Augmented Phosphorylation of Cardiac Troponin I in Hypertensive Heart Failure

Xintong Dong†, C. Amelia Sumandea††, Yi-Chen Chen†, Mary L. Garcia-Cazarin††, Jiang Zhang†, C. William Balke‡, Marius P. Sumandea††,* Ying Ge†§*

From the †Human Proteomics Program, §Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, 53706, ††Department of Physiology, University of Kentucky, Lexington, KY 40536, ‡Clinical and Translational Science Institute and the Department of Medicine, University of California San Francisco, CA 94143

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*Address correspondence to: Ying Ge, PhD, 1300 University Ave., SMI 130, Madison, WI, 53706. E-mail: ge2@wisc.edu, Tel: 608-263-9212, Fax: 608-265-5512. Marius P. Sumandea, PhD, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 56285, E-mail: sumandea_marius_p@lilly.com

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Background: Phosphorylation of cardiac troponin I (cTnI) is critical in modulating contractility. cTnI phosphorylation is hyperphosphorylated at Ser22/23 and Ser42/44 in spontaneously hypertensive rat of heart failure.

Results: cTnI phosphorylation is hyperphosphorylated at Ser22/23 and Ser42/44 in spontaneously hypertensive rat of heart failure.

Conclusion: The augmented phosphorylation of cTnI in hypertensive heart failure is correlated with elevated protein levels of PKC-α and –δ.

Significance: This is the first in vivo evidence of PKC-specific phosphorylation of cTnI in an animal model of hypertensive heart failure.

SUMMARY

Altered cardiac myofilaments’ response to activating Ca\(^{2+}\) is a hallmark of human heart failure. Phosphorylation of cardiac troponin I (cTnI) is critical in modulating contractility and Ca\(^{2+}\) sensitivity of cardiac muscle. cTnI can be phosphorylated by protein kinase A at Ser22/23 and protein kinase C (PKC) at Ser22/23, Ser42/44, and Thr143. While the functional significance of Ser22/23 phosphorylation is well understood, the role of other cTnI phosphorylation sites in the regulation of cardiac contractility remains a topic of intense debate, in part, due to the lack of evidence of in vivo phosphorylation. In this study, we utilized top-down high resolution mass spectrometry (MS) combined with immunoaffinity chromatography to quantitatively determine the cTnI phosphorylation changes in spontaneously hypertensive rat (SHR) model of hypertensive heart disease and failure. Our data indicate that cTnI is hyperphosphorylated in the failing SHR myocardium compared with age-matched normotensive Wistar-Kyoto rats. The top-down electron capture dissociation MS unambiguously localized augmented phosphorylation sites to Ser22/23 and Ser42/44 in SHR. Enhanced Ser22/23 phosphorylation was verified by immunoblotting with phospho-specific antibodies. Immunoblot analysis also revealed upregulation of PKC-α and –δ, decreased PKC-ε, but no changes in PKA or PKC-β levels in the SHR myocardium. This provides direct evidence of in vivo phosphorylation of cTnI-Ser22/44 (PKC-specific) sites in an animal model of heart failure, supporting the hypothesis that PKC phosphorylation of cTnI may be maladaptive and potentially associated with cardiac dysfunction.

Heart disease is the leading cause of morbidity and mortality in industrialized societies and is becoming a worldwide epidemic (1-2). The molecular and cellular mechanisms underlying heart failure are complex and not fully understood (2-3). Cardiac troponin I (cTnI), the inhibitory subunit of cardiac troponin complex, is a key
element in the Ca\textsuperscript{2+}-dependent regulation of contraction and relaxation of cardiac muscle (4-5). Since its identification in 1972 by Stull and others (6-7), it has been widely recognized that phosphorylation of cTnI changes its conformation and modulates cardiac contractility (5,8-9). Altered cTnI phosphorylation status has been proposed as an underlying mechanism in heart failure (10-12).

Cytoskeletal protein cTnI is phosphorylated by various protein kinases, most notably protein kinase A (PKA) and protein kinase C (PKC) (8,13-19). Extensive studies have demonstrated that PKA phosphorylates cTnI at Ser 22/23, generating the “fight or flight” response to β-adrenergic stimulation (8,20). Phosphorylation at these sites reduces myocardial Ca\textsuperscript{2+} sensitivity and increases cross-bridge cycling rate (8). PKC can phosphorylate cTnI at Ser22/23, Ser42/44 and Thr143 (also known as Ser23/24, Ser43/45 and Thr144 if counting the N-terminal methionine) following activation of G-protein coupled receptors by angiotension II (AngII), endothelin-1 and the α-adrenergic agonist phenylephrine (8,21). Ser42/44 and Thr143 are traditionally viewed as PKC-specific phosphorylation sites. Phosphorylation of Ser42/44 (sites common for all PKC isozymes especially PKC-α) reduces the maximum Ca\textsuperscript{2+} activated force, Mg-ATPase rate and cross-bridge cycling rate (8,16,22). Recent studies suggest that Thr143 is a good in vitro substrate for PKC-β and Tyr-phosphorylated PKC-δ (16,19,23-24). PKC isozymes α/β/δ also cross-phosphorylate Ser22/23 (14,16,19,24). Upregulation of PKC-α and δ are correlated with cardiac dysfunction whereas PKC-ε seems to have a cardioprotective role (3,25-26). While the functional significance of the PKA phosphorylation sites has been thoroughly investigated, the PKC phosphorylation sites in cTnI and their roles in regulation of patho-physiological cardiac function remain controversial, in part, due to the lack of in vivo phosphorylation evidence (27).

Top-down mass spectrometry (MS) is a powerful technique for comprehensive analysis of protein post-translational modifications (PTMs) since it can universally detect all existing modifications without a priori knowledge and accurately map PTM sites with full sequence coverage (28-39). This top-down MS method eliminates the need for proteolytic digestion. Instead, it analyzes whole proteins directly in the mass spectrometer to obtain accurate molecular weight measurements of all forms of the protein in the first step. Subsequently, the protein’s modified form can be isolated in the mass spectrometer (like “gas-phase” purification) and fragmented by various tandem MS (MS/MS) techniques for reliable mapping of the PTM sites. Electron capture dissociation (ECD), a non-ergodic MS/MS technique, can preserve labile PTMs during its fragmentation process and is, thus, especially suited for studying protein phosphorylation (32-34,40). We have extensively studied the basal phosphorylation level of cTnI purified from healthy human and animal heart tissues using such a top-down approach and unambiguously identified Ser22/23 as the basally phosphorylated sites in human, monkey, pig, rat, and mouse cTnI (32-33,41-42). Hyperphosphorylation of Ser42/44 and Thr143 have not been identified in healthy hearts with normal cardiac function. Thus, a comprehensive study of endogenous cTnI modifications in animal models of heart failure is imperative to our understanding of protein kinase-dependent regulation of cardiac contractile dysfunction.

Herein, we combined top-down high resolution MS/MS, affinity purification, and Western blot analysis to study the phosphorylation of cTnI in SHR. Since its development from Wistar-Kyoto rats (WKY) in 1963 (43), SHR has been the most extensively studied model of hypertension-induced heart failure (43-44). Similar to patients with hypertension, SHR animals progress from persistent hypertension (around 2 months of age) to stable compensated hypertrophy (around 6 months of age) to cardiac dysfunction and heart failure (around 18 months of age) (45). The transition from compensated hypertrophy to heart failure is accompanied by marked changes in cardiac function associated with altered mechanical properties of cardiac myofilaments. In this study, we have thoroughly characterized the PTMs of cTnI from SHR and WKY hearts. Quantitative MS and immunoblot analysis showed augmented phosphorylation of cTnI in SHR. The top-down ECD MS/MS unambiguously mapped the up-regulated phosphorylation sites to Ser22/23 and Ser42/44. Additionally, immunoblot analysis also revealed
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up-regulation of PKC-α and δ, decreased PKC-ε, but no changes in PKA or PKC-β levels in the failing SHR myocardium. Our data provide direct evidence of in vivo hyper-phosphorylation of cTnI-Ser42/44 sites in an animal model of hypertensive heart failure, supporting the hypothesis that PKC phosphorylation of cTnI may be maladaptive and potentially associated with cardiac dysfunction. To our knowledge this is the first in vivo evidence documenting phosphorylation of cTnI-Ser42/44 in a pathologic condition.

EXPERIMENTAL PROCEDURES

Materials— All reagents were obtained from Sigma Chemical Co. (St Louis, MO) unless noted otherwise. Protease inhibitor cocktail was purchased from Roche Diagnostics Corporation (Indianapolis, IN). All solutions were prepared in Milli-Q water (Millipore Corp., Billerica, MA).

Animals and Tissue Preparation— Male SHR and WKY rats were purchased from Charles River (Wilmington, MA) or Harlan (Indianapolis, IN) animal research laboratories. Blood pressure was monitored weekly in non-anesthetized animals by the tail-cuff method. All animals were handled in accordance with the guidelines of the University of Kentucky Animal Care Committee. Left ventricular tissues were prepared from hearts of 5 week-old and 80 week-old SHR and age-matched WKY controls. SHR and WKY hearts were quickly removed under deep anesthesia (sodium pentobarbital, 100 mg/kg IP) and rinsed free of blood in ice-cold saline (0.9% NaCl) or Tyrodes buffer. The tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Western Blot Analysis— Male SHR and WKY rats were purchased from Charles River (Wilmington, MA) or Harlan (Indianapolis, IN) animal research laboratories. Blood pressure was monitored weekly in non-anesthetized animals by the tail-cuff method. All animals were handled in accordance with the guidelines of the University of Kentucky Animal Care Committee. Left ventricular tissues were prepared from hearts of 5 week-old and 80 week-old SHR and age-matched WKY controls. SHR and WKY hearts were quickly removed under deep anesthesia (sodium pentobarbital, 100 mg/kg IP) and rinsed free of blood in ice-cold saline (0.9% NaCl) or Tyrodes buffer. The tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Immunoaffinity Chromatography— The cTn complexes were purified from left ventricles of individual rat hearts by immunoaffinity chromatography as previously described (10,33,41,48) with minor modifications (42,49). The entire purification process was conducted at 4°C to minimize the enzymatic activity and preserve the endogenous state. The cTn complex was eluted from CNBr-activated Sepharose CL-4B conjugated with an anti-troponin I monoclonal antibody (MF4, Hytest, Finland) with 100 mM glycine HCl (pH 2). A total of 6-7 eluted samples with 400 mL each were collected into 1.5 mL microcentrifuge tubes containing 40 mL MOPS (pH 9). The flow-through, wash and all elutions were analyzed on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for an estimation of concentration before desalting.

Top-down Mass Spectrometry— Purified cTn complexes were desalted with offline reverse phase C18 protein microtrap (Michrom Bioresources Inc., CA, USA) and eluted with 1% acetic acid in 30:70, 50:50 and 75:25 methanol:water elution buffer. The sample was then introduced to a linear trap/Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Thermo Scientific, Bremen, Germany) by an automated nano electrospray ionization (ESI) source (Triversa NanoMate, Advion BioSciences, Ithaca, NY, USA) as described previously (33,42). 2% - 3% electron energy and 55 ms duration time were used for ECD. Approximately 2000 scans with 2 microscans were averaged for each ECD spectrum to ensure data quality.
All MS and MS/MS data were analyzed and assigned to rat cTnI sequence (Swiss-Prot P23693, TNNI3_RAT, http://www.uniprot.org/uniprot/P23693) using in-house developed Ion Assignment software. The signal to noise threshold was set at 3 and fit factor at 40%. The tolerance for mass error was 10 ppm for precursor ions and 20 ppm for fragment ions, respectively. All reported mass values are most abundant masses. Allowances were made for PTMs such as removal of N-terminus methionine, acetylation and phosphorylation. The mass lists generated for ECD analysis were manually validated and assigned to fragments with or without modifications.

Quantitative analysis was performed as described previously (32-33,41-42). Briefly, the integrated peak heights of the top five isotopomers were used to calculate the relative abundance of each observed protein or fragment ion. The percentage of the total phosphorylated cTnI species (%P_{total}) is defined as the summed abundances of all phosphorylated (including both mono-, bis-, and tris-phosphorylated) cTnI species over the summed abundances of the entire cTnI population. The percentage of the mono- (%P_{mono}) and bis-(%P_{bis}) cTnI species is defined as the summed abundance of mono- (p_cTnI) and bis-phosphorylated cTnI (pp_cTnI) species over summed abundances of the entire cTnI population, respectively. To calculate the quantitative value of moles Pi/moles cTnI, \( \%P_{total} = \%P_{mono} + 2 \times \%P_{bis} + 3 \times \%P_{tris} \). Samples were run in triplicates to ensure technical consistency and averages were taken for statistical analysis. The percentages of phosphorylation were analyzed and plotted using Prism 5.0 (GraphPad Software, Inc., CA, USA).

**Statistical Analysis**— Data are expressed as mean ± SEM. Student’s t-tests were performed between group comparisons to evaluate statistical significance of variance. Differences among means were considered significant at \( p < 0.05 \).

**RESULTS**

**Characterization of Spontaneously Hypertensive Heart Failure Rat**— SHR animals develop high blood pressure early on (approximately 8-10 weeks) and remain hypertensive throughout their lifespan. At approximately 80 weeks, SHRs (referred to as SHR-HF hereafter) showed significantly higher systolic blood pressure (BP) compared with normotensive WKY (Fig. 1A). SHR-HFs also demonstrated progressive increase in heart weight (HW) and a decrease in body weight (BW) compared with age matched WKY. The ratio of HW/BW is significantly higher in SHR than that of age-matched WKY, indicating severe hypertrophy (Fig. 1B).

**SDS-PAGE and High Resolution MS Analysis of cTnI from WKY and SHR**— We employed immunoaffinity chromatography for the purification of cTn complex from both WKY and SHR-HF left ventricular tissue samples. Representative SDS-PAGE of the affinity purification process is shown in Fig. S1. As the three subunits of cTn strongly interact with each other, cTnT and cTnC co-elute with cTnI that binds tightly to the anti-cTnI antibody. The three bands at approximately 37, 26, and 18 kDa correspond to cTnT, cTnI and cTnC, respectively.

In parallel to SDS-PAGE analysis, the affinity purified cTn heterotrimer was separated with offline reverse-phase protein chromatography and analyzed by top-down MS. MS spectra of cTnI from the WKY and SHR-HF groups exhibit large difference in the phosphorylation level (Fig. 2). In WKY rats, cTnI is present in three forms: un-, mono- and bis-phosphorylated, with mass increment of 80 Da (the mass of added phosphate, HPO_4^2-) between each other. The most abundant mass of 24069.97 Da, matches well with the un-phosphorylated cTnI based on the rat cTnI sequence (Swiss-Prot P23693, TNNI3_RAT) with the removal of initial methionine and N-terminal acetylation (Calc'd: 24069.73 Da). The other two masses, 24149.93 and 24229.87 Da, are consistent with mono- and bis-phosphorylated cTnI (Calc'd: 24149.69 and 24229.66 Da), respectively. Evidently, the majority of cTnI exists as un-phosphorylated in WKY rats. Tris-phosphorylated cTnI is not detected in WKY myocardium (which is estimated to be <0.1 % of the total cTnI population based on the signal intensity at the expected position of tris-phosphorylated cTnI over the summed intensity of all cTnI protein forms). In contrast, SHR-HF exhibits significant hyperphosphorylation of cTnI. The relative intensity of bis-phosphorylated cTnI is significantly increased in SHR-HF compared with WKY. Tris-phosphorylated cTnI, which has an experimental molecular weight of 24309.85 Da...
and is 80 Da larger than the bis-phosphorylated protein, was confidently detected in SHR-HF. The single amino acid polymorphism of Ser7->Ala7 reported in our previous study in cTnI purified from Sprague-Dawley rats (48) was not detected in WKY nor SHR hearts.

Top-down Quantitative MS Analysis— To satisfy statistical requirements, we have systematically analyzed cTnI purified from 7 WKY and 7 SHR-HF hearts. Three technical replicates were performed for each sample to ensure data reproducibility and consistency. cTnI from all SHR-HF hearts show consistently higher bis-phosphorylation than WKY. Percentages of mono-, bis- and total phosphorylation from WKY and SHR are shown in Fig. 3. The percentages of mono-phosphorylated cTnI (%P\textsubscript{mono}) are comparable between WKY and SHR (WKY 34.3±7.6%, SHR 37.5±4.9%, student’s t-test P'=0.1825), whereas the percentages of the bis-phosphorylated form (%P\textsubscript{bis}) differ drastically between the two groups (WKY 7.2±6.3%, SHR 31.5±8.3%, student’s t-test P<0.001). The tris-phosphorylated form is only present in SHR (3.7±2.4%). The percentage of mono-, bis- and tris-phosphorylated cTnI were added up to calculate total amount of phosphorylated cTnI (%P\textsubscript{total}). The difference is of high statistical significance (WKY 41.5±11.3%, SHR 72.7±7.2%, student’s t-test P<0.001) and shows that SHR cTnIs are hyperphosphorylated compared with WKY.

Localization of cTnI Phosphorylation Sites by ECD— In order to identify the augmented phosphorylation sites and assess the order of phosphorylation, single charge state of monophosphorylated cTnI of WKY, mono- and bis-phosphorylated cTnI of SHR-HF were individually isolated in the mass spectrometer by "gas-phase" purification (Fig. S2). The precursor ions were then dissociated by ECD and generated a series of fragmented product ions. The summary of three ECD datasets for mono-phosphorylated WKY cTnI, mono-phosphorylated SHR-HF cTnI and bis-phosphorylated SHR-HF cTnI indicate full sequence coverage (Fig. 4). Overall, the total numbers of ions generated for monophosphorylated WKY cTnI were 31 e ions and 63 z\textsuperscript{*} ions (Fig. 4A); 64 e ions and 85 z\textsuperscript{*} ions for mono-phosphorylated SHR-HF cTnI (Fig. 4B); and 49 e ions and 78 z\textsuperscript{*} ions for bis-phosphorylated SHR cTnI (Fig. 4C). Note that e ions counts from the N-terminus and z\textsuperscript{*} ions from the C-terminus (e.g., e\textsubscript{21} covers the first 21 amino acids from the N-terminus (residues 1-21) and z\textsuperscript{*}\textsubscript{21} covers the first 21 amino acids from the C-terminus (residues 190-210)).

Fig. S3 displays some key ions used for mapping the phosphorylation sites. The top panels (Fig. S3, A1-H1) show fragment ions from mono-phosphorylated cTnI of WKY and the lower two panels (Fig. S3, A2-H2, A3-H3) show fragments from mono- and bis-phosphorylated cTnI of SHR-HF, respectively. In mono-phosphorylated WKY spectra, no ions before e\textsubscript{21} are phosphorylated (Fig. 4A and Fig. S3, A1). e\textsubscript{22} remains un-phosphorylated (Fig. S3, B1) whereas all ions after e\textsubscript{23} are detected in their mono-phosphorylated forms (Fig. S3, C1–H1). These results suggest that Ser23 is the primary phosphorylation site in WKY mono-phosphorylated cTnI.

We analyzed the ECD spectra of SHR-HF mono- and bis-phosphorylated cTnI (Fig. 4 B,C). Similar to that of WKY, e ions before e\textsubscript{21} are exclusively un-phosphorylated in SHR mono-phosphorylated cTnI (Fig. 4B, and Fig. S3, A2). e\textsubscript{22} ion was also detected in its un-phosphorylated form (Fig. S3, B2) but only mono-phosphorylated e ions were observed after e\textsubscript{23} (Fig. S3, C2-H2), which suggests Ser23 is the primary phosphorylation site in mono-phosphorylated SHR-HF cTnI. In the ECD of SHR bis-phosphorylated cTnI spectra, c\textsubscript{22} is majorly mono-phosphorylated, with about 35% in the un-phosphorylated form. This suggests that there are other potential phosphorylation sites beyond Ser22/23. Consistently, ions between c\textsubscript{23} and c\textsubscript{41} in these spectra exhibit around 26-36% (31±4%) mono-phosphorylated signal (Fig. 4C, Fig. S3, C3-E3). On the other hand, all fragments larger than c\textsubscript{41} (i.e. c\textsubscript{46}, c\textsubscript{48}, and c\textsubscript{67}) (Fig. S3, F3-H3) show bis-phosphorylated forms exclusively and monophosphorylated peaks were not positively detected. As shown in Fig. S2, the isolation spectra were clean so these signals should come from the bis-phosphorylated protein. Collectively, these results indicate that there are phosphorylation sites between the 41st and 46th amino acids. In this region, Ser42 and Ser44 are the only possible sites for phosphorylation and are also well known targets of PKC.
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In summary, top-down ECD MS identified Ser23 as the phosphorylation site in monophosphorylated cTnI. Ser22/23 is also identified as the major phosphorylation sites in the bisphosphorylated cTnI. However, phosphorylation is not exclusive to these two cTnI sites. Ser42/44 has also been detected as the minor phosphorylation sites in SHR-HF. The fact that Ser42/44 was only phosphorylated in bisphosphorylated cTnI but not in monophosphorylated cTnI suggests a possible interdependency between Ser22/23 and Ser42/44.

Analysis of Site-Specific cTnI Phosphorylation by Immunoblot—We employed immunoblotting to confirm the changes in specific phosphorylation sites in cTnI from SHR and WKY. Analysis of site-specific phosphorylation of cTnI-Ser22/23 in cardiac myofibrils isolated from SHR-HF hearts showed a significantly higher level of phosphorylation compared with age-matched WKY (whereas no significant changes were observed in the normotensive 5 week-old SHR and WKY hearts) (Fig. 5A). Representative immunoblots show reactivity of anti-cTnI-Ser22/23 phosphorylation-specific antibodies with freshly isolated cardiac myofibrils from SHR-HF animals (Fig. 5B). These data confirmed our MS result that Ser22/23 phosphorylation of cTnI is increased in failing SHR hearts. At an intermediate age (12-13 weeks old) where the animals experience sustained hypertension, Boknik et al. observe augmented cTnI phosphorylation at the N-terminal Ser22/23 (50). The attempt to confirm Ser42/44 phosphorylation in SHR-HF hearts was hampered by poor specificity and reactivity of the anti-cTnI-Ser42/44 phosphorylation specific antibodies (Abcam).

Characterization of PKA and PKC Expression Levels in SHR vs. WKY—Since cTnI Ser22/23 is the prototypical PKA substrate, we investigated whether PKA expression levels had changed in SHR and WKY hearts. Both PKA types I and II show no significant changes in SHR-HF hearts compared with age-matched WKY (Fig. 6). Our data suggest that the augmented phosphorylation at Ser22/23 of cTnI is not directly related to PKA levels.

Ser22/23 can also be phosphorylated by PKC–α/β/ε/δ (14,16,19,24) and Ser42/44 is known to be the substrate for PKC–α/ε (8,16,22). Therefore, we used immunoblotting to investigate the expression levels of PKC–α/β/ε/δ in SHR-HF and WKY. Anti-PKC isoform-specific antibodies showed amplified levels of PKC–α and PKC–δ, reduced expression level of PKC–ε, but no change in PKC–β in the SHR-HF hearts compared with age-matched WKY (Fig. 7). It is plausible that the augmented phosphorylation of cTnI may be related to the upregulation of PKC–α/δ.

DISCUSSION

Hyper-phosphorylation of cTnI in SHR—We have demonstrated significant increases in cTnI phosphorylation levels in the failing SHR myocardium (80 weeks old) compared with WKY. cTnI from SHR-HF hearts is present in highly abundant mono- and bis-phosphorylated forms and the detection of tris-phosphorylation in SHR-HF indicates the presence of at least 3 phosphorylation sites in SHR-HF (Fig. 2). Top-down ECD MS unambiguously mapped the cTnI hyperphosphorylation sites to Ser22/23 and Ser42/44.

Ser22/23 are located on the N-terminal extension that is unique to the cardiac form of TnI and have been shown to be the only sites basally phosphorylated in healthy animals and human myocardium (33,41-42,48). cTnI–Ser22/23 phosphorylation dampens myofilament responsiveness to activating Ca\(^{2+}\), enhances the rate of relaxation, augments cross-bridge cycling, and accelerates unloaded shortening velocity (51-53). Immunoblot analysis showed no significant changes in PKA levels between SHR and WKY. Therefore, we hypothesized that augmented phosphorylation of cTnI–Ser22/23 could be due to upregulation of the PKC family of kinases. cTnI–Ser22/23 phosphorylation is ascribed to various PKC isoforms (α, β, δ) or PKC–activated enzymes (8). Our immunoblot data with anti-PKC isoform specific antibodies revealed that protein expression of PKC–α/δ is up-regulated whereas PKC–β remains unchanged and PKC–ε is down-regulated in SHR-HF compared with age-matched WKY. These results suggest that augmented phosphorylation of cTnI–Ser22/23 is most likely due to up-regulation of PKC–α/δ at the myofilaments.

Ser42/44, traditionally viewed as PKC–specific phosphorylation sites, are the common phosphorylation sites for all PKC
isozymes and are known to be preferably regulated by PKC-α (16,22). Ser42/44 are located in the “anchor” region near the N-terminus of cTnI that directly interacts with the C-terminal lobe of cTnC (4,9). The functional effects of Ser42/44 have been extensively studied by both in vitro assays in reconstituted detergent-skinned fiber bundles in which the endogenous cTnI was replaced with specific cTnI mutants, and in vivo in transgenic animal models in which Ser42/44 were rendered unphosphorylatable by mutation to alanine (cTnI-S42A/S44A) or constitutively phosphorylated (i.e. pseudo-phosphorylated) by mutation to glutamic acid (cTnI-S42E/S45E/T144E) (8,15-16,54-58). In vitro reconstituted skinned fiber preparations show Ser42/44 phosphorylation decreases maximal actomyosin Mg-ATPase, Ca\(^{2+}\)-activated force, cross-bridge cycling rate, and Ca\(^{2+}\) sensitivity of force development (8,15-16). These effects may increase the cost of contraction and contribute to the loss of mechanical function and eventually could lead to heart failure (11,16,54). Roman et al. showed that preventing Ser42/44 phosphorylation had beneficial, positive inotropic and lusitropic effects on the heart (59). Scruggs et al. generated a transgenic mouse line by crossing a PKC-ε over-expressing mouse with the cTnI-S43A/S45A and demonstrated that hyperphosphorylation of the Ser42/44 on cTnI is an important contributor to left ventricular dysfunction (56). In addition, Kirk et al. suggests that a modest increase (approximate to 7%) in cTnI pseudo-phosphorylation at Ser42/44 (together with T144) significantly reduced cardiac muscle contractile function (60). Collectively, these studies indicate that phosphorylation of cTnI by PKC has a detrimental effect on myocardial contractile ability whereas inhibition of PKC-dependent phosphorylation at Ser42/44 is beneficial and improves cardiac contractility (59).

While PKA-dependent phosphorylation of Ser22/23 sites is taken as fact, PKC-dependent phosphorylation of Ser42/44, until now, was somewhat controversial, mainly due to the lack of in vivo evidence (27,61). Our previous studies using healthy animal and human heart tissues only detected Ser22/23 phosphorylation in cTnI (33,41-42,48). Moreover, the cTnI phosphorylation level is significantly lower and Ca\(^{2+}\) sensitivity is higher in skinned myocytes isolated from explanted human hearts compared to controls (10,61-62). Consistently, our recent report employed top-down MS also revealed nearly abolished phosphorylation level of cTnI in failing human hearts in comparison to controls with normal cardiac function (49). These findings raised the question whether the other phosphorylation sites such as Ser42/44 are ever phosphorylated in vivo and are of any significance pertaining to pathologies in human hearts (27). Remarkably, in the present study we provide the direct evidence that Ser42/44 phosphorylation is indeed present in vivo, in the myocardium of rats with hypertensive heart failure, suggesting a potentially important role of PKC-mediated phosphorylation in the control of the integrated function of cardiac sarcomeres in certain pathological conditions.

We reason that the discrepancy between the human donor/failing hearts and WKY/SHR-HF hearts can be attributable to species differences (human vs. rat), and more importantly, different disease etiologies. The human heart failure samples are from end-stage ICM/DCM or coronary artery diseases (10,49,61-62) whereas SHR-HF samples studied here are purely from hypertension-induced heart failure. Although direct analysis of human heart samples are ideal since the measurements are closest to human cardiac disorders, admittedly, it is challenging to study the disease mechanisms from human samples due to the lack of adequate and proper controls and complication with multiple confounders (heterogeneous disease etiologies associated with co-morbidities and extensive pharmacological treatment especially for end-stage heart failure patient) (27,63). In contrast, the animal models offer great advantages including the ability to precisely control and define disease etiology and stages as well as greatly reduced complexity and heterogeneity. The similarities in chronic stress responses in the animal and human systems suggest that much can be learned from the well-controlled animal disease models with the proper consideration of the distinctions between animal and human hearts (64). Conceivably, the findings from animal models can be relevant and shed light on the complex underlying molecular mechanisms of human diseases (27).

We did not observe phosphorylation on Ser76/Thr77 in WKY/SHR samples. Ser76/77 is another potential phosphorylation site previously identified in commercially available human cTnI.
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Altered PKC Expression and Activity in heart failure—

Augmented PKC-dependent phosphorylation of Ser42/44 sites of cTnI in SHR-HF, suggests a dysregulation of the PKC signaling pathway may have an important role in failing myocardium. The PKC family contains at least twelve Ser/Thr kinases (65). PKC isoforms α, β, δ, and ε are the most well studied among many PKC isozymes expressed in cardiomyocytes. PKC–α is highly expressed in the adult heart and its expression is elevated in the failing human heart (26) and in end stage heart failure rat myocytes (25). Cardiac expression and activation of PKC–δ is detrimental to the normal cardiac function (65-66). No changes were detected for the PKC–β expression level in the animal models of chronic heart failure (25), which is in agreement with our immunoblot data on PKC–β. Regarding PKC–ε, many studies showed that the activation of PKC–ε is cardioprotective, especially in the context of ischemic preconditioning (65). Inhibition or depletion of PKC–ε results in loss of cardioprotection, impaired cardiac function and the onset of heart failure (67).

Our immunoblot data suggest that PKC–α and δ are up-regulated (whereas PKC–ε is down-regulated and PKC–β protein expression is unchanged in SHR-HF), which contributes, at least in part, to the augmented phosphorylation in Ser22/23 and Ser42/44. Increased activity of PKC–α and PKC–δ together with compromised protection by PKC–ε appears to be associated with the disease phenotype. PKC activity is induced not only by the α1-adrenergic pathway, but also other Gq signaling pathways, most notably AngII (68). The level of angiotensin II is largely increased in SHR (69-71). Previous studies suggested that PKC regulation is altered in the end stage human heart failure (65). Inhibition of PKC phosphorylation of cTnI improves cardiac performance in vivo (59). Thus PKC is believed to be a promising therapeutic target for heart failure (65).

A valuable aspect of the SHR studies is the indication that, just like in humans, blockade of angiotensin converting enzyme (ACE) or AngII production prevents heart failure development (72). PKC inhibitors were also shown to blunt AngII-induced effects in SHR (73). Evidence from animal and human studies strongly support the idea that PKC activation plays a key role in the hypertensive/failure process both by activating signaling pathways that alter gene expression and by directly phosphorylating myofilament proteins that control cardiac function (65,74). McCarty et al. collectively referred to the involvement of PKC in SHR as the “PKC syndrome” (75). In SHR, the transition from compensated hypertrophy to failure is accompanied by marked changes in cardiac function, which are associated with altered active and passive mechanical properties of myocardial tissue resulting in a decreased force of contraction. Studies with isolated papillary muscle preparations from SHR vs WKY (18-24 month) showed that peak active tension is depressed in SHR groups relative to WKY, whereas the peak intracellular calcium concentration did not differ between the two groups (76). However, the mechanisms responsible for this depression in function are poorly understood. We propose here a potential mechanism that involves PKC dependent phosphorylation of cTnI. PKC–dependent cTnI phosphorylation is believed to be particularly important in the development of other hypertrophy/failure syndromes since it is linked to depressed contractile function (5,27,55-56). In agreement with this scenario, our data indicate up-regulation of key PKC isoforms (α and δ) and no change in PKA levels in SHR hearts compared with WKY controls.

PKC activity is kept in balance by protein phosphatases (PPase). While the important role of PPase should be taken into account, the cross-talk between kinases and phosphatases and their sarcomeric targets are poorly understood. Boknik et al. (50) indicate that PP-1 and PP2-A expression concentration did not differ between the two groups (76). However, the mechanisms responsible for this depression in function are poorly understood. We propose here a potential mechanism that involves PKC dependent phosphorylation of cTnI. PKC–dependent cTnI phosphorylation is believed to be particularly important in the development of other hypertrophy/failure syndromes since it is linked to depressed contractile function (5,27,55-56). In agreement with this scenario, our data indicate up-regulation of key PKC isoforms (α and δ) and no change in PKA levels in SHR hearts compared with WKY controls.

Top-down ECD MS for Comprehensive Analysis of Protein Phosphorylation—

Comprehensive analysis of protein phospho-
rylation in vivo is challenging due to its complex and dynamic nature (63). Top-down MS with ECD is becoming a powerful method for studying phosphorylation in vivo (32-34,42). It measures whole proteins, providing a "bird's eye" view of all existing modifications in a protein, which can easily and reliably detect the protein phosphorylation by the mass increment of 80 Da (HPO$_3$). It also offers a relative quantification of protein phosphorylation since the physicochemical properties of large proteins are much less affected by the presence of phosphorylation group in comparison with peptides (30-35). Moreover, ECD generates fragment-ion-rich data that can be used for quantitative analysis of phosphorylated positional isomers (32). We have demonstrated the unique advantages of top-down ECD MS for comprehensive analysis of protein phosphorylation, including the global detection and quantification of all phosphorylation species simultaneously in one spectrum, precise mapping of multiple phosphorylation sites (32-34,41-42). Here we have unambiguously detected and localized all phosphorylation sites in an unbiased manner in WKY and SHR-HF cTnI by the top-down ECD MS approach. The identification of Ser42/44 is especially valuable due to the lack of high specificity and reactivity of the anti-cTnI-Ser42/44 phosphorylation specific antibodies. We have also quantitatively determined the changes in the distribution of phosphorylation between multiple targeted sites in cTnI from control and failing rat myocardium, which is of crucial importance for understanding protein phosphorylation in cardiac disease (27,63).

In summary, we have revealed augmented cTnI phosphorylation levels in SHR-HF in comparison with age-matched WKY controls using immunoaffinity chromatography, high resolution top-down MS, and Western blot analysis. We have unambiguously identified in vivo PKC-specific Ser42/44 phosphorylation in the hyperphosphorylated SHR-HF cTnI in addition to Ser22/23. Collectively, our results suggest that cTnI exists in a hyperphosphorylated state in the rat heart of hypertensive heart failure, which is attributable, in part, to alterations in PKC signaling. This study provides the first direct evidence of in vivo phosphorylation of Ser42/44 sites in cTnI in a clinically relevant animal model of hypertensive heart disease and heart failure. These results support the notion that alterations in PKC signaling may be maladaptive and associated with cardiac dysfunction in hypertensive heart failure.
REFERENCES

Augmented cTnI phosphorylation in SHR


Augmented cTnI phosphorylation in SHR


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FOOTNOTES
*To whom correspondence may be addressed: Ying Ge, Ph.D., 1300 University Ave., SMI 130, Madison, WI 53706. E-mail: ge2@wisc.edu; Tel: 608-263-9212; Fax: 608-265-5512. Marius P. Sumandea, PhD, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 56285, E-mail: sumandea_marius_p@lilly.com
1 Current address: Department of Biology, Stanford University, Stanford, CA 94305
2 Current address: Eli Lilly and Company, Indianapolis, IN 46285
}

The abbreviations used are: cTnI, cardiac troponin I; PKA, protein kinase A; PKC, protein kinase C; SHR, spontaneously hypertensive rat; SHR-HF, SHR with heart failure; WKY, Wistar-Kyoto rat; MS, mass spectrometry; PTMs, post-translational modifications; MS/MS, tandem mass spectrometry; ECD, electron capture dissociation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FTICR, Fourier transform ion cyclotron resonance; ESI, electrospray ionization; BP, blood pressure; HW, heart weight; BW, body weight; AngII, angiotensin II; ACE, angiotensin converting enzyme; SEM, standard error of the mean.
**FIGURE LEGENDS**

**Figure 1.** Characteristics of SHR-HF vs. WKY.  (A) Systolic blood pressure (BP) in SHR vs. WKY at 80 weeks.  (B) The ratio of heart weight (HW) to body weight (BW) of SHR vs. WKY.

**Figure 2.** High resolution FTMS analysis of intact rat cTnI purified from WKY and SHR.  (A) Representative FTMS spectrum of cTnI (M<sup>25+</sup>) purified from age-matched WKY.  Dashed arrow indicates the expected position of tris-phosphorylated cTnI (pppcTnI) which is not observed in this spectrum.  The minor proteolytic fragment, cTnI A[16-205]K, was observed.  (B) FTMS spectrum of cTnI purified from SHR-HF.  ppcTnI was observed in this spectrum.  Circles represent theoretical isotopic abundance distribution of the isotopomer peaks.  pcTnI, monophosphorylated cTnI; ppcTnI, bisphosphorylated cTnI.  ΔMW, molecular weight difference. +Na, sodium adduct; +K, potassium adduct; +H<sub>3</sub>PO<sub>4</sub>, non-covalent phosphoric acid adduct; +HOAc, non-covalent acetic acid adduct.  Calc’d, calculated most abundant mass; Expt’l, experimental most abundant mass.

**Figure 3.** Quantification of cTnI phosphorylation in WKY and SHR-HF hearts.  The percentages of mono-phosphorylated cTnI components (%P<sub>mono</sub>) in (A); bis-phosphorylated cTnI (%P<sub>bis</sub>) in (B); and total phosphorylated cTnI (%P<sub>total</sub>) including pcTnI, ppcTnI and pppcTnI over the entire cTnI populations in (C).  Data points indicate average of triplicates.  Average and standard error of the mean (SEM) shown in the graph.  *<i>p</i> < 0.05; **<i>p</i> < 0.001.

**Figure 4.** Identification of cTnI phosphorylation sites in WKY and SHR-HF.  ECD product maps from (A) WKY mono-phosphorylated cTnI, (B) SHR-HF mono-phosphorylated cTnI, and (C) bis-phosphorylated SHR-HF cTnI.  Fragment assignments were made to DNA predicted rat cTnI sequence (Swiss-Prot P23693, TNNI3_rat) with the removal of N-terminal methionine and acetylation at the new N-terminus.  Single dot, only mono-phosphorylated ions observed.  Double dots, only bis-phosphorylated ions observed.  Star, both mono- and bis-phosphorylated ions observed.  Identified phosphorylation sites are labeled as “p”.

**Figure 5.** Differential phosphorylation of cTnI - Ser22/23 in SHR-HF and WKY.  A. Analysis of site-specific phosphorylation of cTnI-Ser22/23 in cardiac myofibrils isolated from 5 week-old (wk) and 80 wk hearts.  Data are means of the values calculated from the optical density of the bands obtained by immunoblotting normalized against pan-cTnI densities (n = 8 hearts/group).  Values marked with asterisk were deemed statistically significant (<i>p</i> < 0.05).  B. Representative immunoblots show reactivity of anti-phosphorylation specific antibodies cTnI-Ser22/23 with freshly isolated cardiac myofibrils from 80 week-old animals (n=8 hearts).  Each lane represents a distinct heart.

**Figure 6.** PKA type I and II protein levels show no significant change in SHR-HF hearts compared with age-matched WKY.  A. Representative immunoblots from two distinct WKY and SHR-HF hearts.  B. Bar graph data are means of the values calculated from the optical density of the bands obtained by immunoblotting normalized against anti-actin densities (n = 6 hearts/group).  No statistical significant difference was observed (<i>p</i> > 0.05).

**Figure 7.** Differential PKC protein levels in the SHR-HF hearts compared with age-matched WKY.  Anti-PKC isoform specific antibodies show augmentation of PKC–α and PKC–δ, decrease in PKC–ε, and no change in PKC–β, protein expression levels in SHR-HF hearts.  A. Representative immunoblots from two distinct WKY and SHR-HF hearts.  B. Bar graph data are means of the values calculated from the optical density of the bands obtained by immunoblotting normalized against anti-actin densities (n = 6 hearts/group).  *,<i>p</i> < 0.05.
Augmented cTnI phosphorylation in SHR

Figure 1

A

B

Systolic BP (mmHg)

HW/BW (mg/g)

WKY

SHR

WKY

SHR

*
Augmented cTnI phosphorylation in SHR

Figure 2

A

Calc'd: 24069.73 Da
Expt'l: 24069.97 Da

cTnI 67.7%

M 25+

963.5 963.7 963.9 964.1 m/z

Calc'd: 24149.69 Da
Expt'l: 24149.93 Da

Calc'd: 24229.66 Da
Expt'l: 24229.87 Da

B

Calc'd: 24069.73 Da
Expt'l: 24069.96 Da

cTnI 15.7%

M 25+

963.5 963.7 963.9 964.1 m/z

Calc'd: 24149.69 Da
Expt'l: 24149.95 Da

Calc'd: 24229.66 Da
Expt'l: 24229.91 Da

Calc'd: 24309.63 Da
Expt'l: 24309.85 Da

ΔMW=79.96 Da
ΔMW=79.94 Da
ΔMW=79.96 Da
ΔMW=79.94 Da

<0.1%
Augmented cTnI phosphorylation in SHR

Figure 3

A

\[
\%P_{\text{mono}}
\]

WKY  SHR

\[
\%P_{\text{bis}}
\]

WKY  SHR

\[
\%P_{\text{total}}
\]

WKY  SHR

**
Figure 4

A

1  Ā D E S S D A A G E P Q P A P A P V R R  191
41  I S A S R K L Q L K T L M L Q I A K Q E  151
61  M E R E A T E E L R R G E K G R V L S T R C  131
81  Q P L V L D L G L G F E L E L Q D L C R Q L  111
101  H A R V D K V L D E L E R Y D V E A L K V T K  91
121  N I T L E I A D L L T L Q L K I Y D L L R L G K F K  71
141  L R P L T L R R L V L R I L S A D L M M Q L A L L L G  51
201  L M L E L G L R L K L K F E I G  1

B

1  Ā D E S S D A A G E P Q P A P A P V R R  191
41  I S A S R K L Q L K T L M L Q I A K Q E  151
61  M E R E A T E E L R R G E K G R V L S T R C  131
81  Q P L V L D L G L G F E L E L Q D L C R Q L  111
121  L N L I L T L E I A D L L T L Q L K I Y L D L L R L G K F K  71
141  L R P L T L R R L V L R I L S A D L A L M M Q A L L L L L G  51
201  L M L E L G L R L K L K F E I G  1

C

1  Ā D E S S D A A G E P Q P A P A P V R R  191
41  I S A S R K L Q L K T L M L Q I A K Q E  151
61  M E R E A T E E L R R G E K G R V L S T R C  131
81  Q P L V L D L G L G F E L E L Q D L C R Q L  111
101  H A R V D K V L D E L E R Y D V E A L K V T K  91
121  N I T L E I A D L L T L Q L K I Y L D L L R L G K F K  71
141  L R P T L L R R L V L R I L S A D L A L M M Q A L L L L L G  51
201  L M L E L G L R L K L K F E I G  1

CO—NH—CHR—ECDD
Augmented cTnI phosphorylation in SHR

Figure 5

A

![Bar graph showing cTnI-Ser22/23 phosphorylation in WKY and SHR at 5 and 80 weeks.](image)

B

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR (80 wk)</th>
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</thead>
<tbody>
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<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td>pan-Tnl</td>
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</table>
Augmented cTnI phosphorylation in SHR

Figure 6
Figur 7

**A**

IB:

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<tr>
<th>Protein</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC-ε</td>
<td></td>
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<tr>
<td>PKC-δ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
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</tr>
</tbody>
</table>

**B**

Normalized O.D. (Relative Units)

- PKC-α
- PKC-β
- PKC-ε
- PKC-δ

* *
Augmented phosphorylation of cardiac troponin I in hypertensive heart failure
Xintong Dong, C. Amelia Sumandea, Yi-Chen Chen, Mary L. Garcia-Cazarin, Jiang Zhang,
C. William Balke, Marius P. Sumandea and Ying Ge

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