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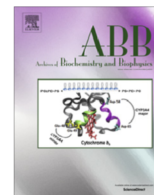

In vitro rescue study of a malignant familial hypertrophic cardiomyopathy phenotype by pseudo-phosphorylation of myosin regulatory light chain

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Pseudo-phosphorylation of cardiac myosin regulatory light chain (RLC) has never been examined as a rescue method to alleviate a cardiomyopathy phenotype brought about by a disease causing mutation in the myosin RLC. This study focuses on the aspartic acid to valine substitution (D166V) in the myosin RLC shown to be associated with a malignant phenotype of familial hypertrophic cardiomyopathy (FHC). The mutation has also been demonstrated to cause severe functional abnormalities in transgenic mice expressing D166V in the heart. To explore this novel rescue strategy, pseudo-phosphorylation of D166V was used to determine whether the D166V-induced detrimental phenotype could be brought back to the level of wild-type (WT) RLC. The S15D substitution at the phosphorylation site of RLC was inserted into the recombinant WT and D166V mutant to mimic constitutively phosphorylated RLC proteins. Non-phosphorylatable (S15A) constructs were used as controls. A multi-faceted approach was taken to determine the effect of pseudo-phosphorylation on the ability of myosin to generate force and motion. Using mutant reconstituted porcine cardiac muscle preparations, we showed an S15D-induced rescue of both the enzymatic and binding properties of D166V-myosin to actin. A significant increase in force production capacity was noted in the *in vitro* motility assays for S15D-D166V vs. D166V reconstituted myosin. A similar pseudo-phosphorylation induced effect was observed on the D166V-elicited abnormal Ca^{2+} sensitivity of force in porcine papillary muscle strips reconstituted with phosphomimic recombinant RLCs. Results from this study demonstrate a novel *in vitro* rescue strategy that could be utilized *in vivo* to ameliorate a malignant cardiomyopathic phenotype. We show for the first time that pseudo-RLC phosphorylation can reverse the majority of the mutation-induced phenotypes highlighting the importance of RLC phosphorylation in combating cardiac disease.

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Introduction

Familial hypertrophic cardiomyopathy (FHC)[1] is a heritable form of cardiac hypertrophy caused by mutations in genes encoding for all major sarcomeric proteins [1–4]. The clinical manifestations of FHC range from asymptomatic to progressive heart failure and sudden cardiac death (SCD), and can vary from individual to individual even within the same family. Several mutations in the myosin regulatory light chain (RLC) have been implicated in the development of FHC and some of them are among the most prevalent mutations capable of affecting the thick filament structure and sarcomeric protein organization [5–7]. The RLC wraps around the α-helical neck region of the myosin head (lever arm) [8], and plays an important role in stabilizing its structure and function [9]. Considering the importance of the RLC in cardiac muscle contraction, it is understandable why subtle structural alterations in the RLC sequence would lead to cardiac disease. In particular, the D166V (aspartic acid to valine) mutation in the RLC was found to result in hypertrophic cardiomyopathy and SCD phenotypes [10,11]. The mutation occurs at the last amino acid residue of the human ventricular RLC (Swiss-Prot: P10916) and substitutes valine for the normally occurring aspartic acid (Fig. 1). In the three dimensional structure of RLC, the site of mutation lies close

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[1] *Abbreviations used:* FHC, familial hypertrophic cardiomyopathy; SCD, sudden cardiac death; RLC, regulatory light chain of myosin; D166V, aspartic acid to valine mutation; MLCK, myosin light chain kinase; WT-RLC, human ventricular wild-type RLC, Swiss-Prot: P10916; MHC, myosin heavy chain; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PC, porcine cardiac; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; Tm–Tn, tropomyosin–troponin complex; PIA, pyrene iodoacetamide; CDTA, cyclohexane-1,2-diaminetetraacetic acid; TnC, troponin C; TCA, trichloroacetic acid; ABP, actin-binding protein; TRITC, tetramethylrhodamine isothiocyanate.

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to serine 15 (Ser15), a recognized site for the myosin light chain kinase (MLCK)-dependent RLC phosphorylation. As shown by many, myosin RLC phosphorylation plays important functional and structural roles in cardiac muscle contraction [12–16], and any changes in RLC phosphorylation are expected to affect heart performance and lead to cardiac disease [17–19]. Besides a highly conserved N-terminal phosphorylatable Ser15, the RLC also contains a Ca^{2+}–Mg^{2+} binding site [20] which is thought to be occupied by Mg^{2+} when muscles are in the relaxed state [21] and partially saturated with Ca^{2+} during contraction [22]. The D166V mutation is located close to these two functionally important RLC domains and is also positioned in the elbow of the myosin heavy chain (MHC) region that links the cross bridge lever arm with the rod portion of myosin [8] (Fig. 1). Our previous studies on transgenic (Tg)-D166V mice revealed a mutation specific functional change in the Ca^{2+} dependent cardiac muscle contraction, which coincided with a significant decrease in the endogenous level of RLC phosphorylation [23]. This result implied a possible communication between all functional regions of RLC and in particular between the site of the D166V mutation and phosphorylatable Ser15 (Fig. 1). It also suggested an importance of RLC phosphorylation in the manifestation of the FHC-linked RLC phenotype. Our further study of this mutation showed that an MLCK-induced phosphorylation of Tg-D166V cardiac muscle preparations was able to partially reverse the negative functional consequences of the D166V mutation tested in myofibrils and skinned papillary muscle strips [24].

In the current report we explore a novel rescue mechanism via constitutive RLC phosphorylation, using pseudo-phosphorylated RLC mimetic proteins. Porcine cardiac preparations were reconstituted with recombinant RLC constructs containing the following mutations: (1) S15D substitution to mimic a constitutively phosphorylated RLC; (2) S15A to mimic a non-phosphorylatable RLC; (3) S15D-D166V to mimic a constitutively phosphorylated RLC in D166V background; (4) S15A-D166V to mimic D166V-RLC protein that is incapable of phosphorylation; and (5) WT-RLC and D166V-RLC proteins as controls. The advantage of using porcine czardiac preparations reconstituted with phosphomimic RLCs was 2-fold. First, in contrast to transgenic mouse cardiac samples expressing the α-MHC, this study was performed on porcine preparations expressing the β-MHC, the same isoform of myosin found in the human heart. Hence, our experiments provide an essential control in studying human disease by using the proper MHC background. Second, the preparations of fixed levels of RLC phosphorylation allowed us to overcome any potential discrepancies between the samples due to potential varied levels of MLCK-induced phosphorylation of RLC.

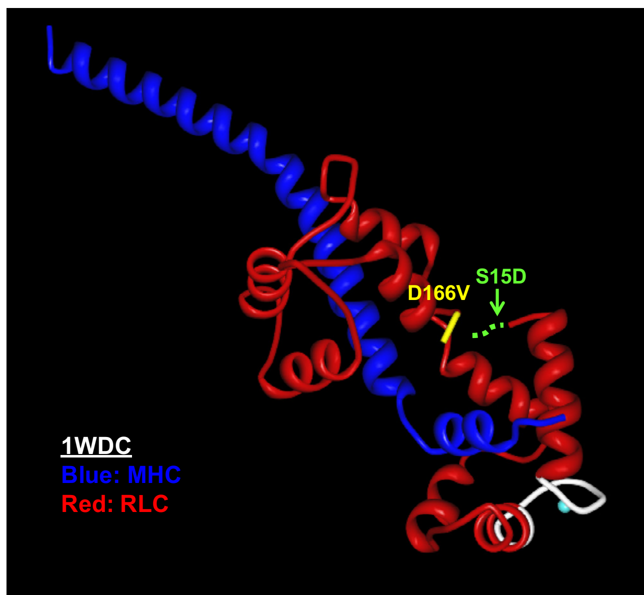

Fig. 1. The location of the D166V mutation in myosin RLC pictured in the regulatory domain of scallop myosin (1WDC) [49]: the MHC is labeled in dark blue and the RLC in red, the D166V mutation (in yellow) and the Ca^{2+}–Mg^{2+} binding site (in white). The hypothetical serine phosphorylation site has been indicated (dashed green line) since the region of the RLC containing the serine 15 site has not been resolved in any of the available myosin crystal structures.

In this study, we demonstrate the ability of the S15D phosphomimic RLC proteins to reverse or partially rescue the majority of the detrimental effects induced by the D166V mutation. We show an S15D-induced rescue of V_{max} of the actin-activated myosin ATPase activity which was observed to be decreased due to the D166V mutation. A significant increase in force production capability was noted in the *in vitro* motility assays for S15D-D166V vs. D166V reconstituted myosin. In porcine papillary muscle strips reconstituted with the S15D-D166V construct, we monitored a similar pseudo-phosphorylation induced rescue of the D166V-elicited increase in Ca^{2+} sensitivity. Importantly, effects of pseudo-phosphorylation of D166V observed in this report are in accord with the results obtained in Tg-D166V preparations phosphorylated with Ca^{2+}-calmodulin activated MLCK [24] suggesting that the mechanism by which RLC phosphorylation counteracts the effects of the D166V mutation is specific to the phosphorylation-induced RLC conformation rather than the enzymatic activity of the MLCK or its counteractive phosphatase.

Materials and methods

Mutation, expression, and purification of wild-type (WT) human cardiac RLC and the FHC mutants

The cDNA for WT human cardiac RLC was cloned by reverse transcription-polymerase chain reaction using primers based on the published cDNA RLC sequence (GenBank™ Accession No. AF020768) using standard methods as described previously [20]. The RLC mutants: S15D, S15A, D166V, S15D-D166V and S15A-D166V were generated using overlapping sequential polymerase chain reaction [25]. Briefly, WT and mutant cDNAs were constructed with an *Nco*I site at the N-terminal ATG and a *Bam*HI site following the stop codon to facilitate ligation into the *Nco*I–*Bam*HI cloning site of the pET-3d (Novagen) plasmid vector and transformation into DH5α cloning host bacteria for expression. The cDNAs were transformed into BL21 expression host cells and all proteins were expressed in large (16 l) cultures. Expressed proteins were purified using a S-Sepharose column followed by a Q-Sepharose column, both equilibrated with 2 M urea, 25 mM Tris–HCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.001% NaN_3, pH 7.5. The proteins were eluted with a salt gradient of 0–450 mM NaCl. The final purity of the proteins was evaluated using 15% SDS–PAGE.

Preparation of porcine cardiac (PC) myosin

PC myosin was purified as described by Pant et al. [26]. Briefly, left ventricular muscle was chilled on ice, washed clear of blood with ice-cold dH_2O, and minced. The muscle mince was rinsed in ice-cold dH_2O until clear and extracted on ice with stirring for 1.5 h in 300 ml/100 g of muscle in Edsall–Weber solution (0.012 M Na_2CO_3, 0.04 M $NaHCO_3$, and 0.6 M KCl, pH 9.0). The homogenate was then centrifuged at 13,000*g* for 20 min, and the supernatant was precipitated with 13 vol of water containing 1 mM EDTA (ethylenediaminetetraacetic acid) and 1 mM DTT,

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