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Cardiac-specific deletion of protein phosphatase 1β promotes increased myofilament protein phosphorylation and contractile alterations

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ABSTRACT

There are 3 protein phosphatase 1 (PP1) catalytic isoforms (α, β and γ) encoded within the mammalian genome. These 3 gene products share ~90% amino acid homology within their catalytic domains but each has unique N- and C-termini that likely underlie distinctive subcellular localization or functionality. In this study, we assessed the effect associated with the loss of each PP1 isoform in the heart using a conditional Cre-loxP targeting approach in mice. *Ppp1ca-loxP*, *Ppp1cb-loxP* and *Ppp1cc-loxP* alleles were crossed with either an *Nkx2.5-Cre* knock-in containing allele for early embryonic deletion or a tamoxifen inducible α-myosin heavy chain (αMHC)-MerCreMer transgene for adult and cardiac-specific deletion. We determined that while deletion of *Ppp1ca* (PP1α) or *Ppp1cc* (PP1γ) had little effect on the whole heart, deletion of *Ppp1cb* (PP1β) resulted in concentric remodeling of the heart, interstitial fibrosis and contractile dysregulation, using either the embryonic or adult-specific Cre-expressing alleles. However, myocytes isolated from *Ppp1cb* deleted hearts surprisingly showed enhanced contractility. Mechanistically we found that deletion of any of the 3 PP1 gene-encoding isoforms had no effect on phosphorylation of phospholamban, nor were Ca^{2+} handling dynamics altered in adult myocytes from *Ppp1cb* deleted hearts. However, the loss of *Ppp1cb* from the heart, but not *Ppp1ca* or *Ppp1cc*, resulted in elevated phosphorylation of myofilament proteins such as myosin light chain 2 and cardiac myosin binding protein C, consistent with an enriched localization profile of this isoform to the sarcomeres. These results suggest a unique functional role for the PP1β isoform in affecting cardiac contractile function.

1. Introduction

Protein phosphatase 1 (PP1) is a serine/threonine protein phosphatase that plays a fundamental role in many cellular processes [1]. The three isoforms of the catalytic subunits (PP1α, PP1β, and PP1γ) are encoded by distinct genes that are highly homologous to one another, although their divergent N- and C-termini are believed to underlie some diversification in target selectivity [2–4]. However, isoform-specific regulation and functions of specific PP1 isoforms are complex and remain poorly understood. For example, PP1α and PP1γ have been reported to selectively interact with neurabin to target the actin cytoskeleton [5,6], although this interaction specificity is not likely due to the more divergent N- or C-termini of PP1 [7]. PP1β also forms a selective complex with the targeting subunit 2 of myosin phosphatase (MYPT2) [8]. In the heart, increased PP1 activity is implicated in heart failure progression, and PP1β was reported to be preferentially associated with the sarcoplasmic reticulum (SR) where it influences Ca^{2+} cycling [9,10].

Dynamic phosphorylation and dephosphorylation of key regulatory proteins are major determinants of cardiac Ca^{2+} cycling and myofilament protein force production and cross-bridge cycling. For example, sympathetic stimulation generates cAMP in the heart that activates protein kinase A (PKA) resulting in the phosphorylation of nodal Ca^{2+} handling proteins, such as ryanodine receptor 2 (RyR2) and phospholamban (PLN), which then augment contractility and lusitropy to facilitate greater cardiac output [11,12]. Several myofilament proteins are also regulated at the level of phosphorylation to affect contractile or lusitropic activity [8,13], such as myosin light chain 2 V (MLC2V) at serine 14/15 by the myosin light chain kinase (MLCK) [14,15]. Indeed, transgenic mice overexpressing a phosphorylation-deficient MLC2V mutant protein in the heart showed aberrant cardiac function [16,17]. Cardiac myosin binding protein C (cMyBPC), a 140-kDa thick filament protein that regulates the binding of the myosin head to actin [18],

Abbreviations: αMHC, α-myosin heavy chain; cMyBPC, cardiac myosin binding protein C; FS, fractional shortening; I-1, inhibitor 1; I-2, inhibitor 2; IP, intraperitoneal; fl, loxP site; LV, left ventricle; MLC2V, myosin light chain 2 V; MLCK, myosin light chain kinase; PKA, protein kinase A; PP1, protein phosphatase 1; PLN, phospholamban; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; shRNA, short hairpin RNA; SR, sarcoplasmic reticulum.

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can be phosphorylated at serines 273/282/302 by PKA, protein kinase C and Ca^{2+}/calmodulin-dependent kinase [19]. Transgenic mice overexpressing phosphorylation deficient or phosphomimetic cMyBPC mutant proteins in the heart demonstrate that phosphorylation of this protein is a critical regulatory mechanism for altering cardiac function [20], and very similar observations have also been made for phosphorylation of the troponin proteins in the heart [21,22].

Given the pro-contractile effects associated with kinase-mediated phosphorylation of selected myofilament proteins, regulated inhibition of protein phosphatases is hypothesized to maintain or augment this sympathetic "fight-or-flight" profile of catecholamine-mediated protein phosphorylation. Indeed, studies of inhibitor-1 and inhibitor-2, proteins that specifically inactivate PP1, support such a relationship. For example, overexpression of either inhibitor-2 [23] or a constitutively active inhibitor-1 mutant [24] in the hearts of transgenic mice resulted in increased cardiac function. Conversely, overexpression of a catalytic PP1α isoform in the heart led to reduced cardiac function and heart failure [11]. However, other data generated in mice where PP1 activity has been manipulated suggest a more complicated picture, such that inhibition of PP1 by overexpression of inhibitor-2 led to more severe heart failure in mice with pressure overload [25], while inhibitor-1 overexpressing transgenic mice showed cardiac hypertrophy with depressed cardiac function [26]. Thus, despite the clear nodal position of PP1 in regulating cardiac function, it remains unclear how this phosphatase might be modulated to treat heart disease [27], although one attractive possibility is to more selectively target only one of the 3 known PP1 gene-encoding isoforms: *Ppp1ca* (PP1α), *Ppp1cb* (PP1β), or *Ppp1cc* (PP1γ).

In the present study, we employed a novel approach to address this long-standing issue as to how PP1 activity might be manipulated to affect cardiac contractility and propensity towards heart failure. We employed Cre-loxP technology to achieve cardiac-specific deletion of each of the 3 PP1 gene isoforms in the mouse. Our results show that while deletion of PP1α or PP1γ had no effect on the heart at baseline, loss of PP1β promoted ventricular remodeling and heart failure, in association with a dramatic change in myofilament protein phosphorylation, but without a change in Ca^{2+} handling dynamics or PLN phosphorylation.

2. Materials and methods

2.1. Mice and tamoxifen administration

All mice were bred and utilized according to procedures approved by the Animal Care and Use Committee at the Cincinnati Children's Hospital Medical Center. *Ppp1cα-loxP* (*fl*), *Ppp1cb-fl*, and *Ppp1cc-fl* mice, in which exon 3 was flanked by Cre recombinase-dependent loxP recognition sequences, were generated in collaboration with Lexicon Genetics as described previously [28]. These mice were then crossed with the Cre recombinase knock-in line *Nkx2.5-Cre* [29] or the tamoxifen-inducible α-myosin heavy chain (αMHC)-MerCreMer transgene [30] to achieve efficient deletion of each PP1 isoform in the heart. Tamoxifen (Sigma, T-5648) was dissolved in peanut oil (5 mg/ml) and administered to $Ppp1c^{\alpha MHC\text{-}MerCreMer}$ mice via intraperitoneal (IP) injections for five consecutive days (0.5 mg/day), after which mice were analyzed 2, 6, and 8 weeks later.

2.2. Isolation of adult mouse cardiomyocytes

Adult ventricular myocytes were isolated as described previously [31]. In brief, hearts from 2 month-old mice were removed after treatment with heparin (0.35 units) under anesthesia (Nembutal, 100 mg/kg), and cannulated for retrograde perfusion with a solution containing liberase blendzyme (Roche, 05,401,151,001) followed by a gentle mechanical disassociation using sterile plastic pipettes to generate individual myocytes. After transferring to a new tube, myocytes were allowed to settle by gravity followed by $CaCl_2$ re-introduction. The cell pellet was resuspended in MEM (Modified Eagles Medium) plus 5% fetal bovine serum, and cells were counted and plated on laminin-coated dishes.

2.3. Ventricular myocyte protein subfractionation

Subcellular protein fractions were prepared as described previously [32]. In brief, cells were washed and collected in lysis buffer containing 50 mM Tris–HCl pH 7.4, 5 mM EGTA, 2 mM EDTA, 5 mM DTT, 0.05% digitonin, and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78,440). Cell lysates were centrifuged at 14,000 ×*g* for 15 min, and the supernatant was collected as a cytosolic fraction. The pellet was resuspended in lysis buffer containing 1% Triton x-100 for 10 min and centrifuged for 15 min at 14,000 ×*g* to collect the supernatant as the membrane fraction. The subsequent triton-insoluble pellet contained the myofilament fraction. This insoluble pellet was resuspended with PBS buffer containing 0.5 M NaCl for 20 min on ice and centrifuged to collect supernatant as the final myofilament protein fraction. Protein samples from each fraction were quantified with a Bradford assay (Bio-Rad) and subjected to 10% SDS-PAGE for Western blot detection of PP1α (Santa Cruz Biotechnology, sc-6104), PP1β (Millipore, 07–1217), PP1γ (Santa Cruz Biotechnology, sc-6108), GAPDH (Fitzgerald, 10–1500), Troponin I (Cell Signaling Technology, 4002), pSer 23/24-Troponin I (Cell Signaling Technology, 4004), Caveolin-3 (BD Biosciences, 610,421), I-1 (Abcam, ab40877), and I-2 (R&D Systems, AF4719).

2.4. Myofilament protein isolation, Pro-Q Diamond staining and Phos-Tag westerns

Mouse hearts were minced into small pieces with scissors and homogenized in F60 solution (60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78,440)). Tissues were collected by centrifugation for 3 min at 8000 ×*g*, resuspended in F60 solution two more times, followed by 2 washes with F60 solution containing 1% Triton x-100. The pellet was washed 3 times with F60 and eluted with buffer (20 mM HEPES, 1% Triton, 0.5 M NaCl, 1 mM EDTA, and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78,440)). Protein samples were quantified and equal amounts of protein was subjected to 12% SDS-PAGE. For Pro-Q Diamond staining, the acrylamide gel containing the separated proteins was fixed, washed and stained with Pro-Q Diamond solution (Invitrogen, P-33,300). Images were visualized through UV transillumination (Bio-Rad). For Phos-Tag gels, Phos-Tag (Wako Chemicals, 304–93,526) and $MnCl_2$ solution were added to the 12% acrylamide gel to reach a final concentration of 50 μM, and 0.1 mM respectively. The gel was washed with transfer buffer containing 1 mM EDTA, transferred, and blotted with MLC2V antibody (Proteintech, 10,906–1-AP).

2.5. Ca^{2+} and cell shortening measurements

Ca^{2+} measurement assays in adult cardiac myocytes have been described previously [33]. In brief, cells were loaded with 2 μM Fura-2 acetoxymethyl ester (Invitrogen, C-2938) for 15 min, and then placed in Tyrode's solution containing: 130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4). The Fura-2 fluorescence ratio was determined with a Delta scan dual-beam spectrofluorophotometer (Photon Technology International) operated at an emission wavelength of 510 nM and excitation wavelengths of 340 and 380 nM. The myocyte stimulation frequency for Ca^{2+} transient measurements was 0.5 Hz. For caffeine-induced Ca^{2+} release, myocytes were perfused with a Tyrode's solution and stimulated at 0.5 Hz until stabilization of the transients, after which caffeine was acutely added. Ca^{2+} traces from healthy myocytes sensitive to caffeine treatment