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Original article

Cardiac myosin binding protein-C is a potential diagnostic biomarker for myocardial infarction

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ABSTRACT

Cardiac myosin binding protein-C (cMyBP-C) is a thick filament assembly protein that stabilizes sarcomeric structure and regulates cardiac function; however, the profile of cMyBP-C degradation after myocardial infarction (MI) is unknown. We hypothesized that cMyBP-C is sensitive to proteolysis and is specifically increased in the bloodstream post-MI in rats and humans. Under these circumstances, elevated levels of degraded cMyBP-C could be used as a diagnostic tool to confirm MI. To test this hypothesis, we first established that cMyBP-C dephosphorylation is directly associated with increased degradation of this myofilament protein, leading to its release *in vitro*. Using neonatal rat ventricular cardiomyocytes *in vitro*, we were able to correlate the induction of hypoxic stress with increased cMyBP-C dephosphorylation, degradation, and the specific release of N′-fragments. Next, to define the proteolytic pattern of cMyBP-C post-MI, the left anterior descending coronary artery was ligated in adult male rats. Degradation of cMyBP-C was confirmed by a reduction in total cMyBP-C and the presence of degradation products in the infarct tissue. Phosphorylation levels of cMyBP-C were greatly reduced in ischemic areas of the MI heart compared to non-ischemic regions and sham control hearts. Post-MI plasma samples from these rats, as well as humans, were assayed for cMyBP-C and its fragments by sandwich ELISA and immunoprecipitation analyses. Results showed significantly elevated levels of cMyBP-C in the plasma of all post-MI samples. Overall, this study suggests that cMyBP-C is an easily releasable myofilament protein that is dephosphorylated, degraded and released into the circulation post-MI. The presence of elevated levels of cMyBP-C in the blood provides a promising novel biomarker able to accurately rule in MI, thus aiding in the further assessment of ischemic heart disease.

1. Introduction

Coronary heart disease is the most common cause of myocardial infarction (MI), which afflicts approximately 5 million people in the U.S. each year [1]. The infarct is associated with altered Ca^{2+} handling and myofilament protein phosphorylation that leads to lower cross-bridge cycling rates, and a loss of cardiac contractility [2, 3]. Changes in calcium sensitivity have been found to be associated with development of heart failure [4, 5]. Furthermore, increased intracellular Ca^{2+} activates the protease calpain, which leads to proteolytic degradation of contractile proteins [6, 7]. These changes correspond to the detection of degraded contractile proteins in the blood [8–11], such as cardiac troponin (cTn) I and T, which are used clinically to determine the severity of myocardial injury [12]. Recent studies have also shown that alterations in the state of contractile proteins, such as changes in phosphorylation status, might also contribute to cardiac dysfunction after an ischemic period [13–15]. The cardiac sarcomere consists of thick and thin filament proteins in which thick filaments are composed of titin, myosin, cardiac myosin binding protein-C (cMyBP-C) and myosin light chains, whereas thin filaments consist of actin, cTnI, cTnT, cTnC, and α-tropomyosin (α-TM). During MI, degradation

Abbreviations: cTnI, cardiac troponin I; cTnT, cardiac troponin T; cMyBP-C, cardiac myosin binding protein-C; LV, left ventricular; MI, myocardial infarction, kDa, kilo Dalton; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AW, anterior wall; PW, posterior wall; IP, immunoprecipitation; ng, nanogram; ml, milliliter; α-TM, α-tropomyosin; ICM, ischemic cardiomyopathy.

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of the thin filament proteins, such as cTnI and cTnT, has been extensively studied [6–12]. However, the degradation profile of thick filament proteins, in particular, cMyBP-C, and the resulting contractile dysfunction have not been systematically characterized.

cMyBP-C is an assembly protein and myosin stabilizer that is involved in regulating sarcomeric structure and function in the heart [16–19]. Increasing evidence suggests that cMyBP-C plays a critical role in regulating myosin function and cardiac contraction [14, 19, 20]. Given that mutations in this protein have been linked to familial hypertrophic cardiomyopathy in more than 60 million people worldwide [21], elucidation of its function is clinically imperative [19]. cMyBP-C comprises 2% of the total contractile proteins in the heart, belongs to the intracellular immunoglobulin super family, and is highly soluble due to its hydrophilic properties [22]. Previous literature has shown that cMyBP-C phosphorylation at Ser-273, Ser-282 and Ser-302 regulates myocardial function [14, 23, 24] and confers resistance to proteolysis and protection against MI [14, 20]. On the other hand, the degradation of cMyBP-C during MI correlates with contractile dysfunction [14, 25]. We previously demonstrated that cMyBP-C is a substrate for calpains and that calpains cleave cMyBP-C during MI [26]. This event results in the release of 40 kDa fragments, polypeptides that could potentially cause pathogenic cardiac muscle damage by interacting with myosin and inhibiting its function. At this time, the interrelationship of cMyBP-C phosphorylation, degradation, and contractile dysfunction is poorly understood. Therefore, it is necessary to identify mechanisms for cMyBP-C phosphorylation and degradation post-MI to better understand the pathological processes [8].

The objectives of this study were to determine whether cMyBP-C is an easily releasable protein, whether degradation is associated with its phosphorylation status, and whether cMyBP-C is released into the circulation post-MI. To accomplish this, we utilized an *in vitro* system, an *in vivo* rat model of MI, and samples from post-MI patients. Our data show that cMyBP-C is rapidly degraded and released in a time-dependent manner from myocardial tissue *in vitro* and that its degraded products appear in the circulation post-MI *in vivo*. In addition, our data associate cMyBP-C degradation with its dephosphorylation, which establishes a firm rationale for using elevated plasma cMyBP-C titers as a novel biomarker of myocardial injury.

2. Materials and methods

An expanded methods section is available in the online-only supplement.

2.1. Determining cMyBP-C degradation, phosphorylation and plasma levels

To determine whether cMyBP-C is an easily releasable myofilament *in vitro*, left ventricular (LV) tissue from normal male Sprague–Dawley rat hearts was incubated in phosphate buffered saline (PBS) for different durations at 37 °C. The released proteins in the effluent were analyzed by Western blot using rabbit polyclonal antibodies against cMyBP-C residues 2–14 (cMyBP-C$^{2-14}$) [27], domains C0 (Santa Cruz), C0–C1 [23, 25], C5 [28] and C8–C9 [28], and a custom-made rabbit polyclonal antibody against C-terminal 1119–1212 residues (ProSci, Inc.). Release of other sarcomeric proteins, such as myosin, actin, cTnI, and α-TM, was determined by Western blot, as described previously [20]. To determine the proteolytic profile of cMyBP-C degradation, hypoxia was induced in three-day-old neonatal rat ventricular myocytes (NRVMs) for 12 h, and total proteins were analyzed by Western blot using rabbit anti-cMyBP-C$^{2-14}$ antibodies [27]. To investigate cMyBP-C degradation in MI heart tissue, total proteins were separated on 4–15% precast Tris–HCl gels (Bio-Rad Laboratories), followed by Western blot analyses using rabbit anti-cMyBP-C$^{2-14}$ antibodies [20]. Site-specific phospho antibodies against Ser-273, Ser-282, and Ser-302 (number refers to mouse Uniport O70468) were used to determine cMyBP-C phosphorylation levels [29]. Plasma levels of cMyBP-C were quantified by sandwich ELISA using capture antibody (monoclonal anti-cMyBP-C antibody, E-7, Santa Cruz) and detection antibody (rabbit polyclonal anti-cMyBP-C^{C0-C1}) [23]. Plasma cTnI levels were measured by sandwich ELISA according to the manufacturer's recommendations (rat, Life Diagnostics; human, Calbiotech).

2.2. Rat model of myocardial infarction

Ten-week old male Sprague–Dawley rats were used to induce MI as described [30]. Three days post-MI, LV structure and function was measured in MI, sham, and naïve animals by non-invasive M-mode echocardiography, and blood and tissue samples were collected for analyses [30]. 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 Loyola University Medical Center approved all experimental procedures.

2.3. Human samples

Echocardiography and blood samples were collected from normal controls and patients admitted to the Scott &White Hospital, Temple, Texas, who were diagnosed with MI based on ECG findings and elevations in cTnI levels (cutoff >5 ng/ml). Plasma was separated immediately after blood collection, and plasma cTnI, glucose and creatine levels were measured within 1 h in a clinical lab; aliquoted and stored at −80 °C prior to determining cMyBP-C levels. In addition, explanted heart samples (non-ischemic, viable region) were obtained from patients with ischemic cardiomyopathy undergoing heart transplantation and non-failing donor hearts from the Gift of Hope from the tissue repository of the Cardiovascular Institute at Loyola University Medical Center, Maywood, IL. These samples were used to determine the phosphorylation status of cMyBP-C and its degradation in human hearts. All human samples were obtained with informed consent and de-identified with Institutional Review Board approval.

2.4. Statistical analysis

Results are presented as mean ± SEM. Comparisons between groups (sham vs. MI) were made using a Student's *t*-test. Differences within and between groups were analyzed with Two-Way Repeated-Measures ANOVA, followed by a Tukey post-hoc test using SigmaPlot V11. $P<0.05$ was considered significant.

3. Results

3.1. cMyBP-C is an easily releasable sarcomeric protein in vitro, and cMyBP-C dephosphorylation is associated with its degradation

To determine whether cMyBP-C is an easily releasable myofilament protein, LV heart tissue from wild-type rats was incubated in PBS at 37 °C for 1 s, 0.5, 1, 3, 6 and 12 h. During these incubations, releasable proteins from the LV tissue were allowed to freely diffuse into the PBS effluent. SDS-PAGE analyses showed that the release of total cardiac protein increased over time (Fig. 1A). To determine whether full-length cMyBP-C and its proteolytic fragments were released into the PBS effluent, Western blot analysis was performed with N′-specific rabbit anti-cMyBP-C$^{2-14}$ antibodies that recognize full-length and all N′-fragments of cMyBP-C (Figs. 1B and D). Results show that cMyBP-C and its fragments are released into the effluent as early as 1 s with a time-dependent increase in release up to 12 h. After 0.5 h, the predominant cMyBP-C fragments (40 kDa) were also released in a time-dependent manner. For comparison, other cardiac sarcomeric proteins, such as actin (Fig. 1C), myosin, cTnI and α-TM (Online Supplemental Fig. 1), were measured in the effluent. Results show the presence of myosin and actin, and a time-dependent increase in α-TM and cTnI. Antibodies