaments were less Ca^{2+} -sensitive (37% increase in EC_{50}) than failing RV myofilaments. Expression and activation of PKC- α was increased twofold in failing RV myocardium and relative to the RV, PKC- α was twofold higher in the failing LV, while PKC- β expression was unchanged by CHF. PKC- α -dependent

phosphorylation and PP1-mediated dephosphorylation of failing RV myofilaments increased EC_{50} and increased F_{max} , respectively. Phosphorylation of cTnI and cTnT was greater in failing LV myofilaments than in failing RV myofilaments. RV myofilament function is depressed in experimental CHF in association with increased PKC- α signaling and myofilament protein phosphorylation. Furthermore, myofilament dysfunction is greater in the LV compared to the RV due in part to increased PKC- α activation and phosphorylation of cTnI and cTnT.

Keywords

Heart; Muscle mechanics; Force-pCa relation; Myofilaments; Protein kinase C; Hypertrophy

Introduction

Mechanical overload of the left ventricle (LV) due to myocardial infarction (MI) or chronic hypertension, elicits LV contractile dysfunction, and ultimately, congestive heart failure (CHF) [22]. In patients with CHF, LV dysfunction may also beget contractile dysfunction of the right ventricle (RV) due to elevated pulmonary venous and arterial pressures, ventricular interdependence, and LV dilation [45]. The molecular mechanisms contributing to RV contractile dysfunction in end-stage CHF secondary to mechanical LV overload are currently unknown.

The RV and LV differ structurally and functionally in several important ways. The RV is thin-walled owing to its ejection into the low pressure pulmonary circuit, while the LV is thick-walled due to its ejection into the high pressure systemic circuit [44]. Thus, RV and LV cells manifest different embryologic, structural, metabolic, and electrophysiologic characteristics [13, 19, 45, 46]. RV-LV differences are not limited to nondiseased cardiac muscle. Reports indicate that mechanical overload of the LV produces concentric and eccentric hypertrophy of this chamber, but only concentric hypertrophy of the RV [2]. Also, in experimental and human CHF, induced by primary LV dysfunction, RV-LV differences exist regarding (1) contractile function [15]; (2) myosin light chain (MLC) phosphorylation [21]; (3) calcium handling [1]; (4) small G-protein expression [35]; (5) β -adrenergic receptor density, adenyl cyclase activity, and cAMP [36]; (6) myocardial norepinephrine/epinephrine levels [12]; and (7) protein kinase C (PKC) isozyme expression and activity [45]. Taken together, there is convincing evidence that interventricular differences in contractile function of failing myocardium are likely due to differences in mechanical and biochemical signaling. Also, we and others have shown that myofilament function is depressed in experimental and human CHF [3, 4, 9, 11, 15, 17, 18, 20, 26, 27, 30]. Furthermore, several studies indicate that expression and activity of PKC- α and β is selectively increased in experimental and human hypertrophy/heart failure and that increased activity of these isozymes may contribute to contractile dysfunction of the failing heart [3, 5, 6, 16, 37–40]. At present, in experimental CHF, it is unclear whether (1) myofilament function differs across ventricular chambers, (2) whether the etiology of CHF explains RV-LV differences, or (3) what the molecular basis for such alterations is. Our hypothesis is that in end-stage CHF due to chronic LV pressure overload (PO) or MI, regional differences in myofilament function exist with dysfunction being more pronounced in the LV relative to the RV. Accordingly, we compared active myofilament function, PKC- α/β expression and activation, and myofilament protein phosphorylation (cardiac troponin I (cTnI), cardiac troponin T (cTnT), MLC-2) in the right and left ventricles isolated from rats with CHF due to chronic PO or MI.

Methods

Animal models

Ascending aortic banding or MI was induced in 4-week female Sprague–Dawley rats as described earlier [3, 4]. Animals were followed for a period of 32–36 weeks until they transitioned to a stage of PO-induced left ventricular hypertrophy (LVH) or MI-induced CHF. Morphologic and hemodynamic parameters were used to define CHF as described previously [4]. Clinical signs of end-stage CHF in our rat models were adjudicated using liver and lung weights. RV and LV weights were determined postmortem. Unoperated age-matched animals served as controls. In previous studies, we found no difference between sham-operated and age-matched, unoperated control animals [3, 4, 11]. Ventricular structure and function were examined invasively and non-invasively, as described previously [41]. In an effort to directly compare regional differences in myofilament function, PKC- α expression/activation, and myofilament protein phosphorylation (*interventricular RV–LV studies*) in diseased and nondiseased myocardium, RV and LV myocardium was isolated from the same animal/heart.

Mechanical measurements in isolated ventricular myocytes

Mechanical function in isolated RV and LV myocytes was examined as described in detail previously [3, 4, 9, 11, 17]. Myofilament protein phosphorylation status was preserved during cardiac myocyte isolation and experimentation by adding okadaic acid, calyculin A, and protein kinase inhibitor cocktail (Sigma Aldrich) to all bathing and study solutions as described previously [3, 4].

PKC expression and activity

Approximately 30 to 40 mg of left and right ventricular LVH, CHF, and age-matched control myocardium was homogenized in homogenization buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 15% [vol/vol] glycerol, 5 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 100 mmol/L NaF, 10 mmol/L Napyrophosphate, 1% [vol/vol] Triton X-100, 1% [vol/vol] Na-deoxycholate, 1 mmol/L dithiothreitol, 0.1% [vol/vol], 1 mmol/L 4-[2-aminoethyl]benzenesulfonyl fluoride, 50 μ g/mL aprotinin, 5 mmol/L pepstatin A, and 50 μ g/mL leupeptin) on ice. The homogenate was centrifuged at 4°C and 100,000 $\times$ g for 1 h. The protein concentration of the supernatant was determined and the samples were stored at –80°C. A total of 150 μ g of protein was loaded onto 10% SDS-PAGE gels. A human carcinoma cell line (20 ng total protein loaded per gel) was used as the positive control to identify the PKC isozymes. The electrophoresed proteins were transferred to poly(vinylidene difluoride) membranes. Membranes were incubated with primary antibodies against PKC- α , phospho-specific PKC- α , PKC- β , and phospho-specific PKC- β (Upstate Biotechnology). Secondary anti-mouse and anti-rabbit IgG peroxidase conjugates (Sigma-Aldrich) were used. The relative abundance of single proteins was detected using enhanced chemiluminescence (Amersham Biosciences). Suitable films were scanned and PKC- α , PKC- β , phospho-specific PKC- α /PKC- β , band density was quantified using commercially available software (Image J; NIH) [3]. Scanning units (optical density) within each blot were compared as illustrated in Fig. 3. The relative intensity of the diseased bands (e.g., CHF-RV) was normalized to the intensity of control bands (e.g., CON-RV) and the fold increase above control illustrated.

Expression and purification of recombinant PKC- α

Expression and purification of recombinant human PKC- α was carried out as described previously in detail [37].

Phosphorylation/dephosphorylation of myofilament proteins

The functional effect of PKC- α -mediated phosphorylation and PP1-dependent dephosphorylation of myofilament proteins was performed as described in detail earlier [3]. Treatment times and doses were determined based on in vitro studies examining the capacity of bacterially expressed PKC- α to significantly phosphorylate bacterially expressed cardiac troponin or troponin I and T within native myofilaments of cardiac myofibers. From these studies, it was determined that at a 0.1- μ g/mL PKC- α and 0.15 U/mL PP1 when incubated for 60 min was sufficient for these enzymes to phosphorylate or dephosphorylate, respectively, their myofilament targets. The relevance of these incubation times to the diseased state is that in control right and left ventricular myofilaments, incubation with PKC- α induced sufficient phosphorylation of myofilament proteins such that control myofilament function became blunted to a similar degree observed in failing myofilaments that had not been treated with PKC- α . Also, PP1 dephosphorylation under the above conditions was sufficient to remove enough phosphate within the myofilament proteins such that myofilament function could be restored toward control levels. In brief, an attached cell was washed with PKC- α buffer less the enzyme for 2 min. The cell was then incubated in PKC- α buffer (1 mmol/L NaF, 1 mmol/L Na₃VO₄, 0.5 mmol/L MgCl₂, 100 mmol/L leupeptin, 100 mmol/L pepstatin, 150 mmol/L PMSF-EtOH, 1 mmol/L dithiothreitol, 0.8 μ mol/L Ca²⁺, 20 μ mol/L diacylglycerol, 0.3 mmol/L phosphatidylserine, and 0.1 μ g/mL recombinant PKC- α) at 22°C to 25°C for 60 min. For PP1-induced dephosphorylation, myocytes were incubated in relaxing solution containing the catalytic subunit of PP1 (0.15 U/mL; Upstate Biotechnology) along with 1 mmol/L dithiothreitol at 22°C to 25°C for 60 min. Following the incubation, the attached cell was washed and exposed to five submaximal Ca²⁺ concentrations. Cell data were included only if the %rundown was less than 20% from the beginning to the end of the experiment.

Myofilament protein phosphorylation

The protocol for myofilament protein phosphorylation analysis has been described in detail in previous studies [37–40]. In an effort to preserve endogenous cardiac myofilament protein phosphorylation, all solutions used for cardiac muscle cell isolation contain protease and phosphatase inhibitors. All muscle samples were then rapidly flash-frozen in liquid nitrogen. Cardiac myofibrils were isolated from 50 mg of flash-frozen rat ventricles. Briefly, tissue was homogenized first in ice cold relax buffer, then in ice cold standard rigor buffer with Triton X-100 containing protease and phosphatase inhibitors. Myofibrils solubilized in urea/thiourea loading buffer (without SDS) were separated on 15% polyacrylamide gels (8 mg/lane). All gels were run in triplicate. Gels were fixed and stained with Pro-Q Diamond phosphoprotein stain (to detect phosphorylated proteins) (Invitrogen) followed by Sypro Ruby protein stain (to detect total proteins) (Invitrogen). Gels were imaged on a Typhoon 9410 molecular imager and analyzed using the Image-Quant v2005 software. Relative phosphorylation was determined from the ratio of ProQ Diamond/Sypro ruby signal as determined via densitometry. For immunoblot analysis, myofibrillar proteins were transferred to Hybond-LPF membrane (GE Healthcare), incubated with appropriate antibodies, and developed, using the ECF Western Detection Kit (GE Healthcare). The following antibodies were used: anti-TnT (clone JLT-12, Sigma), anti-phospho-cTnT (custom made) [38], and anti-cTnI (cTnI—pS23/24 and cTnI—Cell Signaling). The phosphocardiac TnI (Ser 23/24) is specific only for cTnI when it is phosphorylated at Ser 23/24.

Data and statistical analysis

Cell data were analyzed as described earlier [3, 4, 11, 17]. Data are expressed as mean $\pm$ SEM. Statistical differences between structural and functional myocyte data (Table 2) were determined using one-way ANOVA. Statistical differences in PKC- α and PKC- β expression

and phosphorylation in CON, LVH, and CHF right and left ventricular preparations were determined using unpaired Student's *t* test (Instat 3). The impact of PKC- α and PP1 on myofilament function was determined by a paired Student's *t* test. Differences in phosphorylation of cardiac myofilament proteins were determined using unpaired Student's *t* test (SPSS 14.0 for Windows). A $P < 0.05$ was considered statistically significant.

Results

Ventricular structure and function in experimental CHF

End-stage CHF in our animal models was confirmed by clinical evidence of lung engorgement and evidence of pleural effusion on postmortem dissection as we have described previously [4]. Wet lung weight/body weight ratios were 0.64 ± 0.08 , 1.63 ± 0.16 , and 1.94 ± 0.15 in control, CHF, and LVH rats, respectively ($P < 0.05$ for LVH and CHF rats). Similarly, dry lung weight/body weight ratios were 0.16 ± 0.03 , 0.42 ± 0.09 , 0.41 ± 0.09 in control, CHF, and LVH rats, respectively ($P < 0.05$). The extent of RV remodeling was determined via right ventricle weight (RVW)/body weight (BW) ratios (milligram per gram). RVW/BW ratios were 0.53 ± 0.03 , 1.17 ± 0.23 , and 1.37 ± 0.11 in control, CHF, and LVH rats, respectively ($P < 0.05$). The extent of LV remodeling was determined using LVW/BW ratios (for POLVH studies) and IVSW/BW ratios (for congestive heart failure myocardial infarction (MICHF) studies), where IVSW denotes interventricular septal weight. LVW/BW ratios (milligram per gram) were 9.9 ± 0.52 and 16.1 ± 0.91 for control and LVH rats, respectively ($P < 0.05$). IVSW/BW ratios (milligram per gram) were 9.6 ± 1.2 and 18.2 ± 1.8 for control and CHF rats, respectively ($P < 0.05$). IVSW was used for MICHF rats to gauge the degree of LV hypertrophy given that most of the LV free wall in these animals was infarcted and scarred from the surgery. End-stage CHF was associated with systolic and diastolic dysfunction and marked dilation of the left atria and ventricles (Table 1).

Right and left ventricular myofilament function

Intraventricular comparison—Myofilament function in RV myocytes isolated from control, LVH, and CHF rats is summarized in Fig. 1. Relative to control RV cells, the Ca^{2+} -saturated maximal force ($F_{\max}$) parameter in LVH-RV and CHF-RV cells was reduced by 45% and 47%, respectively ($P < 0.001$). The EC_{50} (myofilament Ca^{2+} sensitivity index) and Hill coefficient (myofilament cooperativity index) were not different between control and failing RV myocytes (Fig. 1). In contrast, compared to controls, LV myofilament function was markedly depressed in experimental LVH and CHF as shown by a significant reduction in the $F_{\max}$ together with an increase in the EC_{50} (Table 2).

Interventricular comparison—To compare myofilament function between right and left ventricular muscles, we isolated RV and LV myocytes from CON, LVH, and CHF rats. In order to reduce variation and increase data fidelity, we obtained and studied RV and LV cells from the same animal/heart. In some instances, only LV myocytes were studied from a given rat heart, and we observed no statistically significant difference between matched (LV and RV myocytes from the same heart) and unmatched LV cell data (data not shown). Figure 2 summarizes the force- $[\text{Ca}^{2+}]$ relations and averaged curve fit parameters for RV and LV myocytes obtained from LVH (LVH-RV, LVH-LV) and CHF (CHF-RV, CHF-LV) rats. The $F_{\max}$ parameter was not different between RV or LV myofilaments of either disease model. In contrast, there were significant differences in the EC_{50} parameter. Relative to LVH-RV myofilaments, LVH-LV myofilaments displayed a 16% increase in the EC_{50} (decreased myofilament Ca^{2+} sensitivity) ($P = 0.047$) (Fig. 2c, Table 2). Similarly, the EC_{50} parameter was 37% higher in CHF-LV myofilaments relative to matched CHF-RV myofilaments ($P < 0.0001$) (Fig. 2f, Table 2). Of note, RV myofilament function (both $F_{\max}$ and EC_{50}) was not statistically different between LVH-RV and CHF-RV myocytes (Table

2). These results indicate that in two different animal models of end-stage CHF, regional differences in myofilament function exist with dysfunction being more severe in failing LV myofilaments than RV myofilaments.

PKC- α expression and activation in RV and LV myocardium

PKC- α expression was significantly increased by approximately twofold in chronically remodeled right ventricles from both LVH and CHF rat hearts (Fig. 3a, c). Likewise, PKC- α activity, as indexed by the quantity of phosphorylated PKC- α , was significantly increased by approximately twofold in LVH and CHF right ventricles (Fig. 3b, d). In chronically diseased CHF ventricles, we found a significant approximately twofold increase in activated PKC- α in the LV relative to the RV (Fig. 3f). No major differences in PKC- β expression and activation in LVH and CHF right or left ventricular myocardium were observed (Fig. 3g–j). Collectively, our results indicate that expression and activation of PKC- α is upregulated in failing RV myocardium and that, relative to the RV, PKC- α is more active in the failing LV, whereas PKC- β expression is unchanged in chronic LVH or CHF.

Impact of PKC- α -dependent phosphorylation on RV myofilament function

To determine whether altered PKC- α -dependent phosphorylation explains the decrease in RV myofilament function in end-stage CHF, we examined the impact of in vitro PKC- α treatment on myofilament function in detergent-permeabilized nonfailing and failing RV myocytes (Fig. 4). In control RV cells, PKC- α -dependent myofilament phosphorylation resulted in a 35% decline in $F_{\max}$ and 49% increase in the EC_{50} parameter (Fig. 4a–c). In failing RV myocytes, PKC- α treatment elicited no significant change in the $F_{\max}$ parameter and a significant 57% increase in the EC_{50} parameter (Fig. 4d–f). These results indicate that PKC- α -dependent phosphorylation elicits depression of RV myofilament function in nonfailing cells and that preserved myofilament Ca^{2+} sensitivity in failing RV myofilaments may relate, in part, to lower PKC- α -mediated phosphorylation as compared to failing LV myofilaments.

Impact of PP1-dependent dephosphorylation on RV myofilament function

In control cells, PP1-dependent dephosphorylation was without a marked effect on RV myofilament function (unaltered $F_{\max}$; EC_{50}) (Fig. 5a–c). However, in end-stage CHF cells, PP1-elicited dephosphorylation induced a ~20% increase in the $F_{\max}$ parameter and no change in Ca^{2+} sensitivity in failing RV myofilaments (Fig. 5d–f). These results suggest that increased myofilament protein phosphorylation explains, in part, the reduced $F_{\max}$ observed in end-stage failing RV myocytes.

Myofilament protein phosphorylation

Total myofilament phosphoprotein expression—To determine whether altered myofilament protein phosphorylation was associated with regional differences in myofilament function between failing right and left ventricular myocytes, we examined total cTnI, cTnT, and MLC-2 phosphorylation using phospho-specific staining (Fig. 6). In failing right ventricles, total cTnI and cTnT phosphoprotein levels were significantly increased by ~50% relative to controls, $P < 0.05$ (Fig. 6a). In contrast, MLC-2 phosphorylation was decreased by ~30% in failing RV relative to controls, $P < 0.05$ (Fig. 6a). In failing left ventricles, total cTnT, cTnI, and MLC-2 phosphorylation levels were increased by ~50% ($P = 0.053$), 102% ($P < 0.05$), and 50% ($P = 0.06$), respectively, relative to controls (Fig. 6b). In interventricular analyses, comparing failing RV and LV muscles, failing LV myocardium displayed a 47% increase in cTnT phosphorylation and a smaller, yet significant, 28% increase in cTnI phosphorylation (Fig. 6c). MLC-2 phosphorylation was not different between failing RV and LV preparations.

Site-specific phosphorylation of myofilament proteins—Finally, we examined phosphorylation-specific sites within cTnI and cTnT using phospho-specific antibodies (Fig. 7). Phosphorylation of cTnT at threonine residue 206 was increased 87% in failing RV muscles relative to control (Fig. 7a) and increased 24% ($P=0.054$) in failing LV muscles compared to control (Fig. 7b). In LV myocardium, compared to control, failing LV muscles displayed a ~300% increase in phosphocTnI (Ser 23, 24) (Fig. 7b). In failing ventricles, interventricular comparison revealed that LV muscles displayed a 47% increase in phospho-cTnI (serine 23, 24) and ~250% increase in phospho-cTnT (Thr 206) (Fig. 7c). Of note, phosphorylation of cTnT at serine 278 was not altered by heart failure or ventricular region (Fig. 7a–c). Collectively, these studies indicate that in experimental end-stage CHF in the rat, (1) there is increased phosphorylation of cTnI and cTnT and (2) RV-LV differences in total and site-specific phosphorylation of cTnI and cTnT occur, with phosphorylation being greater in failing LV myocardium.

Discussion

Our study is the first to examine the existence and underlying molecular basis for interventricular differences in myofilament dysfunction in end-stage experimental CHF of diverse etiology. Our data demonstrate the likelihood that chronic pressure overload or myocardial infarction of the LV activates neurohumoral and mechanical signals that are selectively more robust in the LV than the RV. Ultimately, the differences in signaling beget greater LV myofilament biochemical and functional remodeling in CHF. Here, we report that, in end-stage CHF, while RV myofilament function is depressed, the degree of depression, as indexed by the $F_{\max}$ and EC_{50} , is greater in LV myofilaments. Furthermore, we report that this RV–LV difference relates, in part, to higher PKC- α activation and phosphorylation of cTnI and cTnT in failing LV myocardium.

Right and left ventricular myofilament function in CHF

Mechanical dysfunction of the ventricular myocyte is centrally involved in pump failure observed in CHF [23]. Furthermore, the cardiac myofilaments are emerging as central players in ventricular myocyte dysfunction in the failing heart. In the present study, we found a 45% reduction in the $F_{\max}$ in skinned RV cells and 45% decline in $F_{\max}$ and 50% increase in EC_{50} in skinned LV cells. Our findings are in agreement with several other studies that have likewise documented right and left ventricular myofilament dysfunction in experimental and human CHF [3, 4, 8, 9, 11, 15, 17, 18, 20, 26, 27, 30]. Thus, there is a growing literature indicating that myofilament activity is depressed in ventricular failure. In the spontaneously hypertensive heart failure prone rat, Janssen and colleagues reported impaired isoproterenol response, force frequency response, and contractile function in the LV; of note, these parameters were relatively normal in the failing RV [15]. Importantly, RV and LV myofilament activation was not examined in their study. In our mechanical analyses of right and left ventricular cells isolated from the same failing hearts, we found that left ventricular myofilaments were less sensitive to Ca^{2+} compared to failing RV myofilaments independent of CHF etiopathology. In control (nonfailing) myofilaments, we found that RV myofilaments were less sensitive relative to LV myofilaments, results similar to those reported by Perreault and colleagues in the rat [31]. Given these data, the question becomes what is/are the molecular basis for these regional differences in myofilament function?

PKC expression, activation, and signaling in CHF

Several studies have reported upregulation of PKC isozyme expression, activation, and signaling in experimental and human CHF [3, 5, 6, 26, 27, 45]. Furthermore, increases in PKC signaling beget dysfunction of the cardiac myofilaments in several animal models of cardiac failure [7, 14, 25, 32, 33]. In the current study, we observed a modest (twofold)

increase in PKC- α expression and activation in failing RV myofilaments. Our finding is that PKC- α -induced phosphorylation of failing RV myofilaments caused myofilament desensitization (increased the EC₅₀), while PP1 dephosphorylation increased the $F_{\max}$ suggests that RV myofilament protein phosphorylation is increased, yet the increase is modest in that $F_{\max}$ is depressed, but myofilament Ca²⁺ sensitivity is unaffected in RV failure. Previously, we reported a fourfold increase in PKC- α expression and activation in failing LV myofilaments [3]. In failing LV myocytes, PKC- α phosphorylation had no effect on myofilament function (unaltered $F_{\max}$ or EC₅₀), while PP1 dephosphorylation partially restored LV myofilament function (increased $F_{\max}$, decreased EC₅₀). Following PP1 treatment, the $F_{\max}$ did not completely normalize, and this may be due to phosphorylated myofilament sites which cannot be dephosphorylated by PP1, but perhaps are more appropriate targets of other protein phosphatase isozymes, for example PP2. In support of this notion, Noguchi and colleagues reported increased maximal Ca-saturated force development following PP2A1-mediated dephosphorylation of myofilaments isolated from failing human hearts [27]. Collectively, these data suggest that RV–LV differences in PKC- α activation, signaling to cardiac myofilaments, and myofilament protein phosphorylation exist in experimental CHF. Indeed, direct comparison of failing right and left ventricular myocardium showed that the level of PKC- α activation in the LV is approximately twofold greater than in RV muscles isolated from the same heart. Relatively lower PKC- α activation in failing RV myofilaments may result in this enzyme selectively targeting cardiac troponin I and/or T that when phosphorylated have a greater impact on blunting calcium-activated maximal force. Our finding of regional differences in PKC isozyme expression in cardiac failure is similar to the work by Wang and colleagues who reported interventricular differences in expression of PKC- α and ζ in the rat [45]. Interventricular differences in PKC- α activation and signaling to the myofilaments can arise from either altered neurohumoral signaling to the RV or LV or altered ventricle-specific response to upstream neurohormonal signals. In support of this thesis, studies in small animal models of CHF revealed that failing RV and LV muscles manifested differences in norepinephrine and epinephrine levels as well as norepinephrine turnover [12]. Also, in the mouse, phenylephrine treatment induced an increase in LV myofilament Ca²⁺ sensitivity while causing a decrease in RV myofilament Ca²⁺ sensitivity [46]. On the whole, our data are exciting as they illustrate, for the first time, that interventricular differences exist in PKC- α activation, signaling to the cardiac myofilaments, and myofilament protein phosphorylation in different rat models of experimental end-stage CHF.

Myofilament protein phosphorylation in CHF

Several studies have shown that increased PKC isozyme activation induces functionally important phosphorylations in myofilament proteins thus resulting in myofilament depression, ventricular myocyte dysfunction, and reduced cardiac pump function [3, 7, 14, 16, 25, 28, 32, 33]. Within the cardiac myofilaments, there exist several targets for PKC-dependent phosphorylation. Specifically, cTnI can be phosphorylated by PKC on Ser 23, 24, 43, and 45 and Thr 144 [28]. cTnT can be phosphorylated by PKC at Thr 197, Ser 201, Thr 206, Ser 279, and Thr 287 [37–40]. Sumandea et al. showed that select phosphorylation of the Thr 206 site in cTnT by PKC- α causes myofilament depression [37–39]. Also, recent studies by Sumandea and colleagues using mass spectroscopy and custom-made antibodies revealed that PKC- α phosphorylates Thr 206 and Ser 279 of cTnT [38]. In this study, we chose to examine the phosphorylation status of both Thr 206 and Ser 279 to verify that despite the high level of PKC- α activation observed in our failing models, the enzyme still remained specific in targeting the Thr 206 residue, while other potential sites of phosphorylation were unaltered. Finally, studies suggest that regulatory MLC-2 phosphorylation increases the level of steady-state force generation at submaximal [Ca²⁺] [29].

Cardiac troponin I phosphorylation—In RV myofilaments, we found increased total cTnI phosphorylation without changes in cTnI phosphorylated at Ser 23, 24, whereas in LV myofilaments, we observed increased total and Ser 23, 24 phosphorylated cTnI. These observations have several implications. First, they explain, in part, why we do not see a change in RV myofilament Ca^{2+} sensitivity, but do observe a decrease in LV myofilament Ca^{2+} sensitivity in CHF. Second, they suggest that other sites within the cTnI molecule are phosphorylated, which contribute to decreased $F_{\max}$ in RV and LV myofilaments. In support of this postulate, Burkart et al. reported that phosphorylation of cTnI at serine 43 and 45 and threonine 144 induces a marked depression in maximum force generation and myofilament Ca^{2+} sensitivity in muscle fibers [7]. Importantly, these sites can be phosphorylated by PKC- α [16]. Additionally, studies in chronic experimental models of CHF have demonstrated phosphorylation of potentially novel sites within cTnI that appear to contribute to contractile failure of the myofilaments [3, 33]. Our finding that increased cTnI Ser 23, 24 phosphorylation, at putative protein kinase A (PKA) sites, occurs in failing LV may seem somewhat surprising given the reports that cTnI phosphorylation at these sites may decrease due to β -adrenergic receptor downregulation and decline in PKA activation [23, 24]. However, it is possible that downregulation of β -receptor signaling and PKA phosphorylation of cTnI exposes sites for phosphorylation to an already hyperactive PKC- α which then hyperphosphorylates cardiac troponin I at Ser 23, 24 and thus promotes myofilament decompensation. Indeed, previous studies in reconstituted filaments show that PKC isozymes can cross phosphorylate cTnI at Ser 23, 24 sites [16, 28]. Direct comparison of failing right and left ventricular myofilaments illustrated greater total and Ser 23, 24 phosphorylated cTnI in the LV; these results therefore provide a molecular basis for the greater decrease in LV myofilament Ca^{2+} sensitivity compared to the RV in experimental CHF.

Cardiac troponin T phosphorylation—In failing RV myofilaments, we observed an increase in total and Thr 206-phosphorylated cTnT, which potentially explains the decrease in $F_{\max}$ and provides molecular credence to upstream signaling through PKC- α . So, why do we not observe a decrease in RV myofilament Ca^{2+} sensitivity given the changes in cTnI and cTnT phosphorylation? It should be pointed out that most studies examining cardiac troponin physiology have focused on a single cTn subunit (e.g., cTnI or cTnT) and probed phosphorylation at specific sites [7, 25, 33, 37]. Thus, because we found phosphorylation of cTnI and cTnT at different sites in experimental CHF, what we may be observing, functionally, is the integrated impact of multiple phosphorylations in multiple myofilament proteins. Also, cTnI may be phosphorylated at novel residues in CHF [3, 33]; the functional effect of which remains to be determined. Direct comparison of failing right and left ventricles revealed an increase in total and Thr 206 cTnT phosphorylation in LV relative to RV. These results coupled with greater PKC- α activation in failing LV relative to RV suggests that PKC- α -dependent phosphorylation of cTnT contributes to interventricular differences in myofilament Ca^{2+} sensitivity in end-stage experimental CHF.

Myosin light chain-2 phosphorylation—Our finding that MLC-2 phosphorylation was blunted in failing RV myofilaments and increased in LV myofilaments suggests a potentially important role for the molecule in explaining RV and LV myofilament dysfunction in CHF. A recent study by Scruggs and coworkers showed that transgenic overexpression of a nonphosphorylatable regulatory MLC caused decreased contractility, decreased maximal force, and altered phosphorylation of myosin binding protein C and cTnI [34]. Thus, it appears that lack of MLC-2 phosphorylation decreases myofilament activation and may alter phosphorylation of neighboring thinfilament constituents in relevant ways which have functional consequences. Support for this notion can be derived from studies in which phenylephrine stimulation induces differences in myofilament Ca^{2+} sensitivity due to

differences in the level of MLC-2 phosphorylation between right and left murine ventricles [21]. Our finding of increased MLC-2 phosphorylation in phase with decreased LV myofilament Ca^{2+} sensitivity seems in contrast to these observations. However, our contention is that the elevated phosphorylation of cTnI and cTnT and consequent myofilament desensitization prove to “outweigh” the elevated MLC phosphorylation and presumed enhanced myofilament sensitization in the failing LV.

Limitations—There are several limitations in the current study. First, we did not determine the phosphorylation profile of other myofilament constituents (e.g., myosin binding protein C, tropomyosin), which, when phosphorylated, can modulate myofilament activation [43]. Of note, a recent study indicates that phosphorylation of myosin binding protein C is reduced in experimental and human CHF [10]. Additionally, studies in murine LV muscles indicate that phosphorylation of α -tropomyosin begets decreases of myofilament function [42]. However, a detailed myofilament proteomic analysis is beyond the scope of this paper and future studies in this area are warranted. Second, altered proteolysis of myofilament proteins may also explain some of the findings reported here. For example, a decline in the quantity of cTnI has been reported in experimental CHF in parallel with myofilament depression [20]. Future studies are warranted in order to determine whether such proteolytic events occur in experimental CHF and whether regional differences exist in myofilament protein proteolysis. Finally, we and others reported that phosphorylation of additional sites within cTnI occurs in chronic animal models of CHF [3, 33]. Careful examination of these sites, their functional significance in diseased myocardium, and perhaps variation by ventricular chamber are needed in the future.

Nevertheless, our findings are novel and exciting in that they indicate that (1) increased PKC- α expression and activation as well as signaling to the myofilaments in phase with phosphorylation of cTnI and cTnT contributes to RV myofilament dysfunction independent of animal model and (2) qualitative and quantitative right and left ventricular differences exist, in myofilament dysfunction, PKC- α activation, and phosphorylation of cTnI, cTnT, and MLC-2 in end-stage CHF in the rat.

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Fig. 1.

a Average force-[Ca²⁺] relations for RV cells from CON ($n=22$ cells, 12 right ventricles), LVH ($n=8$ cells, 5 right ventricles), and CHF ($n=23$ cells, 10 right ventricles) rats. **b, c** Bar graphs of averaged curve fit parameters: maximal Ca²⁺-saturated force ($F_{\max}$) and [Ca²⁺] at 50% of $F_{\max}$ (EC_{50}). * $P<0.05$, defined as significant relative to CON-RV

Fig. 2. Average force- $[Ca^{2+}]$ relations for LVH (a) and CHF (d) right ventricular (LVH-RV, CHF-RV) and left ventricular (LVH-LV, CHF-LV) skinned myocytes. LVH data were obtained from 8 cells from 5 right ventricles and 19 cells from 7 left ventricles of LVH rats. CHF data were obtained from 23 cells from 10 right ventricles and 25 cells from 12 left ventricles of CHF rats. b, c, e, f Bar graphs of averaged curve fit parameters: F_{max} and EC_{50} . * $P < 0.05$, defined as significant relative to LVH-RV or CHF-RV

Fig. 3. PKC- α expression and activation in right and left ventricular homogenates. *Inset* for all subfigures illustrates representative western blots. All PKC- α /phospho-PKC- α signals were normalized to an internal positive control (A4321 cells, 20 ng protein load) to insure even loading of gel lanes. **a, b** Expression of PKC- α and phosphorylated PKC- α in CONRV ($n=5$) and LVH-RV ($n=4$) homogenates. **c, d** Expression of PKC- α and phosphorylated PKC- α in CON-RV ($n=4$) and CHF-RV ($n=5$) homogenates. **e, f** Expression of PKC- α and phosphorylated PKC- α in CHF-RV ($n=5$) and CHF-LV ($n=5$) homogenates. **g, h** Expression of PKC- β and phosphorylated PKC- β in CON-RV ($n=8$) and LVH-RV ($n=8$) homogenates.

i, j Expression of PKC- β and phosphorylated PKC- β in CON-RV ($n=7$) and CHF-RV ($n=6$) homogenates. * $P<0.05$, regarded as significant relative to CON-RV or CHF-RV

Fig. 4.

a Average force- $[Ca^{2+}]$ relations for control right ventricular (CON-RV) myocytes ($n=6$) before (CON-RV-PKC-α) and after (CON-RV+PKC-α) incubation with recombinant PKC-α. **b, c** Histograms of averaged curve fit parameters (F_{max} and EC_{50}) from CON-RV cells before and after treatment with bacterially expressed PKC-α. **d** Average force- $[Ca^{2+}]$ relations for CHF right ventricular (CHF-RV) myocytes ($n=7$) before (CHF-RV-PKC-α) and after (CHF-RV+PKC-α) incubation with recombinant PKC-α. **e, f** Histograms of averaged curve fit parameters (F_{max} and EC_{50}) from CHF-RV cells before and after treatment with bacterially expressed PKC-α. * $P<0.05$, regarded as significantly different versus CON-RV-PKC-α or CHF-RV-PKC-α.

Fig. 5.

a Average force- $[Ca^{2+}]$ relations for control right ventricular (CON-RV) myocytes ($n=10$) before (CON-RV-PP1) and after (CON-RV+PP1) incubation with the catalytic subunit of protein phosphatase type 1 (PP1) (0.15 U/mL). **b, c** Bar graphs of averaged curve fit parameters (F_{max} and EC_{50}) from CON-RV cells before and after treatment with PP1. **d** Average force- $[Ca^{2+}]$ relations for right ventricular (CHF-RV) myocytes ($n=8$) before (CHF-RV-PP1) and after (CHF-RV+PP1) incubation with the catalytic subunit of PP1 (0.15 U/mL). **e, f** Bar graphs of averaged curve fit parameters (F_{max} and EC_{50}) from CHF-RV cells before and after treatment with PP1. * $P<0.05$, considered significant versus CHF-RV-PP1

Fig. 6.

ProQ diamond-stained SDS gels illustrating myofilament protein phosphorylation. *Histograms* summarize the quantity of phosphorylated cTnT, cTnI, and MLC-2-phosphorylated protein in failing RV and LV preparations. **a** Right ventricular myofilament protein phosphorylation in CHF ($n=10$) normalized to CON ($n=5$). **b** Left ventricular myofilament protein phosphorylation in CHF ($n=4$) normalized to CON ($n=5$). **c** Interventricular comparison of failing RV ($n=4$) and LV (6) myofilament protein phosphorylation. * $P < 0.05$, considered significant versus CON-RV, CON-LV, or CHF-RV. *Tn-P* denotes phosphorylated cardiac Tn. *Tn-WT* denotes bacterially expressed wild-type cardiac Tn (unphosphorylated)

Fig. 7.

Western blots illustrating site-specific cTnI (Ser 23, 24) and cTnT (Thr 206, 279) protein phosphorylation. *Histograms* summarize quantity of phosphorylated cTnI (Ser 23, 24) and cTnT (Thr 206, 279). **a** Right ventricular myofilament protein phosphorylation in CHF ($n=7$) normalized to CON ($n=4$). **b** Left ventricular myofilament protein phosphorylation in CHF ($n=7$) normalized to CON ($n=4$). **c** Interventricular comparison of failing RV ($n=3$) and LV ($n=9$) myofilament protein phosphorylation. * $P<0.05$, considered significant versus CON-RV, CON-LV, or CHF-RV

Table 1

Noninvasive and invasive assessment of ventricular structure and function

	CON	CHF
Echocardiography	<i>n</i> =10	<i>n</i> =10
HR (beats/min)	328±29	334±28
LVEF (%)	72.2±2.4	33.2±5.6 [*]
LVDd (cm)	0.62±0.04	1.01±0.06 [*]
LVSd (cm)	0.30±0.03	0.79±0.07 [*]
FS (%)	52.9±2.9	23.1±2.8 [*]
Vcf (circ/s)	8.3±0.9	3.2±0.4 [*]
LAD (cm)	0.42±0.02	0.62±0.06 [*]
Invasive hemodynamics	<i>n</i> =9	<i>n</i> =12
LVESP (mmHg)	120.9±6.3	108.4±4.3
LVESV (μL)	88.4±8.7	115.9±5.9 ^{**}
LVEDP (mmHg)	4.2±0.9	16.4±3.4 ^{**}
LVEDV (μL)	103.7±9.3	125.9±6.0 ^{**}
Ea (mmHg/μL)	11.2±2.6	6.2±0.42 ^{**}
+dP/dT (mmHg/s)	8,243±521	5,565±527 ^{**}
−dP/dT (mmHg/s)	−7,924±472	−4,309±360 [*]

Data are illustrated as mean±SEM

HR heart rate, LVEF left ventricular ejection fraction, LVDd left ventricular diastolic dimension, LVSd left ventricular systolic dimension, FS fractional shortening, Vcf velocity of circumferential shortening, LAD left atrial dimension, LVESP left ventricular end-systolic pressure, LVESV left ventricular end-systolic volume, LVEDP left ventricular end-diastolic pressure, LVEDV left ventricular end-diastolic volume, Ea arterial elastance, +dP/dT first derivative of the rate of pressure rise, −dP/dT first derivative of the rate of pressure decline

^{*} $P < 0.0001$ or

^{**} $P < 0.05$ relative to control (CON) defined as significant

Table 2

Right and left ventricular myocyte morphology and function

	CON-RV (n=22 cells; 12 ventricles)	CON-LV (n=36 cells; 9 ventricles)	LVH-RV (n=8 cells; 5 ventricles)	LVH-LV (n=19 cells; 7 ventricles)	CHF-RV (n=23 cells; 10 ventricles)	CHF-LV (n=25 cells; 12 ventricles)
F_{max} (mN/mm ²)	28.1±1.7	26.7±1.3	15.5±2.6*	12.2±0.9*	14.9±1.0*	13.3±0.8*
EC ₅₀ (μmol/L)	1.77±0.01	1.36±0.06**	1.71±0.14	2.16±0.20*,**	1.59±0.08	2.42±0.18*,**
Hill coefficient	4.05±0.17	3.74±0.23	3.97±0.40	3.33±0.22	4.04±0.21	3.56±0.21
F_{abs} (mg)	0.87±0.07	1.43±0.10**	1.09±0.11	1.03±0.08*	1.00±0.08	1.12±0.07*
Cell length (μm)	140±4.2	141±3.1	157±5.7*	153±5.5*	154±3.6*	154±2.7*
Cell width (μm)	22±1.4	28±1.4**	31.2±1.8*	35.1±1.2*	32.1±1.0*	36.8±1.3*
Cell height (μm)	14.6±0.81	18.3±0.83**	20.3±1.4*	22±1.1*	19.8±0.7*	22.9±0.76*
Cell CSA (μm ²)/10 ²	3.8±0.36	5.6±0.5**	6.5±0.83*	8.2±0.6*	6.3±0.41*	8.7±0.6*
Cell volume (μm ³)/10 ³	47.2±5.1	81.2±8.9**	92.5±15.4*	133±10.3*	91±6.5*	128±8.6*

Number of cells and ventricles utilized to obtain the data are indicated in parentheses. Data are expressed as mean±SEM. F_{max} represents the Ca^{2+} -saturated maximal force. EC₅₀ indicates the $[Ca^{2+}]$ at 50% of F_{max} and is the myofilament Ca^{2+} -sensitivity index. Myofilament cooperativity is given by the Hill coefficient. F_{abs} represents the maximal cellular force output under Ca^{2+} saturating conditions. Note comparisons are across treatment groups (i.e., CON-RV, LVH-RV, CHF-RV, etc.) and ventricles within treatment groups (i.e. CON-RV, CON-LV, etc.)

* $P<0.05$, considered significant versus respective CON-RV or CON-LV preparation;

** $P<0.05$, considered significant relative to CON-RV, LVH-RV, or CHF-RV