ANALYSIS OF GENE PATHWAYS INVOLVED IN DCIS PROGRESSION IN RESPONSE TO ACIDIC EXTRACELLULAR pH

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Breast cancer is the most commonly diagnosed cancer in women in USA and has a high mortality rate, second only to lung cancer. About 85% of total 63300 new cases of breast cancer are predicted to be ductal carcinoma in situ (DCIS) in 2012. We are interested in identifying markers that are predictive of changes that occur in the breast microenvironment as a result of the presence of premalignant lesions such as DCIS that are poised to develop into breast cancer. A critical barrier to cancer progression is its ability to survive in the acidic microenvironment characteristic of breast cancer. As the breast is comprised of different cell types, we performed gene expression analysis using Affymetrix gene chip HG U133 plus 2.0 array of 3 DCIS cell lines grown in 3D at neutral and acidic pH. We then computed the significantly changed genes at acidic pH for three DCIS cell lines and found 6 common and 121 similar genes. IPA core analysis of these genes revealed the interferon-signaling (IFN) pathway to be significantly altered. STAT1 was one key transcription factor that was upregulated and that might be driving downstream signaling as a response to acidic microenvironment. We are validating some of the downstream targets of the IFN pathway using qPCR. In preliminary studies, genes such as oligadenylate synthase 1 (OAS1) and interferon-induced GTP-binding protein or myxovirus resistance 1 (MX1) that are more specific to type II IFN signaling are upregulated. Nonetheless, involvement of type I pathway cannot be neglected as genes such as interferon regulatory factor 9 (IRF9), common to both pathways are altered as well. We are interested in studying both the IFN pathways including STAT1 and other STATs along with their phosphorylated-unphosphorylated status in regard to the effect of acidic environment in breast cancer progression.

PROTEIN ABUNDANCE OF PTDINS(3,5)P2-METABOLIZING ENZYMES IN BREAST CANCER CELLS: HIGH LEVELS OF THE PTDINS(3,5)P2 PHOSPHATASE SAC3 IN THE BT-20/BT-549 CELL LINES

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Purpose: PIKfyve, the sole phosphoinositide 5-kinase synthesizing PtdIns(3,5)P2 and PtdIns5P, plays a key role in regulating endosome dynamics in mammalian cells. The vital role of PIKfyve for life in mammals is underscored by preimplantation lethality of PIKfyve-null embryos, presumably due to defective cell proliferation (Ikonomov et al. 2011). Genes implicated in embryogenesis are frequently involved in tumorigenesis. Concordantly, PIKfyve was found to assist EGFR nuclear trafficking/cell cycle progression in human bladder carcinoma cells (Kim et al. 2008). Somatic mutations in PIKfyve are reported in all of the examined seven samples with ovarian carcinoma (Sanger Inc.). PIKfyve forms a stable ternary complex with the phosphatase Sac3 that dephosphorylates PtdIns(3,5)P2 and the scaffolding activator ArPIKfyve. This interaction both activates and turns over PtdIns(3,5)P2 (Ikonomov et al. 2009). In this study we determined PIKfyve, ArPIKfyve and Sac3 protein levels and examined levels of the PIKfyve lipid product PtdIns(3,5)P2 in several breast cancer cell lines. **Methods:** The estrogen-independent BT-20, BT-549 and MDA-MB-231 or estrogen-sensitive T-47D and MCF-7 cell lines were used. Cell lysates were subjected to immunoblotting with anti-PIKfyve, anti-Sac3 and anti-ArPIKfyve antibodies. Lysates of PC-12 cells were used to normalize protein levels. HPLC analyses revealed the phosphoinositide profiles in BT-20 cells vs. those of non-transformed cells. **Results:** Low levels of PIKfyve were observed in MCF-7 and T-47D with Sac3 and ArPIKfyve diminished proportionally. PIKfyve was reduced in BT-cells yet Sac3 and ArPIKfyve were markedly increased. Proteins in other cell types had no differences vs. PC-12. HPLC-based quantitation of phosphoinositides in BT-20 revealed an increased PtdIns3P-to-PtdIns(3,5)P2 ratio vs. that in non-transformed cells. **Conclusions:** The data indicate disproportionate expression of ArPIKfyve-Sac3 proteins relative to PIKfyve in both BT cell lines. We speculate that the increased PtdIns3P/PtdIns(3,5)P_2 ratio in the BT cell lines leads to disturbed phosphoinositide signaling and uncontrolled cell proliferation.

A NOVEL ROLE OF H2-CALPONIN IN THE DEVELOPMENT OF ANTI-GPI SERUM-INDUCED ARTHRITIS

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Purpose: H2-calponin plays a role in regulating the motility and phagocytosis of macrophages. Here we investigated the role of h2-calponin in the development of anti-GPI serum-induced arthritis. **Methods:** H2-calponin knockout (KO) and wild type mice were injected with anti-GPI mouse serum. Delta-ankle circumference and arthritis clinical score were determined on a series of time points after injection. The ankles were analyzed for histology by H&E staining. IL-1β concentration in ankle homogenates and serum were determined by ELISA. Elicited peritoneal macrophages were also examined for their cytokine production in response to LPS stimulation in culture using quantitative PCR. **Results:** Significantly ($P<0.05$) lower delta-ankle circumference and clinical arthritis score were observed in h2-calponin KO mice in comparison to WT mice. Histology of ankle joints showed significantly ($P<0.05$) lower degrees of inflammation and bone erosion in h2-calponin KO group than that of wild type controls. In contrast, there was no difference in IL-1β in joint tissues of H2-calponin KO and WT mice. Further, compared with the WT controls, h2-calponin-null macrophages demonstrated increased *in vitro* synthesis of IL-1β, IL-6, IL-10, IL-12, TNFα, MIP-2, IFNγ, HIF-1α, and INOS both at baseline and upon LPS stimulation. The levels of TLR2 and VEGF-A in h2-calponin-null cells were also higher than WT cells at baseline but were not different after LPS stimulation. TLR4, TGF-β, VEGF-R1 and VEGF-R2 were unchanged in the KO macrophages. **Conclusion:** H2-calponin plays a novel role in the development of anti-GPI serum-induced arthritis. The function of h2-calponin in regulating macrophage activity may form a basis for its role in inflammation and autoimmune responses. Since there was no reduction of IL-1β in joint tissue, a critical cytokine in this model of passive arthritis, the results may suggest that the mechanisms downstream of the induction of cytokines contributed to the reduction of arthritis development despite the elevation of IL-1β and TNFα. The increased phagocytotic activity of h2-calponin-null macrophages may facilitate the clearance of autoimmune complexes to reduce the severity of arthritis. Further studies are underway to investigate the underlying mechanisms and explore the value of inhibiting h2-caloponin expression for the treatment of arthritis.

cGMP INDUCES THE DEGRADATION OF INTERNALIZED NKCC2 IN THICK ASCENDING LIMBS: ROLE OF UBIQUITIN

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NaCl reabsorption by the thick ascending limb (TAL) is mediated by the Na/K/2Cl cotransporter NKCC2. cGMP decreases NKCC2 activity by decreasing the amount of NKCC2 in the apical membrane. Furthermore, internalized NKCC2 is degraded slowly (0.33%/min) but it is not known whether NKCC2 degradation is regulated. We hypothesized that internalized NKCC2 is degraded by the ubiquitin-proteasome system in a process stimulated by cGMP. TAL surface proteins were biotinylated, allowed to internalize and biotin remaining in surface proteins stripped away. Internalized NKCC2 was measured by western blot. cGMP enhanced the rate of disappearance of internalized NKCC2 by 83 % and this was blocked by a proteasomal (MG132) but not lysosomal (leupeptin) inhibitor (Control: 0.29 ± 0.04; cGMP: 0.53 ± 0.10 ; cGMP + MG132: 0.10 ± 0.10 ; cGMP + Leupeptin: 0.44 ± 0.06 %/min; $p < 0.05$). Ubiquitination of membrane proteins is involved in their degradation. We found that NKCC2 immuno-precipitated with ubiquitin. Proteasome inhibition induced the accumulation of ubiquitin-NKCC2 and this was enhanced by cGMP (MG132: 59 ± 14%, MG132+cGMP: 111 ± 25%; $p<0.05$). We concluded that internalized NKCC2 is degraded *via* the proteasome pathway in a process stimulated by cGMP. cGMP induced degradation of internalized NKCC2 may contribute to decreased NKCC2 trafficking to the apical membrane of TALs.

THE VESICLE FUSION PROTEIN VAMP2 BINDS THE CARBOXY-TERMINUS OF NKCC2 AND MEDIATES cAMP-STIMULATED EXOCYTIC INSERTION IN THICK ASCENDING LIMBS

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Objectives: In the kidney, NaCl absorption by the apical Na-K-2Cl co-transporter NKCC2 in the thick ascending limb (TAL) is essential for salt, water and blood pressure homeostasis. NKCC2 is delivered to the apical membrane of TALs and cAMP stimulates this process. Cleavage of vesicle fusion proteins VAMPs with tetanus toxin inhibits cAMP-stimulated apical surface NKCC2 levels. The mechanism by which VAMPs mediate NKCC2 exocytic delivery is unknown. We hypothesize that VAMP2 binds NKCC2 and mediates cAMP-stimulated exocytic delivery to the apical membrane of TALs. **Methods:** We isolated rat TALs and immunoprecipitated (IP) NKCC2 and VAMP isoforms. To map the interacting motif in NKCC2, we generated GST-fusion proteins coding for the amino (N)-terminus of NKCC2 or the carboxy (C)-terminal region. We tested the role of VAMP2 in TALs by shRNA-mediated silencing using adenovirus-mediated transduction *in vivo*. We measured NKCC2 exocytic delivery by surface biotinylation. **Results:** NKCC2 co-IP with VAMP2 but not VAMP7/8. VAMP2 bound the C-terminus of NKCC2 but not the N-terminus. To test whether VAMP2 co-localized with NKCC2 at the apical surface, we expressed VAMP2-GFP facing the extracellular space in primary cultures of TALs and labeled VAMP2 at the cell surface. Endogenous surface NKCC2 was labeled with an antibody against an extracellular epitope. NKCC2 was restricted to discrete domains of 0.14±0.04 μm^2 in area. After stimulation by cAMP, 45±7% of NKCC2 surface clusters also contained VAMP2. Finally we tested whether VAMP2 mediates cAMP-stimulated exocytic delivery. *In vivo* delivery of VAMP2-shRNA decreased VAMP2 expression in TALs by 69±7% and completely blocked cAMP-stimulated NKCC2 exocytic delivery without affecting baseline "constitutive" delivery (baseline: 100, cAMP: 91±12%, n=5). In control TALs (scrambled shRNA), cAMP enhanced NKCC2 exocytic delivery by 88±17% ($p<0.05$). **Conclusion:** VAMP2 binds NKCC2 and mediates cAMP-stimulated NKCC2 exocytic delivery. In addition, NKCC2 and VAMP2 co-localize in clusters at the apical surface of TALs.

TRANSCRIPTIONAL MECHANOREGULATION OF H2-CALPONIN GENE

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Objectives: The essential role of mechanical signaling in regulating the cellular function of living organisms has been widely recognized. However, how mechanical signals are transduced in cells to regulate gene expression and other biochemical activities is not well understood. Our previous studies have demonstrated that the gene encoding h2-calponin (Cnn2) is regulated by mechanical tension (Hossain et al., JBC 280:42442-53, 2005). The present study quantitatively studied the transcriptional regulation of h2-calponin gene by mechanical tension in the cytoskeleton. **Methods**: To explore the regulatory mechanism, mouse genomic DNA containing the Cnn2 promoter was cloned and a nested set of 5' deletion reporter genes was constructed. Transcriptional activity of the Cnn2 promoter constructs and their responses to the stiffness of cultural substrates were examined through transfective expression in NIH/3T3 and HEK293 cells. **Results**: The results showed significant levels of transcriptional activities for the -1.00-kb and -1.24-kb constructs whereas the -0.61-kb construct had no detectable activity. The -1.38-kb, -1.57-kb and -2.12-kb constructs exhibited higher transcriptional activity, of which the -1.57-kb and -2.12-kb constructs showed a suppression of expression in cells growing on low stiffness substrate. Selective deletion of the segment between -1.57-kb and -1.38-kb in the -2.12-kb construct abolished this low cytoskeleton tension-produced suppression. **Conclusions**: The data indicated that cis-regulatory element(s) located between −1.6-kb and −1.4-kb of the mouse Cnn2 gene is responsible for the mechanical tension-regulation. Potential trans-active binding sites within this region were explored, specifically HES1 and the Notch signaling pathway. To identify specific trans-regulatory factors involved in the cellular signaling pathway will elucidate the mechanotransduction pathway that controls the h2-calponin expression.

DERLIN ISOFORM SELECTIVELY REGULATES CFTR DEGRADATION

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Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent Cl channel at the apical membranes of most epithelial cells. Mutations of the gene encoding CFTR lead to cystic fibrosis (CF), a most common life threating disease in the Caucasian. The most common mutation is the deletion of the phenylalanine residue at position 508 (ΔF508CFTR), which is present in more than 90% CF patients. Instead of trafficking to the plasma membrane, ΔF508CFTR is retained in the endoplasmic reticulum (ER) and rapidly degraded by ubiquitin-proteasome system. However, restoration of this mutant trafficking results in a substantial Cl channel activity at the plasma membrane. Previous studies from our group and others showed that an ER resident protein, Derlin-1, promotes the efficient degradation of wt CFTR and the folding mutant ΔF508CFTR by facilitating their retrotranslocation from the ER to the cytosol. To further gain insights of the role of Derlin isoform in ΔF508CFTR biogenesis, we performed co-expression experiments. When co-expressing wt and ΔF508 CFTR with Derlin-1 and Derlin-2 in HEK293 cells we found that Derlin-1 virtually eliminated both mature and immature wt and ΔF508 CFTR, by ~80% and 95%, respectively. However, Derlin-2 has a different effect on CFTR biogenesis. Derlin-2 co-expression resulted in an 80% reduction in ΔF508CFTR, but had no significant effect on wt CFTR expression level. The cellular locations of CFTR and Derlin-1 and-2 in human airway epithelia CFBE4o1- wt and -ΔF cells examined with immunofluorescence microscopy also suggested that Derlin-2 might be selective for ΔF508CFTR degradation by preferentially interacting with the mutant protein. To investigate Derlin-1 domain specific mediated CFTR degradation, we generated Derlin-1 deletion and point mutations and co-expressed them with wt and ΔF508 CFTR. Deletion of the first 17 amino acids from the N-terminus or the last 74 amino acids from its C-terminus did not markedly reduce the ability of Derlin-1 to mediate wt and ΔF508 CFTR degradation, indicating that the membrane anchored regions are crucial for CFTR degradation. When co-expressing wt and ΔF508 CFTR with Derlin-1 point mutants we found that M9 mutant, which is located in the 2nd loop of the ER lumen, effectively eliminated the effect of Derlin-1 mediated wt and ΔF508 CFTR degradation. Interestingly enough, M9 mutant did not completely reverse its degradation effect on wtCFTR whereas it served as a dominant negative effect and increased ΔF508CFTR expression nearly 5-fold. Our data indicate that Derlin-1 efficiently degrades wt and ΔF508 CFTR whereas Derlin-2 selectively eliminates ΔF508CFTR. Therefore, Derlin-1 acts as a general gate keeper for ER quality control of this protein, while Derlin-2 is specialized for the ΔF508CFTR folding. The finding that Derlin-1 M9 mutant serves as a dominant negative will allow us to further characterize the early steps of ΔF508CFTR biogenesis and to provide a roadmap for restoration of this mutant function.

REAL-TIME MEASUREMENT OF TRANSCELLUAR SODIOM TRANSPORT MEDIATED BY NA,K-ATPASE IN PROXIMAL TUBULE CELLS

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The binding of angiotensin II (Ang II) to the angiotensin receptor 1 (AT_1) stimulates Na,K-ATPase activity that directly drives sodium reabsorption in the proximal tubule, which reabsorbs 2/3 of the filtered sodium. To determine how quickly Ang II can stimulate Na,K-ATPase activity, we have developed a new experimental system that has a time resolution on the order of seconds. A proximal tubule cell line (opossum kidney cells) stably co-expressing the wild-type rat kidney α-1 subunit of Na,K-ATPase and the AT_{1a} receptor were grown on permeable supports so that the cells can polarize and form a high resistance barrier. These cells were then mounted at 37^{o} in an Ussing Chamber with oxygenated normal Ringer's solution on both sides of the cells. In this system the short-circuit current (Isc) across the cells is proportional to the net movement of sodium from the apical to basolateral sides, which is actively driven by the NaK-ATPase. To test for Na,K-ATPase activity we added 200 μM nystatin to the apical membrane to increase its permeability to sodium and thereby increase the concentration of intracellular sodium, which is the rate-limiting substrate for the Na,K-ATPase. Approximately 30 sec after the addition of nystatin Isc began to increase and reached its plateau in approximately 2 min. The subsequent addition of 600 μM ouabain, a specific inhibitor for Na,K-ATPase, to the basolateral side of the cells reduced Isc to its original baseline value. Therefore, the observed increase in Isc was directly due to Na,K-ATPase activity and its contribution to net sodium movement across the basolateral membrane under control conditions. In other experiments the addition of 10 μM Ang II to the basolateral side of the cells in the absence of nystatin increased Isc as quickly as could be measured (≤ ~10 sec). The peak Ang II-dependent Isc was observed in ~20 sec and was ~50% greater than the response to nystatin in control cells. The subsequent addition of nystain increased Isc similar to that under control conditions, which suggests that the Ang II-dependent stimulation of transcellular sodium transport in the absence of nystain occurred at concentration of intracellular sodium that was rate-limiting for the Na,K-ATPase. The subsequent addition of 600 μM ouabain in Ang II-stimulated cells reduced Isc to its original baseline value. Thus, Ang II significantly stimulated transcellular sodium transport and very likely Na,K-ATPase activity in 10 seconds or less. We

hypothesize that the ability of Ang II to rapidly simulate sodium reabsorption in the proximal tubule is part of a mechanism for the body to recover from a sudden drop in blood pressure.

POLYUBIQUITIN CHAINS DIFFERENTIALLY REGULATE DF508CFTR BIOGENESIS

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The conjugation of ubiquitin (Ub) chains to specific protein substrates is necessary for Ub-mediated proteasomal degradation. Ub contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), and all seven lysine residues of Ub can be conjugated to another to form polyUb chains on substrates with different topologies, which provide sorting signals for different pathways. To gain further mechanistic insights of Ub-mediated ΔF508CFTR degradation, we have examined all seven lysine residues of Ub in ΔF508CFTR biogenesis by making Ub containing lysine only mutants (the rest by lysine-to-arginine mutation) in position 6 (UbK6), 11 (UbK11), 27 (UbK27), 29 (UbK29), 33 (UbK33) 48 (UbK48), 63 (UbK63) and found that two lysine-linked polyUb chains (UbK11 and UbK48) facilitate ΔF508CFTR degradation and three lysine-linked polyUb chains (UbK63, UbK33 and UbK6) protect ΔF508CFTR from degradation. UbK11, instead of K48, is more potent for ΔF508CFTR degradation. Overexpression of UbK11 resulted in a profound reduction of ΔF508CFTR expression (~80%, $p<0.01$). Surprisingly, UbK33 and UbK63 protected ΔF508CFTR from degradation. Overexpression of UbK33 and UbK63 increased ΔF508CFTR expression by 4-5 folds. The effect of UbK33 and UbK63 on the kinetics of ΔF508CFTR biogenesis was examined in cycloheximide chase experiments. Under control conditions, ΔF508CFTR had a half life of approximately 60min. However, when UbK33 or UbK63 was expressed, the Ub mutants prolonged ΔF508CFTR half life to ~7hrs. To further dissect the roles of degradation and protection polyUb chains in ΔF508CFTR biogenesis, we made a Ub degradation vector containing all degradative Ub mutants and a Ub stabilization vector including all protective Ub mutants. Overexpression of the degradation vector virtually eliminated ΔF508CFTR expression, whereas overexpression of the stabilizing mutant yielded a significant protection of ΔF508CFTR from degradation. Relative to the degradation mutant, overexpression of the stabilizing Ub mutant led to ~90-fold increase in ΔF508CFTR steady-state expression, indicating that the site of Ub modification has opposite functional outcomes in ΔF508CFTR biogenesis. UbK33 conjugated ΔF508CFTR was accumulated as detergent-insoluble aggregates and elicited a mild unfolded protein response. Overexpression of the Ub mutants including UbK33 and UbK63 was not observed any impairment of proteasomal degradation function; however, ΔF508CFTR ploychains formed by UbK33 and UbK63 are failed to be recognized by Ub receptors on the proteasome, a prerequisite step for proteasomal degradation. The physiological role of formation the polyUb chains was tested by using UbK48 or UbK63 specific antibodies to perform co-immunoprecipitation and revealed that ΔF508CFTR can be modified by these Ub linkages in CFBE41o-ΔF cells. Taken together, our data highlight novel mechanisms showing that ΔF508CFTR utilizes multiple Ub conjugation pathways for its biogenesis. The finding that UbK33 mediated ΔF508CFTR aggregation formation may provide new insights to understand pathogenesis of cystic fibrosis. [Supported by the CFF grant SUN08G0, and NIH grant HL096800]

ENHANCED RENIN RELEASE AND CONTENT IN JUXTAGLOMERULAR (JG) CELLS FROM VAMP8 KNOCKOUT MICE

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Background: Renin is essential for angiotensin II production and blood pressure control. Renin is stored in granules in juxtaglomerular (JG) cells. We showed that vesicle associated membrane protein 2 (VAMP2), a member of SNARE family, mediates stimulated renin exocytosis in JG cells. However, the VAMP isoform required for constitutive renin release is unknown. In other cells VAMP8 mediates granule maturation and constitutive exocytosis. **Hypothesis:** We hypothesized that VAMP8 mediates constitutive renin release in JG cells. **Method:** We used primary cultures of JG cells from wild type (WT) and VAMP8 knockout (V8-KO) mice and measured VAMP8 localization and renin released to the medium during 1 hr. **Results:** In wild type, VAMP8 was expressed in JG cell granules. Baseline renin release was 5-fold higher in V8-KO than WT (WT=0.06±0.01; KO=0.3±0.05 μgAngI/hr/mg prot.; $p<0.05$). Total renin content was 5-fold higher in V8-KO than WT (WT=2.9±0.2; KO=16.7±0.6 μgAngI/hr/mg prot.; $p<0.05$). The fraction of total renin released to the media was similar in V8- KO and WT JG cells (WT: 1.6±0.3; KO: 1.7±0.5% of total renin) indicating higher number of granules in V8-KO, or higher renin content/granule. Immunolabeling of renin and confocal imaging showed enlarged granules and vacuoles in JG cells from V8-KO. **Conclusions:** We concluded that: 1) VAMP8 does not mediate constitutive renin exocytosis; and 2) VAMP8 may play an inhibitory role in transcription, translation or processing of renin.

N-TERMINAL TRUNCATED CARDIAC TROPONIN I ALTERS THE CONTRACTILITY OF ISOLATED CARDIOMYOCYTES

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Troponin I (TnI) is the inhibitory subunit of the troponin complex and plays an essential function in the regulation of striated muscle contraction. Cardiac TnI has a unique N-terminal extension that is a cardiac specific regulatory structure not present in skeletal muscle TnI. Previous studies from several laboratories have demonstrated that a restrictive proteolytic N-terminal truncation of cardiac TnI occurs in cardiac adaptation to hemodynamic stress or ß-adrenergic deficiency (*Yu et al., JBC, 2001*; *Feng et al., JBC, 2008*). The N-terminal truncated cardiac TnI (cTnI-ND) alters the conformation of the core structure of cardiac TnI, similarly to that produced with PKA phosphorylation (*Akhter et al., AJP-Heart Circ Physiol, 2012*). At organ level, cTnI-ND enhanced ventricular diastolic function (*Barbato et al., JBC, 2005*), improved cardiac function in aging mice (*Biesiadecki et al., JBC, 2010*), and corrected diastolic dysfunction of mouse hearts caused by a restrictive cardiomyopathy cardiac TnI mutation (*Li et al., JMCC, 2010*). **Purpose:** To investigate the functional effect of cTnI-ND at the cellular level avoiding any influence of extracellular matrix. **Methods:** cardiomyocytes were isolated from wild type and cTnI-ND transgenic mouse hearts using a refined protocol eliminating the need of 2,3-butanedione monoxime (BDM) and studied for morphology, sarcomere length and contractility. Paced contraction was examined using edge detection on freshly isolated cardiomyocytes in healthy condition as shown by a positive frequency response. **Result:** cTnI-ND cardiomyocytes had no change in cell width, cell length and sarcomere length indicating an absence of pathological remodeling. cTnI-ND myocytes exhibited higher shortening amplitude and shortening/re-lengthening velocities than that of wild type cells. Isoproterenol treatment significantly increased the contractility of both wild type and cTnI-ND cardiomyocytes and their differences diminished. The non-additive effects of cTnI-ND and β-adrenergic stimulation suggest that cTnI-ND enhances cardiac muscle performance via the same mechanism as that following PKA-catalyzed N-terminal phosphorylation of cardiac TnI. **Conclusion:** The functional effect of cTnI-ND demonstrates a posttranslational regulatory mechanism to modulate cardiac muscle contractility in physiological adaptation and heart failure.

FUNCTIONAL EXPRESSION OF ADENOSINE A_{2B} AND A_{2A} RECEPTORS IN MOUSE CARDIAC VENTRICULAR FIBROBLASTS

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Objectives: The beneficial effects of the four G protein-coupled adenosine receptor (AR) subtypes (A_1, A_{2A}, A_{2B} and A_3) are well documented in acute myocardial infarction (MI), but their roles in chronic MI are unclear. Angiotensin and β-adrenergic receptor (ATR and βR) effects on cardiac fibroblasts (CF) modulate collagen production and remodeling in chronic MI, and signaling through MAP kinase pathways has been demonstrated to play a role in these processes. The aim of this study was to assess the functional expression of A_1, A_{2A} and A_{2B}R on CF in contrast with ATR and βR. **Methods**: CF were isolated from hearts of C57BL/6 mice and cultured for one passage. AR, ATR and βR expression were measured by real time quantitative PCR. CF were treated for 10 min with 500 nM A_{2A} agonist (CGS-21680), A_{2B} agonist (BAY 60–6583), angiotensin (Ang II, 100 nM) and β-agonist isoproterenol (ISO, 1 μM). Western blotting was used to assess phosphorylation and total expression of AKT and ERK. **Results**: Real time qPCR indicated 10-fold greater expression of A_{2B}R vs A_{2A}R and A_{T1}R, 20-fold greater expression vs. β_1R and β_2R, and negligible A_1R and AT_{2A}R expression (as % GAPDH). Both BAY and CGS increased ERK phosphorylation (p44: 167±39% with BAY; 290±104% with CGS, $p<0.05$). Ang II and ISO significantly increased ERK phosphorylation (p44: 413±189% and 449±180% respectively, $p<0.05$). Ang II increased AKT phosphorylation (67±18%, $p<0.05$), whereas BAY and ISO reduced AKT phosphorylation (73±4% for BAY, 33.8±10.5% for ISO, $p<0.05$). CGS had no effect on AKT. **Conclusions**: Our results demonstrate that A_{2B}R and A_{2A}R are functionally present on CF with the A_{2B}R expressed much greater than ATR and βR. All 4 receptors increase ERK phosphorylation, but have differing effects on AKT phosphorylation. The effects of A_{2B}R and A_{2A}R in CF should be considered in the post-MI heart, where AKT and ERK modulate CF proliferation and collagen deposition which can be both beneficial and deleterious.

ACTIVATOR OF MITOCHONDRIAL ALDEHYDE DEHYDROGENASE AMELIORATES HYPERGLYCEMIC STRESS-INDUCED CARDIAC DAMAGE

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Purpose: We plan to investigate whether activation of mitochondrial enzyme, aldehyde dehydrogenase-2 (ALDH2) by Alda-1, a small molecule activator of ALDH2, can ameliorate hyperglycemia-induced cardiac damage. **Methods:** Hyperglycemic stress was induced *in vitro* by subjecting primary cardiomyocytes to high glucose stress (25mM glucose and 1μM Ang-II for 48 hrs) with or without Alda-1 (20 μM). Diabetes was induced by streptozotocin (STZ) in DBA/2J mice at 8 weeks of age and development of hyperglycemia was confirmed at 4 weeks after STZ injection. Diabetic mice were treated for three months without (n=18) or with (n=24) Alda-1 (16mg/Kg/day) using osmotic pumps for subcutaneous delivery after 4 weeks of STZ injection. **Results:** Alda-1 treatment reduced both impairment of ALDH activity and formation of 4-hydroxy-2-nonenal (4-HNE)-protein adducts in cell culture and in mice. Human heart samples from patients with cardiomyopathy and diabetes also showed decreased ALDH activity and increased 4-HNE-protein adducts formation. Activator of ALDH attenuated cardiac damage in mice as evident from reduction in myocardial fibrosis and functional impairment. **Conclusion:** Finally, we propose that ALDH activators can serve as a therapeutic agent for the treatment of diabetes-induced cardiovascular complications.

FAT CELL-SPECIFIC INACTIVATION OF ONE OR BOTH PIKFYVE ALLELES IN MICE CAUSES INSULIN RESISTANCE

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Purpose: PIKfyve is an essential-for-life mammalian phosphoinositide kinase synthesizing two low abundance phosphoinositides PtdIns(3,5)P2 and PtdIns5P, both markedly increasing upon insulin stimulation of 3T3L1 adipocytes. Global PIKFYVE disruption causes early embryonic lethality whereas striated muscle-specific PIKFYVE inactivation results in insulin resistance, hyperinsulinemia and increased adiposity. Noteworthy, PIKfyve protein levels are much higher in fat tissue in comparison with muscle and liver. The adipose cells, via their adipokine secretion, have recently emerged as a major endocrine regulator of whole body metabolism. To test the role of PIKfyve in adipose tissue we generated transgenic mice with fat cell-specific PIKFYVE disruption and began characterizing their metabolic phenotype. **Methods:** Homozygous PIKFYVE$^{fl/fl}$ mice were crossed with mice expressing Cre recombinase under the control of the adiponectin promoter. Both mouse lines were on C57BL6 genetic background. Mice (male and female) with four PCR-determined genotypes were studied: APIfKO (Cre+ fl/fl); HetKO (Cre+ fl/wt); and the Cre-negative controls PIKFYVE$^{fl/wt}$ and PIKFYVE$^{fl/fl}$. The specificity of PIKfyve disruption was validated by measuring PIKfyve activity in immunoprecipitates from different mouse cells, tissues and organs by *in vitro* lipid kinase assay. Body weight, food and water intake were monitored. Relative adiposity was followed non-invasively by EchoMRI. Standard insulin-, glucose- and pyruvate-tolerance tests were used to establish changes in whole-body glucose homeostasis. Plasma adiponectin levels were quantified by western blotting under non-denaturing and denaturing conditions. **Results:** Adipose-specific PIKFYVE disruption caused selective ~60% decrease of PIKfyve activity in the epididymal fat of Het KO mice. Stronger (~90%) and selective PIKfyve activity decrease was found in adipocytes from APIfKO mice. APIfKO and HetKO mice of both genders did not differ from their Cre-negative controls in growth curves, food and water intake, and relative adiposity. Importantly, both APIfKO and HetKO mice demonstrated marked insulin resistance in comparison with PIKFYVE$^{fl/wt}$ and PIKFYVE$^{fl/fl}$ littermates. The possibility that the whole body insulin resistance is mediated through changes in plasma adiponectin was not supported by western blot analysis of adiponectin. **Conclusions:** Targeted PIKFYVE disruption in adipose tissue of mice with one or both floxed alleles causes marked insulin resistance without concomitant changes in adiposity or food and water intake. Taken together with the insulin resistance of mice with muscle-specific PIKFYVE disruption, our data demonstrate that PIKfyve enzyme activities play a central role in insulin signaling and whole-body glucose homeostasis.

A UNIQUE MONOMER OF ADIPONECTIN IS EXCLUSIVE TO THE SECRETED FORM

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Objective: Adiponectin is a major protein in serum, with endocrine actions throughout the body, including major insulin-sensitizing activity. Co-translational and post-translational modifications of adiponectin are responsible for secretion of low-molecular weight (LMW) trimers, medium-molecular weight (MMW) hexamers, and high-molecular weight (HMW) multimers (18 or more monomers). The purpose of this study was to determine cell-biological mechanisms of adiponectin secretion. **Methods:** Adiponectin-flag adenovirus was used to overexpress the protein in mammalian cells, and primary adult rat cardiomyocytes. Overexpressed intracellular adiponectin was compared with the secreted protein. Native oligomeric adiponectin within these cellular systems was analyzed using gel filtration and immunofluorescence, while composite monomers and multimers were examined by SDS-PAGE and western blotting. **Results:** Adiponectin was secreted from every cell type examined, but with roughly half of the protein retained in the luminal compartments, co-localizing with endoplasmic reticulum markers. Secreted adiponectin polymers were composed of monomers that exhibited a range of masses in HEK, COS, and cardiac myocytes. Upon disulfide bond reduction and heat-denaturation, secreted adiponectin was found to be composed of polymers that contained faster-migrating monomeric species that were not detected intracellularly, indicating a posttranslational modification necessary for its secretion. **Conclusion:** Our data indicate that secreted adiponectin may be regulated at the level of the monomer, prior to assembly into polymers for secretion. As levels of adiponectin correlate with chronic disease in patients, these data point to novel biosynthetic steps that may be employed to regulate important endocrine control in insulin biology.

YM201636 DIFFERENTIAL INHIBITION OF THE PIKfyve LIPID PRODUCTS REVEALS PtdIns5P SELECTIVE ROLE IN INSULIN-REGULATED ACTIN REMODELING.

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Purpose: We tested the hypothesis that a preferential inhibition of the two PIKfyve-generated lipid products PtdIns5P and PtdIns(3,5)P2 by the PIKfyve-selective inhibitor YM201636 could reveal a selective role for one lipid over the other in mediating various PIKfyve activities. **Methods:** Mouse 3T3-L1 fibroblasts were differentiated into adipocytes and used after days 5-7 of the differentiation program. CHO-T cells, stably expressing the human insulin receptor, were fixed in 4% form-aldehyde and stained with rhodamine-phalloidin to visualize F-actin cytoskeleton after 30-min YM201636 and 10-min insulin (100 nM) treatments. A HEK293 cell line, stably expressing PIKfyveWT at levels ~2-fold above the endogenous PIKfyve, was generated with a Tet-Off/Tet-On gene expression system. Cells were labeled for 26-40 h with 25 μCi/ml myo-[2-3H]inositol in glucose- and inositol-free DMEM. Radiolabeled phosphoinositides were analyzed by HPLC on a Whatman SAX Partisphere column. **Results:** At low doses (10-25 nM), YM201636 inhibited preferentially PtdIns5P rather than PtdIns(3,5)P2 production in vitro, but at higher doses, the two products were similarly inhibited. In radiolabeled cells,160 nM YM201636 inhibited PtdIns5P synthesis twice more effectively than PtdIns(3,5)P2 synthesis. In 3T3L1 adipocytes, HEK293 and CHO-T, levels of PtdIns5P fell by 62-71% of the untreated controls, whereas those of PtdIns(3,5)P2 fell by only 28-46%. The preferred inhibition of PtdIns5P vs. PtdIns(3,5)P2 at low doses of YM201636 was therefore used to probe contributions of the PIKfyve-catalyzed PtdIns5P pool to actin stress-fiber disassembly in CHO-T cells, GLUT4 translocation in 3T3L1 adipocytes and induction of aberrant cellular vacuolation in these cells. At 800 nM, YM201636 formed profound vacuoles only in HEK293 and CHO-T cells, but not terminally differentiated 3T3L1 adipocytes. At 160 nM YM201636 blocked insulin-stimulated loss of F-actin stress fibers by nearly 100% (but not insulin-regulated GLUT4 surface translocation in 3T3L1 adipocytes). **Conclusions:** Comparing the in vitro and in vivo potency of YM201636 at low doses against PtdIns5P and PtdIns(3,5)P2 provides the first experimental evidence that: 1) the principal pathway for PtdIns5P intracellular production is through PIKfyve and 2) that insulin's effect on actin-stress-fiber disassembly is mediated entirely by the PIKfyve-produced PtdIns5P pool.

COPII DEPENDENT ER EXIT OF SOLUBLE CARGO PROTERINS IN PANCREATIC SECRETORY CELLS

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Objectives: In both exocrine and endocrine pancreas, secretory cells have well-developed ER to adapt to the massive production and secretion of soluble cargo proteins such as proinsulin and digestive enzymes. COPII vesicle is a conserved mechanism for ER export of cargo proteins from yeast to mammals. However, its role in pancreatic secretory cells has not been investigated. Our goal is to understand the molecular mechanism by which pancreatic secretory proteins exit ER. **Methods:** Rat pancreatic AR42J cells and mouse MIN6 cells were used as model system. Recombinant adenoviruses were created expressing human chymotrypsin C (CTRC) and human propreinsulin both fused with mCherry at their C-terminals. These fusion proteins act as reporters to monitor ER to secretory granule (SG) transport. Recombinant adenoviruses expressing wild type (WT) Sar1A and its dominant negative (DN) mutations Sar1A T39N and Sar1A H79G were created to study COPII dependent ER exit. **Results:** At first, the expression of right size fusion proteins and their SG localization were confirmed for both reporters by Western blotting and confocol microscopy. In addition, the viral titer dependent overexpression of WT and DN Sar1A was also verified by WB. Next, the effect of WT and DN Sar1A on ER exit of soluble cargo was examined using the reporter adenoviruses. Confocol images as well as total internal reflection fluorescence images showed that while CTRC-mCherry and proinsulin-mCherry targeted to SGs in WT Sar1A expressing cells, in DN Sar1A expressing cells, they had a homogenous distribution (excluding the nucleus) consistent with an ER localization which was confirmed by co-staining with an ER marker. Moreover, DN Sar1A mutants but not WT Sar1A strongly induced ER stress as indicated by the up-regulation of all ER stress markers tested. **Conclusions:** CTRC-mCherry and proinsulin-mCherry behaved as endogenous soluble cargo and can be used as reporters to study ER-SG transport. DN Sar1A blocked soluble cargo to exit ER and induced ER stress. These data indicate that digestive enzymes and proinsulin exit ER via COP II dependent manner in pancreatic secretory cells.

MOLECULAR CHARACTERIZATION OF ROUGH ENDOPLASMIC RETICULUM SUBPROTEOME IN PANCREATIC BETA CELLS

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Objectives: Insulin plays a central role in the regulation of glucose homeostasis. Pancreatic beta cells have well-developed endoplasmic reticulum (ER) to accommodate for the massive production and secretion of insulin. ER homeostasis is vital for normal beta cell functions. Perturbation of ER homeostasis and chronic activation of ER stress contribute to beta cell dysfunction in both type 1 and type 2 diabetes. Our goal is to systematically characterize the molecular machinery responsible for proinsulin biogenesis and maintenance of beta cell ER homeostasis. **Methods:** A widely used mouse pancreatic beta cell line, MIN6 cell, was used to purify rough ER. Two different purification schemes were compared, one with differential ultracentrifugation and the other using step sucrose gradient. MIN6 cells from two different sources were also compared for their ER protein compositions. In each experiment, the rER pellets were solubilized and separated on one dimensional SDS-PAGE. Thirty five gel slices were excised from each lane followed by in-gel tryptic digestion and LC-MS/MS analyses using Dionex nanoHPLC and QSTAR XL mass spectrometer. **Results:** A total of 1696 proteins were identified in three experiments with ≥99% confidence, among which 1279 proteins were found in at least two separate experiments. Preliminary analysis revealed a comprehensive coverage of all known and many novel players in main functional categories for preproinsulin processing including protein translation/translocation (25% of total), protein folding (12%), calcium/other ion homeostasis (8%); ER export/trafficking (24%) and ER associated protein degradation (8%). Representative proteins include ERO1A&B, zinc transporter 7 & 8, Hrd1, EDEM3, WFS1, UFM1-protein ligase 1 and atlastin 2. **Conclusions:** This study represents the first comprehensive characterization of the molecular machinery responsible for proinsulin biogenesis in the ER. A preliminary molecular function model is developed from these results and will serve as a foundation for subsequent studies to characterize the alteration of ER homeostasis under diabetes causing conditions.

RESVERATROL INDUCES ACUTE ENDOTHELIUM-DEPENDENT RENAL VASODILATION

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Purpose: Resveratrol (3,5,4′-trihydroxystilbene), a dietary polyphenol, has been shown to exhibit anti-inflammatory and cardioprotective effects. Resveratrol has also been shown to upregulate endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production. Thus, we hypothesized resveratrol can act as an acute endothelium-dependent vasodilator in the kidney. **Methods**: Inactin-anesthetized male Sprague Dawley rats were instrumented for acute renal hemodynamic studies. Protocol 1: Renal blood flow (RBF), mean arterial pressure (MAP), heart rate (HR), and renal vascular resistance (RVR) were measured following i.v. injections of vehicle, 0.5, 2.0, and 5.0 mg/kg resveratrol. Protocol 2: Bolus injections of vehicle and 5.0 mg/kg resveratrol were given before and after a NOS inhibitor, L-NAME (10mg/kg). **Results**: Protocol 1: We found only 5.0 mg/kg resveratrol resulted in a significant (8%) but transient increase in RBF from 6.98±1.20 to 7.54±0.491 ml/min/g k.w (n=8 $p<0.025$). RVR decreased 18% from 14.99±4.69 to 12.24±3.47 ARU ($p<0.003$). HR and MAP remained unchanged; 339±81 to 341±79 bpm and 100±12 to 97±13 mmHg, respectively. Protocol 2: L- NAME increased MAP from 97±10 to 139±15 mmHg, decreased HR from 346±37 to 277±19 bpm andincreased RVR from 12.81±2.11 to 34.07±9.23 ARU. L-NAME significantly diminished but did not eliminate resveratrol-induced vasodilation. The increase in RBF was only 43% of the original magnitude of the untreated resveratrol-induced response (4.35±1.13 to 4.59±.15 ml/min/g k.w. n=7 $p<0.001$) and the decrease in RVR was only 25% of control; 34.07±9.23 to 25.48±8.77 ARU ($p<0.002$). **Conclusion**: We conclude resveratrol can act as an acute and transient renal vasodilator. Resveratrol-induced renal vasodilation is partially mediated endothelial NO production, and partially by a mechanism yet to be defined.

FUNCTION OF THE HIGHLY CONSERVED C-TERMINAL SEGMENT OF TROPONIN I STUDIED WITH MUTATIONS FOUND IN RESTRICTIVE CARDIOMYOPATHY

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Objective: The C-terminal segment of troponin I (TnI) containing the last 27-33 amino acids is highly conserved which is critical for its inhibitory function. Indicating a potential mechanism, we recently showed a Ca^{2+}-regulated interaction between the C-terminal segment of TnI and tropomyosin (*Zhang et al. FEBS J, 2011*). Several mutations identified in or near the C-terminal segment of cardiac TnI (K178E, R192H and R204H) have been found to cause restrictive cardiomyopathy in humans and the pathogenic impact of these mutations have also been confirmed in transgenic mice over-expressing R193H and K179E mutation (*Du J et al. Am J Physiol Heart Circ Physiol, 2008, Jean-Charles et al. 56th Biphys Society meeting, 2011*, *Doolan et al. J Mol Cell Cardiol, 2005, Gambarin et al. Heart, 2008*). Our goal was to understand the function of the TnI C-terminal region in the regulation of muscle contraction and the pathogenesis of cardiomyopathy. **Method:** We constructed mouse cardiac TnI containing these mutations and purified recombinant proteins from *E.coli*. ELISA based monoclonal antibody epitope analysis and solid phase protein binding assay were used to determine the effects of the mutations on the overall conformation of cardiac TnI and on the interaction of TnI with TnT and TnC respectively. **Results:** Monoclonal antibody epitope analysis demonstrated that R193H abolished a C-terminal structure highly conserved in all TnI isoforms across vertebrate species. R205H resulted in a significant conformational change in the middle region troponin C-binding helix of cardiac TnI. Mutations R193H and K179E had similar but weaker effects. Solid phase protein binding assays showed that mutations R193H and K179E slightly increased the binding affinity of cardiac TnI for troponin T with statistic significance, whereas R205H drastically decreased the binding to troponin T. The binding affinity for troponin C was also very much decreased in the R205H mutation. **Conclusion:** These results provided novel evidence for the function of the highly conserved C-terminal segment of TnI, in which myopathic mutations may alter local and long-range effects on the function of the troponin complex.

ADDITIVE COMPENSATORY EFFECTS OF CARDIAC TROPONIN I AND CARDIAC TROPONIN T N-TERMINAL TRUNCATIONS ON THE DISEASE PHENOTYPES OF A FAMILIAL HYPERTROPHIC CARDIOMYOPATHY MUTATION (E180G) OF α-TROPOMYOSIN

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Objectives: A naturally occurring proteolytic N-terminal truncation of cardiac troponin I (TnI) has been shown to compensate for functional abnormalities caused by cardiomyopathic mutations (Barbato et al. JBC, 2005; Li et al. JMCC, 2010). Proteolytic N-terminal truncation of cardiac TnT (cTnT-ND) also showed functional benefit by elongating ventricular ejection time to compensate for cardiac output against high pressure load (Feng et al., J Physiol, 2008). To investigate the mechanisms for these troponin modifications to modulate the function of cardiac muscle thin filaments, we produced transgenic mouse lines co-expressing cTnI-ND and/or cTnT-ND with a hypertrophic cardiomyopathy mutation of cardiac α-tropomyosin (TM-E180G) (Prabhakar et al., JMCC 2001). **Methods and Results**: The over expression of TM-E180G resulted in ~90% replacement of endogenous α-tropomyosin in the cardiac muscle with maintained normal total stoichiometry. Functional studies in *ex vivo* working hearts of 2-month-old TM-E180G mice showed lower diastolic velocity and higher left ventricular diastolic pressure, indicating higher Ca^{2+} sensitivity. β-MHC expression together with myocardial fibrosis was found in the hearts of 28 days and 2 months old TM-E180G mice with early failing phenotypes (lower stroke volume and lower systolic velocity). In double and triple transgenic mice, expression of cTnI-ND and/or cTnT-ND decreased the occurrence of β-MHC in TM-E180G mouse hearts. Functional studies showed cTnI-ND and cTnT-ND did not override the contractile phenotype of TM E180G mutation but had beneficial effects on improving stroke volume and reducing fibrosis, with additive effects in the triple transgenic hearts. While TM-E180G mice usually die between 4 and 5 months of age (Prabhakar et al., JMCC 2001), cardiac function of TM-E180G+cTnI-ND+cTnT-ND triple transgenic mice remained apparently normal at 10-11 months of age as shown by the compensated heart function, improved cardiac efficiency, and minimal fibrosis and β-MHC occurrence. **Conclusions**: These results demonstrated compensatory effects of posttranslational modifications of troponin on the functional abnormality of tropomyosin for potential applications in the treatment of heart failure.

AN UNUSUAL ALPHA ACTIN TO TARGET CARDIAC REMODELING

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Purpose: A naturally occurring N-terminal truncation of cardiac troponin I (cTnI-ND) has been shown as an adaptation to compensate for heart failure. Over-expression of cTnI-ND in the heart of transgenic mice showed functional benefit and its effect on myocardial remodeling remains to be investigated. Alpha smooth muscle actin (α-SMA) expression is one of the genes specifically upregulated in cTnI-ND hearts and we examined whether it reflects an increase in vasculature and/or a change in cardiomyocytes. **Methods**: Relative levels of α-SMA in transgenic and WT mouse hearts (age, 2 months) were compared by immunoblot analysis. Frozen sections of the hearts were examined for immunohistochemical analysis using a rabbit anti-α-SMA antibody (Abcam). Images were analyzed using ImageJ to determine the density, and size of blood vessels and the relative level of α-SMA in cardiomyocytes. Isolated cardiomyocytes were examined using immunofluorescence microscopy for the expression and cellular distribution of α-SMA. **Results:** An increase in α-SMA expression was detected by Western blots on total heart homogenate of cTnI-ND transgenic mice ($P<0.05$). An increase in the density of blood vessels was observed, which, however, was also seen in the hearts of another line of transgenic mice in the absence of α-SMA upregulation. There was no change in vessel diameter or wall thickness. On the other hand, cTnI-ND transgenic mouse hearts exhibited increased α-SMA in cardiomyocytes as shown by immunohistochemistry staining ($P<0.05$) and fluorescence microscopy in isolated cardiomyocytes ($P<0.05$). **Conclusions**: We determined that α-SMA is upregulated in cardiomyocytes of young cTnI-ND transgenic mouse hearts. We are currently investigating whether this up-regulation of α-SMA is merely an early marker of myocardial remodeling, or has a functional impact, which could be targeted to modulate myocardial remodeling.

CARDIAC OUTPUT DURING MYOCARDIAL ISCHEMIA/REPERFUSION AND INFARCTION IN CHRONICALLY INSTRUMENTED, INTACT, CONSCIOUS MICE

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Multiple systems and regulatory strategies interact to control cardiac homeostasis. In fact, regulated systems, feedback controls, and redundant control mechanisms dominate in whole animals. Accordingly, molecular and cellular tools and techniques must be utilized in complex models with multiple systems and regulatory strategies to fully appreciate the physiological context. Currently, these techniques are mainly performed under conditions remote from the normal *in vivo* condition; thus the extrapolation of molecular changes to the *in vivo* situation, and the facilitation of translational aspect of the findings are limited. A major obstacle has been the reliance on preparations that do not mimic the clinical or physiological situation. To address these concerns, we used a permanently implanted Doppler ultra-sonic flow probe on the ascending aorta for repeated measurements of cardiac output in conscious mice before and during coronary artery occlusion/reperfusion and infarction. The conscious mouse model permits repeated measurements of cardiac output during occlusion and reperfusion of the left anterior descending coronary artery in an intact, complex model free of the confounding influences of anesthetics, surgical trauma and restraint stress. The use of this chronic model, with currently available spontaneous or engineered mouse mutants, has the potential to be of major importance for advancing the concepts and methods that drive cardiovascular research.

DIFFERENTIAL MODULATION OF CARDIAC β_1 AND β_2 ADRENERGIC RECEPTOR CONTRACTILE EFFECTS BY ADENOSINE A_1 AND A_{2A} RECEPTORS

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Objective: The cardiac adenosine A1 receptor (A_1AR) anti-adrenergic effect and its modulation by the adenosine A2A receptor (A_{2A}AR) has been described using the non-selective β-adrenergic receptor (βR) agonist isoproterenol (ISO). However, limited studies have been done to address the contractile effects of β_1 vs. β_2 adrenergic receptors, and no studies have investigated their modulation by adenosine receptors. The purpose of this study was to determine how the contractile effects of selective β_1R vs. β_2R activation are altered by the A_1AR and A_{2A}AR. **Methods:** Dose response studies were performed in isolated perfused hearts from wild type (WT), A_1KO, and A_{2A}KO male mice measuring LV systolic pressure (LVSP) and +dP/dt (n≥3/group). **Results:** The responses to a single dose of β_1R partial agonist xamoterol (Xam) and β_2R agonist procaterol (Pro) are shown in Table 1. Similar responses were seen to Xam in WT, A_1KO and A_{2A}KO hearts, but the response to Pro was potentiated in A_{2A}KO compared to WT and A_1KO hearts (*p<0.05). This contrasts with previously reported effects of ISO in A_1KO and A_{2A}KO hearts. The A_1AR agonist CCPA exerted significant anti-adrenergic effects on both Xam and Pro responses (% increase in LVSP and +dP/dt with 100 nM Xam- 4 ± 2, 10 ± 5; with 10 μM Pro 17 ± 3, 25 ± 5). **Conclusions:** β_1R and β_2R positive inotropic effects, when activated individually or in combination, are differentially modulated by A_1AR and A_{2A}ARs. This may be due to ligand-specific effects and/or heterodimerization between ARs and βRs.

Table 1- Percent change from baseline with βR agonists

	WT		**A_1KO**		**A_{2A}KO**	
	LVSP	+dP/dt	LVSP	+dP/dt	LVSP	+dP/dt
100 nM Xam	44 ± 5	77 ± 8	38 ± 2	65 ± 7	49 ± 2	76 ± 2
10 μM Pro	48 ± 3	82 ± 7	52 ± 4	81 ± 7	77 ± 4 *	127 ± 8*

PROTECTIVE ROLE OF AC-SDKP AND THYMOSIN-B4 ON CARDIAC RUPTURE AFTER MYOCARDIAL INFARCTION

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Objectives: To test our hypothesis that both thymosin-β4 (Tβ4) and N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) reduce myocardial infarction (MI)-induced cardiac rupture by decreasing excessive inflammatory responses and extracellular matrix (ECM) degradation. **Methods:** 12-week old male C57BL/6J mice were divided into four groups: 1) sham MI, 2) MI + vehicle (VEH), 3) MI + Ac-SDKP, or 4) MI + Tβ4. Ac-SDKP and Tβ4 (1.6 mg/kg/day) were given i.p. *via* osmotic pump 7 days before MI and mice were scarified 7 days after MI. In the infarct border zone, CD68- and Ly-6B.2 alloantigen-positive cells were detected by immunohistochemical staining and gelatinolytic activity by *in situ* zymography and fluorescein-labeled-gelatin. Intracellular adhesion molecule 1 (ICAM-1) was detected by Western blot. Ejection fraction (EF) was assessed by echocardiography. **Results:** Both Tβ4 and Ac-SDKP reduce cardiac rupture induced by MI (from 60.5% in VEH to about 23% in each treatment group, $P < 0.005$). In the border zone of infarct region: 1) macrophage infiltration was markedly inhibited by either Ac-SDKP or Tβ4 (195 ± 17 in VEH vs. 118 ± 11 and 118 ± 12 cells/mm^2 in Ac-SDKP- and Tβ4-treated group, respectively, $P < 0.005$), 2) neutrophil infiltration was inhibited by Tβ4 (109 ± 8 in VEH vs. 62 ± 11 cells/mm^2, $P < 0.05$), but not by Ac-SDKP (100 ± 14 cells/mm^2), and 3) gelatinolytic activity was reduced by either Ac-SDKP or Tβ4 (13.88 ± 0.67 in VEH vs. 8.00 ± 0.95 and 7.98 ± 0.62 A.U. in Ac-SDKP- and Tβ4-treated mice, respectively, $P < 0.005$). ICAM-1 was significantly reduced in the heart by Ac-SDKP or Tβ4 (3.49 ± 0.26 in VEH vs. 1.36 ± 0.14 and 1.24 ± 0.11 fold vs. sham in Ac-SDKP- and Tβ4-treated group, respectively, $P < 0.05$). Treatment with either Ac-SDKP or Tβ4 did not affect EF at day 7 post-MI (24.45 ± 4.49 in VEH vs. about 30% in each group, $P > 0.05$). **Conclusions:** Decline in MI-induced cardiac rupture by Ac-SDKP or Tβ4 may be due to decreased macrophage infiltration and gelatinolytic activityin the infarct border.

DRP1 MITOCHONDRIAL TRANSLOCATION: A POSSIBLE MECHANISM FOR CYTOCHROME C RELEASE, ACTIVATION OF APOPTOSIS AND CELL DEATH IN MYOCARDIAL ISCHEMIA-REPERFUSION INJURY?

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Background: Mitochondrial fission is the dynamic process by which mitochondria are cleaved and fragmented. Recent reports suggest that pharmacologic inhibition of fission attenuates lethal ischemia-reperfusion (I-R) injury, thereby implying that fission contributes to cell death. However, the molecular mechanisms by which fission regulates cell fate are unresolved. **Hypothesis**: We propose that: (i) I-R elicits the translocation of DRP1 (dynamin-related protein 1: a critical mediator of fission) from the cytosol to the mitochondria. Once in position, DRP1 will constrict the mitochondria, thereby (ii) facilitating release of Cytochrome C (Cyto C) into the cytosol and (iii) triggering apoptosis. If so, (iv) inhibition of DRP1 translocation will attenuate Cyto C release and offer cytoprotection. **Methods**: In Protocol 1, cultured HL-1 cells (an immortal cardiac cell line) underwent 2 h simulated I + 5, 30 or 120 min reoxygenation or a matched normoxic period. For each group, subcellular localization of DRP1 and Cyto C was resolved by immunoblotting, and cleaved Caspase 3 (harbinger of apoptotic death) was quantified in the cytosol. Protocol 2 was identical, except cells were pretreated with mdivi-1 (specific DRP1 inhibitor; 50 μM) or vehicle. In Protocol 3, cells were pretreated with mdivi-1 or vehicle and viability quantified by Trypan blue staining at 20 h post-R. **Results**: Our results revealed a rapid and significant redistribution of DRP1 from the cytosol to the mitochondria post-R, a concomitant release of Cyto C into cytosol, and cleavage of Caspase 3 ($P<0.05$ *vs* normoxia for all variables). Treatment with mdivi-1 impaired translocation of DRP1 – and, most notably, attenuated Cyto C release, blunted Caspase 3 cleavage ($P<0.05$ *vs* vehicle for all variables), and enhanced cell viability (71±2% *vs* 55±3% in vehicle controls; $P<0.05$). **Conclusion**: DRP1 translocation (i.e., mitochondrial fission) promotes lethal ischemia-reperfusion injury by facilitating release of Cytochrome C into the cytosol and triggering apoptosis.

METABOLIC SUPPORT OF MITOCHONDRIAL COMPLEX I WITH YEAST NDI1 EVOKES SIGNIFICANT CARDIOPROTECTION IN THE *IN VIVO* RAT MODEL OF ACUTE MYOCARDIAL INFARCTION

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Objective: Lethal myocardial ischemia-reperfusion (I-R) injury has been attributed in part to mitochondrial respiratory dysfunction (including damage to Complex I) and the resultant excessive production of reactive oxygen species. Recent evidence has shown that Ndi1 (the single subunit protein that serves as the surrogate for Complex I in yeast), transduced by addition of the TAT-conjugated protein to culture media and buffer, can preserve mitochondrial function and attenuate I-R injury in isolated myocytes and isolated hearts. However, the *in vivo* efficacy of this novel metabolic treatment strategy has, to date, not been explored. **Methods**: To address this issue, TAT-conjugated Ndi1 and TAT-conjugated placebo control protein were synthesized using a cell-free (non-bacterial) system. After confirming the identity of the proteins by immunoblotting, rats were randomized to receive intraperitoneal injection of either TAT-Ndi1 or TAT-placebo. Two hrs post-treatment, all animals underwent 45 min coronary artery occlusion followed by 2 hrs of reperfusion. Infarct size was delineated by tetrazolium staining and normalized to the volume of at-risk myocardium (the primary determinant of infarct size in this model). **Results**: Risk region was comparable in the two cohorts, averaging 28-32% of the total left ventricular weight (p=ns). Preischemic administration of TAT-Ndi1 was, however, associated with profound cardioprotection: infarct size in TAT-Ndi1-treated rats was 25±7%* of the myocardium at risk, significantly smaller than the value of 63±5% seen in the TAT-placebo control group (*p<0.01 *vs* control). **Conclusions**: These results provide the first *in vivo* evidence that yeast Ndi1 can provide metabolic support to ischemic-reperfused myocardium and reduce myocardial infarct size. Moreover, these data suggest that metabolic support of mitochondrial proteins may provide a novel molecular strategy to protect the human heart against planned ischemia-reperfusion events.

VITAMIN D INCREASES PLASMA RENIN ACTIVITY INDEPENDENTLY OF PLASMA CALCIUM, THROUGH POLYURIA-INDUCED DEHYDRATION

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Rationale: Recent data suggest Vitamin D may play a role in cardiovascular health. 1,25 OH_2 dihydroxycholecalciferol (Calcitriol) is the active form of Vitamin D. Currently, many clinical trials are being conducted to test whether calcitriol can decrease renin-angiotensin activity in hypertension or heart failure. However, Calcitriol also increases plasma Ca^{2+}, which can cause polyuria, dehydration, and volume contraction, all of which can increase renin-angiotensin activity. We hypothesized Calcitriol increases plasma renin activity (PRA) due to hypercalcemia-mediated polyuria and dehydration and elevated sympathetic nervous activity. **Methods:** All experiments were run in male Sprague-Dawley rats over 6 days. **Results:** 500 ng/day Calcitriol, i.p., increased PRA from 4.4±0.5 to 17.4±4.6 ngAngI/ml/hr (p<0.05). Calcitriol increased plasma Ca^{2+} from 1.26±0.02 to 1.64±0.06 mM (p<0.001) and increased urine flow from 9.0±1.1 to 26.3±1.1 ml/day (p<0.01). To test the role of plasma Ca^{2+}, we treated rats with 100 ng/day of the vehicle, Calcitriol, or Paricalcitol, a non-calcemic analog of Vitamin D. 100 ng/day Calcitriol increased plasma Ca^{2+} from 1.24±0.01 to 1.52±0.03 mM (p<0.001), while Paricalcitol plasma Ca^{2+} (1.23±0.02 mM) did not differ from control. However, Calcitriol and Paricalcitol both increased PRA from 3.1±0.3 to 10.3±1.0 and 8.8±2.0 ngAngI/ml/hr, respectively (p<0.01). Also, Calcitriol and Paricalcitol both increased urine flow from 11.3±1.0 to 29.3±3.4 and 24.6±3.2 ml/day, respectively (p<0.01). To test if Calcitriol increased PRA *via* polyuria-induced dehydration, we rehydrated Calcitriol-treated rats subcutaneous 0.9% NaCl. Rehydrating Calcitriol-treated rats completely normalized PRA from 9.2±1.8 to 3.1±0.8 ngAngI/ml/hr (p<0.05) and lowered plasma epinephrine from 1695.0±182.9 to 985.5±139.5 pg/ml (p<0.05). Additionally, β-adrenergic blockade with propranolol decreased calcitriol-stimulated PRA from 12.4±1.9 to 8.7±0.7 ng Ang I/ml/hr (p<0.05). **Conclusions:** Thus, our data demonstrate that Vitamin D increases PRA *independently* of increased plasma Ca^{2+}, *via* polyuria-induced dehydration and sympathetic activity. Our data suggest that treatment with Vitamin D may actually worsen cardiovascular outcomes in cardiovascular disease. More research is needed to determine the effects of Vitamin D in the cardiovascular system.

PHOTOBIOINHITION OF MITOCHONDRIAL COMPLEX IV: A NOVEL APPROACE TO REDUCE CEREBAL OXIDATIVE INJURY ASSOCIATED WITH NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY

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Background: Insufficient oxygen delivery to the infant brain prior to, during, or immediately after birth can promote significant brain damage, resulting in hypoxic-ischemic encephalopathy (HIE) of the neonate. While prompt re-oxygenation is critical in the management of affected infants, the reintroduction of oxygen can potentiate oxidative damage by promoting reactive oxygen species (ROS) generation at the level of the mitochondrion. **Hypothesis:** We propose a model in which dephosphorylation of cytochrome c oxidase (complex IV) during cerebral hypoxia-ischemia leads to increased complex IV activity, in turn, promoting mitochondrial ROS generation during reperfusion. Additionally, in accordance with data from our previous in vitro studies, we hypothesis that infrared light (IRL) may favorably modulate complex IV activity and thus may be effective in mitigating mitochondrial ROS production during the critical period following cerebral hypoxic-ischemia. **Methods:** Hypoxic-ischemia was induced in 7-d-old Sprauge-Dawley rats pups via unilateral common carotid artery ligation followed by 120 min of hypoxia (8% O_2; 92% N_2; 36.5^{o}C). The effect of cerebral hypoxic-ischemia on complex IV activity was assessed in isolated mitochondria by measuring oxygen consumption in the presence of reduced cytochrome c at 25^{o}C. Immediately following hypoxia, a subset of animals were treated with IRL (100 mw/cm^2) for 1 hour. High power IRL diodes emitting a wavelength of 950nm were used as this wavelength of IRL was demonstrated in vitro to inhibit complex IV activity. **Results:** As per our hypothesis, our initial data suggest that: (i) complex IV activity is increased immediately following hypoxic-ischemia, and (ii) photobioinhibition of complex IV using IRL_{950nm} may be an effective strategy to reduce oxidative damage following cerebral hypoxic-ischemia in the neonate.

ROLE OF CARDIAC OUTPUT VS. PERIPHERAL VASOCONSTRICTION IN MEDIATING THE MUSCLE METABOREFLEX PRESSOR RESPONSE DURING DYNAMIC EXERCISE AND POST-EXERCISE MUSCLE ISCHEMIA

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Muscle metaboreflex activation (MMA) during sub-maximal dynamic exercise in normal individuals increases mean arterial pressure (MAP) via increases in cardiac output (CO) with little peripheral vasoconstriction. The rise in CO occurs primarily via increases in heart rate (HR) with maintained or slightly increased stroke volume. When the reflex is sustained during recovery (post-exercise muscle ischemia - PEMI), HR declines yet MAP remains elevated. The role of CO in mediating the pressor response during PEMI is controversial. In six chronically instrumented canines, steady state values with MMA during mild exercise (3.2 kph) were observed by reducing hindlimb blood flow by ~60% for 3-5 minutes. MMA was followed by 60 seconds of PEMI. Control experiments consisted of normal exercise and recovery. MMA during exercise increased MAP, HR and CO by 58.2 ± 4.6 mmHg, 42.1 ± 8.1 bpm, and 2.40 ± 0.40 l/min, respectively. During sustained MMA via PEMI, MAP remained elevated and CO remained well above the normal recovery levels. Neither MMA during dynamic exercise nor during PEMI significantly affected peripheral vascular conductance. We conclude that the sustained increase in MAP during PEMI is driven by a sustained increase in CO not peripheral vasoconstriction. HL-55743 and HL-095819

THE EFFECT OF PHYSICAL (IN)ACTIVITY ON BLOOD PRESSURE AND SPLANCHNIC SYMPATHETIC NERVE RESPONSES TO INHIBITION OF THE RVLM

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A sedentary lifestyle is a major risk factor for cardiovascular disease (CVD). CVD is often associated with enhanced activation of the sympathetic nervous system. The sympathetic nervous system is under tonic and phasic control by the rostral ventrolateral medulla (RVLM). In sedentary rats, there is enhanced sympathoexcitation in response to glutamatergic activation of the RVLM. Since the activity of RVLM is tonically restrained by γ-amino-butyric acid (GABA), we hypothesized that sedentary conditions may also lead to decreased responsiveness to GABA in RVLM when compared to physically active conditions. In Inactin anesthetized, sedentary (SED) or physically active (EX) rats, mean arterial pressure (MAP), heart rate (HR) and splanchnic sympathetic nerve activity (sSNA) were recorded during unilateral microinjections of GABA (30 nl, 0.3-600 mM) into the RVLM. Following GABA injections, the contralateral RVLM was inhibited with 90 nl of 2mM Muscimol and the GABA injections were repeated. There were no significant differences between SED or EX conditions for MAP, HR and sSNA responses to GABA before contralateral blockade of RVLM. However, after contralateral blockade of RVLM, SED rats had enhanced decreases in MAP to GABA compared to EX rats. Our results suggest that the contralateral RVLM plays an important role in buffering responses to inhibition of the ipsilateral RVLM under sedentary but not physically active conditions. We conclude that a sedentary lifestyle not only leads to enhanced sympathoexcitation but also enhanced sympathoinhibition in the RVLM. The enhanced sympathoinhibitory mechanisms may be a compensatory response to offset elevated sympathetic nervous system activity following sedentary conditions. (Supported by R01-HL096787; R01-HL096787-S1)

EFFECT OF BLOCKADE OF V1B RECEPTORS IN THE PARAVENTRICULAR NUCLEUS IN CONSCIOUS SPRAGUE DAWLEY RATS

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The paraventricular nucleus (PVN) of the hypothalamus plays a critical role in the autonomic regulation through axonal projections to the brain stem and spinal cord. Arginine vasopressin (AVP) is a neuropeptide released from axon terminals of magnocellular neurons whose cell bodies are located in the supraoptic nucleus and PVN. AVP is also released from dendrites of the magnocellular neurons. PVN neurons possess V1a receptors, and there are data showing that V1b receptors may also be present. AVP is also known to play a role in autonomic regulation. The role of V1b receptors in autonomic regulation has not been studied. Thus, we tested the hypothesis that the inhibition of V1b receptors within the PVN would inhibit both the pressor response and the increase in renal sympathetic nerve activity (RSNA) to AVP in the PVN of conscious rats. Male Sprague Dawley rats were instrumented with vascular catheters, renal nerve electrodes and a cannula directed into the PVN and studied in the conscious state 24 hr after recovery from anesthesia. Baseline mean arterial pressure (MAP), heart rate (HR) or RSNA did not differ among the groups. Microinjection of 100 ng AVP into the PVN resulted in an increase in MAP from 138.2 ± 3.1 (baseline) to 147.7 ± 3.2 mmHg (5 min, n = 5, $P < 0.01$) and 151.0 ± 3.2 mmHg (10 min, $P < 0.001$). Heart rate was also significantly higher at both time points compared with baseline values. The pressor response tested in the same animals was blocked by pre-injection with 100 ng nelivaptan, 140.9 ± 3.6 mmHg (10 min, $P > 0.05$) as was the tachycardia. RSNA could be recorded in only 4 rats. RSNA increased to 147.8 ± 6.6%baseline 10 min after AVP ($P < 0.01$) and was blocked by the V1b inhibitor. Pre-injection of the PVN with vehicle did not block any of the responses to AVP. We conclude that nelivaptan blocks the pressor, tachycardic and RSNA responses to injection of AVP into PVN supporting the concept that V1b receptors in PVN neurons may be activated by dendritic release of AVP within this nucleus.

VENTILATION AND TRYPTOPHAN HYDROXYLASE 2 KNOCK OUT MICE

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Objectives: To examine the impact of serotonin (5HT) on breathing before, during and after exposure to intermittent hypoxia (IH) in spontaneously breathing mice. **Methods:** Fifteen mice with a null mutation for tryptophan hydroxylase 2 (KO) and 15 wild type mice (WT) were exposed to 12 – 2 min episodes of hypoxia (10% oxygen) on 7-10 consecutive days. Ventilation was measured before, during and after exposure via whole body plethysmography. Data was average for the initial and final two days of the protocol. **Results:** *Baseline measures* - Minute ventilation (V_E) before exposure to hypoxia was similar in the KO and WT on the initial two days. However, the KO mice displayed an elevated tidal volume (V_T) (7.03 ± 0.15 vs. 6.36 ± 0.11 µl/g; $p \leq 0.05$) and a decreased breathing frequency (B_f) (155.0 ± 3.8 vs.166.4 ± 3.0 breaths/minute; $p \leq 0.05$) compared to the WT mice. Carbon dioxide production was also elevated in the KO mice (VCO_2; 0.042 ± 0.002 vs. 0.039 ± 0.001 ml/min/g; $p \leq 0.05$) on the initial days. These relationships were unchanged on the final days of the protocol. *Hypoxic Responses* – The hypoxic ventilatory response (HVR) was attenuated in the KO mice (0.75 ± 0.13 vs. 1.04 ± 0.11 ml/min/%O_2; $p \leq 0.05$) on the initial days. In addition, the HVR was attenuated in the WT and KO mice on the final compared to the initial days. *End Recovery* – Compared to baseline, V_E (1.03 ± 0.02 ml/min vs.1.13 ± 0.03; $p \leq 0.05$) and B_f (166.4 ± 3.0 vs.179.9 ± 3.2 breaths/min; $p \leq 0.05$) were increased after exposure to hypoxia in the WT mice on the initial and final days of the protocol. This was not the case in the KO mice. However, after exposure to hypoxia on the final days, V_E and V_T (1.14 ± 0.01 ml/min and 7.49 ± 0.26 µl/g) were significantly greater than baseline values measured in the KO mice on the initial days. **Conclusions:** 5HT in the central nervous system (CNS) has a role in the resting pattern of breathing and the HVR. 5HT also has a role in initiating forms of respiratory plasticity that are evident after initial exposures to intermittent hypoxia.

A_{2a} ADENOSINE RECEPTORS INHIBIT DIFFERENTIAL CARDIOPULMONARY CHEMOREFLEX CONTROL OF REGIONAL SYMPATHETIC OUTPUTS VIA ACTIVATION OF GABA-ergic NEURONS WITHIN THE CAUDAL PORTION OF THE NUCLEUS OF THE SOLITARY TRACT (cNTS): FUNCTIONAL AND ANATOMICAL EVIDENCE

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The cardiopulmonary chemoreflex (CCR) may contribute to severe hypotension and sudden cardiac death when markedly activated. The reflex may be triggered by activation of polymodal serotonin 5HT3 receptors present on vagal afferents in the cardiopulmonary area which carry afferent information centrally to the cNTS where the CCR is primarily integrated. Since interstitial concentration of adenosine in the cNTS may increase during life threatening ischemia and adenosine may markedly affect release of neurotransmitters from cNTS neurons, we hypothesized that adenosine may serve as a central negative feedback regulator for this reflex. We have shown that CCR evoked differential regional sympathoinhibition (renal>adrenal>lumbar) was uniformly and markedly attenuated after stimulation of inhibitory A_1 (*Am J Physiol Regul Integr Comp Physiol 2012 in press*) and activatory A_{2a} adenosine receptors in the cNTS (*FASEB J* 24: 624.11, 2010). A_{2a} receptor mediated attenuation of the CCR was virtually abolished by GABAergic blockade (*FASEB J* 25: 844.3, 2011). These data imply that GABAergic neurons/terminals within the cNTS express activatory A_{2a} receptors. We investigated this hypothesis using double immunofluorescent staining for A_{2a} receptors (Cy3, red) and GAD67, the GABA synthetic enzyme (FITC, green). We observed extensive colocalization of A_{2a} receptors with GABAergic neurons in the cNTS and no such colocalization in more caudal portions of the medulla (*FASEB J* 26: 1091.28, 2012). Tracing of cardiac vagal afferents with cholera toxin B subunit (Alexa 488, green) revealed absence of activatory A_{2a} receptors (Cy3, red) on vagal terminals in the cNTS consistent with inhibitory (not activatory) role of adenosine A_{2a} receptors in modulating the CCR. These immunohistochemical data further support our functional findings that A_{2a} adenosine receptors inhibit the CCR indirectly, by facilitating GABA release from the nerve terminals in the CCR pathway at the level of the cNTS.

PHYSICAL (IN)ACTIVITY DEPENDENT CHANGES IN THE MORPHOLOGY OF RVLM NEURONS

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The rostral ventrolateral medulla (RVLM) contains neurons critical for cardiovascular regulation. Functional plasticity in RVLM neurons may contribute to increased sympathoexcitation in disease states. Here, we aimed to define morphological differences in RVLM cardiovascular neurons in sedentary vs physically active rats, hypothesizing that increased sympathoexcitation in sedentary rats would correlate with more dendritic branching. Physically active rats were provided with in-cage running wheels for 14 weeks and ran 230 ± 81 km (n=4). We used immunohistochemistry for tyrosine hydroxylase (TH) in 150 µm brainstem sections and tracing software to reconstruct TH-immunoreactive neurons at the caudal pole of the facial nucleus. Comparison of dendrites in 3 sedentary vs 4 active rats showed an average length of 1111±54 µm vs. 686±86 µm ($p<0.05$) and 9.7±0.7 branch nodes vs. 7.0±1.1 ($p=0.058$). A Sholl analysis showed 82±4.2 vs. 52.8±6.2 total intersections ($p<0.05$). These data suggest that greater dendritic branching in RVLM cardiovascular neurons contributes to increased sympathoexcitation and incidence of cardiovascular disease in sedentary individuals. Funding: (F30-HL105003, NAM; NHMRC 480414, ILS; R01- HL096787, PJM).

BLAST INDUCED TINNITUS AND ITS RELATED "INVISIBLE WOUNDS" IN LIMBIC STRUCTURES: A COMBINED BEHAVIORAL AND MEMRI STUDY

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Tinnitus and hearing loss are the frequent auditory-related co-morbidities of blast trauma. The development and persistence of tinnitus is often compounded by brain mechanisms associated with cognitive problems such as anxiety, memory loss, emotional disturbances and depression. We set out to develop a realistic and ecologically valid model to address cognitive status (memory) and psychological state (anxiety and emotion) that are associated with the blast induced tinnitus and the related traumatic brain injury (TBI). In this study, 16 adult rats were randomly divided into blast and non-blast control groups. Blast exposure (14 psi) was carried out to expose the left ears of the rats. Blast-induced tinnitus, changes in anxiety and memory were evaluated using gap detection acoustic startle reflex paradigm, elevated plus maze and Morris water maze, respectively. Using manganese-enhanced magnetic resonance imaging (MEMRI), we investigated blast-induced neural changes in several non-auditory brain structures. Compared to non-blast controls, blasted rats with or without tinnitus demonstrated higher level of anxiety, possibly related to post traumatic stress disorder (PTSD). MEMRI data demonstrated that tinnitus(+) rats exhibited hyperactivity as revealed by increased Mn^{2+} uptake in the contralateral basolateral nuclei of amygdala, but hypoactivity as revealed by decreased Mn^{2+} uptake in the ipsilateral anterior cingulate cortex. These changes may have contributed to psychological sequalea of blast-induced tinnitus, TBI and PTSD.

PHYSICAL (IN)ACTIVITY LEADS TO NEUROPLASTIC CHANGES IN THE ROSTRAL VENTROLATERAL MEDULLA (RVLM).

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Individuals who lead a sedentary lifestyle are at increased risk of cardiovascular disease. C1 neurons in the rostral ventrolateral medulla (RVLM) express tyrosine hydroxylase (TH) and are important in blood pressure regulation. We have hypothesized that the RVLM undergoes neuroplastic changes that increase sympathoexcitation in sedentary (SED) versus physically active animals. To test this hypothesis, TH immunohistochemistry was performed on 150 µm thick sections from SED rats or rats housed with running wheels (WRs, 14 wks). The caudal pole of the facial nucleus (CP7) was used as a reference point for blinded cell counts. TH+ cells were distributed rostral-caudally in both groups, however, SED rats had significantly greater TH+ cells near CP7 (92±14 vs. 31±8 cells, $p<0.05$). TH+ cells were not different 600 µm caudal to CP7 (64±12 vs 60±5 cells). These data suggest that TH+ neurons projecting to the spinal cord but not hypothalamus undergo neuroplasticity due to physical activity, inactivity or both. Knowledge from these studies may lead to treatment options in patients who are unable or unwilling to exercise. (APS Frontiers Program; F30HL105003; R01HL096787).

DIFFERENTIAL PATTERN OF SPLANCHNIC AND LUMBAR SYMPATHETIC NERVE ACTIVITY TO STIMULATION OF RVLM IN SEDENTARY VERSUS PHYSICALLY ACTIVE RATS

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The rostral ventrolateral medulla (RVLM) is involved in normal and pathological increases in sympathetic nerve activity (SNA). For example, enhanced responses to stimulation of the RVLM occur in sedentary compared to physically active rats. RVLM neurons can also drive SNA differentially and may in turn contribute to the "sympathetic signature" in hypertension. However, the impact of physical (in)activity on differential regulation of SNA by the RVLM is unknown. In Inactin anesthetized sedentary or active rats (running wheels, 10-14 wks), mean arterial pressure (MAP), splanchnic (sSNA) and lumbar sympathetic nerve activity (LSNA) were recorded simultaneously. Microinjections of glutamate (30 nl, 10 mM) were performed in a three dimensional grid pattern in the pressor region of the RVLM. Glutamate increased sSNA and LSNA differentially depending on the location of injection and whether animals were sedentary or active. Comparison of absolute changes in nerve activity showed that sedentary rats have significantly enhanced splanchnic nerve activity compared to active rats. These data suggest that differential regulation of sympathetic outflow by the RVLM is altered by sedentary or physically active conditions and may play a role in modifying the sympathetic signature of certain disease states such as hypertension. (R01HL096787)

REGULATION OF HBEGF BIOSYNTHESIS BY MICRORNA IN HUMAN TROPHOBLAST CELLS

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Objective: The epidermal growth factor (EGF) family member, heparin binding EGF-like growth factor (HBEGF), is present in the uterus at the time of embryo implantation and is expressed in invading trophoblast cells of the placenta indicating its central role in implantation and subsequent placentation. Previous work has demonstrated that HBEGF protein levels are dramatically upregulated in trophoblast cells cultured at low (2%) O_2 without a change in HBEGF mRNA, suggesting the hypothesis that HBEGF is translationally regulated by O_2. MicroRNAs (miRNAs) are small non-coding RNA species processed by nuclear proteins Drosha and DGCR8 that regulate translation by targeting the 3' untranslated region (3'UTR) of specific mRNAs. **Methods:** The potential role of miRNA in the targeted regulation of HBEGF biosynthesis was examined using DGCR8 knockdown by siRNA transfection into HTR-8/SVneo human first trimester trophoblast cell. Western blots with antibody against DGCR8 and ELISA was used to quantify HEBGF. Toxicity assays were used to optimize conditions. A dual luciferase reporter construct (psiCHECK-2) containing the intact HBEGF 3'UTR or specific subregions was implemented to examine its translational regulatory potential. **Results:** HBEGF quantified by ELISA did not increase when DGCR8 was knocked down at 20% O_2, indicating that HBEGF expression was not repressed by miRNA. However, the upregulation of HBEGF at 2% O_2 was inhibited. The intact 3'UTR reduced luciferase activity ($p=0.008$). However, the isolated 5' and 3' regions of the 3'UTR increased reporter activity by 4- to 5-fold ($p<0.05$) when the central domain was removed. These findings suggest that miRNAs alter interaction between inhibitory elements in the central domain of the 3'UTR and flanking elements to increase HBEGF translation. **Conclusion:** It is concluded that HBEGF is not transcriptionally regulated by O_2 in human trophoblast cells, but is translationally regulated through the interaction of miRNA with the HBEGF 3'UTR.

ENHANCED PHOSPHODIESTERASE 5 (PDE5) IN THICK ASCENDING LIMBS OF DAHL SALT SENSITIVE RATS BLUNTS NO-INDUCED INHIBITION OF TRANSPORT

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Objectives: In normal rats, NO inhibits thick ascending limb (TAL) NaCl reabsorption via cGMP. In Dahl Salt Sensitive (SS) rats NO fails to decrease TAL NaCl absorption. However, the mechanism for this defect is unknown. NO stimulates soluble guanylyl cyclase to produce cGMP. The intracellular concentrations of cGMP and its effect are limited by PDE5, a cGMP-specific phosphodiesterase. We hypothesize that enhanced PDE5 expression in SS TALs blunts the inhibitory effect of NO on transport by decreasing cGMP concentration. **Methods & Results:** We isolated TALs from SS and Dahl Salt-Resistant (SR) rats fed normal salt and measured PDE5 expression by Western blot. PDE5 expression was 210±20% higher in SS TALs compared to SR (p<0.05). We then studied whether PDE5 inhibition with vardenafil (25 nM) enhances the inhibitory effect of NO on transport by measuring Na transport-related oxygen (O_2) consumption in TALs. In SR TALs, the NO donor spermine-NONOate (NO) decreased O_2 consumption by 7.9±2.3% (p<0.05). Adding vardenafil to the bath further decreased O_2 consumption by 7.7±2.3% (p<0.05). In contrast, in SS TALs NO failed to decrease oxygen consumption (5±2.5% from baseline). However in the presence of a PDE5 inhibitor NO, inhibited oxygen consumption by 15.5±2.5 % (p<0.05, n=7). We then tested whether NO-stimulated cGMP production is decreased in SS TALs. To measure production, we inhibited all phosphodiesterases with IBMX. NO enhanced cGMP production in both SS and SR TALs, but production was higher in SS than SRs (delta SS: 4.0±1.0 vs SR: 1.9±0.6 fmoles/µg, p<0.05). Finally we tested whether the decreased effect of NO in SS TALs is related to enhanced cGMP degradation by PDE5. During PDE5 inhibition NO-stimulated cGMP levels were 4-fold greater in SS than SR TALs (Δ SS: 1.9±0.7 *vs* SR: 0.4±0.2 fmoles/µg, p<0.05). **Conclusions:** We concluded that: a) NO-induced inhibition of transport is blunted in SS TALs due to abnormally enhanced PDE5 expression and cGMP catabolism; and b) the blunted transport response to NO in SS TALs is not caused by decreased cGMP production. PDE5 inhibition in TALs could provide protection against salt-sensitive hypertension.

THICK ASCENDING LIMB TRANSPORT IN ANGIOTENSIN II-INDUCED HYPERTENSION: ROLE OF PHOSPHODIESTERASE 5

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Background: Medullary thick ascending limbs (THALs) absorb 25% of the filtered NaCl load primarily via the Na/K/2Cl cotransporter NKCC2. In Angiotensin (Ang) II-induced hypertension NaCl reabsorption is increased in the THAL. Endothelin-1 (ET-1) inhibits NKCC2 activity via NO. NO increases, whereas cGMP-specific phosphodiesterase 5 (PDE 5) reduces cGMP levels. Ang II elevates PDE5 and reduces cGMP levels in smooth muscle cells; however, whether this occurs in THALs in Ang II-induced hypertension is unknown. **Purpose:** We hypothesized that inhibition of NKCC2 by NO is blunted in Ang II-hypertensive rats in part due to elevated PDE5. **Methods:** Male Sprague-Dawley rats were infused with vehicle or Ang II 200 ng/kg/min. At day 5, mean arterial blood pressure (MAP) was measured in anesthetized rats. NKCC2 activity was recorded in isolated THALs by fluorescence microscopy. **Results:** Chronic Ang II infusion increased MAP from 96 ± 4 to 120 ± 8 mmHg (p<0.02). In vehicle-treated rats 1 nM ET-1 reduced NKCC2 activity from 1.58 ± 0.14 to 0.96 ± 0.16 AU/s (p<0.04) whereas in Ang II-hypertensive rats ET-1 did not significantly decrease NKCC2 activity (control: 1.46 ± 0.24 vs ET-1: 1.23 ± 0.21 AU/s). Similarly, 100 µM of spermine NONOate, a NO donor, reduced NKCC2 activity from 1.67 ± 0.31 to 1.11 ± 0.21 AU/s (p<0.01) in vehicle-treated rats but not in THALs from Ang II-hypertensive rats (control: 1.13 ± 0.17 vs NO: 1.32 ± 0.23 AU/s). Addition of 100 µM dibutyryl cGMP also reduced NKCC2 activity in control rats from 0.97 ± 0.19 to 0.73 ± 0.15 AU/s (p<0.01) but not in THALs from Ang II-hypertensive rats (1.00 ± 0.09 to 1.02 ± 0.30). However, 500 µM dibutyryl cGMP reduced NKCC2 activity in THALs from both, controls (1.20 ± 0.32 to 0.76 ± 0.27 AU/s; p<0.03) and Ang II-hypertensive rats (1.15 ± 0.07 to 0.68 ± 0.10 AU/s; p<0.03). Inhibiting PDE5 with 25 nM vardenafil restored the ability of NO to reduce NKCC2 activity in THALs from Ang II-hypertensive rats (vardenafil: 1.17 ± 0.23; vardenafil plus NO: 0.44 ± 0.08 AU/s, p<0.02). **Conclusions:** Inhibition of NKCC2 activity by NO is blunted in Ang II-induced hypertension due to elevated PDE5. Increased PDE5 activity in the THAL could explain in part the impaired ET-1-induced inhibition of NKCC2 and could contribute to the enhanced THAL NaCl reabsorption seen in this model of hypertension.

THE IMPACT OF AROUSAL STATE, GENDER, AND SLEEP APNEA ON THE MAGNITUDE OF PROGRESSIVE AUGMENTATION AND VENTILATORY LONG-TERM FACILITATION IN HUMANS

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Purpose: Exposure to acute intermittent hypoxia (AIH) elicits two forms of respiratory plasticity known as progressive augmentation of the hypoxic ventilatory response and ventilatory long-term facilitation (vLTF). The present study examined whether arousal state, gender, and sleep disordered breathing have an impact on the manifestation and/or magnitude of progressive augmentation and vLTF. The study also examined whether exposure to AIH during sleep impacts the apnea/hypopnea index (AHI) in individuals with obstructive sleep apnea (OSA). **Methods:** Seventeen participants with OSA (10 males and 7 females) and 20 control participants (10 males and 10 females) were exposed to AIH on two occasions; once during wakefulness and the other during sleep. The OSA participants completed an additional sham study during sleep. Baseline measures of minute ventilation, tidal volume, breathing frequency, and the partial pressure of oxygen ($P_{ET}O_2$) and carbon dioxide ($P_{ET}CO_2$) were obtained. The AIH protocol consisted of 12-2 min. episodes of hypoxia ($P_{ET}O_2$ – 50 mmHg) in the presence of $P_{ET}CO_2$ levels sustained 3 mmHg above baseline. Each episode was followed by a 2 min. normoxic recovery period with the exception of the last recovery period which was 30 min in duration. For the OSA participants, the AHI during the first hour of sleep following exposure to AIH was compared to measures following sham exposure. **Results:** Progressive augmentation was only evident during wakefulness and was enhanced in the OSA participants. vLTF was evident during wakefulness and sleep. When standardized to baseline, the response was greater during wakefulness and was enhanced in the OSA group (Males: wakefulness 1.39 ± 0.08 vs. sleep 1.14 ± 0.03; Females: wakefulness 1.35 ± 0.03 vs. sleep 1.16 ± 0.05; $p \leq 0.001$) compared to control (Males: wakefulness 1.19 ± 0.03 vs. sleep 1.09 ± 0.03; Females: wakefulness 1.26 ± 0.05 vs. sleep 1.08 ± 0.04; $p \leq 0.001$). The AHI following exposure to AIH was increased in males (AIH 75.7 ± 8.0 vs. sham 60.4 ± 8.2; $p \leq 0.02$) as well as females (AIH 69.5 ± 13.2 vs. sham 51.2 ± 12.7; $p \leq 0.02$). Gender related differences were not observed for any of the primary measures. **Conclusion:** We conclude that progressive augmentation is not evident and the magnitude of vLTF is diminished during sleep compared to wakefulness in males and females. However, when present, the phenomena are enhanced in individuals with OSA. The AHI data indicates that even though vLTF may be beneficial in mitigating apnea, its effectiveness is sensitive to prevailing conditions.

AUTOMATED ASSESSMENT OF MICROSCOPIC ORGANISMS IN ENVIRONMENTAL SAMPLES USING FLUORESCEIN DIACETATE

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Background: The experiments tested whether fluorescein diacetate (FDA) (see Reed et al.) can be used in an automated fluorescence detection system to assay live organisms in environmental or ballast water samples. Hydrolytic cleavage of FDA (colorless) produces fluorescein (fluorescent). **Methods:** The prototype automated system pumped water samples through a filter and subsequently backwashed with buffer and FDA into a 3 mL cuvette. Fluorescein emission (515-530 nm) was measured by a fiber optic spectrometer for 10-30 minutes. The reuse of filters and efficiency of cleaning by alternately backwashing with deionized (DI) water was tested manually and assayed on a fluorescent plate reader. Experiments tested an anti-biofilm reagent (ABR) used by Balagadde et al., 2005. Biofilm was created in cuvettes through overnight incubation of phytoplankton or water (control). A computer-controlled relay board operated valves and pumps and fluorescence data were recorded with real-time remote control and monitoring of results using TeamViewer software. **Results:** FDA-hydrolysis in environmental water samples increased linearly over time. Fluorescence in DI water assays was 7.0±8.3% of the average intensity of environmental water samples (n=7). Cellulose acetate 0.2 µm filters could be reused 20 times; nylon filters failed after 6 cycles. Backwashing with >60 mL of DI water cleaned the filter efficiently for reuse. Filters reused with DI, 60%, and 90% strength Detroit River water samples yielded relative fluorescence of 64±5, 694±45, and 1161±21, respectively over many repeats in a typical experiment. The ABR (10 min 2%; 10 min 10 %; 20 min 10%) decreased biofilm residual FDA hydrolysis activity by 25% (n=1), 75% (n=2), and >90% (n=1), respectively. Environmental samples measured after ABR treatment (20 min 10%) followed by DI rinse was 107.2% and 98.4% of corresponding pre-ABR measurements (n=2). **Conclusions:** These studies demonstrated that automated measurements of FDA hydrolysis with a reusable filter backwash system under computer control should be applicable to remote-controlled monitoring of live organisms in ballast water (provisional patent pending).

FIRST OBSERVATION OF *ALISHEWANELLA AESTUARII* IN BALLAST WATER OF A GREAT LAKES FREIGHTER

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Background: Ballast water of ships in the Great Lakes has mediated the transport of numerous non-native organisms into the Great Lakes and facilitated their distribution once introduced. To assess the efficacy of ballast water treatment (BWT) methods designed to reduce this threat to the Great Lakes, we developed DNA –based methods for differentiating live from dead microorganisms in ballast water. Metagenomic methods and quantitative species-specific PCR were used to identify *Alishewanella aestuarii*, an organism previously reported only from South Korea, in ballast water of a Great Lakes ship. **Methods and Results:** Ballast water was obtained from the Great Lakes freighter *Indiana Harbor*, prior to and after control (untreated, 2 ballast tanks) or NaOH (pH 12, 2 tanks) treatment during a 3-day voyage. 16S DNA was amplified with universal bacterial primers. Sequencing and BLAST analysis of DNA isolated from the NaOH-treated ballast water identified *A. aestuarii* as being present. 16S species-specific primers were designed to detect the presence of the *A. aestuarii* via qPCR. The proportion of putative *A. aestuarii* in the discharge waters that was alive (i.e., propridium monoazide resistant) was 78% in the untreated tank and 19% in the treated tank. The increase of total *A. aestuarii* after treatment was 400-fold, while the increase of live (propridium monoazide resis-tant) *A. aestuarii* after treatment was 100-fold. Pyrosequencing comparison of the intake bacterial populations to the NaOH-treated ballast water populations revealed no *Alishewanella* in 10,431 intake sequences; 6% of the 22,498 sequences in the discharge were identified as *Alishewanella* by the RDP classifier. **Conclusions:** *A. aestuarii* was not detected in intake water, indicating that the organism was either not present, or present in significantly lower amounts compared to the discharge. While it is unknown where *A. aestuarii* originated from, it is clear that the organism can be transported through ballast water and can survive highly alkaline treatments better than other bacteria. Further study of the organism and where it originates is needed to determine whether this organism is a ubiquitous marine organism, or a potentially harmful invasive species. Supported by EPA grant GL00E00444-0.

DIFFERENTIATING LIVE FROM DEAD: VERIFICATION OF BALLAST WATER TREATMENT TECHNOLOGIES USING FLUORESCEIN DIACETATE

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Background and Objectives: To reduce the number of non-native species trans-ported into the Great Lakes incipient international and U.S regulations will require ship ballast water treatment (BWT) to decrease viable organisms before its discharge. BWT is predicted to eliminate the source of at least half of the non-native species that might enter the Great Lakes. This project tested fluorescein diacetate (FDA), a membrane permeable fluorogen substrate of hydrolytic enzymes of bacteria and plankton, as a way to differentiate live from dead organisms in ballast water. **Methods:** Plankton cultures and other samples (river water, ballast water) were filtered using a syringe filtration apparatus, backwashed off the filter into a small volume of Jaworski's medium, pH 7, and assayed in a fluorescence plate reader. Assays were performed on cultures with and without chlorine (3 mg/L, 3 hours) or heat (30 minutes, 95°C) treatment. In addition, whole samples of river and ballast water were separated by size, and assayed. **Results:** Experiments revealed linearity between relative amount of fluore-scence and time over 60 minutes, as well as relative amount of fluorescence and the amount of culture within a sample (r^2=0.92, n=2). After plankton and ballast water had been treated with heat or chlorine, respectively, the fluorescence production was low in comparison to untreated samples (1.8% heat treated, 5.9% chlorine treated). For ballast water samples accompanying microbiological tests showed that chlorine effectively reduced the number of live coliform & *E. coli* to <1 per 100 mL. Samples separated by size yielded less fluorescent product (about 20% of the total) for organisms greater than 10 µm, in comparison to smaller organisms (n=3). **Conclusions:** The FDA method is useful in distinguishing live versus dead organisms, and can be used to assess organisms in ballast water, and the effectiveness of ballast water treatment. This method is capable of detecting organisms over a broad size range. The future plan is to compare two methods of verifying ballast water treatment, FDA and PMA-PCR (a DNA-based live/dead method), when organisms are separated by size, and to apply the FDA technology in an automated device (see Akram et al.)