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miR-208A Targeted Suppression of PDE4D Directly Enhances Myocyte Contractile Function via PKA-Mediated Phosphorylation of cTnI and PLN

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Molecular inotropy refers to cardiac myocyte contractile status that can be titrated, positively or negatively, to affect overall heart pump performance. Although inotropic drugs have been in clinical practice for many decades, there is an urgent need to discover new inotropes with unique mechanisms of action for the treatment of heart failure. Here, we investigate the prospect of micro-RNAs to directly modify downstream inotropic signaling pathways for improving contractile function. We focused on miR-208a owing in part to its known restricted expression profile in the myocardium. Results show that acute miR-208a expression, at 4-5 fold over endogenous miR-208a and independent of altered host myosin gene expression, confers significant positive inotropy and faster relaxation compared to miR-208a-mutant and untreated control adult cardiac myocytes ($P<0.05$). Using in vitro cardiac stress testing, miR-208a amplified the inotropic and relaxation responses to increased stimulation frequency. MiR-208a also promoted fast calcium transient decay with no change in peak calcium accounting in part for enhanced relaxation. To gain insight into mechanism, we analyzed in silico putative miR-208a targets, focusing on potential inotropic signaling targets. Interestingly, we provide evidence that miR-208a has a direct effect to negatively regulate expression of PDE4D, but does not affect PDE5A in myocytes. Consistent with these findings, phosphorylation of cTnI and PLN at PKA sites was increased in myocytes after acute miR-208a expression. Taken together, we show for the first time that miR-208a confers positive inotropy and enhances relaxation in myocytes by PKA mediated phosphorylation of cTnI and PLN through a mechanism of direct suppression of PDE4D. Because heart failure is associated with decreased phosphorylation of cTnI and PLN, miR-208a may represent a new therapeutic modality for enhancing ventricular myocyte performance via the PDE4D-cAMP-PKA signaling pathway.

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Regulatory Light Chain Phosphorylation Mimic S15D Causes Partial Rescue of Isometric Force Production in FHC Causing Mutation D166V

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Familial hypertrophic cardiomyopathy (FHC) is characterized by a pathological thickening of the muscle surrounding the heart and is the leading cause for sudden cardiac death in young people. There are numerous mutations in sarcomeric proteins that have been implicated in causing FHC. Myosin, the molecular motor that powers cardiac muscle contraction, consists of a globular domain and an elongated α-helical neck region, which is thought to undergo large conformational changes during muscle contraction. The myosin regulatory light chain (RLC) functions to support the neck region, therefore it is not surprising that several single amino acid substitutions in the RLC have been implicated in FHC.

Here we studied D166V, an RLC point mutation that is associated with increased left ventricular wall thickness, abnormal electrocardiogram, and decreased isometric force and ATPase in skinned fibers from transgenic mouse hearts. We have expressed porcine cardiac β myosin (the same isoform found in humans) and replaced the endogenous RLC with a human RLC. The exchanged RLC contained either wild type (WT), D166V, S15A, or a double mutant D166V/S15A, or D166V/S15D. The S15D mutation served as a phosphorylation mimic. We performed frictional loading assays using a modified *in vitro* motility assay and determined the average force produced by a bed of monomeric myosin. While maximal unloaded velocity was unchanged for all of the mutants studied, the force of D166V and D166V/S15A was significantly reduced compared to WT, with an additive decrease in the double mutant. D166V/S15D resulted in a significant increase in force compared to D166V/S15A and was restored to near WT levels. These results suggest that D166V causes a reduction in myosin isometric force production and phosphorylation may act to recover it.

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Physiological Effects of FHC-Causing K104E Mutation in the Myosin Regulatory Light Chain

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We have studied the physiological effects of the Lysine 104 to Glutamic Acid (K104E) mutation in the ventricular myosin regulatory light chain (RLC), shown to cause familial hypertrophic cardiomyopathy (FHC). *In vitro* and *in vivo* experiments were performed using transgenic (Tg) mouse cardiac muscle preparations carrying the K104E-RLC mutation. We observed a slight but significant decrease in maximal force ($\Delta F_{max} \cong 8 kN/m^2$) and an increase in the Ca^{2+}-sensitivity ($\Delta pCa_{50} \cong 0.1$) of isometric contraction in glycerinated skinned muscle fibers from Tg-K104E compared to Tg-WT mice. No mutant related changes in rigor binding of transgenic K104E mouse myosin to pyrene-actin were observed. Likewise, no changes in actomyosin or myofibrillar ATPase activities were monitored. Histological examination of Masson's trichrome stained Tg-hearts showed signs of fibrosis in 8 mo-old Tg-K104E mice compared to age matched Tg-WT. These observed changes in myocyte organization in Tg-K104E mice could be due to ~4-fold decrease in the myosin heavy chain-RLC interaction determined by the binding of bacterially expressed K104E mutant to RLC-depleted porcine myosin. Echocardiography examination of senescent Tg-K104E mice confirmed a hypetrophic phenotype and showed a significantly enlarged interventricular septum and LVPWd (left ventricular posterior wall in diasole), ~1.6-fold increase in LV mass and significantly decreased LVIDs (LV inner diameter in systole) compared to Tg-WT mice. However, EF (ejection fraction) was higher in Tg-K104E mice ($73 \pm 8\%$) compared to $61 \pm 7\%$ observed in Tg-WT mice. In addition, Doppler E velocity was also 1.3-fold higher in Tg-K104E mice compared to Tg-WT. These results confirm a mutation induced hypertrophic phenotype in Tg-K104E mice, similar to the patients carrying this FHC-mutation. No drastic changes at the level of actomyosin interaction or in cardiac function assessed in Tg-K104E mice suggest that the mutation might be associated with good prognosis. Supported by NIH-HL071778 (DSC).

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Myosin Regulatory Light Chain Phosphorylation Rescues Cardiac Dysfunction Caused by Familial Hypertrophic Cardiomyopathy-Linked Mutations

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In this report, we compared the role of cardiac myosin regulatory light chain (RLC) phosphorylation on cardiac function in skinned muscle preparations containing two RLC mutations, R58Q (arginine to glutamine) and D166V (aspartic acid to valine), both linked with a malignant disease phenotype. Previous studies on D166V-transgenic mice showed that the myosin light chain kinase (MLCK)-induced phosphorylation of D166V mouse myocardium was able to alleviate detrimental functional effects caused by this mutation. In this study, we used recombinant phosphomimetic S15D (serine to aspartate) mutation in the D166V background exchanged into porcine cardiac myosin and investigated the effect of constitutively phosphorylated RLC proteins on the actomyosin interaction. The actin-activated myosin ATPase activity, which was decreased in D166V- exchanged myosin, was partially rescued in S15D-D166V exchanged myosin reaching the level observed for WT-reconstituted myosin. Similarly, myosin reconstituted with S15D-D166V mutant showed an increase in the binding to fluorescently labeled actin compared to D166V-reconstituted myosin, with Kd=1.9 μM and Kd=41 μM, respectively. To further investigate whether MLCK-phosphorylation could rescue the phenotype associated with the R58Q mutation, we studied force development in transgenic R58Q-mouse papillary muscle fibers. Compared to Tg-WT, a drastic reduction in maximal force and myofibrillar ATPase activity was observed in samples carrying the R58Q mutation. However, MLCK induced phosphorylation of R58Q muscle fibers resulted in significantly increased maximal force and myofibrillar ATPase activity. These results suggest that RLC phosphorylation plays an important role not only in the physiological performance of the heart, but also helps to maintain normal cardiac function in the diseased myocardium. Our findings may contribute to the development of targeted cellular therapeutic approaches to limit FHC related cardiac dysfunction. Supported by AHA-10POST3420009 (PM) and NIH- HL071778 and HL090786 (DSC).

2833-Pos Board B603

Novel Phosphorylation Sites and Reduced Phosphorylation Levels in Human Cardiac MyBP-C from Failing Hearts

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Cardiac myosin binding protein-C (cMyBP-C) is a large multidomain protein associated with the thick filaments. Phosphorylation of cMyBP-C is a regulator of cardiac contractility and it is known that the phosphorylation status of