Heart Failure

Myosin Light Chain Phosphorylation Is Critical for Adaptation to Cardiac Stress

Sonisha A. Warren, PhD; Laura E. Briggs, BS; Huadong Zeng, PhD; Joyce Chuang, PhD; Eileen I. Chang, MS; Ryota Terada; Moyi Li, PhD; Maurice S. Swanson, PhD; Stewart H. Lecker, MD, PhD; Monte S. Willis, MD, PhD; Francis G. Spinale, MD, PhD; Julie Maupin-Furlowe, PhD; Julie R. McMullen, PhD; Richard L. Moss, PhD; Hideko Kasahara, MD, PhD

Background—Cardiac hypertrophy is a common response to circulatory or neurohumoral stressors as a mechanism to augment contractility. When the heart is under sustained stress, the hypertrophic response can evolve into decompen-
sated heart failure, although the mechanism(s) underlying this transition remain largely unknown. Because phos-
phosphorylation of cardiac myosin light chain 2 (MLC2v), bound to myosin at the head-rod junction, facilitates actin-myosin interactions and enhances contractility, we hypothesized that phosphorylation of MLC2v plays a role in the adaptation of the heart to stress. We previously identified an enzyme that predominantly phosphorylates MLC2v in cardiomyocytes, cardiac myosin light-chain kinase (cMLCK), yet the role(s) played by cMLCK in regulating cardiac function in health and disease remain to be determined.

Methods and Results—We found that pressure overload induced by transaortic constriction in wild-type mice reduced phosphorylated MLC2v levels by ≈40% and cMLCK levels by ≈85%. To examine how a reduction in cMLCK and the corresponding reduction in phosphorylated MLC2v affect function, we generated Mylk3 gene-targeted mice and transgenic mice overexpressing cMLCK specifically in cardiomyocytes. Pressure overload led to severe heart failure in cMLCK knockout mice but not in mice with cMLCK overexpression in which cMLCK protein synthesis exceeded degradation. The reduction in cMLCK protein during pressure overload was attenuated by inhibition of ubiquitin-proteasome protein degradation systems.

Conclusions—Our results suggest the novel idea that accelerated cMLCK protein turnover by the ubiquitin-proteasome system underlies the transition from compensated hypertrophy to decompen-
sated heart failure as a result of reduced phosphorylation of MLC2v. (Circulation. 2012;126:2575-2588.)

Key Words: heart failure ■ mice, transgenic ■ myocardial contraction ■ myosin light chains ■ phosphorylation

In 2006, the overall death rate from cardiovascular disease was 262.5 per 100,000, of which 1 in 8.6 deaths was due to heart failure. During failure, the heart is unable to pump sufficient blood to meet circulatory demand owing to reduced myocardial force development.1 Pressure development and ejection are achieved by shortening of the minor and long axes of the heart and twisting of the apex (torsion) resulting from differential orientation of ventricular myofibers in roughly 3 layers: inner, mid, and outer.2-5 The dynamics of cardiac contraction and relaxation are fundamentally related to actin-myosin interactions, which are initiated by Ca2+ binding to troponin C and relief of inhibition of cross-bridge binding to actin. A range of factors regulate this process and contribute to the adaptation of cardiac function to physiological or pathological stressors,6-8 in turn suggesting potential therapeutic strategies for heart failure.5-11

Clinical Perspective on p 2588

Myosin is a hexamer composed of 2 heavy chains and 2 pairs of light chains (MLC1 and MLC2) bound to the rod and neck regions of the heavy chain, respectively. Phosphorylation of MLC2v has been shown to potentiate the rate and

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From the Department of Physiology and Functional Genomics (S.A.W., L.E.B., E.I.C., R.T., H.K.), Advanced Magnetic Resonance Imaging and Spectroscopy Facility (H.Z.), and Department of Molecular Genetics and Microbiology (M.L., M.S.S.), College of Medicine, University of Florida, Gainesville; Department of Bioengineering, University of California, San Diego, La Jolla (J.C.); Department of Medicine, Beth Israel Deaconess Medical Center, Boston MA (S.H.L.); Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill (M.S.W.); Cardiovascular Translational Research Center, University of South Carolina, Columbia (F.G.S.); Department of Microbiology and Cell Science, College of Agricultural and Life Sciences, University of Florida, Gainesville (J.M.-F.); Baker IDI Heart and Diabetes Institute, Melbourne, Australia (J.R.M.); and Department of Cell and Regenerative Biology, University of Wisconsin School of Medicine and Public Health, Madison (R.L.M.).

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Correspondence to Hideko Kasahara, MD, PhD, University of Florida College of Medicine, 1600 SW Archer Rd, M540, Gainesville, FL 32610-0274. E-mail hikasahar@ufl.edu

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2575
force of cardiac contraction. Using mutant MLC2v knock-in mice in which 2 phosphorylatable serine residues were mutated to alanine (Ser14/15Ala), our recent study with others demonstrated that phosphorylation increases myosin binding and myosin lever arm stiffness and alters the kinetics of cross-bridge cycling to increase cross-bridge duty ratio (stroke time/cycle time). Furthermore, in addition to the traditional view of Ca\(^{2+}\)-dependent activation of thin filaments, phosphorylated MLC2v can facilitate this process by activating neighboring binding sites on actin. Thus, non-phosphorylatable MLC2v in mice reduced contractility, and reduced phosphorylation of MLC2v has been implicated in human heart disease, in which phosphorylation was reduced from \(\approx 30\%\) to \(\approx 40\%\) in healthy hearts to \(\approx 18\%\) of total MLC2v in failing hearts.

The predominant kinase for MLC2v, cardiac-specific myosin light-chain kinase (cMLCK), encoded by Mylk3, has been identified as a target of transcription factor Nkx2-5 and independently as a gene product that is differentially expressed in failing human hearts. Knockdown of cMLCK in neonatal cardiomyocytes and in zebrafish embryos resulted in abnormal formation of the sarcomere and depressed contraction. A recent study using mice expressing hypomorphic cMLCK confirmed that cMLCK is the predominant MLC2v kinase in the heart and is important for normal cardiac contraction in vivo.

Under physiological conditions, the level of MLC2v phosphorylation is relatively constant because of countervailing actions of cMLCK and protein phosphatase 1\(\beta\) (also called protein phosphatase 1\(\delta\)) in association with the myosin-binding phosphatase-target subunit. The level of MLC2v phosphorylation has been shown to vary through the thickness of the murine ventricular wall, being relatively low in the inner layer and high in the outer layer. This gradient has been proposed to be critical for nonuniform contractile properties of the myocytes across the wall, particularly for left ventricular (LV) torsion, the counterclockwise twisting of the LV apex, and the clockwise twisting of the base during systole when viewed from the apex. It has been thought that the gradient is formed by reduction of phosphatase activity in the outer layer. However, the existence of an MLC2v phosphorylation gradient is still controversial, and if it is present, the participation of MLC2v kinase as a determinant of the gradient remains to be clarified.

From these observations, we sought to investigate the role of cMLCK in the heart by determining its transmural expression and its functional roles through the use of loss- or gain-of-function mutations in mice.

## Methods

### Mouse Models

A conditional null allele of Mylk3 was generated by introducing loxP sites spanning exon 5, which was done through homologous recombination in embryonic stem cells. Mice heterozygous for this Mylk3 allele were bred to mice expressing the ACTB1-Cre transgene, resulting in a germline Mylk3\(^{+/}\)- allele, followed by crossbreeding to generate Mylk3\(^{+/}\)- mice having a mixed genetic background mainly with 129/Sv and C57BL/6. Transgenic mice were generated by injection of hemagglutinin-tagged full-length cMLCK cloned into an \(\alpha\)-MHC promoter plasmid (kindly provided by J. Robbins).

All animal experiments were performed with approval from the University of Florida Institutional Animal Care and Use Committee.

### Human Heart Samples

Human heart samples were obtained from the National Human Tissue Resource Center. All protocols were approved by the University of Florida Institutional Review Board.

Additional experimental procedures are described in the online-only Data Supplement.

## Results

### Regional Expression of cMLCK Protein and Phosphorylation of MLC2v in Mouse and Human Hearts

We recently identified an enzyme, cMLCK, that predominately phosphorylates MLC2v in cardiomyocytes. First, we confirmed that the regional expression of cMLCK and the extent of phosphorylation of MLC2v (pMLC2v) are almost identical in normal mouse hearts, although labeling of each exhibits nonuniform intensity across transverse tissue sections (Figure 1A and Figure 1A in the online-only Data Supplement). The staining of both was below the level of detection in the absence of cMLCK in Mylk3\(^{−/−}\) mice (described later). The specificity of the pMLC2v antibody against pMLC2v was confirmed by Western blotting (Figure II in the online-only Data Supplement).

At higher magnification, cMLCK staining was more diffuse in the cytoplasm compared with the striated staining pattern of pMLC2v (Figure 1B). Globally, expression of cMLCK and expression of pMLC2v were higher in the right ventricle than in the LV (Figure 1C and 1D). In the LV, higher levels of expression were observed in the mid to outer epicardial layers than in the inner endocardial layer at the interventricular septal wall (Figure 1C–1H). A few layers of myofibers at the surface of the endocardium highly expressed cMLCK and pMLC2v (Figure 1I–1L). These cells are Purkinje fibers, which form a ventricular conduction system marked by the presence of the gap junction protein connexin40 (Figure 1M). In human hearts, levels of cMLCK and pMLC2v were also high in Purkinje fibers that were stained for atrial natriuretic factor (Figure 1N). Notably, the antibody against connexin40 was not compatible with the paraffin sections of the human heart.

These data confirm that cMLCK is the predominant kinase for MLC2v, which is highly expressed in the mid and epicardial layers compared with the endocardial layer of the LV, consistent with a previous report, and has even greater expression in the right ventricle and Purkinje fibers.

### Reductions in cMLCK and pMLC2v After Pressure Overload

The transmural expression of cMLCK proteins and pMLC2v was quantified by Western blotting of tissue from hearts subjected to pressure overload by transverse aortic constriction (TAC) for 3 months. The endocardial layers significantly thicken after TAC, making them easier to dissect (Figure 2A). cMLCK expression and MLC2v phosphorylation were higher in the right ventricle than in the LV and were below the level of detection in the endocardial layers (Figure 2B and 2C).
study showed that ≈30% of total MLC2v was phosphorylated in normal mouse hearts. In the remaining experiments described (unless otherwise specified), cMLCK expression and pMLC2v expression were examined in the apical third of the heart to minimize variations caused by regional expression of cMLCK. Reductions in cMLCK and pMLC2v were evident as early as 1 week after TAC, by 85% and 40%, respectively, compared with control mice (Figure 2D and 2E).

One week of TAC resulted in ventricular wall thickening without apparent changes in cardiac function (%FS) or chamber diameters (Figure 2F and 2G, 1 week). Two weeks of TAC significantly reduced %FS and increased systolic dimensions compared with control values or 1 week of TAC in our experimental conditions (Figure 2F and 2G). Thus, the functional transition from compensated to decompensated cardiac hypertrophy occurred between 1 and 2 weeks of TAC, shortly after reductions in cMLCK and pMLC2v.

Deletion of Mylk3 Leads to Moderate Abnormalities in Contraction and Conduction Under Basal Conditions

Data from mice with TAC suggest that reduced cMLCK expression and MLC2v phosphorylation may be involved in both chronic and transitional (from compensated to decompensated) cardiac hypertrophy. To examine this possibility mechanistically, we first generated Mylk3 gene-targeted mice and examined whether the absence of cMLCK affects cardiac function. Germline deletion of floxed exon 5 resulted in elimination of the first coding exon of the catalytic domain and a frameshift of the subsequent downstream exons (Figure 3A and Figure III in the online-only Data Supplement). cMLCK mRNA expression was below the level of detection in Mylk3/−/− hearts by Northern blotting with a cDNA probe that recognizes exons 1 through 6 (1266 bp; Figure 3B). This result is likely attributable to a nonsense-mediated mRNA decay, with targeted cMLCK mRNA containing a prema-
Figure 2. Reduction in cardiac myosin light-chain kinase (cMLCK) in mouse hearts owing to pressure overload. A, Immunostaining of cMLCK and phosphorylated cardiac myosin light chain 2 (pMLC2v; purple) in transverse serial sections of transverse aortic constriction (TAC) heart counterstained with eosin (pink). B, Western blotting of cMLCK, total MLC2v, and GAPDH in right ventricle (RV), the entire left ventricle (LV), and the endocardial layer (endo) dissected from a TAC heart. C, Unphosphorylated MLC2v and pMLC2v in RV, LV, and papillary muscles dissected from TAC hearts examined with 2-dimensional electrophoresis followed by Western blotting with an anti-MLC antibody. D, Western blotting of cMLCK, pMLC2v, total MLC2v, and GAPDH after 1 week of TAC (lanes 6–9) and control (lanes 1–5). E, Fold difference of cMLCK, pMLC2v, and total MLC2v normalized to GAPDH with the value without TAC defined as 1. F, Representative images of sequential M-mode ultrasound of a Mylk3+/− mouse from before operation to 4 weeks of TAC. G, Echocardiographic indexes of Mylk3+/− mice before and during TAC. Time-dependent effects were not significant in the end-diastolic dimension (EDD), and hazard ratio was found with repeated measure ANOVA. %FS indicates percent LV fractional shortening; IVS, interventricular septum; and ES, end systolic. *P<0.05.
Figure 3. Moderate heart enlargement and reduced contractile function in Mylk3^{−/−} mice. A, Polymerase chain reaction genotyping demonstrates ∼1750 bp in Mylk3^{+/+} (lane 1) vs ∼800 bp in Mylk3^{−/−} (lane 2). B, Northern blotting shows that cardiac myosin light-chain
tured termination codon. In Mylk3−/− hearts, MLC2v phosphorylation was below the level of detection (Figure 3C), consistent with immunostaining in Figure 1A. Mylk3−/− mice were born at the expected mendelian ratios and survived through adulthood beyond 1.5 years of age. Mylk3−/− hearts were moderately enlarged predominantly along the long axis with increased heart weight at 3 and 6 months of age (Figure 3D and 3E). Neither cytoarchitectural disarray nor increased interstitial fibrosis was observed (Figure 3F). Cardiac contracture assessed with magnetic resonance imaging (Figure 3G and movies in the online-only Data Supplement) and echocardiography (Figure IV in the online-only Data Supplement) demonstrated a reduction in ejection fraction and increases in the volume of the LV cavity at both end systole and end diastole. Cardiac torsion was also reduced in Mylk3−/− hearts compared with control mice (Figure 3G).

Isolated ventricular cardiomyocytes from adult Mylk3−/− mice were larger than cardiomyocytes from control mice and displayed reductions in contractility and speed of relaxation with no changes in amplitude of the intracellular Ca2+ transient (Figure 3H and 3I).

In the ventricular conduction system, cMLCK and pMLC2v were present at higher levels than in the rest of the LV (Figure 1K–1N). Ambulatory telemetry ECG recordings demonstrated a small but significant prolongation of the QRS complex in Mylk3−/−/− mice compared with age- and sex-matched Mylk3+/+ controls (Figure VA–VC in the online-only Data Supplement), indicating that ventricular depolarization was prolonged in Mylk3−/− mice. This could be due, entirely or in part, to elongation of the ventricular conduction system secondary to enlargement of the heart (Figure VD in the online-only Data Supplement).

**Adaptation to Pressure Overload Requires cMLCK**

In contrast to the finding that Mylk3−/− mice showed a moderate reduction in contractility under basal conditions, 3 months of TAC resulted in profound heart failure in these mice, which was associated with increased heart size (Figure 4A), decreased survival rate (Figure 4B), and increased heart weight/tibial length (Figure 4C). Hereafter, heart weight is normalized to tibial length in reports of the results of TAC.

Swimming Exercise

Role of cMLCK in Response to 4 Weeks of Swimming Exercise

Besides the hypertrophic response to pressure overload, the heart can exhibit hypertrophic growth as an adaptation to exercise. This adaptation results in an increase in absolute force production by the ventricular wall due at least in part to an increase in the total number of actin-myosin motor units working in parallel. To understand the role of cMLCK in the response of the heart to exercise, 3 month-old Mylk3−/− and Mylk3+/+ mice were subjected to 4 weeks of swim training (Figure 5A).

Wild-type mice tolerated 4 weeks of swimming, whereas 25% of Mylk3−/− mice died during or shortly after exercise (Figure 5B). Both Mylk3+/+ and surviving Mylk3−/− mice demonstrated an increased ratio of heart weight to body weight (Figure 5C); however, exercise failed to improve cardiac contractive function, evident as a reduction in %FS in Mylk3−/−/− mice compared with control Mylk3+/+ (Figure 5D) and sedentary Mylk3−/−/− mice at a similar age from 26% to 19% (Figure IV in the online-only Data Supplement, 3 months of age). In Mylk3+/+ but not in Mylk3−/−/− mice, swimming increased pMLC2v to 39% (33% without swimming; Figure 5E) and increased the expression of cMLCK protein (Figure 5E and 5F). These results indicate that cMLCK is the predominant kinase mediating increased MLC2v phosphorylation in response to swim training.

Increased expression of fetal genes such as atrial natriuretic factor and brain natriuretic peptide is often observed in failing hearts but was not seen in sedentary Mylk3−/− mice (no swimming), consistent with moderate cardiac dysfunction without an increase in interstitial fibrosis (Figure 3F). Exercise-induced hypertrophy normally does not increase the expression of atrial natriuretic factor and brain natriuretic peptide (Figure 5G, Mylk3+/+ no swim versus swim).
Figure 4. Heart failure in Mylk3−/− mice after pressure overload. A, Representative images of hearts after 3 months of TAC. Bar = 2 mm. B, Survival analysis during 3 months of transverse aortic constriction (TAC). Several mice died shortly after the operation (1 week) and were eliminated from these studies in consideration of postoperative complications. C, Heart weight (HW)/tibial length ratio (mg/mm) after 3 months of TAC. D and E, Representative images of M-mode ultrasound and echocardiographic indexes after 3 months of TAC. F and G, Representative tracing of left ventricular pressure (LVP), dP/dt, and summarized data from hemodynamic measurements after 3 months of TAC. H, Sequential echocardiographic indexes from before operation to 4 weeks of TAC. ED indicates end-diastolic; ES, end-systolic; %FS, percent left ventricular fractional shortening; and IVS, interventricular septum. *P < 0.05.
Figure 5. Effects of cardiac myosin light-chain kinase (cMLCK) on cardiac adaptations to physiological stress. A, Representative images of hearts dissected after 4 weeks of swimming exercise. Bars=2 mm. B, Survival analysis during 4 weeks of swimming exercise. C, Heart weight/body weight ratio (HW/BW) with or without 4 weeks of swimming exercise at 4 months of age. D, Echocardiographic indexes after swimming exercise. E, Unphosphorylated and phosphorylated myosin light chain 2 (MLC2v) examined with Western blot analysis. F, Western blot analysis of cMLCK, p-MLC2v, MLC2v, and GAPDH. G, Real-time PCR analysis of ANF and BNP with or without 4 weeks of swimming exercise.
Despite the reduction in %FS in Mylk3−/− mice after exercise, there was no substantial increase in atrial natriuretic factor or brain natriuretic peptide before versus after exercise in Mylk3−/− mice.

Overexpression of cMLCK Protects Against Pressure Overload–Induced Cardiac Dysfunction

Profound heart failure in pressure-overloaded Mylk3−/− mice supports our hypothesis that reduced cMLCK expression and MLC2v phosphorylation are involved in decompensated TAC-induced cardiac hypertrophy. We further examined whether excess cMLCK protects the heart during short- and long-term pressure overloading using a second mouse model that overexpresses cMLCK under the control of an α-MHC promoter/enhancer. Among 5 transgenic lines, 3 lines expressed cMLCK relatively homogenously in the LV (data not shown) and were examined further. The level of transgenic protein expression relative to the endogenous cMLCK in nontransgenic mice ranged from 6.5-fold in transgenic line 1 (TG1) to 21-fold in TG2 and TG3 (Figure 6A), leading to a moderate increase in pMLC2v from 34% of total MLC2v in nontransgenic to 42% to 50% in the transgenics (Figure 6B). Basal cardiac function in the 3 transgenic lines was not statistically different from that in the nontransgenic mice, except for a slight reduction in end-diastolic dimensions in TG3 (Figure VIIA in the online-only Data Supplement).

One week of TAC reduced transgenic proteins expression, similar to endogenous cMLCK proteins (Figure 6C), despite endogenous cMLCK and transgenic cMLCK having different promoter/enhancers, exon-intron structures, and 3’ poly-A tails. These results suggest that the reduction in cMLCK protein by pressure overload is regulated by posttranscriptional mechanisms.

Although nontransgenic and transgenic mice were subjected to similar degrees of pressure overload (Figure 6D), the increase in heart weight (determined by the ratio of heart weight to tibial length) was attenuated in transgenics, including the TG3 line with higher cMLCK expression (Figure 6D). Attenuation was accompanied by a parallel reduction in cardiomyocyte cell size and preservation of contractility at 2 and 4 weeks after TAC (Figure VIIIB and VIIC in the online-only Data Supplement). After long-term pressure overload (3 months; similar degree in all groups; Figure VIII in the online-only Data Supplement) and reductions in both endogenous and transgenic cMLCK proteins (Figure VIIIIB in the online-only Data Supplement), cardiac function was significantly preserved in the high-expressor TG3 line compared with the nontransgenic mice with respect to contractility and chamber size (Figure 6E and 6F), interstitial fibrosis (Figure 6G), and apoptosis (Figure 6H and Figure IX in the online-only Data Supplement).

These results further support our hypothesis that elevated expression of cMLCK protein (6.5-fold–21-fold higher than endogenous) protects the heart against cardiac dysfunction during short- and long-term pressure overload.

Involvement of the Ubiquitin-Proteasome System in cMLCK Protein Degradation

To understand the potential mechanisms underlying the reduction in cMLCK protein during pressure overload, we explored the possible role of the ubiquitin-proteasome system, the major nonlysosomal pathway for intracellular protein degradation. Recently, altered activity of the ubiquitin-proteasome system has been emphasized in studies of cardiac hypertrophy. Target proteins are polyubiquitinated by concerted action of 3 enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) before degradation by the 26S proteasome composed of the 20S catalytic and 19S regulatory cores. Both synthetic and natural proteasome inhibitors block proteasome activity; Synthetic peptide aldehyde such as MG132 mimics proteasome substrates, binds to the proteasome active site, and transiently disrupts its activity. A natural inhibitor, lactacystin, covalently binds to the β subunit of the 20S unit and irreversibly and more specifically blocks its activity.

We first examined whether endogenous cMLCK expression was upregulated by 2 different proteasome inhibitors, MG132 (Figure X in the online-only Data Supplement) and lactacystin (Figure 7A). Both inhibitors increased cMLCK expression in cardiomyocytes. Next, mice were treated with lactacystin, MG132, or both MG132 and lactacystin 3 hours before the TAC operation, followed by daily injection of the inhibitors for 1 week. Compared with the 80% reduction in cMLCK protein after 1 week of TAC without proteasome inhibitors (Figure 7B, lane 1 versus 2), the addition of proteasome inhibitors attenuated the reduction of cMLCK proteins by 40% to 60% in all 3 groups and was most effective when the 2 inhibitors were used in combination (Figure 7B). In contrast to reduced cMLCK expression after TAC, the expression of MLC2v and myosin binding protein C assessed on the same membrane was unchanged with or without TAC (Figure 7C). Proteasome inhibitors increased the expression of myosin binding protein C but not MLC2v. Thus, TAC-induced reductions and partial rescue by proteasome inhibitors were observed for cMLCK but not MLC2v or myosin binding protein C.

Third, in the fraction of ubiquitylated proteins enriched by TUBE (tandem ubiquitin binding entity)–conjugated beads from the same amount of heart lysate, cMLCK antibodies preferentially recognized proteins with several different molecular weights in the heart lysate from pressure-overloaded Mylk3−/+ hearts in the presence of proteasome inhibitors (Figure 7D).

Substrate specificity of ubiquitin-ligation is determined by E3 ligases. Among ~10 muscle-specific E3 ligases, muscle-specific RING finger protein 1 (MuRF1) and atrogin-1/MAFbx are the most intensively studied muscle-specific E3 ligases and are upregulated in rodent hypertrophic models. Overexpression of E3 ligases, including MuRF1 and atrogin-1, has been shown to promote degradation of target proteins expressed by pressure overload.

Figure 5 (Continued). 2-dimensional electrophoresis followed by Western blotting and relative amounts of phosphorylated (p) to total MLC2v. F. Western blotting of cMLCK, pMLC2v, total MLC2v, and GAPDH with or without swimming exercise. Fold difference of cMLCK and pMLC2v normalized to total MLC2v with the value in Mylk3+/− without swimming exercise defined as 1. G. Real-time reverse transcriptase–polymerase chain reaction demonstrates relative expression of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNA normalized to β-actin mRNA with the value in Mylk3+/− without swimming exercise defined as 1. *P<0.05.
Figure 6. Overexpression of cardiac myosin light-chain kinase (cMLCK) attenuates pressure overload–induced pathological hypertrophic responses. A, Schematic of the transgenic construct. Western blotting demonstrates transgenic (TG) protein expression in 3 lines of transgenics using antibodies against cMLCK (top) and hemagglutinin (bottom). Relative expression compared with the endogenous cMLCK proteins is shown. B, Unphosphorylated and phosphorylated (p) myosin light chain 2 (MLC2v) examined with 2-dimensional electrophoresis followed by Western blotting and relative amounts of pMLC2v to total MLC2v (n = 4 hearts from each line). C, Western blotting of heart lysates with or without 1 week of transverse aortic constriction (TAC) using antibodies against cMLCK, HA, pMLC2v, MLC2v, and cMLCK. D, LV systolic pressure (mm Hg) and HW/tibial length (mg/mm) measured in 3 lines of transgenics (n = 4 hearts from each line). E, Aortic banding (TAC) 3 months. F, Echocardiographic indices (TAC 3M). G, TAC 3M. H, TAC 3M as determined by TUNEL and DAPI staining.

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proteins in cultured cardiomyocytes but not cMLCK by adenovirus infection into mouse neonatal cardiomyocytes from ∼5 to 20 multiplicities of infection (Figure 7E and 7F).

Collectively, these results suggest that ubiquitin-proteasome–dependent protein degradation and/or processing participates in the reduction of cMLCK protein resulting from TAC, which was not seen for 2 other proteins and was independent of MuRF1 or atrogin-1 in cultured cardiomyocytes.

**Discussion**

Potential mechanism(s) underlying the transition from compensated to decompensated heart failure are largely unknown. In this study, we demonstrated that functional transition from compensated to decompensated hypertrophy occurred between 1 and 2 weeks of TAC. Reduced cMLCK expression and MLC2v phosphorylation occurred as early as 1 week and continuously during TAC, suggesting a role for MLC2v phosphorylation in this transition and under long-term pressure overload. Second, this possibility was examined mechanistically in loss- and gain-of-function cMLCK mutant mouse models. TAC led to severe heart failure in Mylk3−/− mice but did not affect cardiac contractility in cMLCK-overexpressing mice when the level of cMLCK expression exceeded that of the normal heart. Third, this process involved ubiquitin-proteasome–dependent protein degradation. Fourth, increased cMLCK expression and MLC2v phosphorylation were observed in exercise-induced cardiac hypertrophy after 4 weeks of swim training in Mylk3+/− mice with unchanged contractility, whereas an induction of MLC2v phosphorylation was not observed in Mylk3−/− mice, which exhibited 25% mortality and reduced contractility. Collectively, these data demonstrate that reduced cMLCK expression and the consequent reduction of pMLC2 play a pivotal role in mediating the transition from compensated to decompensated hypertrophy. In addition, induction of cMLCK is critical for the cardiac adaptive response to exercise stress, presumably as a result of MLC2v phosphorylation-dependent facilitation of cross-bridge formation, as discussed in our earlier study.

Transgenic mice overexpressing cMLCK did not display an obvious cardiac phenotype (based on size or function) under basal conditions even with an ∼21-fold increase in expression. After TAC, however, beneficial effects were evident, including maintenance of contractility without fibrosis or apoptotic cell death. Levels of exogenous, transgenically expressed cMLCK protein were also substantially decreased by TAC. Thus, a >6.5-fold induction of cMLCK under basal conditions causes protein synthesis to exceed degradation during TAC, and the increased levels of cMLCK are sufficient to have a beneficial effect on cardiac function after TAC.

Proteasome inhibitors could attenuate, at least in part, the fall in cMLCK protein after TAC; however, inhibition of proteasomal processing does not necessarily improve cardiac function in rats. The proteasome inhibitor bortezomib is the first approved by the Food and Drug Administration for clinical application. Patients with multiple myeloma treated with bortezomib demonstrated cardiotoxicity, including heart failure, as did normal rats. Thus, a more specific methodology to attenuate cMLCK protein degradation during overloading must be identified. Because substrate specificity is defined by E3 ligases, we reasoned that identification of an E3 ubiquitin-ligase specific for cMLCK might be a step in this direction. However, the 2 best-characterized muscle-specific E3 ligases, MuRF1 and atrogin-1, failed to reduce cMLCK expression in cardiomyocytes. Thus, identifying cMLCK-specific E3 ligases and determining how proteasomal processing works on cMLCK, whether by degradation and elimination or a process yielding biological active polypeptide fragments of cMLCK, remain to be accomplished.

Exercise is a stressor of the heart resulting in regional adaptive hypertrophy and alterations in contractility and biochemical properties of myocardial proteins. Typically, greater responses to exercise are observed in endocardial than epicardial layers. In this study, swimming increased the expression of cMLCK and phosphorylation of MLC2v. Mylk3−/− mice exhibited cardiac dysfunction in response to long-term exercise and reduced survival compared with control Mylk3+/+ mice. These findings are in contradistinction to the beneficial effects of exercise reported in other genetic mouse models with cardiac dysfunction in which exercise improved cardiac function and/or lifespan. Collectively, this finding suggests that cMLCK plays an essential role by inducing a beneficial adaptation of the heart to exercise, which is consistent with pMLC2v playing a critical role in cardiac adaptation to increased loading of the heart such as that which occurs with regular exercise.

We also found that spatial distributions of pMLC2v and cMLCK expression were nearly identical and were neither homogeneous nor simple gradients between the inner and outer layers of the rodent heart. Nonuniform distributions of cMLCK and pMLC2v are consistent with previous observations that the ventricular walls are not homogeneous in terms of size, electrophysiological coupling, or active wall stress under basal conditions. It is possible that expression of cMLCK is related to spatial orientation of fibers and corresponding variations in fiber stress, which may confer to myocytes across the wall variable contractile properties that contribute to LV torsion. In this regard, torsion was substantially reduced in Mylk3−/− and MLC2v (Ser14/15Ala) knock-in mutant mice. cMLCK expression is higher in right ventricular than in LV myocardium, exhibiting an inverse relationship to systolic pressure (∼17 mm Hg in the right ventricle versus ∼100 mm Hg in the LV). cMLCK expression in the LV was

**Figure 6 (Continued).** GAPDH. D, Summarized data of left ventricular (LV) systolic pressure and heart weight (HW)/tibial length ratio with or without 1 week of TAC. Representative M-mode ultrasound images (E) and echocardiographic indexes (F) after 3 months of TAC. G, Masson trichrome staining of heart sections and area size of fibrosis (percent relative to the total area size examined) after 3 months of TAC. Bar=50 μm. H, Representative costaining for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), DAPI, and troponin T of LV after 3 months of TAC, and summary for TUNEL-positive nuclei (percent of total nuclei). Bar=20 μm. Total number of nuclei counted is indicated. NTG indicates nontransgenic; %FS, percent LV fractional shortening; ED, end-diastolic; and ES, end-systolic. *P<0.05.
markedly downregulated as a result of TAC. Furthermore, cMLCK is highly expressed in the specialized cardiomyocytes of the ventricular conduction system, even though the contractile protein content in these cells is substantially less than in contractile myocytes. These results could be interpreted as showing that increased mechanical stress reduces net expression of cMLCK by increasing ubiquitin-proteasome activity. It is also possible that cMLCK expres-
sion and pMLC2v levels are related to diastolic pressure, decreasing with long-term increases in diastolic pressure as proposed previously. Of course, both active and passive stresses vary within the wall as a function of the radius of curvature, which in turn varies with depth within the wall and position along the base-apex axis. Viewed this way, an inverse relationship between wall stress and cMLCK and pMLC2v expression might provide a regulatory mechanism by which variations in stress are normalized.

With respect to the ventricular conduction system, our observation of high levels of cMLCK expression and pMLC2v in normal mouse and human hearts was unexpected. It seems unlikely that increased cMLCK and pMLC2v would influence the activity of the conduction system, although our experiments do not rule out this possibility.

Our finding of decreased cardiac contraction in a nearly complete absence of MLCK2 phosphorylation in our Mylk3−/− mice is consistent with results of a recent study using hypomorph cMLCK mutant mice. However, our Mylk3−/− mice demonstrated milder cardiac dysfunction without an increase in interstitial fibrosis and only a marginal increase in fetal gene expression, including atrial natriuretic factor and brain natriuretic peptide, under basal conditions. The milder phenotype displayed in our Mylk3−/− mice might be attributed to the absence of Nemo8 cassettes or to differences in mouse genetic backgrounds and other experimental conditions.

A limitation of this study is that cardiac function was different between the mouse models (mutants versus wild type) under basal conditions before being subjected to a cardiac stress. This was particularly true for Mylk3−/− mice. However, because we observed reciprocal phenotypes in mice with constitutively less or more cMLCK, our conclusions appear to be sound.

Conclusions

This study demonstrates that cMLCK plays essential roles in cardiac adaptations to exercise and pressure overload. Development of heart failure under pressure overload is attenuated by greater expression of cMLCK without apparent adverse effects. The overall pattern of cardiac cMLCK and enhancement of its function is a potentially important therapeutic strategy for the treatment of heart failure resulting from pressure overload (ie, hypertensive heart failure).

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Disclosures

None.

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Circulation. November 27, 2012


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**CLINICAL PERSPECTIVE**

Cardiac hypertrophy is a common response to circulatory or neurohumoral stressors as a mechanism to augment contractility. When the heart is under sustained stress, the hypertrophic response can evolve into decompensated heart failure, although the mechanism(s) underlying this transition remain largely unknown. The dynamics of cardiac contraction and relaxation are fundamentally related to actin-myosin interactions: Initiated by Ca\(^{2+}\)-induced Ca\(^{2+}\) release, Ca\(^{2+}\) binds to troponin C, which releases tropomyosin from preventing actin participating in cross-bridge formation. A range of factors regulate this process and contribute to the adaptation of cardiac function to physiological or pathological stressors, which could suggest a prominent therapeutic strategy for heart failure. Myosin is a hexamer composed of 2 heavy chains and 2 pairs of light chains (MLC1 and MLC2) located on the rod and neck regions of the heavy chain, respectively. Phosphorylation of MLC2v has been shown to potentiate the rate and force of cardiac contraction. In this study, we demonstrated that the kinase that phosphorylates MLC2v and cardiac myosin light-chain kinase play essential roles in cardiac adaptations to pressure overload. Development of heart failure under pressure overload is attenuated by greater expression of cardiac myosin light-chain kinase without apparent adverse effects on normal cardiac function in the absence of increased loading. Thus, an increase in the expression of cardiac myosin light-chain kinase or an enhancement of its function is a potentially important therapeutic target for the treatment of heart failure resulting from pressure overload (ie, hypertensive heart failure).
Myosin Light Chain Phosphorylation Is Critical for Adaptation to Cardiac Stress
Sonisha A. Warren, Laura E. Briggs, Huadong Zeng, Joyce Chuang, Eileen I. Chang, Ryota Terada, Moyi Li, Maurice S. Swanson, Stewart H. Lecker, Monte S. Willis, Francis G. Spinale, Julie Maupin-Furlowe, Julie R. McMullen, Richard L. Moss and Hideko Kasahara

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SUPPLEMENTAL MATERIAL

Supplemental Methods:

Transverse aortic constriction (TAC), pressure measurement and swimming exercise

TAC and left ventricular pressure measurements using a Millar catheter were performed using standard methods as described using Mylk3+/+, Mylk3−/−, transgenic and non-transgenic mice

1. For chronic swim training, groups of 14–16 Mylk3+/+ and Mylk3−/− mice were swum in water tanks (32°C) 7 days a week for 4 weeks 2. Three-month-old mice were utilized in the experiments with the exception of 6.5-month-old Mylk3+/+ and Mylk3−/− mice used for 4 weeks of TAC.

Echocardiogram and MR imaging

Mice were anesthetized with 1.5 to 2% isoflurane for M-mode ultrasound imaging of left ventricles 3, and for MR imaging. MR imaging of the hearts was acquired with a 33 cm clear bore 4.7 T MRI scanner with ECG gating. Heart rate during imaging was between 400 and 450 bpm. Tagged images were acquired using the following parameters: flip angle, 50º; data matrix, 128 x 128, field of view, 3.0 cm; slice thickness, 1 mm; averages of 12 hearts. A long axis (four chamber) view of the left ventricle was first acquired, then tagged short axis images (at base and apex) parallel to the mitral valve were acquired. Torsion was calculated as the maximum net degree of change between base and apex divided by the distance between the two slices.

Administration of proteasome inhibitors, adenovirus infection, and enrichment of ubiquitylated proteins by TUBE (tandem ubiquitin binding entity)-conjugated beads
Neonatal mouse cardiomyocytes were isolated by serial trypsin-digestion and attached to laminin-coated plates in medium (DMEM F-12, 10% fetal bovine serum, 0.5% of Insulin-Transferrin-Selenium) (Gibco-Invitrogen). Lactacystin (5 or 10 µM) or MG132 (1 or 5 µM) was added on the second day (and third day for 48 hr incubation) and incubated for an additional 24 hrs before harvesting. Infection of adenovirus-GFP, MuRF1 \(^4\)\(^6\), or Atrogin1 \(^7\) into neonatal mouse cardiomyocytes with or without MG132 (5 µM) was performed on the second day and the cells were incubated for an additional 24 hrs before harvesting.

Mice were injected with diluted MG132 or lactacystin in PBS (1 µmole/kg/day, i.p.) or both in combination (MG 132 for 3 days followed by lactacystin for 4 days) 3 hrs prior to the TAC operation followed by daily injection for 1 week.

One mg of heart lysates [25 mM HEPES, pH7.6, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, 1% triton, 10% glycerol, protease inhibitor cocktail (Complete, Roche), 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM Na\(_2\)P\(_2\)O\(_7\), 20 µM ubiquitin/ubiquitin-like isopeptidase inhibitor PR-619 (LifeSensors)] was precleared by control agarose (LifeSensors) for 1hr, followed by incubation with Agarose-TUBE2 (LifeSensors) overnight at 4°C. After extensive washing with the protein lysis buffer, samples eluted by SDS-sample buffer were analyzed by Western blotting.

**Northern and Western blotting, immunostaining and histological analyses**

Northern blotting was performed using the cMLCK RT-PCR product (1266 bp, F, 5’-TGGCAGCACTCCCCCAACC-3’; R, 5’-CCAAAACCGACCCCCCTCCCTAAG-3’). Western blot analyses and immunostaining were performed with the following antibodies: HA (C29F4 or 5E2, Cell Signaling), Myc (1814150, Roche), MLC2 (F109.3E1, BioCytex, Marseille, France), phospho-MLC2\(_\gamma\) (gift from Dr. N. Epstein, NIH)\(^8\), cMLCK\(^9\), connexin40 (Cx40-A, Alpha
Diagnostic International), ANF (NMM1717334, Millipore), MyBP-C (gift from Dr. N. Sato, Chiba University)\(^\text{10}\), GAPDH (Research Diagnostics Inc.), troponin T (T6277, Sigma), and ubiquitin (VU101, LifeSensors). Polyclonal anti-human cMLCK antibodies were generated against purified human GST-cMLCK proteins (aa 28-203) with cleavage of GST proteins by thrombin.

Whole-mount immunostaining was performed as follows: after fixation with 4% paraformaldehyde, hearts were dissected in half perpendicular to the base-apex axis, followed by treatment with a solution containing 80% methanol, 20% DMSO, 3% H\(_2\)O\(_2\) for 3-4 hrs, then rehydrated with 75, 50, and 20% methanol in PBS with 0.5% Tween (PBST) for 30 min each. After blocking with PBST including 2% BSA and blocking reagents (Roche) for 1 hr, antibody was added and the hearts were incubated at 4°C overnight. After washing with PBST including BSA and blocking reagents, immunoreaction was performed using an ABC kit (Vector Laboratory). Digitalized images from tissue-staining were utilized for measurement using Image J software as described previously\(^\text{3,11}\).

**Real-time RT-PCR**

Real-time RT-PCR was performed using inventoried Taqman Gene Expression Assays (Applied Biosystems): ANF Mm01255748 and BNP Mm00435304 followed by normalization to β-actin expression (No. 4352933E). Triplicate experiments were averaged.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was performed as described previously, with the exception here of using a Protean IEF System with IPG strips (pH4-7, 163-2001, BIO-RAD)\(^\text{9}\).
Measurements of cardiomyocyte cell size, simultaneous measurements of cell shortening and intracellular free calcium

Isolated adult cardiomyocytes attached to glass coverslips were imaged under a microscope and digitized for measurements of cell surface area. Rod-shaped cardiomyocytes with clear cross-striations, staircase ends and surface membranes free from blebs were utilized for simultaneous measurements of cell shortening and intracellular free calcium performed as described previously.

Telemetry ECG recording

Recording of telemetry ECG (Data Sciences International) was performed 3 days after implantation of wireless radiofrequency telemetry devices.

Statistical analyses

Data presented are expressed as mean values ± S.E.M. The data including more than two levels of a repeated measures factor were analyzed for normal distribution by Bartlett’s (StatView version 5.01) or Mauchly’s test of sphericity (SPSS ver. 17.0 for Windows). Results were compared using ANOVA with or without repeated measures analyses and Fisher’s post-hoc test (StatView version 5.01 or SPSS ver. 17.0 for Windows). Kaplan-Meier survival plots followed by Logrank test for trend were utilized for survival analyses (SPSS ver. 17.0 for Windows). $p < 0.05$ was considered significant.
Supplemental Figures:

Figure S1: Representative immunostaining (purple) of MLC2v and pMLC2v in transverse serial sections of normal hearts (A); cMLCK and pMLC2v (B) in transverse serial sections following 3 months of TAC.

Figure S2: Specificity of anti-phospho-MLC2 antibody.
2D-gel electrophoresis followed by Western blotting using anti-phospho-MLC2 (top panel) vs. anti-MLC2 (bottom panel) using mouse or human ventricular tissues.
Figure S3: Generation of MyLK3<sup>−/−</sup> mice.

(A) A genomic fragment for Mylk3 from a 129 mouse genomic library was cloned and a targeting vector was constructed by insertion of diphtheria toxin-A-chain (DT) gene in intron 3 for negative selection, floxed-neomycin-resistant gene (NeoR) in intron 4 for positive selection and an additional loxP site in intron 5. The targeting vector was electroporated into ES cells. Three ES cell clones were injected into blastocysts and two were successfully transmitted into the germline. By crossing with ACTB-Cre transgenic mice (Jackson Laboratory, stock number 003376), the floxed-NeoR cassette and exon 5 were deleted. (B) Southern blotting hybridized with the 5′ probe demonstrates 20.2 kb SacI-digested genomic DNA in wild-type alleles (lanes 1, 2), and 11.6 kb fragments in the targeted alleles (lanes 3-5). PCR product (425 bp, F, 5′-AAGGAGGACATACCTGTGCGAAC-3′; R, 5′-CCCAACACAGAAGAACATCCTTACC-3′) was used for 5′ probe.
Figure S4: Echocardiographic indices of $\text{Mylk3}^{+/+}$ vs. $\text{Mylk3}^{-/-}$ at 3 months of age (mean ± S.E.).
Figure S5: Prolongation of QRS duration in Mylk3<sup>+/−</sup> mice.

(A) Representative telemetry ECG recordings from Mylk3<sup>+/+</sup> (top) and Mylk3<sup>−/−</sup> (bottom) mice at 5 months of age. (B) Representative averaged ECG recording from a stable 10-20 beats with comparable heart rates (between 676 to 796 bpm). (C) HR (heart rate), PR interval, QRS and QTc duration (mean ± S.E.) are shown. Duration of QRS was significantly prolonged in Mylk3<sup>−/−</sup> vs. Mylk3<sup>+/+</sup> hearts. (D) Whole mount connexin40 immunostaining demonstrates ventricular conduction system (brown staining, arrowheads) in both Mylk3<sup>+/+</sup> and Mylk3<sup>−/−</sup> hearts. Bar = 1 mm.

<table>
<thead>
<tr>
<th></th>
<th>Mylk3&lt;sup&gt;+/+&lt;/sup&gt; (n=7)</th>
<th>Mylk3&lt;sup&gt;−/−&lt;/sup&gt; (n=9)</th>
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<tr>
<td>HR (bpm)</td>
<td>730 ± 8</td>
<td>727 ± 14</td>
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<td>PR (msec)</td>
<td>31 ± 1</td>
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<td>QRS (msec)</td>
<td>12 ± 1</td>
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<td>QTc (msec)</td>
<td>37 ± 1</td>
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Figure S6: Schematics of experimental protocol (A), sequential echocardiographic indices from pre-operation to 4 weeks after TAC (B) and HW/tibial length (C) in Mylk3+/+ and Mylk3−/− mice. One knockout mouse died shortly after anesthetized at 2 weeks after TAC, which was eliminated from echocardiographic analyses using repeated measures ANOVA, but was included in the HW/tibial length. Of note, heart rate of Mylk3−/− mice showed a significant difference in Mauchly’s test of sphericity ($p = 0.012$).
Figure S7: Overexpression of cMLCK attenuates pressure overload-induced pathological hypertrophic responses.

(A) Echocardiographic indices of NTG and TG1, 2 and 3 mice at 3 months of age without TAC (mean ± S.E.). (B) Representative images of cardiomyocytes isolated from NTG, TG1 and 3 with or without TAC. Summarized data of cell area (µm²) of cardiomyocytes isolated from NTG, TG1 and TG3 mice with or without 1 week of TAC. Number of cells examined is indicated. Bar = 50 µm. (C) Echocardiographic indices at 0, 2 and 4 weeks after TAC from NTG and TG3.
Figure S8: LV end-systolic pressure and expression of cMLCK proteins 3 months following TAC.

(A) LV end-systolic pressure (mean ± S.E.). (D) Western blotting of the heart lysates from NTG, TG1 and TG3 mice with or without 3 months of TAC using antibodies against cMLCK, HA and GAPDH.
Figure S9: Representative image of co-staining for TUNEL, DAPI and troponin T of LV following 3 months of TAC.
Figure S10: Effects of proteasome inhibitor MG132 on cMLCK expression in cultured cardiomyocytes.

Representative Western blotting of cMLCK and GAPDH using cultured neonatal mouse cardiomyocytes with or without MG132 treatments (1 and 5 µM) for 24 hrs.
Video files: Representative MRI images of 4-chamber hearts from $\text{Mylk3}^{+/+}$ (wild heart) and $\text{Mylk3}^{-/-}$ (KO heart) mice at 3 months of age.

Cardiac contraction assessed using MRI demonstrated a reduction in ejection fraction and increases in the volume of the left ventricular cavity both at end-systole and end-diastole. Scale = 25 mm x 25 mm.
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