An Endogenously Produced Fragment of Cardiac Myosin-Binding Protein C Is Pathogenic and Can Lead to Heart Failure

Md. Abdur Razzaque, Manish Gupta, Hanna Osinska, James Gulick, Burns C. Blaxall, Jeffrey Robbins

_Rationale_: A stable 40-kDa fragment is produced from cardiac myosin-binding protein C when the heart is stressed using a stimulus, such as ischemia-reperfusion injury. Elevated levels of the fragment can be detected in the diseased mouse and human heart, but its ability to interfere with normal cardiac function in the intact animal is unexplored.

_Objective_: To understand the potential pathogenicity of the 40-kDa fragment in vivo and to investigate the molecular pathways that could be targeted for potential therapeutic intervention.

_Methods and Results_: We generated cardiac myocyte-specific transgenic mice using a Tet-Off inducible system to permit controlled expression of the 40-kDa fragment in cardiomyocytes. When expression of the 40-kDa protein is induced by crossing the responder animals with tetracycline transactivator mice under conditions in which substantial quantities approximating those observed in diseased hearts are reached, the double-transgenic mice subsequently experience development of sarcomere dysgenesis and altered cardiac geometry, and the heart fails between 12 and 17 weeks of age. The induced double-transgenic mice had development of cardiac hypertrophy with myofibrillar disarray and fibrosis, in addition to activation of pathogenic MEK–ERK pathways. Inhibition of MEK–ERK signaling was achieved by injection of the mitogen-activated protein kinase (MAPK)/ERK inhibitor U0126. The drug effectively improved cardiac function, normalized heart size, and increased probability of survival.

_Conclusions_: These results suggest that the 40-kDa cardiac myosin-binding protein C fragment, which is produced at elevated levels during human cardiac disease, is a pathogenic fragment that is sufficient to cause hypertrophic cardiomyopathy and heart failure. (Circ Res. 2013;113:553-561.)

_Key Words_: cardiomyopathies • contractile proteins • sarcomeres

Myosin-binding protein C (MyBP-C) is a thick-filament-associated protein consisting of 1274 amino acid residues (149 kDa), which is localized to the crossbridge-containing C zones in the sarcomeres of striated muscles. Three isoforms of MyBP-C, fast skeletal, slow skeletal, and cardiac, are present in humans and mice. Although the 3 isoforms have similarities, the cardiac isoform of MyBP-C (cMyBP-C) differs from the skeletal isoforms in that it contains an additional domain at the N-terminus (C0) and a phosphorylatable domain located between the C1 and C2 domains (Figure 1B). The ATPase-binding regions of the actin and myosin heavy chains lie at the amino terminus of the protein. The importance of understanding the structure–function relationships of cMyBP-C is underscored by the identification of numerous MYBPC3 mutations that are responsible for an estimated 20% to 35% of verified familial hypertrophic cardiomyopathy (FHC) cases. Although mutation of MYBPC3 is one of the most frequent causes of hypertrophic cardiomyopathy on a per-gene basis, most of these mutations (=60%) result not in a full-length mutated protein, but rather in truncated peptides.

We recently confirmed that cardiac stress can trigger the production and accumulation of a 40-kDa truncated fragment derived from the amino terminus of cMyBP-C. The fragment seems to be generated as a result of dephosphorylation that unmasks a μ-calpain site, resulting in cleavage of intact cMyBP-C. A recent report using neonatal rat ventricular cardiomyocytes showed that hypoxic stress resulted in decreased levels of cMyBP-C phosphorylation, its specific cleavage, and the subsequent production of N’-terminal fragments. The 40-kDa fragment can be detected in diseased and stressed mouse and human hearts. The fragment is apparently stable; however, the functional consequences in terms of normal cardiac function are unknown. Appreciable quantities of a
truncated fragment of cMyBP-C in the diseased human heart raises the possibility of potential pathogenic consequences because the fragment has been shown to effectively compete for the normal protein’s sites of binding to the head regions of myosin and actin.10

Considering the frequency with which truncated cMyBP-C protein can serve as a poison peptide,² we sought to determine the potential pathogenicity of the 40-kDa fragment in vivo. We generated cardiac myocyte–specific transgenic mice using a Tet-Off inducible system to permit controlled expression in cardiomyocytes.¹¹ When the expression of the 40-kDa protein is induced in the hearts by crossing the responder animals with tetracycline transactivator (tTA) mice (double-transgenic [DTG]) in the absence of doxycycline, the DTG mice undergo sarcomere dysgenesis, show altered cardiac geometry, and display signs of heart failure by 3 weeks of age, even though intact cMyBP-C expression is unaffected. Expression of the 40-kDa fragment in cardiomyocytes led to development of significant cardiac hypertrophy with myofibrillar disarray and fibrosis. Because hypertrophy seemed to be directly caused by this fragment’s expression, we wished to determine whether normal pathogenic signaling was activated or whether some novel pathway was involved. Mitogen-activated protein kinase is one of the major signaling pathways involved in cardiac hypertrophy and heart failure, and we subsequently explored the role of this pathway in the developing pathology. MEK–ERK hypertrophic signaling pathways were activated in the 40-kDa fragment-bearing mice; treating the animals with intraperitoneal injections of U0126, a MEK–ERK pathway inhibitor, effectively improved cardiac function and prolonged survival compared with the untreated control mice.

Methods

DNA Constructs and Transgenic Mice

For cardiomyocyte-specific inducible transgene expression, 2 lines of mice are needed. The driver line (tTA) contains the α-myosin heavy chain promoter fused to tet-VP16 protein, which has been described previously.¹² We generated the responder line containing the myosin heavy chain promoter driving the 40-kDa N-terminal fragment of cMyBP-C (amino acids, 1–271).⁷,¹³,¹⁴ An N-terminal c-myc tag encoding the human c-myc peptide (EQKLISEEDL), which has no effect on cMyBP-C function and stability, was inserted after the

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Figure 1. Inducible transgene expression. A, Human heart samples showing the levels of cardiac myosin-binding protein C (cMyBP-C) and the 40-kDa fragment in normal (nonfailing [NF]), diseased, nonischemic (NI), and ischemic (I) samples. B, cMyBP-C structure and transgenic (TG) constructs. cMyBP-C is a 149-kDa protein comprising 8 immunoglobulin-type (ovals) and 3 fibronectin-like (octagon) domains. The protein binds multiple components of the thick and thin filaments and the location of the 40-kDa fragment is shown. One mouse line contains the cardiomyocyte-specific promoter driving the fusion tet-VP16 protein (tetracycline transactivator [tTA]) that, in the absence of tetracycline, will bind to the responder promoter and drive expression of the 40-kDa fragment. We generated multiple lines of cardiomyocyte-specific TG mice, choosing a line that showed reasonably tight control of the transgene and producing levels that approximated those in human disease. B and C, When expression of the 40-kDa protein is induced by crossing the responder animals with the tTA mice, the double-TG (DTG) mice show protein expression in the absence of Dox and show almost no protein when Dox is present. C, Three independently isolated samples from each set of animals were prepared and electrophoresed. D, Phosphorylation status of c-MyBP-C at 273, 282, and 302 sites. Expression was examined at 12 weeks in nontransgenic (NTG) animals, single-TG (TG) mice carrying the 40-kDa fragment (40 kDa) or tTA transgene (tTA), and DTG mice expressing the 40-kDa transgene (DTG). MHC indicates myosin heavy chain.
initiation methionine codon to differentiate transgenic protein from the endogenous one. Earlier studies confirmed that introduction of the c-myc epitope was benign because when we fused c-myc to the wild-type cMyBP-C, we did not see any effects on structural, functional, or hemodynamic parameters.13,14 In the presence of doxycycline, the protein was not expressed. Animals were handled in accordance with the principles and procedures of the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital approved all experimental procedures.

Human Sample Collection
Tissue was harvested from the left ventricular free wall near the cardiac apex (age, 59±3.1 years) in patients with end-stage (stage D) heart failure at the time of left ventricular assist device placement (HeartMate or HeartMate II; Thoratec, Pleasanton, CA) as either destination therapy or bridge to transplantation. Samples from patients with a diagnosis of ischemic or nonischemic heart failure etiology were analyzed. Nonfailing human heart tissue was obtained from the left ventricular free wall of male organ donor hearts rejected for transplantation because of physical incompatibility. Left ventricular tissue obtained from surgery was immediately frozen in liquid nitrogen and stored at −140°C. All procedures related to harvest and use of human tissue were performed in accordance with the guidelines of the National Institutes of Health, University of Rochester Medical Center, and Cincinnati Children’s Hospital Medical Center Institutional Review Boards.

Protein Analyses
To identify modifications at the protein level, enriched myofibrillar proteins were isolated using F60 buffer (60 mmol/L KCl; 30 mmol/L imidazole; 7.2 mmol/L MgCl$_2$; pH 7.0) with protease/phosphatase inhibitors (Cocktails I and II; Sigma), as described previously.13 The presence of the 40-kDa protein was confirmed by SDS-PAGE (12.5% criterion Tris-glycine precast gels; Bio-Rad), followed by Western blots using an anti-c-myc monoclonal antibody (clone 9E10; Roche) and an anti-cMyBP-C rabbit polyclonal antibody raised against the C0–C1 domains.16,17

Evaluation of cMyBP-C Phosphorylation
To define cMyBP-C phosphorylation status, myofibrillar proteins were purified from nontransgenic and 40-kDa single-transgenic and DTG mouse hearts, electrophoresed by SDS-PAGE, and subjected to Western blotting using phospho-specific cMyBP-C antibodies, as described previously.18

Histochemistry, Immunohistochemistry, and Transmission Electron Microscopy
For histopathologic examinations, beating hearts were removed from anesthetized mice, the blood was allowed to drain, and the tissues were fixed in 10% formalin. Hearts were bisected longitudinally, dehydrated through a graded series of alcohol, and laid open before being embedded in paraffin. Step-serial sections (5 μm) were taken from embedding embedded in paraffin. Step-serial sections (5 μm) were taken from paraffin, dehydrated in a series of acetone baths, and embedded in a Poly/Bed 812 resin mixture. Thin sections, counterstained with uranium and lead salts, were examined using a Hitachi 7600 transmission electron microscope equipped with an Advanced Microscopy Techniques digital camera. Multiple sections were cut from 2 to 3 mice of mixed sexes, and >50 fields were observed by a blinded observer.

Cardiac Function
To observe changes in left ventricular chamber size and fractional shortening, echocardiography was performed by noninvasive M-mode echocardiography.18

MEK Inhibitor Treatment
Mice were treated with U0126, a pharmacological inhibitor of mitogen-activated protein kinase/ERK, which blocks phosphorylation of ERK1/2.18 We injected U0126 intraperitoneally on a daily basis (5 mg/kg body weight per day) into pregnant dams until delivery and continued the injections during the perinatal and neonatal periods to the nursing females up to postnatal day 9 and then to individual pups until postnatal day 24 at 2-day intervals. After weaning, individual mice were treated until ≤12 weeks of age.

Statistical Analyses
All biochemical and functional assays were performed in mice with mixed-sex controls. For comparisons of data from 2 groups, Student t test was used. For comparisons of multiple groups, 1-way ANOVA with post hoc Tukey multiple comparisons test was used. Paired data were evaluated by Student t test. Results are shown as mean ± SE or SD. A value of P < 0.05 was considered significant.

Results
Inducible Cardiomyocyte-Specific Expression of the 40-kDa Fragment
Previously, we found that cleavage of cMyBP-C by μ-calpain produced a stable 40-kDa fragment. This fragment, which was either undetectable or present in very small amounts in normal mouse hearts, was easily detectable in both mouse and human hearts that had been subjected to ischemia-reperfusion injury or general cardiovascular stress.7 Using isolated systems, we found that this fragment was able to interact efficiently with the other filament systems in the sarcomere and, in fact, could compete effectively with endogenous cMyBP-C for binding to the thick and thin filaments.10,21 On the basis of these data and the presence of cMyBP-C in hearts collected from human heart failure patients, we wanted to determine whether production of the 40-kDa fragment was merely a consequence of cardiac disease or whether its expression was sufficient to cause cardiac pathology and dysfunction. It was therefore imperative to be able to rigorously control expression of the 40-kDa fragment independent of exogenously induced cardiac stress. We therefore chose to express the 40-kDa fragment inducibly using a Tet-Off system.

Before initiating the transgenic studies, we confirmed the levels of the 40-kDa fragment in normal and failing human hearts to titrate transgenically driven expression to the approximate levels observed in our human samples. Although low levels could be detected in our nonfailing samples, the amount of 40-kDa fragment seen in hearts with either ischemic or nonischemic disease was substantially increased (Figure 1A). To investigate the role of the 40-kDa fragment of cMyBP-C in cardiac function and its intrinsic ability to
cause cardiac disease, we generated cardiac-specific transgenic mice using our inducible transgenic system, which is bigenic and consists of 1 line (the driver) producing the tTA, under the control of the α-myosin heavy chain promoter, and another line carrying the cDNA encoding the 40-kDa fragment, under the control of the responder promoter, which is only active in the absence of doxycycline (Figure 1B). The amino terminus of cMyBP-C binds multiple components of the thick and thin filaments, interacting with actin and myosin, and the 40-kDa fragment contains elements of both these binding sites, as schematically shown in Figure 1B. When these 2 transgenic lines were crossed and in the absence of tetracycline/doxycycline, the 40-kDa fragment was expressed specifically in cardiomyocytes (Figure 1C). The fragment’s synthesis had no visible effect on normal protein synthesis patterns (Figure 1C), and normal levels of the intact wild-type cMyBP-C protein seemed to be present.

We had previously shown that cMyBP-C function is highly dependent on its phosphorylation state. To determine whether expression of the 40-kDa fragment had any effect on the overall phosphorylation status of intact cMyBP-C, we performed Western blotting analyses with phospho-site-specific antibodies that we had developed and used previously, which are able to detect the phosphorylation status of 3 specific residues—serines 272, 282, and 302 in the amino terminus of cMyBP-C. No significant changes in phosphorylation were observed in any of the mice, ruling out posttranslational modifications of the endogenous protein as playing a role in any functional deficits that might be observed (Figure 1D).

**Cardiac Phenotype and Sarcomere Architecture**

The mice began displaying overt signs of cardiac dysfunction in early adulthood. Removal of hearts from nontransgenic, single-transgenic, and induced DTG mice at 12 weeks revealed grossly aberrant cardiac morphology in hearts expressing the 40-kDa fragment (Figure 2). Chamber arrangement and overall geometries were grossly perturbed, with apparent ventricular and atrial enlargement.

To determine whether the 40-kDa fragment is incorporated normally into the sarcomere, we isolated the myofibrillar proteins from nontransgenic and the 40-kDa double-transgenic (DTG) mice were isolated, electrophoresed on acrylamide, and subsequently transferred for Western blot analyses with cardiac myosin-binding protein C (cMyBP-C) antibody. Actin was used as a loading control. Western blot analyses with cardiac myosin-binding protein C (cMyBP-C) antibody. Actin was used as a loading control.

**Figure 3. Incorporation of 40-kDa protein in the sarcomere.**

A. Myofibrils from nontransgenic (NTG), 40-kDa-bearing single-transgenic, tetracycline transactivator (tTA) single-transgenic, and 40-kDa double-transgenic (DTG) mice were isolated, electrophoresed on acrylamide, and subsequently transferred for Western blot analyses with cardiac myosin-binding protein C (cMyBP-C) antibody. Actin was used as a loading control. B. Quantification of total full-length MyBP-C. No significant differences were found between the NTG, 40-kDa-bearing, tTA, and DTG groups. C. Quantification of 40-kDa protein expression. Values represent mean ± SE for each group (n=3). ***P<0.0001 DTG vs NTG, 40-kDa-bearing, and tTA controls. D. Incorporation of myc-tagged 40-kDa cMyBP-C was confirmed in NTG, tTA, 40-kDa, and DTG mice by immunofluorescent staining of cMyBP-C with either an anti-myc (red) or an anti-cMyBP-C antibody (green).

no significant changes in the levels of full-length MyBP-C expression among nontransgenic 40-kDa fragment-bearing single-transgenic, tTA, and DTG mice (Figure 3B). Significantly higher (P<0.0001) levels of the 40-kDa fragment were detected only in myofibril protein preparations from DTG hearts (Figure 3C), suggesting that the 40-kDa fragment was incorporated into the sarcomere. Some leakage was observed in expression of the 40-kDa fragment in the single-transgenic mice carrying that construct (Figure 1B; 40 kDa). To confirm localization of the 40-kDa fragment, antibodies against cMyBP-C and c-myc were used for immunohistochemistry coupled with confocal microscopy. Results showed that the 40-kDa fragment colocalized with cMyBP-C in the DTG hearts, indicating that the fragment was incorporated in a manner indistinguishable from normal endogeneous cMyBP-C at the resolution used (Figure 3D).

Myocardial fibrosis, a hallmark of FHC, can contribute to sudden cardiac death, ventricular tachyarrhythmia, left ventricular dysfunction, and heart failure. We assessed fibrosis at 4, 12, and 16 weeks (Figure 4A, 4B, and 4C, respectively). Fibrosis was seen as early as at 4 weeks in the DTG heart.
mice that expressed the 40-kDa fragment and progressively increased as the animals aged, with no fibrosis observed in the nontransgenic and the 40-kDa-bearing and tTA transgenic mice (Figure 4). The ratio of heart weight to body weight also was significantly increased ($P<0.0001$) in the DTG mice compared with that in nontransgenic, 40-kDa-bearing, and tTA transgenic mice (Figure 4D). These anatomical and histological alterations were accompanied by visible changes at the ultrastructural level as well. Disorganized sarcomeres are commonly observed in cMyBP-C–related FHC; thus, we examined the DTG myocardial ultrastructure at 12 weeks. In the unaffected animals, the sarcomeres were aligned with one another, precisely aligned, and the mitochondria were highly organized (Figure 5). In contrast, sarcomeres derived from the 40-kDa DTG mice were consistently out of register, normal pattern organization was significantly disrupted, and the overall architecture of the mitochondria was severely disrupted (Figure 5). Thus, expression of the 40-kDa fragment, even in the presence of wild-type levels of normal protein (Figure 3B), resulted in overt cardiac pathology and sarcomeric disruption, indicating that this fragment can act in a dominant-negative poison-peptide fashion and directly result in cardiac pathology in the context of the whole animal.

**MEK–ERK Pathways Are Activated in Hearts Expressing the 40-kDa Fragment**

Enhanced MEK and ERK activation in cardiomyocytes is strongly implicated in the pathogenesis of FHC. Therefore, we wished to determine whether the 40-kDa fragment-induced pathogenesis resulted in activation of these pathways as well. On phosphorylation, MEK becomes activated and subsequently signals downstream to ERK, eventually resulting in specific transcriptional programs being upregulated. Hearts were isolated from 5- and 10-week-old mice and the phosphorylation status of MEK and ERK was determined.
by Western blot analysis using phospho-specific antibodies. MEK–ERK pathways are highly activated in the 40-kDa DTG mice compared with nontransgenic and 40-kDa-bearing single-transgenic mice. Moreover, DTG mice displaying obvious signs of heart failure (anasarca, fluid retention, and listlessness) showed the highest levels of MEK–ERK activation (Figure 6).

We hypothesized that aspects of the pathogenic phenotype being observed were because of upregulation of this pathway. Pathogenicity therefore might be ameliorated by MEK–ERK inhibition. We treated the 40-kDa DTG mice with the MEK inhibitor U0126. The untreated 40-kDa DTG mice began to die as early as 23 days after birth, with a majority of the mice deceased by 8 weeks. In contrast, only 1 MEK inhibitor-treated animal died within 8 weeks and there were significant increases in lifespan and probability of survival relative to the untreated cohort (Figure 7A).

After 12 weeks, cardiac function was measured by M-mode echocardiography. Fractional shortening in the untreated DTG mice was significantly decreased compared with the nontransgenic, 40-kDa-bearing, and tTA mice (P<0.0001). However, the U0126-treated cohort showed significantly higher values (P<0.01) relative to the untreated induced DTG mice (Figure 7B). Left ventricular mass and conservation of normal diastolic volumes were also significantly improved (P<0.0001; Figure 7C and 7D). These results suggest that activation of the MEK–ERK pathway plays a significant role in early mortality but clearly, although mitogen-activated protein kinase inhibition can improve overall cardiac function, it cannot rescue the animal in the face of continued production of the 40-kDa fragment.

Discussion

This report significantly extends earlier data concerning the origin and function of an endogeneously produced peptide fragment derived from cMyBP-C. The 40-kDa fragment, derived from the N-terminus of cMyBP-C, contains the C0 domain (99 residues), a Pro/Ala-rich region (51 residues), the C1 domain (104 residues), and 17 residues of the M domain (Figure 1B). Previously, we found that the 40-kDa fragment is released via cleavage at a \( \mu \)-calpain site and that steady-state levels were increased after cardiac stress, such as surgically induced pressure overload or ischemia-reperfusion injury. We also found high levels of the fragment in a number of our genetically induced models of cardiac disease (data not shown) and, recently, the fragment was detected in the circulatory systems of rodents and humans after acute myocardial infarction.7,8 A recent report showed that the presence of the 40-kDa fragment correlated with increased cytotoxicity, impairment of Ca\(^{2+}\) handling, and altered contractile function of the sarcomere in vitro, whereas, in contrast, a fragment derived from the carboxyl terminus of cMyBP-C had no detrimental effects.28 In this article, we detail for the first time the results of dosage-controlled expression in vivo in the cell type in which the fragment accumulates in human...
disease. We show that at high doses, the fragment is sufficient to cause cardiac disease, heart failure, and premature death. Significantly, in some human heart failure patients, we can detect significant levels of the fragment in both ischemic and nonischemic conditions (Figure 1A), raising the possibility that the 40-kDa fragment may play a role in the natural history of human cardiac disease.

Under normal conditions in the mouse, in our experience, only \( \approx 80\% \) of the cMyBP-C is fully phosphorylated, holding the possibility that low levels of the fragment might accumulate at baseline with no detectable ill effects. This appears to be the case in humans as well; the nondiseased samples contain detectable amounts of the fragment (Figure 1A). Consistent with these data is the observation that in our mouse model in which transgene expression is not induced but in which there is a slight amount of leaky expression (Figure 3A), we detected no pathology. The data are consistent with a dose-dependent pathogenic effect of the fragment, which comes about as a consequence of its ability to interfere with the interactions of normal cMyBP-C with thick and thin filaments.

Consistent with the fragment’s specificity for causing the observed pathogenic effects, when we turned off expression of the 40-kDa fragment by administering doxycycline postbirth, we did not see any heart enlargement or sign of heart failure in those animals (data not shown).

Considering our data and the particularly elevated levels of the 40-kDa fragment in cardiac samples derived from patients with heart failure, we thought it would be important to determine its potential pathogenicity. Is the 40-kDa fragment sufficient to cause cardiac disease or even failure when expressed in a cardiomyocyte-specific fashion in vivo? When very low levels of the 40-kDa fragment were expressed (ie, when the transgene was present but uninduced, no pathology was presented). However, on induction of expression of the 40-kDa transgene to levels approaching those detected in stressed hearts (Figure 1A), we observed sarcomere dysgenesis, altered cardiac geometry, and cardiac failure between 3 and 17 weeks after birth. The mice also had development of significant cardiac hypertrophy, with myofibrillar disarray and fibrosis. These symptoms developed despite the presence of normal amounts of intact cMyBP-C protein, indicating that, in vivo, accumulation of the 40-kDa fragment results in a dominant pathogenic effect. These data are consistent with more recent studies performed in vitro by Sadayappan et al. In those studies using rabbit cardiomyocytes and adenovirus infection, the pathogenic properties of the 40-kDa fragment in vitro were confirmed, whereas fragments derived from other portions of cMyBP-C stably expressed in rabbit cardiomyocytes were benign.

We next addressed the question of mechanism: where is this dominant effect concentrated? The N-terminal 40-kDa region of the cMyBP-C is highly conserved across species and its generation is pathogenic in mammalian hearts. Reductionist approaches have confirmed its ability to affect crossbridge kinetics and effectively compete with intact cMyBP-C for actin and myosin binding. These results are consistent with our data demonstrating its correct placement in the sarcomere (Figure 3). Bacterially expressed 40-kDa fragment effectively inhibited actin translocation over a surface of monomeric mouse cardiac myosin in an in vitro motility assay. The 40-kDa fragment also stereospecifically and reversibly bound to actin, effectively serving as a brake or drag to slow myosin kinetics. However, we have not ruled out the possibility that the 40-kDa fragment might affect thick and thin filament kinetics by also binding directly to myosin.

The direct effect of the fragment’s placement on sarcomere function is supported by the effect on structure (Figure 5) where altered sarcomeric architecture is readily apparent. Expression of the fragment had no effects on the steady-state levels of full-length cMyBP-C, implying that the 40-kDa fragment exerts its effect in a dominant fashion, consistent with a proposed pathogenic role in general cardiac disease, even when cMyBP-C levels are unaffected. All of the histological and functional data are consistent with the hypothesis that the pathogenesis of the 40-kDa fragment stems directly from its intercalation into the sarcomere and interference with normal thick and thin filament movement and kinetics. This results in altered cardiac output, cardiomyocyte remodeling and dropout, and subsequent fibrosis, all of which are observed in the 40-kDa fragment model. At this point, we believe that the observed downstream effects have their primary source in altered filament mechanics. Currently, we have neither data nor evidence that the fragment can effectively signal outside of the sarcomere in terms of its ability to activate/inhibit other protective or pathogenic mechanisms.

Enhanced MEK and ERK activation in cardiomyocytes often accompanies development of cardiac pathology in FHC, but there is a paucity of data with reference to altered MEK–ERK pathway contribution to cMyBP-C-related pathology. In the 40-kDa fragment-bearing mice, we detected significant activation of this pathway (Figure 5). Because depletion of ERK1/2 with antisense oligonucleotides or pharmacological inhibition of MEK1/2 attenuates the hypertrophic response to agonist stimulation in cultured cardiomyocytes, we asked whether downregulation of the pathway’s activity might be sufficient to ameliorate overall pathology and even prolong life. Treatment with the MEK inhibitor U0126 significantly increased lifespan and substantially improved heart function. These data provide evidence for a critical role of the MEK–ERK pathway in augmenting the development of cardiac disease in the 40–kDa fragment-bearing mice, although they clearly do not rule out the role of other pathways in the pathogenesis of the disease resulting from sarcomeric dysfunction. We hypothesize that with continued alterations of cardiac hemodynamics, multiple signaling pathways that contribute to the overall pathogenicity of the secondary response are altered. In this study, the MEK inhibitor was administered during the perinatal and neonatal periods, before any detectable structural or functional cardiac abnormalities. A critical question to be addressed is whether the MEK inhibitor would be effective in improving heart function in 40-kDa fragment-bearing mice when initiated after the onset of cardiac disease, which would be more analogous to the potential for affecting the natural history of the disease in patient populations.
Although this study shows that relatively high levels of the 40-kDa fragment are sufficient to cause cardiac failure and death, its role in the natural history of cMyBP-C–induced human cardiomyopathy and cardiac disease is not yet defined. Clearly, in some human hearts in which the fragment is present, the levels of intact cMyBP-C are dramatically decreased (Figure 1A; ischemic hearts) and cMyBP-C haplinsufficiency could, by itself, cause increased pathology. At the same time, in the nonischemic hearts shown in Figure 1A, although the cMyBP-C levels are somewhat reduced, there are still appreciable amounts. In mouse hearts, expression of the 40-kDa fragment with normal amounts of cMyBP-C still causes disease and premature death, leading us to hypothesize that, in humans, appreciable amounts of this fragment will negatively affect the natural history of preexisting cardiac disease.

Disclosures

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References


**Novelty and Significance**

**What Is Known?**

- A small fragment derived from the N-terminal region of cardiac myosin-binding protein C is endogenously produced in stressed hearts and appears to be stable.
- This fragment is capable of interacting with the contractile apparatus in isolated systems in vitro and can affect thick and thin filament mechanics.
- Levels of this fragment appear to be increased in failing human hearts and in mouse models of cardiac disease.

**What New Information Does This Article Contribute?**

- Transgenic mice that express significant levels of the fragment, specifically in cardiomyocytes, develop heart disease and die prematurely, showing that, in vivo, the presence of significant levels of the fragment is sufficient to cause cardiac disease and death.
- Expression of the fragment, even when normal amounts of wild-type cardiac myosin-binding protein C are present, still leads to heart failure and death within 15 weeks, showing that the peptide acts in a dominant fashion.
- The natural history of the disease is characterized by dramatic remodeling of the gross cardiac architecture and activation of pathogenic hypertrophic pathways.
- These findings suggest that because the fragment can be found in human hearts with ischemic and nonischemic disease, it may be a contributing factor in the development of decompensated heart failure in human cardiac disease.

This study was designed to extend data obtained in isolated systems and in cell culture to the whole heart and whole animal contexts. This N-terminal fragment is stable and can accumulate to relatively high levels (20% to 30% of the endogeneous, intact cardiac myosin-binding protein C levels). Studies using isolated thick and thin filament proteins show that the fragment influences contractile filament interactions, raising the possibility that it might affect cardiac hemodynamics. However, its role in the intact heart remained unknown. We have now defined a pathogenic role for this fragment in vivo. Using cardiomyocyte-specific transgenic expression in the mouse, we show that detectable accumulations of the fragment are sufficient to cause cardiac disease, leading to heart failure and premature death by 12 to 15 weeks, even when normal amounts of cardiac myosin-binding protein C remain. The fragment associates with the sarcomeres at a location that is consistent with it playing a dominant role in influencing normal thick and thin filament interactions. These data, along with the detection of appreciable amounts of the fragment in hearts derived from human heart failure patients, point to a potential role for this fragment in the pathogenic natural history of human heart failure.
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