To address this issue, a selective exchange procedure was used in which 50% and 70% of the endogenous cTn complex in permeabilized human cardiomyocytes was exchanged with recombinant unphosphorylated human cTn. Cardiomyocytes isolated from healthy donor hearts showed almost saturated phosphorylation levels at the ser23/24 of cTnI. Endogenous phosphorylated cTn of donor cardiomyocytes (pCa_{50}= 5.45 ± 0.03) was exchanged with 0.5 and 1.0 mg/ml unphosphorylated recombinant human cTn (cTn-U), which resulted in an increase in Ca^{2+}-sensitivity (ΔpCa_{50}=0.08). Subsequent incubation of the cells with PKA reversed Ca^{2+}-sensitivity to baseline levels (pCa_{50}= 5.46 ± 0.03).

To study if the effect of PKA-mediated phosphorylation on cTnI ser23/24 depends on phosphorylation of other contractile proteins, failing human cardiac tissue was used in which phosphorylation of cTnI and cMyBP-C is depressed. Cells from failing tissue showed increased Ca^{2+}-sensitivity (pCa_{50} 5.56 ± 0.03) compared to donor cells. Endogenous cTn of failing cardiomyocytes was exchanged with 0.5 and 1.0 mg/ml cTn pre-treated with PKA to fully saturate ser23/24 (cTn-bisP). However, upon exchange with the cTn-bisP complex, Ca^{2+}-sensitivity did not decrease. Subsequent PKA incubation reduced pCa_{50} back to the level observed in donor myocardium. This indicates that the effect of cTnI ser23/24 bis-phosphorylation on Ca^{2+}-sensitivity is dependent on PKA-mediated phosphorylation of other contractile protein(s). Preliminary protein phosphorylation data point towards the involvement of cMyBP-C.

2585-Pos Board B555

EM and 3D-Reconstruction of Thin Filaments Reconstituted with Truncated Troponin I Associated with Myocardial Stunning

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Myocardial ''stunning'', a condition resulting from a short period of ischemia followed by reperfusion, can cause cardiac dysfunction, despite the absence of tissue necrosis. Stunning can be associated with rapid proteolytic truncation of the C-terminal 17 acids of TnI (to form ''$TnI_{1\text{-}192}$''). Expression of $TnI_{1\text{-}192}$ in transgenic mice is sufficient to account for the stunning phenotype (Murphy *et al.*, 2000), where for example myofibrils containing $TnI_{1\text{-}192}$ and otherwise normal troponin-tropomyosin display increased Ca^{2+}-sensitivity (Narolska *et al.*, 2006; also Foster *et al.*, 2003). In the current study, electron microscopy and 3D-image reconstruction of thin filaments containing $cTnI_{1\text{-}192}$ and control TnC, TnT and tropomyosin was performed to determine if the truncation causes an imbalance in the tropomyosin distribution between different regulatory states. Negatively stained ''mutant'' filaments showed characteristic periodic troponin projections and tropomyosin strands. Both helical reconstruction and single particle analysis indicated that at low-Ca^{2+} the tropomyosin localized on the inner aspect of the outer domain of actin. As expected, tropomyosin moves to the inner domain of actin in Ca^{2+} (Foster *et al.*, 2003). However, truncated TnI appears to promote an extra transition of tropomyosin from the Ca^{2+}- induced, closed position on actin toward the myosin-induced, open-state position. Here, tropomyosin in myosin-free thin filaments appears biased towards the open-state in the presence of only Ca^{2+}. Cross-correlation of filament segments to models of the blocked-, closed-, and open-states (as in Pirani *et al.*, 2005) confirms this open-state bias, which correlates well with the increase in Ca^{2+}-sensitivity observed in *in vitro* and in fiber assays of function.

2586-Pos Board B556

Impact Of N-terminal Truncation Of Cardiac Troponin I On Myofilament Chemo-mechanical Transduction: Implications For The Enhanced Cardiac Function In Hemodynamic Adaptation

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The deletion of N-terminal extension of cardiac troponin I($cTnI_{ND}$) by restricted proteolysis has been recently proposed to be a novel mechanism to regulate cardiac function during hemodynamic adaptation. In vivo and isolated working heart from transgenic mice overexpressing $cTnI_{ND}$ revealed an enhanced rate of relaxation and reduced end diastolic pressure. However, the functional effect of $cTnI_{ND}$ on myofilament properties has not been fully evaluated. Accordingly, we determined the functional effects of $cTnI_{ND}$ on cardiac tension cost(cross-bridge cycling), maximal tension development(F-max) and Ca^{2+}-sensitivity(EC_{50}) using mechanical force- and enzyme-coupled UV absorbance measurements. Wild-type(WT) or $cTnI_{ND}$ containing recombinant troponin(cTn) complexes were exchanged for endogenous cTn in skinned rat cardiac trabeculae. $cTnI_{ND}$ induced a significantly reduction in Fmax and Ca^{2+}-sensitivity but increased cross-bridge cycling rate. In addition, by using steady-state fluorescence measurements, we found that the decreased myofilament Ca^{2+} sensitivity is due to a decrease in Ca^{2+} binding affinity of the regulatory site of cTnC in the thin filament. We conclude that increased cross-bridge cycling rate by $cTnI_{ND}$ may underlie, in part, the modulation of cardiac function and hemodynamic adaptation associated with $cTnI_{ND}$.

Summarized Table

	WT (N=10)	cTnIND (N=8)	p value
Fmax (mN/mm2)	42.9 ± 5.1	17.8 ± 1.7	0.0007*
Hill	4.1 ± 0.7	5.1 ± 0.6	0.2587
EC50 (uM)	2.6 ± 0.2	3.7 ± 0.2	0.0022*
Tension Cost	7.5 ± 0.6	11.4 ± 1.3	0.0140*

2587-Pos Board B557

Structural and Proteomic Analysis of the Drosophila Cardiac Tube

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Drosophila melanogaster possess a simple linear heart tube which is an efficient in vivo system for studying basic developmental and physiological processes as well as for investigating potentially conserved pathogenic mechanisms of genetically inherited cardiac disorders. Human cardiomyopathies commonly arise from cytoarchitectural mutations. We previously showed that, as in humans, Drosophila exhibit disparate cardiac responses to depressed or enhanced myosin biomechanical properties. Here, we further characterized the morphological and ultrastructural consequences of altered myosin function on the Drosophila heart. Fluorescent microscopy revealed D45 flies, expressing myosin with depressed ATPase and in vitro sliding properties, show cardiac dilation with relatively normal myofibrillar organization. However, Mhc5 fly hearts, expressing myosin with enhanced molecular properties show centrally located restricted regions, a loss of contractile material and myofibrillar disarray. Moreover, electron microscopy revealed perturbed sarcomeric organization of the cardiomyocytes in both mutants. Mitochondria appeared swollen with apparent increased matrix volume and membranic rupture resulting in a prevalence of vacuolization. These cardiac phenotypes bear similarity to those observed in human cardiomyopathies and imply the existence of conserved pathological responses to altered myosin motor function. To further substantiate the use of the Drosophila heart as a model for investigating developmental, physiological and pathological processes and to identify conserved and potentially unique molecular components, we have undertaken preliminary proteomic analysis of isolated hearts. LC-MS/MS analysis identified ~450 proteins with high confidence. The cardiac proteins derive primarily from the sarcomere, cytoskeleton and the mitochondria. Many of the major cardiac components appear conserved between flies and humans. We ultimately seek to use quantitative proteomic studies to identify how specific lesions of myosin perturb protein networks within the Drosophila heart, and to determine how these perturbations contribute to the pathogenesis of cardiomyopathy.

2588-Pos Board B558

In-Solution Proteomic Workflow for Purification of Endogenous Sarcomeric Proteins and Identification of Distinct Charged Variants of Regulatory Light Chain

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The molecular conformation of the myosin motor is modulated by intermolecular interactions with the light chains, C-protein and titin, and governed by post-translational modifications (PTMs). These PTMs are important in regulation of function in ejecting ventricles as mechanisms downstream of Ca^{2+} fluxes at the level of the sarcomere appear to dominate ejection and sustain

ventricular elastance during systole. Therefore it is paramount to identify specific sites of PTMs. In-gel digestion has classically been used for PTM identification, however this approach is limited by protein size, pI, and difficulties in peptide extraction. We report a solution-based workflow for purifying endogenous sarcomeric proteins designed to enrich for peptides containing low-abundance PTMs. We focus particular attention on regulatory light chain (RLC), which was shown first by W.T. Perrie and S.V. Perry to be phosphorylated *in vivo*, but the specific sites have been unclear. Simplification of our sample with sub-cellular fractionation followed by OFFGEL electrophoresis (OGE) resulted in discriminatory purification of thick filament proteins including regulatory and essential light chains, myosin heavy chain, and myosin binding protein-C. Digestion and HPLC profiling of OGE-separated charge variants identified unique peptides suggestive of protein modifications, thus effectively enriching for endogenous PTMs which are low in abundance and have been historically difficult to identify with mass spectrometry. In addition, UV detection provided an additional unbiased quantitative analysis of peptides without having to explore more time-intensive quantitative MS methods. Using LC/MS/MS we unequivocally identified three distinct endogenous charge variants of cardiac RLC in unique OGE fractions, thus providing explanation for isoelectric point shifts observed, both in OGE and 2D-PAGE. The singly- versus doubly-phosphorylated RLC may evoke unique conformational states and thus may be functionally distinct in regulating cardiac contraction.

2589-Pos Board B559

High Resolution Top-Down MS/MS Reveals Single Amino Acid Sequence Polymorphisms in Rat Cardiac Troponin

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Heterotrimeric cardiac troponin (cTn) is a critical component of the thin filament regulatory complex. Two of the three subunits, cTnI and cTnT, are expressed only in cardiac muscle and are widely used in the clinic as serum biomarkers of cardiac injury. cTnI and cTnT are subject to extensive post-translational modification such as proteolysis and phosphorylation, but linking modification patterns to function remains a major challenge. In order to obtain a global view of the state of post-translational modification of cTn, we are performing high resolution top-down mass spectrometry on cTn subunits isolated from native tissues. Whole cTn complexes affinity purified from a single rat heart were analyzed in a 7 Tesla Thermo LTQ-FT-ICR mass spectrometer equipped with an ESI source. High resolution MS spectra of cTn from healthy adult rats showed molecular ions for intact cTnT and cTnI as well as phosphorylation and acetylation patterns similar to human cTnI (Zabrouskov et al., 2008 *Mol Cell Proteomics*, in press). 'Shadow peaks' of similar intensity to parent peaks were detected exhibiting masses of cTnI + 16 Da and cTnT + 128 Da, suggestive of single amino acid polymorphisms. Tandem mass spectrometry (MS/MS) analysis by ECD and CAD fragmentation of intact and protease-digested cTn subunits localized an Ala/Ser polymorphism at residue 7 of cTnI, and an additional Gln within a 3 residue alternative splice site beginning at residue 192 of cTnT. High resolution top-down MS/MS has revealed intriguing heterogeneity not only in the extent of phosphorylation, but also in amino acid sequences of cTnI and cTnT even within a single rat heart. Supported by NIH, AHA, UW-CVRC & Wisconsin Partnership for a Healthy Future.

2590-Pos Board B560

Cytotoxicity of non-myofilament-incorporated troponin T fragments

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Troponin T (TnT) is a striated muscle-specific protein and an abundant component in skeletal and cardiac myofilaments. Forced expression of TnT protein in non-muscle cells or undifferentiated myoblasts in the absence of myofibrils indicated cytotoxicity (Wang *et al., J. Biol. Chem.* 280:13241-9, 2005). To investigate the cytotoxic effect of non-myofibril-incorporated TnT, we constructed non-fusion co-expression vectors encoding green fluorescence protein tracer and different regions of the TnT polypeptide chain. Transient transfection in culture was studied in HEK293 non-muscle cells and undifferentiated C_2C_{12} myoblasts. Cytotoxicity of the TnT fragments was examined by the viability of the transfected cells. The results revealed distinct toxic effects of different regions of TnT. The evolutionarily conserved middle and C-terminal segments of TnT were highly toxic to cells whereas the N-terminal variable region was not. The cytotoxicity of the middle and C-terminal regions of TnT was associated with apoptotic cell death. Although muscle cells have high capacity of proteolysis to rapidly remove non-myofilament incorporated TnT protein, peak releases of TnT or TnT fragments from myofibrils may occur in the events of myocardial ischemia reperfusion and skeletal muscle fatigue or injuries. When the level of non-myofilament-associated TnT and TnT fragments exceeds the protective capacity of proteolytic removal in the muscle cell, they may impose cytotoxic effect and cause cell death. Therefore, the activity of non-myofilament-associated TnT or TnT fragments in inducing apoptosis and cell death is a potential pathogenic factor, particularly important in adult cardiac myocytes that lack the ability of regeneration.

2591-Pos Board B561

Proximity mapping of troponin T and troponin I in cardiac troponin using molecular dynamics simulations

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The intersite distances from cTnT residues 240, 276, and 288 to cTnI N-terminal residues 5, 17, 27, 40, and to the cTnI C-terminal residues 131, 145, 152, 160, and 167 in reconstituted cTn were determined in the presence of Mg^{2+} and Mg^{2+} plus Ca^{2+} using FRET. The distances from cTnT residues 276 and 288 to cTnI residues 5, 17, 27 were also determined in the presence of Ca^{2+} with cTnI bisphosphorylated at Ser^{23} and Ser^{24}. The results showed that the transition of troponin from the Mg^{2+} state to the Ca^{2+} state was accompanied by small to moderate changes in distances, suggesting small global conformational changes. The distance changes were accompanied by changes in the half-width of the distributions of the distances. To clarify the structural basis for population broadening, we performed MD simulations with explicit solvent. The published NMR structure of the N-terminal region of cTnI was docked and integrated into cTn. The average ensemble structure showed interactions of the cTnI N-terminal region with cTnC. In the Mg^{2+} state, the cTnI N-terminal segment interacted with the defunct Ca^{2+}-binding site I and the functional site II in cTnC. In the Ca^{2+}state, non-phosphorylated cTnI interacted with the helix A and site I of cTnC. These interactions stabilized the open hydrophobic pocket in the N-domain of cTnC, and the cTnI regulatory region was constrained within the hydrophobic pocket. The bisphosphorylated segment of cTnI was bent, interrupting its interaction with Ca^{2+} site I of cTnC. This loss of interaction resulted in depressed opening of the cTnC N-domain, forcing the regulatory region of cTnI to move out from the hydrophobic pocket.

2592-Pos Board B562

The Rate Of Calcium Dissociation From The Cardiac Thin Filament Is Affected By Multiple Modulatory Factors

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The rate of cardiac muscle relaxation is dynamically modulated. We hypothesize that the rate of Ca2+ dissociation from the thin filament is at least one of the factors that can strongly influence the rate of relaxation. Thus, we would expect the various physiological and pathological stimuli that affect the kinetics of cardiac muscle relaxation to likewise affect the rate of Ca2+ dissociation from the thin filament. In this study, we investigated various modulators of the Ca2+ exchange kinetics in the physiologically relevant biochemical model systems of reconstituted thin filaments and rabbit ventricular myofibrils such as: 1) PKA phosphorylation of TnI, 2) ischemia-reperfusion associated truncation of TnI, 3) familial cardiomyopathy related mutations of TnI and TnT, 4) the calcium sensitizing compound bepridil, 5) rationally engineered TnC mutations, and 6) tropomyosin isoforms. Consistent with the effects of PKA on accelerating relaxation, the rate of Ca2+ dissociation from the thin filament was accelerated by TnI mutations (S23D,S24D) mimicking PKA phosphorylation. Additionally, the rate of Ca2+ dissociation was slowed by truncation of TnI (residues 1-192), consistent with ischemia-reperfusion slowing the rate of cardiac relaxation. The hypertrophic and restrictive cardiomyopathy mutations (TnIS166F and TnIR192H) slowed the rate of Ca2+ dissociation from