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Mitochondrial Physiology and Medicine

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SHEY-SHING SHEU

*Mitochondrial Calcium Entry Pathways. **DAVID CLAPHAM**, *HHMI, Children's Hospital Boston and Harvard Medical School*

During intracellular Ca^{2+} signaling, mitochondria accumulate significant amounts of Ca^{2+} from the cytosol. Mitochondrial Ca^{2+} uptake controls the rate of energy production, shapes the amplitude and spatiotemporal patterns of intracellular Ca^{2+} signals, and is instrumental to cell death. This Ca^{2+} uptake is primarily via the mitochondrial Ca^{2+} uniporter (MCU) located in the organelle's inner membrane. By patch-clamping the inner mitochondrial membrane, we identified the MCU as a highly Ca^{2+}-selective ion channel (MiCa; Mitochondria Ca channel). Single MiCa channels had multiple subconductance states between 3 and 5 pS at −160 mV. The open probability of the channel was ~99% at −200 mV and declined to ~11% at −80 mV. This unique channel binds Ca^{2+} with extremely high affinity, enabling high Ca^{2+} selectivity despite relatively low cytoplasmic Ca^{2+} concentrations, and is especially effective for Ca^{2+} uptake into energized mitochondria. Currently we are attempting to identify the gene encoding this ion channel.

In parallel with the search for MiCa, we searched for genes that regulate mitochondrial Ca^{2+} and H^+ levels using a genome-wide *Drosophila* RNAi screen. The mammalian homolog of one *Drosophila* gene identified in the screen, called Letm1, was found to specifically mediate coupled Ca^{2+}/H^+ exchange. RNAi knockdown, overexpression, and liposome reconstitution of the purified protein demonstrate that it is a mitochondrial Ca^{2+}/H^+ antiporter. I will present new data on Letm1 and its function.

1. Mitochondrial Fusion Dynamics in Skeletal Muscle. VERONICA EISNER and **GYORGY HAJNÓCZKY**, *Thomas Jefferson University*

Skeletal muscle (SM) physiology depends on mitochondrial function. Mitochondrial dysfunction has been linked to myopathies including alcoholic myopathy. Mitochondrial fusion–fission dynamics has been recently recognized as a determinant of mitochondrial function but was difficult to evaluate in adult skeletal muscle. Here, we applied mitochondria targeted DsRed (mtDsRed) and photoactivable GFP (mtPAGFP) to study mitochondrial fusion in rat FDB SM fibers and satellite cell–derived myotubes. In vivo electroporated fibers were enzymatically isolated and imaged by confocal microscopy. When we tagged the mitochondria in ~5% of total cellular area with two photon photoactivated-GFP, rapid spreading of GFP fluorescence showed subsets of interconnected mitochondria, mostly in longitudinal direction. Matrix fusion occurred with a rate of 0.5 and 6.4 events/min in adult fibers and skeletal myotubes, respectively. Interestingly, when mitochondrial fusion proteins (Opa-1, Mfn1 or Mfn2) or their mutants were co-expressed with the fluorescent proteins, the mitochondrial fusion activity decreased. Fusion activity in fibers isolated from ethanol-fed (6 months) rats decreased to 33% of the control. Furthermore, in vitro incubation with ethanol (80 mM, 48 h) induced 97% decrease in the fusion rate. Thus, our work revealed that SM mitochondria undergo fusion to support complementation of the matrix components. Fusion activity is dependent on the differentiation stage and is affected by fusion protein expression or alcohol exposure. Suppression of fusion might contribute to the pathogenesis of myopathy evoked by genetic diseases or alcoholism.

2. Mitochondrial Dynamics and Diabetes: The Fat Cell and the Beta Cell. GUY LAS, LINSEY STILES, KIANA MAHDAVIANI, SAM SEREDA, ANTHONY MOLINA, GILAD TWIG, JAKOB WIKSTROM, MARC LIESA, GYORGY HAJNOCZKY, DAVID CHAN, and **ORIAN SHIRIHAI**, *Boston University, Boston, MA; Thomas Jefferson University, Philadelphia, PA; CalTech, Pasadena, CA*

Beta cell mitochondria undergo continuous cycles of fusion and fission. We have previously shown that exposure of beta cells to high levels of glucose and free fatty acids, an in vitro model of diabetes (glucolipotoxicity), leads to a decrease in mitochondrial fusion capacity. To determine the effects of nutrition on mitochondrial dynamics in vivo, we studied the levels of a mitochondrial fusion protein in mice fed a high fat diet. We find that diet induced obesity was accompanied by decreased levels of mitofusin 2 (Mfn2), an outer mitochondrial membrane fusion protein, in islets of C57BL/6 mice. To assess the functional significance of this finding in vivo, we generated a beta cell specific knockout of Mfn2 (βMfn2KO) by utilizing the Cre/LoxP system, with Cre expression driven by the rat insulin promoter. Examination of mitochondrial structures in βMfn2KO beta cells reveals that Mfn2 deficiency by itself is sufficient to cause fragmentation of the mitochondrial network and an increase in heterogeneity of mitochondrial membrane potential. βMfn2KO mice display hyperglycemia and striking obesity compared to littermate controls. βMfn2KO mice display early glucose intolerance with subsequent insulin resistance emerging with age and weight gain. Islets isolated from βMfn2KO mice display a 2.5-fold increase in basal insulin secretion with blunted 1st phase glucose stimulated insulin secretion. Lack of beta cell Mfn2 represents a scenario where initial beta cell

mitochondrial dysfunction leads to whole body metabolic dysfunction, identifying beta cells as having an essential role in the development of obesity and type 2 diabetes.

Long term exposure of beta cells to excessive concentrations of nutrients results in arrest of mitochondrial fusion and in increased subcellular heterogeneity. Is glucolipotoxocity inducing compensatory autophagy? Studies in our lab demonstrate that glucotoxicity and lipotoxicity inhibit the turnover of autophagosomes by interfering with lysosomal function. This represent a situation where both fates of the mitochondrion, fusion and autophagy are inhibited, leading to the expansion of the pre-autophagic pool, consisting of non-fusing, depolarized mitochondria. In this presentation I will also discuss the methodologies available for the study of autophagic flux and their employment in the study of beta cell dysfunction.

3. Physiological Significance of Mitochondrial Dynamics. **YISANG YOON**, *University of Rochester School of Medicine and Dentistry*

Mitochondria are dynamic organelles, changing their shapes through fission and fusion. Cells utilize energy for these morphological changes, suggesting that mitochondrial fission and fusion are important cellular processes. However, the mechanistic correlation between mitochondrial functionality and morphology is poorly understood. We found that the production of reactive oxygen species (ROS) from mitochondria in hyperglycemic conditions is associated with morphological change of mitochondria. Our studies demonstrated that inhibition of mitochondrial fission decreases the level of ROS produced from mitochondria. Our current studies include investigating the mechanisms of how mitochondrial fission plays a role in regulating insulin secretion and mitochondrial ROS generation. The current development regarding the potential therapeutic value of mitochondrial fission in oxidative stress–associated pathologies will be further discussed.

4. Mitochondrial Physiology and Dynamics During P19 Embryonal Carcinoma Cell Differentiation. JENNA R. ERICKSON,[1] IGNACIO VEGA-NAREDO,[2] LUDGERO C. TAVARES,[2] ANA C. BURGEIRO,[2] ANA F. BRANCO,[2] JON HOLY,[1] EDWARD L. PERKINS,[3] and PAULO J. OLIVEIRA,[2] *[1]University of Minnesota, Medical School, Duluth, MN, USA; [2]Center for Neuroscience and Cell Biology, University of Coimbra, Portugal; [3]School of Medicine, Mercer University, Savannah, GA*

A subpopulation of stem cells has been identified in many types of cancer, and the ability of these cells to evade treatment may provide a source for tumor re-growth. Our working hypothesis is that the differential physiology of mitochondria in tumor stem cells versus differentiated tumor cells results in altered susceptibility to chemotherapeutics. The present work was conducted in a model cell line to compare mitochondrial morphology, function, and dynamics between these two distinct cell populations searching for differences that could provide novel drug targets unique to cancer stem cells.

Undifferentiated P19 cells obtained were used as the model for cancer stem cells (StemTC). P19 cells differentiated with retinoic acid during 4 d were investigated as a model for differentiated tumor cells (DiffTC).

StemTCs showed the highest protein levels in the pluripotency marker Oct 3/4, while DiffTCs had an increase in neuron-specific betaIII-tubulin when differentiated with RA. Mitochondrial remodeling during StemTC to DiffTC transition was characterized by the conversion from small-round bodies to long filaments, although no alterations were observed in mtDNA copy number. StemTCs were found to have decreased ATP levels, oxidative stress and respiration. The functional results suggest lower mitochondrial activity in StemTCs, which may contribute to higher resistance to apoptosis. The increased quantity of fusion proteins in DiffTCs supports the observed elongation of mitochondria that may account for heightened mitochondrial activity in this specific population. Together, the present work provides evidence that tumor stem cells may survive chemotherapeutic treatments due to lower mitochondrial activity which can result in decreased susceptibility to apoptosis.

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5. Alignment of SR-Mitochondrial Junctions with Mitochondrial Contact Points. CECÍLIA GARCÍA-PÉREZ, TIMOTHY SCHNEIDER, and GYÖRGY CSORDÁS, *Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107*

Propagation of ryanodine receptor-derived Ca^{2+} signals to the mitochondrial matrix supports oxidative ATP production or facilitates mitochondrial apoptosis in cardiac muscle. Ca^{2+} transfer likely occurs locally at focal associations of the SR and mitochondria, which are secured by tethers. The outer and inner mitochondrial membrane (OMM and IMM) also form tight focal contacts (contact points) that are enriched in VDAC, the OMM's gate for Ca^{2+}. Contact points could offer the shortest Ca^{2+} transfer route to the matrix; however their alignment with the SR-OMM associations remains unclear.

Here, in rat heart we have studied the distribution of mitochondria-associated SR in submitochondrial membrane fractions and evaluated the colocalization of SR-OMM associations with contact points using

transmission electron microscopy (TEM). In a sucrose gradient designed for OMM purification, biochemical assays revealed lighter fractions enriched in OMM only and heavier fractions containing OMM, IMM and SR markers . Pure OMM fractions were enriched in MFN2, an ~80 kD mitochondrial fusion protein and SR-mitochondrial tether candidate, whereas fractions of OMM+IMM+SR contained only a lighter (~50 kD) band detected by antibodies raised against the N-terminus of MFN2. TEM revealed mandatory presence of contact points at the junctional SR-mitochondrial interface versus a random presence along SR-free OMM segments of matching size. For each SR-mitochondrial junction at least one tether was attached to contact points. MFN2, an ~80 kD OMM-resident protein and SR-mitochondrial tether candidate was enriched in the fractions containing IMM-free OMM. In contrast, the fractions of OMM+IMM+SR contained only a lighter (~50 kD) band that was detected by two different antibodies raised against the N terminus of MFN2.

These data together point toward a preferential role of the OMM-IMM contact points as anchorage sites for the jSR-mitochondrial physical coupling. Close coupling of the jSR, OMM and IMM is likely to provide a favorable spatial arrangement for microdomain Ca^{2+} signaling.

6. Expression of a Dominant-Negative Mitochondrial Fission GTPase, DLP1-K38A, Suppresses ROS Production and Oxidative Damage in a Diabetic Murine Model. CHAD A. GALLOWAY,[1,3] SOUAD NEJJAR,[1] TIANZHENG YU,[5] WEI HSU,[4] and YISANG YOON,[1,2,3] *[1]Department of Anesthesiology, [2]Department of Pharmacology and Physiology, [3]Mitochondrial Research and Innovation Group, and [4]Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, NY*; *[5]Alfaisal University College of Medicine, Riyadh, Kingdom of Saudi Arabia*

Morphological and functional abnormalities of mitochondria have long been associated with diabetic complications but their underlying mechanisms remain to be elucidated. Mammalian mitochondria are observed in various forms ranging from small spheres to reticular networks composed of filamentous tubules undergoing continuous change in a process termed "mitochondrial dynamics". Mitochondrial fission requires the dynamin-like protein DLP1. Our previous studies correlated enhanced mitochondrial fission with increases in ROS abundance in hyperglycemic incubation of both hepatic and cardiac cells (Yu et al. 2006. *Proc. Natl. Acad. Sci. USA*. 103:2653–2658). Enhanced production of ROS was associated with an increased incidence of mitochondrial permeability transition (MPT) and apoptotic cell death both alleviated when expression of a dominant negative form of DLP1, DLP1 K38A, suppressed mitochondrial fission (Yu et al. 2008. *Cardiovasc. Res.* 79:341–345). Pathologically, enhanced mitochondrial ROS production is linked to hyperglycemic complications arising from diabetes in multiple target tissues. The total ablation of DLP1 expression is embryonic lethal (Wakabayashi, J., et al. 2009. *J. Cell. Biol.* 186:805–816.), therefore we have created inducible DLP1-K38A transgenic mice to evaluate the role of mitochondrial fission in diabetic tissue damage. Transgene expression was found in multiple tissues with the most pronounced expression in renal and hepatic tissue. Diabetes was induced by streptozotocin i.p. injection of these mice. In renal proximal tubular cells, DLP1-K38A expression suppressed an observed rounded and "swollen" mitochondrial appearance in diabetic mice. We found that DLP1-K38A expression in diabetic mice significantly decreased cellular ROS levels in both renal and hepatic tissues. Importantly, transgene expression suppressed enhanced oxidative damage in diabetic mice indicating that mitochondrial morphology can be a novel target of intervention in combating diabetic tissue damage.

7. Mitochondrial Fission is an Essential Cellular Process for Glucose-Stimulated Insulin Secretion. BONG SOOK JHUN,[1,3] HAKJOO LEE,[1,3] and YISANG YOON,[1,2,3] *[1]Department of Anesthesiology, [2]Department of Pharmacology and Physiology, and [3]Mitochondrial Research and Innovation Group, University of Rochester School of Medicine and Dentistry, Rochester, NY*

Fission and fusion of mitochondrial tubules are the major processes regulating mitochondrial morphology. However, physiological significance of mitochondrial shape change is still poorly understood. Glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells requires mitochondrial ATP-production, which evokes Ca^{2+} influx through the plasma membrane, triggering insulin vesicle exocytosis. Therefore, we used the GSIS as functional readout to test the physiological role of mitochondrial fission. Using rat pancreatic β-cell line INS-1E, we found that sustained inhibition of mitochondrial fission by the dominant-negative fission mutant DLP1-K38A abolished the glucose-stimulated insulin secretion. However, Ca^{2+}-induced insulin secretion was unaffected by the fission inhibition. These results indicate that the fission inhibition alters an upstream event of the GSIS involving mitochondrial function. We found that cells expressing DLP1- K38A were not able to increase the ATP level upon glucose stimulation. Measuring cellular respiration revealed that inhibiting mitochondrial fission causes an increase of the mitochondrial inner membrane proton leak. In conclusion, our data demonstrate that maintaining normal fission of mitochondria is essential for insulin secretion in pancreatic β-cells. Mechanistically, our findings indicate that sustained inhibition of mitochondrial fission causes an increased mitochondrial uncoupling that limits the mitochondrial capacity to increase ATP production in glucose stimulation.

8. Perturbation of Mitochondrial Fission Induces Mitochondrial Uncoupling in Primary Mouse Hepatocytes. HAKJOO LEE and YISANG YOON, *Department of Anesthesiology, University of Rochester School of Medicine and Dentistry, Rochester, NY*

Fission and fusion of mitochondrial membranes mediate mitochondrial shape change. Despite circumstantial evidence for a close relationship between mitochondrial morphology and bioenergetic activity, its mechanistic link is not fully understood. Previous studies showed that inhibition of mitochondrial fission by a dominant-negative fission mutant, DLP1-K38A, decreased hyperglycemic cell injury by normalizing ROS levels. To gain mechanistic insight for bioenergetic role of mitochondrial fission, we expressed DLP1-K38A in primary mouse hepatocytes and assessed mitochondrial bioenergetic parameters. We found that cells expressing DLP1-K38A showed higher rate of the steady-state respiration than control cells. This difference in oxygen consumption rate became even larger upon the addition of the ATP synthase inhibitor oligomycin, indicating an increased proton leak in DLP1-K38A cells. The maximum respiration rate induced by FCCP in DLP1-K38A–expressing cells was indistinguishable to that of control cells. The inner membrane potential measured by the potentiometric dye TMRE appeared normal in DLP1-K38A cells. Remarkably, however, time-lapse imaging of TMRE fluorescence in DLP1-K38A–expressing cells revealed a large-scale oscillation of TMRE in the subpopulation of mitochondria. The abrupt depolarization was accompanied by the hyperpolarization in the remaining mitochondria in the cell. It is assumed that these flickering mitochondria in DLP1-K38A cells are connected together due to the inhibited mitochondrial fission. The TMRE flickering continued in the presence of cyclosporin A, indicating that the conventional cyclosporin A-sensitive PT pore was not involved in this process. Additional inhibitors DIDS, 4-chlorodiazepam, and MnTMPyP that have been shown to inhibit the similar TMRE oscillation in cardiomyocytes had no effect on the TMRE flickering in DLP1-K38A cells. In summary, our data demonstrate that the inhibition of mitochondrial fission by DLP1-K38A induces flickering of the mitochondrial inner membrane potential, which is functionally reflected in mitochondrial uncoupling, providing the underlying mechanism that decreases ROS levels in hyperglycemic conditions.

9. Phosphoproteome Analysis Reveals Regulatory Sites in Major Pathways of Cardiac Mitochondria. **PEIPEI PING**, *UCLA School of Medicine*

Mitochondrial functions are dynamically regulated in the heart. In particular, protein phosphorylation has been shown as a key mechanism modulating mitochondrial function in diverse cardiovascular phenotypes. However, phosphorylation information about amino acid residues remains scarce for this organ. Accordingly, we performed a comprehensive characterization of murine cardiac mitochondrial phosphoproteome in the context of mitochondrial functional pathways. A platform using the complementary fragmentation technologies of collision-induced dissociation (CID) and electron transfer dissociation (ETD) demonstrated successful identification of a total 236 phosphorylation sites in the murine heart; 210 of these sites were novel. These 236 phosphorylation sites were mapped to 181 phosphoproteins and 203 phosphopeptides. Among those identified, 45 phosphorylation sites were captured only by CID, whereas 185 phosphorylation sites, including a novel modification on ubiquinol-cytochrome c reductase protein 1 (Ser-212), were identified only by ETD, underscoring the advantage of a combined CID and ETD approach. The biological significance of the cardiac mitochondrial phosphoproteome was evaluated. Our investigations illustrate key regulatory sites in murine cardiac mitochondrial pathways as targets of phosphorylation regulation, including components of the electron transport chain complexes (ETC) and enzymes involved in metabolic pathways (e.g., tricarboxylic acid cycle). Furthermore, calcium overload injured cardiac mitochondrial ETC function; whereas enhanced phosphorylation of ETC via phosphatase inhibitors restored calcium attenuated ETC complex I and complex III activities, demonstrating positive regulation of mitochondrial ETC function by phosphorylation. Moreover, in silico analyses of the identified phosphopeptide motifs illuminated molecular nature of participating kinases, which include several known mitochondrial kinases (e.g., pyruvate dehydrogenase kinase) as well as kinases whose mitochondrial location were not previously appreciated (e.g., Src). In conclusion, the phosphorylation events defined herein contribute to advance our understanding of cardiac mitochondrial biology, facilitating the integration of still fragmentary knowledge about mitochondrial signaling networks, metabolic pathways, and intrinsic mechanisms of functional regulation in the heart.

10. Mechanisms of Cytoplasmic-Mitochondrial Redox Balance and Interaction with Mitochondrial Energetics: A Computational Study. MIGUEL A. AON, JACKELYN KEMBRO, BRIAN O'ROURKE, NAZARENO PAOLOCCI, and SONIA CORTASSA, *Johns Hopkins University, School of Medicine, Division of Cardiology, Baltimore, MD*

The Redox-Optimized ROS Balance (R-ORB) hypothesis postulates that the redox environment determines reactive oxygen species (ROS) levels in mitochondria and cells. Maximal rates of respiration and ATP synthesis and minimal ROS levels occur at intermediate redox environments. Overflow of ROS

happens: *(i)* at more reduced redox environments when mitochondrial ROS production exceeds scavenging, and *(ii)* under more oxidizing conditions when antioxidant defenses are compromised.

Herein we study the role of the antioxidant defenses in determining mitochondrial redox balance dynamics under different energetic conditions. The computational model utilized accounts for the production of ROS in the respiratory chain and ROS scavenging, in the mitochondrial matrix and extramitochondrial compartments. This version of the mitochondrial model includes glutathione- and thioredoxin-scavenging systems, and was built upon the mitochondrial energetics one that comprises energy metabolism and protons, Ca^{2+}, Na^{+} and Pi dynamics (Wei et al. 2011. *Biophys. J.*).

The stability analysis of the model as a function of cytoplasmic ADP (ADPi) exhibits the typical transition between respiratory states 4 to 3. With the exception of NADH, ROS species and other redox variables did not change significantly with ADPi. The model displays oscillations in membrane potential ($\Delta\Psi_m$) and redox intermediates under state 4 but not state 3 respiration. The dynamics in the oscillatory regime, driven by excess superoxide ($O_2^{\cdot -}$) production or insufficient scavenging, involves large amplitude cycles of $\Delta\Psi_m$, NADH, cytoplasmic $O_2^{\cdot -}$, and H_2O_2 in both compartments. Oscillatory periods ranging from 14 s to 5 min, and an inverse correlation between the amplitude and the period of the oscillations were found.

Model simulation results obtained are in agreement with the prediction by the R-ORB hypothesis under oxidizing redox environments. In addition, it shows that mitochondrial oscillatory dynamics tend to happen under more energized and reduced conditions, when respiratory rates are lower.

11. Computational Model for ROS Regulation in Cardiac Mitochondria. RASHMI KUMAR[1] and M. SALEET JAFRI,[1,2] *[1]School of Systems Biology, George Mason University, Manassas, VA; [2]Biomedical Engineering and Technology, University of Maryland, Baltimore, MD*

Mitochondria play an important role in the control of ATP production, cellular redox potential and ROS (reactive oxygen species such as superoxide and peroxide).generation. ROS generation by the electron transport chain is a natural result of aerobic mitochondrial respiration under normal conditions. Excessive ROS leads to oxidative stress and is linked to various pathological conditions like diabetes, neurodegenerative diseases, and ischemia reperfusion and is related to aging. Experimental evidence indicates that the two important regions donating electron toward ROS production are the Complex I (NADH: ubiquinone oxidoreductase) and Complex III (Ubiquinol: cytochrome C oxidoreductase) of mitochondrial respiratory chain. To better understand the regulation of ROS production, we present a mechanistic model of the electron transport chain based on principles of reaction kinetics that includes the dominant production sites for superoxide and the pathways for the removal of superoxide (through its conversion to hydrogen peroxide). In the model we analyze the mechanism of electron transfer across respiratory complex I, II and III. By means of principles of chemical kinetics, we model the transfer of electrons from NADH to Ubiquinone (Q) across complex I, from succinate to Q across complex II and from QH_2 to cyt C across complex III. Electrons can diverge along the pathway of electron flow across complex I and III resulting in single electron reduction of oxygen atom and forming superoxide radical and thereafter peroxide across matrix and extramitochondrial space. In addition the computational model also presents a very detailed mechanism of various matrix and extramitochondrial antioxidant defenses (described by GSH/GSSG and $NADPH/NADP^{+}$ redox pairs). Our findings offer a mechanistic understanding of the underlying ROS triggered changes in mitochondrial membrane potential and offers explanation for experimentally observed phenomenon of ROS burst in cardiac myocytes.

12. Real-Time and Label-Free Monitoring of Bioenergetics: Mitochondrial, Glycolysis and Cell Impedance/Adhesion for Long- and Short-Term Cell-Based Functional Assays In Vitro. CARSTEN HABER, SABINE DRECHSLER, AXEL KOB, MARCUS WEGO, RALF EHRET, and STEFANIE ORTINAU

For the prediction of compound toxicity in early phases of development and the identification of pharmacological properties of substances advanced in vitro methods are applied. Most relevant for pharmaceutical studies are the investigation of long-term effects mediated by toxins and xenobiotics and the evaluation of short-term effects involving membrane transporter mechanisms or receptor signaling. Therefore the functional analysis of living cells in a physiologically controlled environment which provides information about pharmacological and toxicological as well as metabolic properties may serve as an alternative method for animal experiments.

The Bionas® analyzing systems detect physiological parameters of cell lines and primary cells in label-free and non-invasive assays. The multi-parametric sensor chip generates profiles of bioenergetics (glycolysis and mitochondrial respiration) and cellular impedance/adhesion and monitors the action of substances and their cytotoxic effects including potential regeneration of cells in the area of toxicology, oncology and drug discovery, respectively. Therefore specific data from the drug/cell interaction are determined. These data are either generated by studies over hours to days or via

short-term detection of cellular responses. In the field of drug development, membrane transporter-mediated mechanism and receptor interaction studies are the major focus.

In summary, the Bionas® analyzing systems are the only available devices capable of analyzing extracellular acidification, respiration and cell impedance/adhesion in short and long term studies. The combination of the cellular parameters with the functionality of the temporal resolution of compound effects provides a valuable tool for a variety of cell-based functional studies.

13. A Mitochondrial ATP-Sensitive Potassium Channel from the ROMK Family. **D. BRIAN FOSTER,**[1] ALICE S. HO,[2] ANDERS GARLID,[4] JASMA RUCKER,[1] MARJAN GUCEK,[3] ROBERT N. COLE,[3] KEITH D. GARLID,[4] and BRIAN O'ROURKE,[1] [1]*The Institute of Molecular Cardiobiology,* [2]*Department of Biomedical Engineering, and* [3]*Johns Hopkins Proteomics Core Facility, The Johns Hopkins University School of Medicine, Baltimore, MD;* [4]*Department of Biology, Portland State University, Portland, OR*

D.B. Foster and A.S. Ho contributed equally.

Mitochondrial potassium transport plays a role in the modulation of bioenergetics and the existence of resident potassium channels in the mitochondrial inner membrane has been amply validated, yet the pore-forming subunits of these channels remain unidentified. We therefore undertook an in-depth proteomic analysis of the mitochondria using repeated fractionation at the organellar, protein, and peptide levels. In brief, density-purified inner membranes were extracted with 1% lauryl maltoside, and fractionated by sucrose gradient centrifugation. Each fraction was digested with trypsin and subjected to strong-cation exchange HPLC before reversed-phase LC-coupled tandem mass spectrometry. 964 proteins were identified, of which 684 were classified as mitochondrial in UniProtKB and/or MitoCarta databases. From the inner membrane fraction, the ROMK (renal outer-medullary potassium) channel, was identified by 6 spectra matching two overlapping peptides. Matches were statistically validated at >95%. Reverse-Transcription/Polymerase Chain Reaction (RT-PCR) confirmed identification of 3 isoforms (ROMK1, ROMK2 and ROMK6) in the adult rat hearts and NRVMs. Subsequent bioinformatic analysis using TargetP and Mitopred detected a mitochondrial localization sequence near the N-terminus of ROMK. This mitochondrial targeting sequence was confirmed experimentally by 2-photon microscopy, as fusion of N-terminus of either ROMK1 or ROMK2 with GFP confers colocalization with TMRM-stained mitochondria. In neonatal rat ventricular myocytes, full length ROMK 2 fused with a V5 tag showed partial colocalization with to mitochondria in H9C2 cells. To determine whether a ROMK isoform might mediate mitochondrial potassium uptake, we measured K^+-dependent swelling in rat heart mitochondria. In preliminary studies, cromakalim-induced mitochondrial swelling of liver mitochondria was abrogated by Tertiapin-Q, a high-affinity ROMK channel toxin, with half-maximal inhibition in the picomolar range. Finally, in cell culture, knockdown of ROMK confers a differential response to tert-butyl hydroperoxide, an oxidative stress inducer, implying a role for mitoROMK in cytoprotection. Collectively the data support a role for ROMK as a candidate for the pore-forming subunit of mitoKATP.

14. Uncoupling Protein of Brown Fat: Electrophysiologist's Perspective. **YURIY KIRICHOK**, *Department of Physiology, University of California, San Francisco, CA*

Uncoupling proteins (UCP1–UCP5) are integral transport proteins of the inner mitochondrial membrane (IMM). They mediate transmembrane ion leak, thus dissipating the electrochemical proton gradient across the IMM and uncoupling mitochondrial respiration and ATP synthesis. UCPs are involved in thermogenesis, reducing fat deposition, and attenuating reactive oxygen species production by mitochondria to protect the cell against oxidative damage and ageing. The mechanism of ion conductance of UCPs has long remained elusive due to the lack of direct methods for measuring ion currents produced by them. To resolve this problem, we applied the patch-clamp technique to the whole inner membrane of mitochondria from the brown adipose tissue to identify and characterize currents produced by the family's founding member, UCP1. In our experiments, both the cytoplasmic and matrix faces of the IMM were exposed to solutions that did not contain any ions normally permeable through ion channels or transporters, except for H^+ and OH^-. Under these conditions, we identified a current that showed all signature properties of UCP1: it was strongly potentiated by micromolar concentrations of unsaturated fatty acids, inhibited by removing endogenous membrane fatty acids with bovine serum albumin, and blocked by micromolar concentrations of purine nucleotides. The current was absent in the IMM of the kidney COS-7 cells, in agreement with the fact that UCP1 is specifically expressed in the brown adipose tissue. Finally, the current disappeared in UCP1 knockout mice. In this presentation, I will discuss the dependence of UCP1 current on the membrane potential, matrix and cytosolic pH, as well as on the nature of the fatty compound activating the current. The ultimate goal of this work is to elucidate the molecular mechanism used by UCP1 to uncouple the mitochondrial oxidative phosphorylation in brown fat.

15. BCL-Xl Regulates ATP Synthase and Synaptic Efficiency. KAMBIZ N. ALAVIAN, HONGMEI LI, PANAH NABILI, EMMA LAZROVE, J. MARIE HARDWICK, and **ELIZABETH A. JONAS**

Anti-apoptotic BCL-2 family proteins such as BCL-xL play a crucial role in protecting cells from death. High levels of expression of BCL-xL are also key to the maintenance of life of certain cancer cells. Healthy adult neurons also contain high levels of BCL-xL, suggesting that BCL-xL plays a role in daily neuronal function. We have found previously that over-expression of BCL-xL in cultured neurons causes an increase in the number and size of synapses and an increase in synaptic activity, providing evidence that BCL-xL participates in the onset of long term changes in synaptic efficacy and structure. We now describe that in cultured hippocampal neurons, BCL-xL overexpression enhances the availability of total cellular ATP by specifically enhancing mitochondrial ATP production even while producing a marked decrease in cellular oxygen use. Although BCL-xL is usually thought to function in the mitochondrial outer membrane, our findings suggest that it creates an increase in the efficiency of cellular energy metabolism by direct protein–protein interaction with the ATP synthase at the inner membrane. We find that recombinant BCL-xL protein increases native brain ATP synthase enzymatic activity and that pharmacological inhibitors of BCL-xL decrease the enzymatic activity of the synthase complex. In patch clamp recordings of submitochondrial vesicles enriched in ATP synthase, ATP seals a membrane ion leak that could decrease synthase efficiency. In contrast, inhibition of BCL-xL increases the leak. The leak is separate from the oligomycin-sensitive H^+ ion translocation pathway, and is not regulated by the membrane-permeant ANT inhibitor bongkrekic acid, or by inhibitors of MitoKATP. Our findings suggest that BCL-xL improves the efficiency of mitochondrial metabolism by helping to seal a leak conductance within the ATP synthase complex itself. This allows for increased availability of ATP after intense synaptic activity and may be crucial for determining long term changes in synaptic efficiency.

16. Molecular Definition of the Mitochondrial Ca^{2+} Signalling Machinery. DIEGO DE STEFANI,[1] ANNA RAFFAELLO,[1] ENRICO TEARDO,[2] ILDIKO SZABÒ,[2] and **ROSARIO RIZZUTO,**[1] *[1]Department of Biomedical Sciences and [2]Department of Biology, University of Padua, Italy*

In the last two decades, a large body of experimental work has revealed the physiological and pathophysiological significance of mitochondrial Ca^{2+} handling, highlighting its key role in the control of aerobic metabolism and cell survival. The molecular identity of the transporters, and in particular of the uptake route (the mitochondrial calcium uniporter, MCU), has remained elusive, since neither genetic nor biochemical investigations have provided candidates that could be validated in functional analyses. We have performed, and will describe in the presentation, an in silico screening for the MCU, and validated the positive hit with a variety of in vitro and in vivo assays.

17. Comparative Genomics and Physiology of Mitochondria. **VAMSI MOOTHA**, *Harvard Medical School*

In recent years, we have used a mix of tandem mass spectrometry, large-scale GFP tagging, and computational analysis to systematically characterize the mitochondrial proteome. In this talk I will describe our recent efforts to couple this proteomic inventory with comparative physiology to infer the function of poorly characterized proteins, focusing on calcium transport.

18. Unusual Permeation and Blockage Properties of the Light-Sensitive Conductance of Amphioxus Photoreceptors. CAMILA PULIDO,[1] GERARDO MALAGÓN,[1] CAMILO FERRER,[1] JUN KUICHEN,[3] MARÍA DEL PILAR GOMEZ,[1,4] and ENRICO NASI[2,4], *[1]Departamento de Biología and [2]Instituto de Genética, Universidad Nacional de Colombia, Bogotá; [3]Hunter College, NY; [4]Marine Biological Laboratory, Woods Hole*

Two types of microvillar photoreceptors in the neural tube of amphioxus, an early chordate, sense light via melanopsin, the same photopigment as in 'circadian' light detectors of higher vertebrates. In these cells, melanopsin activates a G_q/PLC cascade to open ion channels, similar to phototransduction in arthropods and mollusks. However, unlike all other microvillar photoreceptors, the photocurrent cannot be reverted under physiological conditions. Reversal can be attained (≈ -27 mV) upon replacement of extracellular Na with NMDG (but not Tris); in addition to Na, Ca must also be permeant, because a small inward photocurrent is measured in Na-free solution provided that Ca is present, and $[Ca]_o$ manipulations modestly shift V_{rev}. The odd reversal is accounted for by an uncommonly low permeability of the light-dependent channels to K ions, as $[K]_o$ only marginally affects the photocurrent amplitude and its reversal. Lanthanum and ruthenium red, two well-known antagonists of TRP-class channels, reversibly suppress the response to flashes of moderate intensity; the melanopsin-initiated cascade therefore may recruit ion channels of the same family as those of invertebrate rhabdomeric photoreceptors. Curiously, the effectiveness of La^{3+} and RuR decline with brighter photostimulation, so that both drugs induce a right-shift in the sensitivity curve without a reduction of its asymptote. Nonetheless, the possibility that these antagonists may act on the transduction cascade, rather than directly on the channels, was ruled out on the basis of a distinct voltage dependency of the blockade and the lack of effects of intracellular application of the same substances. The mechanisms of actions of these

antagonists thus entail a state-dependent blockade, in which the apparent affinity is higher when the channel is in the closed conformation. The results help clarify the evolutionary history of microvillar photoreceptors as ancestral to circadian receptors, and highlight their representation amongst chordates.

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19. BAX Insertion, Oligomerization, and Outer Membrane Permeabilization in Brain Mitochondria. TATIANA BRUSTOVETSKY,[1] YOUYUN YANG,[1,3] JIANG-TING ZHANG,[1,3] BRUNO ANTONSSON,[4] and NICKOLAY BRUSTOVETSKY[1,2] *[1]Department of Pharmacology and Toxicology, [2]Stark Neuroscience Research Institute, and [3]Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202; [4]Merck Serono, Geneva Research Center, Geneva, Switzerland*

BAX works in concert with truncated BID and calcium in permeabilizing the mitochondrial outer membrane and leading to the release of mitochondrial apoptogenic proteins located in the intermembrane space. The mechanism of BAX-induced outer membrane permeabilization is still unclear. In isolated brain mitochondria, recombinant BAX readily self-integrated and self-oligomerized in the outer membrane producing only a minute cytochrome *c* release. This suggested that BAX insertion and oligomerization in the outer membrane was not sufficient to produce substantial outer membrane permeabilization. Calcium in a permeability transition pore-dependent and recombinant truncated BID in a permeability transition pore-independent fashion promoted BAX insertion/oligomerization in the outer membrane and increased cytochrome *c* release. Neither truncated BID nor calcium triggered BAX oligomerization in the solution without mitochondria, suggesting that BAX oligomerization required interaction with the membrane and followed rather than preceded BAX insertion in the outer membrane. Recombinant Bcl-xL failed to prevent BAX insertion and oligomerization in the outer membrane but strongly attenuated cytochrome *c* release. Conversely, a reducing agent, dithiothreitol, inhibited BAX insertion and oligomerization augmented by truncated BID or calcium and suppressed the BAX-mediated efflux of cytochrome *c* and Smac/DIABLO. At the same time, dithiothreitol failed to inhibit calcium-induced swelling. Altogether, these data suggest that in brain mitochondria, BAX insertion and oligomerization can be dissociated from outer membrane permeabilization. Calcium and truncated BID stimulate BAX insertion/oligomerization and BAX-mediated outer membrane permeabilization by different mechanisms, in which permeability transition pore induced by calcium and modulation of the SH-redox state play important roles.

20. Pathological and Physiological Properties of Mitochondria BK Channel in Heart. OLHA KOVAL,[1] JUDITH HERLEIN,[2] BRIAN FINK,[2] JINGDONG LI,[1] PAARI DOMINIC SWAMINATHAN,[1] JINYING YANG,[1] CHANTAL ALLAMARGOT,[3] ANDREA MEREDITH,[4] PETER J. MOHLER,[1] WILLIAM I. SIVITZ,[2] MARK E. ANDERSON,[1] and MEI-LING A. JOINER,[1] *[1]Division of Cardiovascular Medicine, [2] Division of Endocrinology, and [3]Central Microscopy Research Facility, Department of Internal Medicine, University of Iowa, Iowa City, IA; [4]Department of Physiology, University of Maryland School of Medicine, Baltimore, MD*

Mouse hearts from the large-conductance K^+ (BK) channel knockout mice show an intensified response to ischemia reperfusion. Mitochondria from hearts lacking the BK channel are more susceptible to swelling from excess Ca^{2+}. Cyclosporin A mostly restores these adverse responses, suggesting a role for the BK channel in regulating stress response through the mitochondria permeability pore and possibly the Bax apoptotic pathway (Cheng, Gulbins, and Siemen. 2011. *Cell Physiol. Biochem.* 27:191–200). Mitochondria contain a subcellular environment marked by Ca^{2+} and ROS oscillations. Excessive mitochondrial Ca^{2+} or ROS elevations lead to apoptosis. Both Ca^{2+} and ROS activate the BK channel, suggesting that this channel may function to restore Ca^{2+} or ROS levels in mitochondria and promote cardiomyocyte survival. While studies to date, using pharmacological intervention, suggest that BK channel activity protects cardiomyocytes from apoptosis (Xu et al. 2002. *Science.* 298:1029–1033), other findings are contradictory, likely because of the imperfect nature of available pharmacological agonists and antagonists.

A physiological role for BK channels in heart is controversial. In wild-type hearts and hearts lacking the BK channel, we use Western analysis, cryo-immuno electron micrography and electrophysiology to establish the presence and to characterize the properties of the mitochondrial BK channel. We find that the K^+ current across the inner mitochondrial membrane is conducted largely by the BK channel. Kinetic analysis of inner membrane potential, simultaneous with respiration under conditions set so that oxygen consumption is proportional to proton pumping in isolated mitochondria, suggests an increase in proton leak in mitochondria lacking the BK channel. How the mitochondrial physiological changes we see in BK knockout mice influence heart function are under study. One possibility is the mitochondrial BK channel governs the mitochondrial membrane potential, a driving force of ATP synthesis.

Overall, our work describes differences between mitochondria from BK-knockout mouse hearts and control hearts that point to a mechanistic role of BK channel in heart disease. The protection from apoptosis afforded by BK channel described earlier likely works

through a mechanism involving mitochondrial BK channel activity.

21. The Pathophysiology of LETM1. KARIN NOWIKOVSKY,[1] PAOLO BERNARDI,[2,3,4] TULLIO POZZAN,[2,3,4] ROSARIO RIZZUTO,[2,4] and LUCA SCORRANO,[3,5,6] *[1]Department of Internal Medicine 1, Medical University Vienna, Austria; [2]Department of Biomedical Sciences, University of Padova, Italy; [3]Venetian Institute of Molecular Medicine, Padova, Italy; [4]Neuroscience Institute of the National Research Council (CNR), Italy; [5]Dulbecco-Telethon Institute, Padova, Italy; [6]Department of Cell Physiology and Medicine, University of Geneva, Switzerland*

Mdm38p/LETM1 is part of a novel well conserved mitochondrial protein family. A crucial function of the LETM1 proteins is the cation/proton exchange across the mitochondrial inner membrane. Compelling evidence indicates that Letm1p is essential for the mitochondrial K^+/H^+ exchange; however the cationic substrate of the exchanger has become a matter of discussion, since new data suggested a role of LETM1 in mitochondrial Ca^{2+}/H^+ exchange. Moreover, it is still not clear whether LETM1, with one single transmembrane domain, can catalyze the exchange per se.

Inactivation of Letm1p abolished essential mitochondrial functions by deregulating ion and volume homeostasis and by triggering mitophagic degradation. Since haploinsufficiency of human LETM1 correlates with seizures in Wolf-Hirschhorn syndrome patients, the unambiguous identification of the Letm1p substrate(s), and the distinction between its role as regulator or exchanger are of prime importance.

In spite of the fact that the K^+/H^+ exchanger nigericin reverted all mitochondrial defects caused by the absence of LETM1, the nature of the substrate has become controversial. However, this debate raised the possibility that mitochondrial K^+ and Ca^{2+} homeostasis could be linked, with K^+ fluxes regulating Ca^{2+} trans-mitochondrial exchange by modulating the membrane potential. Combining various techniques available in our laboratories we present data that analyse the correlation of LETM1-dependent mitochondrial K^+ and Ca^{2+} homeostasis.

22. Searching for the Molecular Correlate of Cardiac mitoBK$_{Ca}$. HARPREET SINGH, RONG LU, PEDRO FELIPE GARDEAZÁBAL RODRÍGUEZ, ENRICO STEFANI, and LIGIA TORO, *Department of Anesthesiology, Division of Molecular Medicine, University of California, Los Angeles, Los Angeles, CA 90095*

Large conductance calcium- and voltage-activated potassium channel (BK$_{Ca}$) is not expressed at the plasma membrane of cardiomyocytes but an agonist promotes cardioprotection from ischemic injury whilst an antagonist has the opposite effect. However, the molecular correlate of cardiac mitoBK$_{Ca}$ is unknown. Since cardiac mitoBK$_{Ca}$ shares conductance, Ca^{2+}-sensitivity, and pharmacological properties with its plasma membrane counterpart, we hypothesized that mitoBK$_{Ca}$ may be an isoform of BK$_{Ca}$. We first investigated its presence in cardiac mitochondria using immunochemistry approaches followed by RT-PCR analysis of cardiomyocyte mRNAs and reconstitution using heterologous expression. Labeling of adult mouse cardiomyocytes with specific anti-BK$_{Ca}$ antibodies directed against its C-terminus, mitotracker and a surface membrane marker (wheat germ agglutinin) yielded remarkable colocalization between BK$_{Ca}$ and mitochondria but not with plasma membrane signals. Nanoscale fluorescence microscopy (Stimulation emission depletion, STED) revealed 7–15 BK$_{Ca}$ clusters of ~22–30 nm per mitochondria in isolated mitochondria (n = 5), as well as in neonatal (n = 3) and adult cardiomyocytes (n = 3). Western blot analysis of purified mitochondria showed the presence of a full length ~125 kD protein (n = 4). Analysis of cardiomyocyte mRNAs by RT-PCR scanning demonstrated the presence of full-length BK$_{Ca}$ alpha subunit sequences with a predicted protein mass of ~125 kD and identified three C-terminus splice inserts STREX, SV27 and DEC. Real-time PCR quantification established that DEC is the most abundant of the three splice inserts in the heart. Insertless-BK$_{Ca}$ when expressed in adult cardiomyocytes robustly localized to the plasma membrane but when a C-terminal splice insert (DEC) was present BK$_{Ca}$ was readily targeted to the mitochondria of CHO cells (n = 3). Hence, cardiac mitoBK$_{Ca}$ is likely composed by full-length BK$_{Ca}$ protein but with splice inserts which facilitate its targeting to mitochondria.

23. Properties of a Selective Ca^{2+} Release Channel in Mitochondria of *Drosophila melanogaster*. SOPHIA VON STOCKUM,[1] EMY BASSO,[1] VALERIA PETRONILLI,[1] MIKE FORTE,[2] and PAOLO BERNARDI,[1] *[1]Department of Biomedical Sciences and CNR Institute of Neurosciences, University of Padova, Italy; [2]Oregon Health and Sciences University, Portland, OR*

The mitochondrial permeability transition (PT) describes a process of Ca^{2+}-dependent, tightly regulated increase in the permeability of the inner mitochondrial membrane due to the opening of a high-conductance channel, the mitochondrial permeability transition pore (PTP). PTP opening causes collapse of the mitochondrial membrane potential ($\Delta\psi_m$) and Ca^{2+} release through the pore itself, an event that for short "open" times may be involved in physiological Ca^{2+} homeostasis and for prolonged opening is known to induce cell death through matrix swelling, outer mitochondrial membrane rupture and release of apoptogenic proteins like cytochrome *c*. In spite of its importance as a model organism, remarkably little is known about the properties of Ca^{2+} transport in mitochondria of *Drosophila melanogaster*, and on whether

these mitochondria can undergo a PT. We have studied the pathways for Ca^{2+} transport in mitochondria of permeabilized *Drosophila* S_2R^+ cells. We demonstrate the presence of ruthenium red (RR)-sensitive Ca^{2+} uptake, of RR-insensitive Ca^{2+} release and of Na^+-stimulated Ca^{2+} release in energized mitochondria, which match well-characterized transport pathways of mammalian mitochondria. Following matrix Ca^{2+} loading *Drosophila* mitochondria underwent RR-insensitive Ca^{2+} release, an event that in mammals is due to opening of the PTP. Ca^{2+} release could also be triggered by uncoupler, diamide and N-ethylmaleimide, indicating the existence of regulatory voltage- and redox-sensitive sites. Unlike PTP-mediated Ca^{2+} release in mammals, however, mitochondrial Ca^{2+} release in *Drosophila* was (i) insensitive to CsA, ubiquinone 0 and ADP; (ii) inhibited by Pi, as is the PTP of yeast mitochondria; and (iii) not accompanied by matrix swelling and cytochrome *c* release even in KCl-based media. We conclude that *Drosophila* mitochondria possess a selective Ca^{2+} release channel with features intermediate between the PTP of yeast and that of mammals.

24. Role of the Permeability Transition Pore in Controlling Ca^{2+} Exchange within Mitochondria. JOHN W. ELROD and **JEFFERY D. MOLKENTIN**, *Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center, Howard Hughes Medical Institute, Cincinnati, OH 45229*

Cyclophilin D (*Ppif* gene) is a mitochondrial matrix peptidyl-prolyl isomerase known to modulate opening of the mitochondrial permeability transition pore (MPTP). Outside of regulating necrotic cell death, the physiologic function of the MPTP is largely unknown. We have more recently shown that $Ppif^{-/-}$ mice exhibit substantially greater cardiac hypertrophy, fibrosis, and reduction in myocardial function in response to pressure overload stimulation compared with control mice. In addition, $Ppif^{-/-}$ mice showed greater hypertrophy, lung edema and death in response to sustained exercise stimulation. Transgene-mediated expression of cyclophilin D within myocytes of the $Ppif^{-/-}$ heart rescued the enhanced hypertrophy, reduction in cardiac function, and rapid onset of heart failure following pressure overload stimulation. Mechanistically, the maladaptive phenotype in the hearts of $Ppif^{-/-}$ mice was associated with a significant alteration in MPTP-mediated Ca^{2+} efflux resulting in elevated levels of mitochondrial matrix Ca^{2+} and enhanced activation of Ca^{2+}-dependent dehydrogenases. Elevated matrix Ca^{2+} led to increased glucose oxidation relative to fatty acids, thereby limiting the metabolic flexibility of the heart that is critically involved in compensation during stress. These findings suggest that the MPTP controls homeostatic mitochondrial Ca^{2+} levels to match metabolism with alterations in myocardial workload, thereby suggesting a physiologic function for the mitochondrial permeability pore. More recently we have manipulated other mitochondrial Ca^{2+} handling transporters or exchangers that have further shown the importance of the MPTP in regulating mitochondrial metabolic activity and cell death propensity.

25. Calcium Signaling in Neuronal Mitochondria. **JORGINA SATRÚSTEGUI**,[1] IRENE LLORENTE-FOLCH,[1] LAURA CONTRERAS,[1] PATRICIA MÁRMOL,[1] SANTIAGO CAVERO,[1] JAVIER TRABA,[1] IGNACIO AMIGO,[1] CARLOS RUEDA,[1] ARACELI DEL ARCO,[2] and BEATRIZ PARDO,[1] *[1]Departmento de Biología Molecular, Centro de Biología Molecular "Severo Ochoa" CSIC-UAM, and CIBER de Enfermedades Raras (CIBERER), Universidad Autónoma de Madrid, Spain; [2]Área de Bioquímica, Centro Regional de Investigaciones Biomédicas (CRIB), Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha, Toledo, Spain*

The calcium-binding mitochondrial carriers of glutamate/aspartate (AGCs) and ATP-Mg/Pi (SCaMCs) provide a mechanism to transmit Ca^{2+} signals to mitochondria independent of the entry of calcium to the organelle. The AGCs Aralar/AGC1 and citrin/AGC2 are components of the malate aspartate NADH shuttle and citrin is also a component of the urea cycle. The presence of calcium binding motifs facing the intermembrane space in both AGCs results in the activation by extramitochondrial calcium of these shuttles at calcium levels below those required by the calcium uniporter. The $S_{0.5}$ for Ca^{2+} activation of the shuttle in tissues where aralar is the predominant isoform (brain, skeletal muscle, insulin-secreting beta cells) is about 300 nM, and Aralar mutants blocking calcium binding fin aralar fully prevent this activation. In neurons utilizing glucose, small calcium signals activating the malate aspartate shuttle appear to "push" pyruvate into mitochondria and thus greatly stimulate mitochondrial NAD(P)H production independently of Ca^{2+} entry in mitochondria. However, this mechanism is blunted with large Ca^{2+} signals. SCaMCs are the only mechanism for calcium signalling in Saccharomyces cerevisiae mitochondria where they play a role in controlling the total content of adenine nucleotides. Mammalian SCaMCs have a lower affinity for Ca than the AGCs but, unlike AGCs, are strictly Ca dependent. The physiological role of these transporters obtained through the use of cells with silenced expression and KO animals will be discussed.

26. Mitochondrial Ion Channels: The Gateways into Cell Death. **K.W. KINNALLY**

Ion channels located in the mitochondrial outer and inner membranes are key regulators of cellular signaling for life and death. Permeabilization of mitochondrial membranes is critical to the progression of some cell

death pathways, e.g., apoptosis. The mitochondrial apoptosis-induced channel (MAC) and the mitochondrial permeability transition pore (mPTP) play major roles in these processes. Formation of MAC, whose pore diameter is >3 nm, can be triggered in the mitochondrial outer membrane by BH3-only proteins. MAC then allows the release of pro-apoptotic factors like cytochrome c from the intermembrane space. In contrast, the matrix space undergoes a rapid and large scale swelling upon opening of mPTP in the inner membrane; this swelling ruptures the outer membrane and causes an indiscriminant spillage of the contents of the intermembrane space into the cytosol. Release of these factors through either mechanism causes cell death. Cyclosporine A has long been recognized as a high affinity inhibitor of mPTP. Recently, several small molecules that inhibit MAC (iMACs) at nanomolar concentrations have been identified and their mechanisms of action are under investigation. Thus, cyclosporine A and iMACs will enable a dissection of the crosstalk between MAC and mPTP mediated by means such as cytosolic Ca^{2+} signaling, Bcl-2 family proteins, or other mitochondrial ion channels. We recently observed that opening of mitochondrial ion channels can lead to a collateral spread of apoptosis to nearby cells. We refer to this phenomenon as a bystander effect—a process integral to tissue homeostasis and a challenge in anti-cancer therapies. The roles of MAC and mPTP in bystander effects in vivo and in vitro are being evaluated by single-cell microinjection of cytochrome c and exogenous expression of pro-apoptotic proteins like GFP-Bax. In some systems, the bystander effects rely on gap junction intercellular communication, as bystander death is abrogated either by pharmacological or molecular inhibition of connexin 43. In contrast, an extracellular pathway seems to underlie bystander effects in other systems. Understanding the interplay between MAC and mPTP in these processes will impact development of novel therapeutic strategies to selectively eliminate tumors or minimize the size of tissue injury in degenerative or traumatic cell death.

27. The Uncoupling Protein 3 Is Not A Mitochondrial Ca^{2+} Uniporter but Alters the Activity of Sarco/Endoplasmic Reticulum Ca^{2+} ATPases by Decreasing Mitochondrial ATP Production. UMBERTO DE MARCHI and NICOLAS DEMAUREX, *Department of Physiology and Cell Metabolism, University of Geneva, Switzerland*

The mitochondrial Ca^{2+} uniporter catalyzes Ca^{2+} entry across the inner membrane of mitochondria. The uncoupling proteins UCP2 and UCP3 have been postulated to catalyze Ca^{2+} entry across the inner membrane of mitochondria (Trenker. 2007. *Nat. Cell Biol.*), but this proposal is disputed (Brookes. 2008. *Nat. Cell Biol.*) and two other, unrelated proteins have since been proposed to facilitate mitochondrial Ca^{2+} uptake (Jiang. 2009. *Science*; Perocchi. 2010. *Nature.*). To clarify the role of UCPs in mitochondrial Ca^{2+} handling, we down-regulated the expression of the only uncoupling protein of HeLa cells, UCP3, and measured Ca^{2+} and ATP levels in the cytosol and in organelles with genetically encoded probes. UCP3 silencing did not alter mitochondrial Ca^{2+} uptake in permeabilized cells. In intact cells however, UCP3 depletion increased mitochondrial ATP production and strongly reduced the cytosolic and mitochondrial Ca^{2+} elevations evoked by histamine. The reduced Ca^{2+} elevations were due to inhibition of store-operated Ca^{2+} entry and reduced depletion of endoplasmic reticulum (ER) Ca^{2+} stores. UCP3 depletion accelerated the ER Ca^{2+} refilling kinetics, indicating that the activity of sarco/endoplasmic reticulum Ca^{2+} pumps (SERCA) was increased. Accordingly, SERCA inhibitors reversed the effects of UCP3 depletion on cytosolic, ER, and mitochondrial Ca^{2+} responses. Our results indicate that UCP3 is not a mitochondrial Ca^{2+} uniporter, but instead negatively modulates the activity of SERCA by limiting mitochondrial ATP production. The effects of UCP3 on mitochondrial Ca^{2+} thus reflect metabolic alterations that impact on cellular Ca^{2+} homeostasis. The sensitivity of SERCA to mitochondrial ATP production suggests that mitochondria control the local ATP availability at ER Ca^{2+} uptake and release sites.

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28. The Novel Mode of Ca^{2+} Coupling from Mitochondria to the Endoplasmic Reticulum and the Plasmalemma in Rodent Brown Adipocytes. R. HAYATO,[1] Y. HIGURE,[1] M. KUBA,[1] H. NAGAI,[1] H. YAMASHITA,[2] and K. KUBA,[1] *[1]Lab. Anat. Physiol., Sch. Nutritional Sci., Nagoya Univ. Arts and Sci., Nissin 470-0196, Japan; [2]Dept. Biomed Sci., Coll. Life and Health Sci., Chubu Univ., Kasugai 487-8501, Japan*

Mitochondria take up Ca^{2+} released from the endoplasmic reticulum (ER), preventing Ca^{2+} reuptake into the ER and so maintain store-operated Ca^{2+} entry (SOC). We propose here the new mode of Ca^{2+} coupling from mitochondria to the endoplasmic reticulum. In brown adipocytes, β_3-adrenergic lipolysis activates an uncoupling protein, UCP1, via free fatty acids, while, α_1-adrenergic activation elicits Ca^{2+} release from the ER and subsequently SOC. We report here that these two modes of actions are closely coupled via the novel mechanism of the Ca^{2+} coupling of mitochondria to the ER and the plasmalemma. Ca^{2+} concentrations in the cytosol, ER and mitochondria and of mitochondrial membrane potential were measured by fluorometry. β_3-adrenergic activation elicited di- or tri-phasic rises in the cytosolic Ca^{2+} in wild-type cells, but not in UCP1-knockout cells. The initial phase was accompanied by a decrease in mitochondrial Ca^{2+} and mitochondrial

depolarization. The late phase was accompanied by a decrease in the Ca^{2+} concentration in the ER and depressed partially by removing external Ca^{2+}, preferentially by intracellular loading BAPTA and fully by the knockdown of type 2 IP_3 receptors and the action of a blocker of phospholipase C. Thus, mitochondrial uncoupling by β_3-adrenergic activation releases Ca^{2+} from mitochondria, which causes Ca^{2+} release from the ER via a Ca^{2+}-induced Ca^{2+} release mechanism under the actions of IP_3, and elicits SOC. This enduring rise of the cytosolic Ca^{2+} concentration that keeps mitochondrial Ca^{2+} high would play indispensable roles in maintaining thermogenesis and thus regulate body temperature and energy balance.

29. Volume-sensitive Cl^- Current Is Activated by Mitochondrial ROS in Simulated Cardiac Ischemia/Reperfusion. WU DENG, JIN YIN, EDWARD J. LESNEFSKY JR., and CLIVE M. BAUMGARTEN, *Department of Physiology and Biophysics and Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA 23298*

ROS upregulate volume-sensitive Cl^- current ($I_{Cl,swell}$) in cardiac myocytes in response to swelling, stretch and signaling, and both NADPH oxidase and mitochondrial ROS are implicated (Browe and Baumgarten. 2004. *J. Gen. Physio*l. 124:273–287; Deng, Baki, and Baumgarten. 2010. *Cardiovasc. Res.* 88:93–100; Deng, Baki, Yin, Zhou, and Baumgarten. 2010. *J. Molec. Cell. Cardiol.* 49:746–752). We tested whether ischemia/reperfusion (I/R) injury also modulates $I_{Cl,swell}$ and identified the source of ROS. Rabbit ventricular myocytes and mouse atrial HL-1 myocytes were incubated in mock ischemia media (0.9% O_2, 0 mM glucose, pH 6.5, 37°C) for 45 min (ventricular myocytes) or 4 h (HL-1) and then reperfused for 1–5 h (21% O_2, 11 mM glucose, pH 7.4, 37°C) before patching. In time-matched control ventricular myocytes maintained in reperfusion solution, basal $I_{Cl,swell}$ was 0.8 ± 0.1 pA/pF at +60 mV and was activated by H_2O_2 (100 µM; 3.6 ± 0.3 pA/pF). H_2O_2-induced current outwardly rectified with physiologic and symmetrical Cl^- gradients and was blocked by $I_{Cl,swell}$ inhibitors DCPIB and tamoxifen. In contrast, $I_{Cl,swell}$ already was strongly activated after I/R (4.3 ± 0.3 pA/pF) and was suppressed by DCPIB (0.4 ± 0.1 pA/pF) and ebselen (1.0 ± 0.1 pA/pF), a ROS scavenger. I/R-induced $I_{Cl,swell}$ was not dependent on NADPH oxidase; it was insensitive to the fusion peptide gp91ds-tat, whereas this agent blocks $I_{Cl,swell}$ elicited by swelling, angiotensin-II and endothelin-1. On the other hand, rotenone, a complex I blocker, strongly suppressed I/R-induced $I_{Cl,swell}$ (0.6 ± 0.2 pA/pF). As expected upon blocking $I_{Cl,swell}$, DCPIB shortened action potential duration after I/R but not in time-matched controls. Parallel studies on HL-1 myocytes recapitulated results in ventricular myocytes, although densities of H_2O_2- and I/R-induced $I_{Cl,swell}$ were more than 10-fold greater in HL-1 cells. These findings suggest that $I_{Cl,swell}$ is stimulated by mitochondrial ROS production in I/R and may contribute to cardiac dysfunction.

30. Comparative Effects of Ryanodine Receptor Inhibitors on Mitochondrial Ca^{2+} Uptake Profiles in Control and Malignant Hyperthermia Mouse Heart, POLINA GROSS,[1] NIINA SOKOLOVA,[2] SARAH PROVAZZA,[3] GISELA BEUTNER,[3] and SHEY-SHING SHEU,[4] *[1]Department of Biotechnology Engineering, ORT Braude College, Karmiel 21982 Israel; [2]Institute of Cybernetics, Tallinn University of Technology, Tallinn 12618 Estonia; [3]Department of Pharmacology and Physiology, University of Rochester, Rochester, NY 14642; [4]Department of Medicine, Thomas Jefferson University, Philadelphia, PA 19107*

Malignant hyperthermia (MH) is a severe hypermetabolic response triggered by exposure to volatile anesthetics and caused by mutations in the genome of ryanodine receptor subtype 1 (RYR1) in the skeletal muscle. The presence of mitochondrial ryanodine receptor (mRYR) in heart, which behaves immunologically and pharmacologically like RyR1, raises the question of its primary cardiac pathological role in MH.

To address this, we used genetically modified mice containing RyR1 with a single point mutation at the position 522 (tyrosine versus serine; YS mice, obtained from Dr. Susan Hamilton, Baylor College of Medicine) and wild-type (WT) mice. Ca^{2+} uptake of isolated heart mitochondria from both animal groups was measured (n = 3) using ArsenazoIII and RYR specific inhibitors (100 µM ryanodine, 10 µM dantrolene sodium, and 10 µM azumolene). Our data show, that in 16-mo-old control mice, azumolene, dantrolene, and ryanodine reduced the Ca^{2+} uptake capacity. In age-matched YS mice, the RyR1 specific inhibitors azumolene and dantrolene appeared more efficient to lower the Ca^{2+} uptake capacity than ryanodine. The time constant (T), calculated from the velocity of Ca^{2+} uptake during the first 2 min after applying a Ca^{2+} pulse of 5 µM showed faster Ca^{2+} uptake in WT than in YS (104.2 ± 11.1 s and 57.1 ± 6.2 s, respectively). All inhibitors decreased T in YS and WT, and most significantly with azumolene and dantrolene (188.1 ± 29.1 s and 276.1 ± 55.8 s, respectively in WT). Surprisingly, YS showed less inhibition due to the difficulty of blocking leaky channels (100.4 ± 15.8 s and 166.8 ± 43.9 s, respectively).

Measuring the membrane potential with TMRE showed that baseline fluorescence of YS mitochondria was significantly lower, indicating that these mitochondria are depolarized (1.6 a.u. vs. 2.1 a.u. in WT). The most significant effect was observed with dantrolene in YS and WT (8.4 vs. 9.5%, respectively).

Depolarized mitochondrial membranes due to leaky mRYR suggest a primary role of this protein in heart pathology in MH.

31. Action Potentials in Nerve Terminals Evoke Intrinsic Fluorescence Changes Which Can Be Modulated by Krebs Cycle Substrates. P. KOSTERIN,[1] A.L. OBAID,[1] and B.M. SALZBERG,[1,2] [1]*Department of Neuroscience and* [2]*Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104*

We have previously studied the properties of the action potential triggered by electrical stimulation of peptidergic nerve terminals in the vertebrate neurohypophysis, and established that Na^+ and Ca^{2+} influxes are responsible for its upstroke (Salzberg et al. 1983. *Nature.* 306:36–40; Obaid et al. 1985. *J Gen Physiol.* 85:481–489). The entrance of these ions depolarizes the membrane, increases $[Ca^{2+}]_i$ by evoking Ca^{2+} release from intracellular stores, and activates NaK- and Ca-ATPases. Together, these processes signal the mitochondria to increase oxidative phosphorylation, which can be monitored by recording the changes in FAD and NADH fuorescence. When excited at 450 nm, FAD is quite fluorescent, while its reduced form $FADH_2$ is only weakly so; in contrast, when NADH is excited at 350 nm, it is fluorescent, while its oxidized form, NAD, is not (Chance and Williams. 1955. *J Biol Chem.* 217:395–407). Thus, flavoprotein-derived and pyridine nucleotide-derived fluorescence changes tend to be opposite in sign. Changes in FAD and NADH fluorescence emission in mammalian neurosecretory terminals are triggered by the $\Delta[Ca^{2+}]_i$, and by the increase in the ADP/ATP ratio that results from turning on the NaK-ATPase (Kosterin et al. 2005. *J. Membr. Biol.* 208:113–124). Because mitochondria in nerve terminals make ATP, both to render secretory granules fusion-competent, and to supply ion pumps with the source of energy required for the restoration of ionic gradients altered during the action potential, we wanted to test whether modulating the capacity of mitochondria to produce ATP, by changing the concentrations of substrates of the Kreb's cycle, such as glucose or pyruvate, would affect the FAD and NADH changes evoked by stimuli applied to the neurohypophysial infundibular stalk. Our results confirmed our hypothesis, revealing an intricate connection between electrical activity, oxidative phosphorylation and Kreb's cycle substrates.

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32. Spatio-Temporal Changes in Intracellular ATP and Mitochondrial Activation During Sea Urchin Fertilization. TATSUMA MOHRI[1] and NORITAKA HIROHASHI,[2] [1]*Division of Correlative Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, 444-8585, Japan;* [2]*Department of Biology, Ochanomizu University, 2-1-1 Bunkyo-ku, Tokyo 112-8610, Japan*

Little is known about how mitochondria become activated or how changes in intracellular adenosine triphosphate ($[ATP]_i$) take place at fertilization in sea urchin eggs. Previous studies on ATP measurements have reported no significant increase or only a gradual increase in $[ATP]_i$ until hatching after fertilization. It also remains unclear whether mitochondrial activation and $[ATP]_i$ are related to fertilization-induced changes in intracellular calcium ($[Ca^{2+}]_i$). In this study, we examined the precise spatio-temporal relationships between $[Ca^{2+}]_i$, the mitochondrial inner membrane potential ($\Delta\Psi m$), and $[ATP]_i$. We measured $\Delta\Psi m$ as an indicator of mitochondrial activation using MitoTracker Red CMXR (MTR-CMXR) in eggs of sea urchin, *H. pulcherrimus* upon fertilization. We found an unequivocal increase in $\Delta\Psi m$ indicating a hyperpolarization of mitochondrial inner membrane potential. Simultaneous measurements of $[Ca^{2+}]_i$ and $\Delta\Psi m$ using Calcium Green-1 dextran and MTR-CMXR showed an occurrence of hyperpolarization within 10-20 s after $[Ca^{2+}]_i$ rise and sustained increase at least 30 min after insemination. To examine $[ATP]_i$ dynamics at fertilization, we microinjected an ATP probe (ATeam), which is a fluorescence resonance energy transfer (FRET)-based ATP indicator (Imamura et al. 2009. *PNAS.* 106:15651–15656). Surprisingly, simultaneous measurements of $[Ca^{2+}]_i$ and $[ATP]_i$ exhibited a drastic $[ATP]_i$ rise within 10-20 s after $[Ca^{2+}]_i$ rise similar to changes in $\Delta\Psi m$ and this increase was sustained at least 100 min before the first cell division. We also observed a small transient decrease in $[ATP]_i$ before the large increase, indicating a rapid ATP consumption before a large production of ATP by mitochondrial activation corresponding to a large $[Ca^{2+}]_i$ increase. Furthermore, we found that $[ATP]_i$ apparently started to increase at the sperm attachment site in a way similar to the $[Ca^{2+}]_i$ wave although the $[ATP]_i$ wave was not so distinct. These results suggest that sperm-triggered $[Ca^{2+}]_i$ rise initiates rapid ATP production via mitochondrial activation in sea urchin eggs.

33. The Permeability Transition Pore Controls Cardiac Mitochondrial Maturation and Myocyte Differentiation. JENNIFER R. HOM, RODRIGO A. QUINTANILLA, DAVID L. HOFFMAN, KAREN L. DE MESY BENTLEY, JEFFERY D. MOLKENTIN, SHEY-SHING SHEU, and GEORGE A. PORTER JR., *Departments of Pediatrics Division of Cardiology, Anesthesiology, Pharmacology and Physiology, Aab Cardiovascular Research Institute, Pathology and Laboratory Medicine, and the Electron Microscope Research Core University of Rochester School of Medicine and Dentistry, Rochester, NY; Department of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center and Howard Hughes Medical Institute,*

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Little is known about cardiac energetics and mitochondrial function in the embryo, and we hypothesize that the mitochondrial permeability transition pore (mPTP) controls mitochondrial structure and function during embryonic cardiac development and is critical for normal myocyte differentiation and cardiac morphogenesis. To test this hypothesis, we examined mitochondrial structure and function in cultured myocytes and whole heart using light and electron microscopy. Mitochondria of embryonic day (E) 9.5 ventricular myocytes displayed less dense cristae and were shorter in length and less branched. By E13.5, mitochondria had abundant cristae, were longer, branched and networked, and were more closely associated with the contractile apparatus. Functional measurements demonstrated dramatic increases in mitochondrial membrane potential, an increased reliance on complex I, and a decrease in oxidative stress as the heart developed. These structural and functional data suggested an increase in inner mitochondrial membrane permeability, and closure of the mPTP using cyclosporin A or cyclophilin−D null embryos caused premature maturation of mitochondrial structure, mitochondrial function, and myocyte differentiation, while differentiation was inhibited upon long term opening of the mPTP. Furthermore, differentiation was inhibited and enhanced with oxidant and antioxidant treatment, respectively, independent of mPTP state, suggesting that redox signaling pathways lie downstream of mitochondria to regulate cardiac myocyte differentiation.

Sponsor: George A. Porter Jr.

34. Alkalinization Transients in Individual Mitochondria. JAIME SANTO-DOMINGO and NICOLAS DEMAUREX, *Department of Cell Physiology and Metabolism, University of Geneva, Switzerland*

The pH within the mitochondrial matrix (pH_{mito}) is an important bioenergetic parameter well studied in isolated mitochondria but poorly characterized in intact cells, and all in situ studies available report spatially averaged measurements of pH_{mito}. We used a new pH-sensitive GFP-based fluorescent probe targeted to the mitochondrial matrix, mito-SypHer, to study pH_{mito} homeostasis at the level of single mitochondria in intact living cells. We observed that individual mitochondria undergo spontaneous alkalinization transients. The pH transients occurred randomly in time and space and had a characteristic profile, with a rapid onset (time to peak 1.6 ± 0.1 s), a slower decay (τ = 8.5 ± 0.6 s), and an average amplitude of 0.38 ± 0.05 pH units. The pH transients were absent in *Rho 0* cells that lack a functional respiratory chain, were abrogated by protonophores, occurred concomitantly with transient mitochondrial depolarization events measured with TMRE, and their frequency was strongly decreased by respiratory chain inhibitors. These kinetics and functional properties resemble the "superoxide flashes" previously reported in single mitochondria with a pericam-derived probe (Wang et al., 2008). However, the fluorescence of purified mito-SypHer was not altered by all the ROS tested including superoxide, and in live cells increasing the pH buffering power of mitochondria with NH4Cl decreased the amplitude and slowed the kinetics of the transients, confirming that they were caused by protons. The pH spikes were not spatially restricted to single mitochondria but were also observed in clusters of interconnected mitochondria and their spatial extent was altered by enforced fusion or fission of mitochondria. Our data indicate that in live cells individual mitochondria undergo spontaneous basification transients that require functional OXPHOS machinery. The synchronicity between the spontaneous pH spikes and depolarization events suggests that H^+ extrusion by the respiratory chain complexes or by the reverse mode of the ATP-synthase might be transiently enhanced during bouts of depolarization.

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35. Mitofusin2 Is Important for Mitochondrial Ca^{2+} Uptake During Intense Muscle Activity. ALINA AINBINDER and ROBERT T. DIRKSEN, *University of Rochester School of Medicine and Dentistry, Department of Pharmacology and Physiology, Rochester, NY*

Skeletal muscle contraction requires ATP and Ca^{2+}, and thus, is under direct control of two major intracellular organelles: mitochondria and sarcoplasmic reticulum (SR). In adult skeletal muscle, mitochondria are connected to the SR by small ~10 nm electron dense linkages, or tethers, that bridge the outer mitochondrial membrane to the SR. While this structural connection between mitochondria and SR is thought to play an important role in the cross talk between these two organelles, the molecular component of the tether and the role of this connection in SR Ca^{2+} release, mitochondrial Ca^{2+} uptake, and Ca^{2+}-stimulated ATP production in skeletal muscle are unclear. Mitofusin 2 (Mfn2) is a dynamin-related transmembrane GTPase present in both mitochondria and ER/SR membranes that forms trans-homodimers and participates in mitochondrial fusion. Here we propose that Mfn2 plays central role in functional SR-mitochondrial crosstalk in skeletal muscle by linking the two organelles together. We achieved a significant reduction (84 ± 11%, n = 3) in Mfn2 protein levels in flexor digitorum brevis (FDB) muscles following in vivo electroporation of 200nM Mfn2 siRNA into mouse footpads. We loaded Mfn2 KD and control fibers with Rhod2AM (5 μM) and Fluo4AM (10 μM) to measure mitochondrial Ca^{2+} uptake and

myoplasmic Ca^{2+} transients, respectively, following repetitive high frequency tetanic stimulation (5 tetani; 500 ms, 100 Hz, 2.5 s start to start). Our results show that stimulation-dependent increases in mitochondrial Ca^{2+} uptake is significantly ($P < 0.05$) reduced in FDB fibers from mice electroporated with Mfn2 siRNAs (peak Rhod2 $(F_{Iband}-F_{Aband})_t/(F_{Iband}-F_{Aband})_{t=0} = 2.6 \pm 0.2$, $n = 7$) compared to scrambled, non-targeting siRNAs (peak Rhod2 $(F_{Iband}-F_{Aband})_t/(F_{Iband}-F_{Aband})_{t=0} = 5.4 \pm 0.8$, $n = 10$). In addition, a significant ($P < 0.05$) increase in the global myoplasmic Ca^{2+} transient was also observed following Mfn2 KD (peak Fluo4 $F_t/F_{t=0}$ was 7.5 ± 0.7 and 9.5 ± 0.7 for control and KD, respectively). However, Mfn2 knockdown did not significantly alter mitochondrial membrane potential or morphology as assessed from tetramethylrhodamine ethyl ester fluorescence. Together, these results indicate that Mfn2 is required for efficient mitochondrial Ca^{2+} uptake, and thus, Ca^{2+}-stimulated ATP production during intense muscle activity.

36. Species-Related Differences in Mitochondrial Calcium Influx and Efflux Rates in Guinea Pig and Mouse: Implications for Excitation-Contraction-Bioenergetic Coupling. AN-CHI WEI,[1] TING LIU,[2] RAIMOND L. WINSLOW,[1] and BRIAN O'ROURKE,[1,2] *[1]Institute for Computational Medicine and Department of Biomedical Engineering, and [2]Institute of Molecular Cardiobiology, Division of Cardiology, The Johns Hopkins University, Baltimore, MD*

Ca^{2+} is an important regulator of both myocardial excitation-contraction coupling and mitochondrial ATP production and is coupled to Na^+ regulation through sarcolemmal and mitochondrial Na^+/Ca^{2+} exchangers. Alterations of cytosolic Na^+ will not only change cytosolic calcium levels but also the rate of mitochondrial Ca^{2+} accumulation. Different ranges of cytosolic Na^+ levels have been reported among species; however, the impact of these differences, as well as possible variations in intrinsic Ca^{2+} transport rates on mitochondrial function have not been investigated. Because such species-related alterations could impact excitation-contraction-bioenergetic coupling, in the present study, initial velocities of mitochondrial Ca^{2+} uptake rate and extrusion rates were compared between isolated guinea pig and mouse heart mitochondria. The fluorescent probe Calcium Green-5N was used to monitor the extra-mitochondrial calcium concentration and the unidirectional mitochondrial calcium influx and efflux rates were determined. Under the zero-Na^+ conditions a pulse of 15 μM free Ca^{2+} gave mitochondrial Ca^{2+} uptake rates of 0.5 nmol/s/mg in guinea pig and 0.3 nmol/s/mg in mouse. This was followed by an addition of Ru360 (to block uptake) and 5 mM NaCl, to measure Na^+-independent and Na^+-dependent Ca^{2+} efflux rates, respectively. Na^+-dependent Ca^{2+} efflux rates through the mitochondrial Na^+/Ca^{2+} exchanger were 0.02 nmol/s/mg in guinea pig and 0.025 nmol/s/mg in mouse. The observed lower mitochondrial Ca^{2+} uptake rate and the higher mitochondrial Na^+-dependent Ca^{2+} extrusion rates coupled with the known higher resting Na^+ level in mouse (15 mM in mouse and 5 mM in guinea pig) is likely to explain reported species-dependent differences in mitochondrial Ca^{2+} transients during excitation-contraction coupling.

37. An Interaction between Anti-Apoptotic Bcl-X_L and VDAC1 Regulates Mitochondrial Ca^{2+} Handling in the Absence of Apoptotic Stimuli. HUIYA HUANG,[1] KRISTEN OLBERDING,[2] CHI LI,[2] and CARL WHITE,[1] *[1]Department of Physiology and Biophysics, Rosalind Franklin University of Medicine and Science, North Chicago, IL; [2]Molecular Targets Group, James Graham Brown Cancer Center, University of Louisville, Louisville, KY*

In the present study, the role of the anti-apoptotic protein Bcl-X_L in regulating mitochondrial Ca^{2+} handling was examined in mouse embryonic fibroblasts (MEF) derived from wild-type (WT) and Bcl-X_L knockout (Bcl-X_L-KO) mice. In cells co-loaded with Rhod-2 and Fluo-4 to simultaneously monitor $[Ca^{2+}]_{mito}$ and $[Ca^{2+}]_{cyto}$, respectively, application of $InsP_3$-generating agonists evoked larger $[Ca^{2+}]_{cyto}$ transients in Bcl-X_L-KO cells compared with WT, consistent with the established role of Bcl-X_L as a modulator of steady-state ER Ca^{2+} levels. Despite the smaller ER Ca^{2+} release in the WT, however, $[Ca^{2+}]_{mito}$ uptake was larger in these cells compared to Bcl-X_L-KO. In permeabilized cells, stepping external $[Ca^{2+}]$ from 0 to 1.25 μM also produced a larger $[Ca^{2+}]_{mito}$ uptake in the WT, suggesting that enhanced uptake was dependent on mitochondrial Bcl-X_L rather than altered ER-mitochondria coupling. Consistent with this, the $[Ca^{2+}]_{mito}$ uptake capacity of Bcl-X_L-KO cells was restored only by re-expression of mitochondrially and not ER-targeted Bcl-X_L. Examination of mitochondrial Ca^{2+} removal upon stepping back to zero $[Ca^{2+}]$ in permeabilized cells revealed slower kinetics in the WT, suggesting that increased $[Ca^{2+}]_{mito}$ uptake may be due to impaired removal rather than enhanced influx. The outer membrane-localized voltage-dependent anion channel (VDAC) is a known Ca^{2+} permeability that directly interacts with Bcl-X_L, although the functional consequences have not been fully established. siRNA knock-down of VDAC1 restored $[Ca^{2+}]_{mito}$ uptake capacity and slowed $[Ca^{2+}]_{mito}$ removal in Bcl-X_L-KO cells, but was without effect in WT. In addition, the VDAC inhibitors, S18 randomer and DIDS, had similar effects on $[Ca^{2+}]_{mito}$ uptake. Furthermore, application of peptides shown to disrupt Bcl-X_L-VDAC interactions reduced $[Ca^{2+}]_{mito}$ uptake and increased removal rate in the WT but were without effect in Bcl-X_L-KO cells. Collectively, these data suggest that an interaction with

Bcl-X_L regulates the VDAC1 Ca^{2+} permeability to facilitate mitochondrial Ca^{2+} loading in the absence of apoptotic stimuli.

38. MitoTimer: A Novel Tool to Identify Mitochondrial Subpopulations. GIOVANNI QUARATO, RAQUEL CARREIRA, CHRISTINE THORNTON, GENARO HERNANDEZ, and **ROBERTA A. GOTTLIEB**, *The Donald P. Shiley BioScience Center, San Diego State University, San Diego, CA*

Timer is a fluorescent protein developed by Terskikh et al. (2000. *Science*. 290:1585–1588). Timer molecules transition from green fluorescence to a more stable red conformation over a span of 48h. We targeted Timer to the mitochondrial matrix (MitoTimer) under control of the CMV promoter, to monitor mitochondrial turnover in vivo. HL-1 cells transfected with pCMV-MitoTimer display green mitochondria 8 h after transfection, while red mitochondria are first detected at 20 h. The percentage of cells with green mitochondria decreases and the percentage with red mitochondria increases over 48 h. In HL-1 cells transfected with MitoTimer and exposed to FCCP, which depolarizes mitochondria, new mitochondrial protein import is disrupted and many mitochondria are removed by autophagy, reflected by a low percentage of cells with green mitochondria at 20 h. A robust increase in the number of cells with green mitochondria (reflecting import of newly synthesized MitoTimer) is apparent at 26 and 30 h. To more precisely regulate its expression, we subcloned MitoTimer into a tetracycline-inducible promoter construct, then tested its utility for monitoring new-onset protein import as an indication of mitochondrial biogenesis. Cells were transfected with rtTA (transcription factor activated by doxycycline (dox)) and pTRE-MitoTimer and treated with dox for 3h. After 48 h, when all MitoTimer protein had aged to red, cells were treated with drugs or vehicle and a second pulse of dox for 30 min followed by washout, then imaged 2 and 6 h later. While relatively little MitoTimer protein was incorporated into mitochondria in vehicle-treated cells, it was much more robustly incorporated into mitochondria in cells treated with agents that stimulate autophagy/mitophagy, namely chloramphenicol, the adenosine A1 receptor agonist CCPA, and the uncoupler FCCP. Ratiometric image analysis allows visualization of mitochondrial subpopulations which are enriched for newly synthesized (green) MitoTimer. Incorporation of green MitoTimer during the second dox pulse was inhomogeneous, revealing for the first time, mitochondrial subpopulations that are highly active for protein import. We suggest that this novel tool will permit real-time visualization of mitochondrial biogenesis and turnover in living cells.

39. Establishing the Role of MTCH2/MIMP in Apoptosis and Metabolism. **ATAN GROSS**, *Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel 76100*

Apoptosis is a crucial process for proper embryonic development and maintenance of tissue homoeostasis. In the extrinsic death pathway, apoptosis is initiated through activation of the Fas/TNF death receptors. The BH3-only BID protein plays a critical role in the death-receptor pathway in the liver by triggering mitochondrial outer- membrane permeabilization. MTCH2/MIMP was identified in our lab as part of a complex with activated tBID in cells signaled to die by Fas/TNF. MTCH2/MIMP is a novel and previously uncharacterized 33-kD protein, which is related to members of the mitochondrial carrier protein family. To study MTCH2/MIMP's activity in vivo we generated MTCH2/MIMP liver-specific knockout (LKO) mice, and found that LKO mice are less sensitive to Fas-induced apoptosis and show less mitochondrial targeting of tBID in the liver. We also examined the behavior of these mice on high fat diet since MTCH2/MIMP was recently identified as a new gene locus associated with obesity in humans. Interestingly, we found that the LKO mice gain less weight than the wild-type littermate mice, and that their serum triglyceride levels were significantly lower than the levels in wild-type mice. Moreover, using shotgun lipidomics we have found that MTCH2/MIMP deletion leads to a profound effect on the hepatic levels of fatty acids. Thus, our future aims are to further understand and establish the role of MTCH2/MIMP in fatty acid metabolism and to determine the connection between this activity and the regulation of apoptosis at the mitochondria.

40. Essential Regulation of Cell Bioenergetics by Constitutive $InsP_3$ Receptor Ca^{2+} Transfer to Mitochondria. C. CÁRDENAS,[1] R.A. MILLER,[3] I. SMITH,[5] T.I. BUI,[4] J. MOLGÓ,[6] M. MÜLLER,[1] H. VAIS,[1] K. CHEUNG,[1] J. YANG,[1] I. PARKER,[5] C. THOMPSON,[4] M. BIRNBAUM,[3] K.R. HALLOWS,[7] and **J. KEVIN FOSKETT**,[1,2] *[1]Department of Physiology, [2]Department of Cell and Developmental Biology, [3]Institute for Diabetes, Obesity and Metabolism, and [4]Abramson Cancer Institute and Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA 19104; [5]Department of Neurobiology and Behavior, University of California, Irvine, CA 92697; [6]CNRS, Institute de Neurobiologie Alfred Fessard, FRC2118, Laboratoire de Neurobiologie Cellulaire et Moléculaire, UPR 3294, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France; [7]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261*

Mechanisms that regulate cellular metabolism are a fundamental requirement of all cells. Most eukaryotic cells rely on aerobic mitochondrial metabolism to generate ATP. Nevertheless, regulation of mitochondrial

activity is incompletely understood. Here we identified an unexpected and essential role for constitutive $InsP_3R$-mediated Ca^{2+} release in maintaining cellular bioenergetics. Macroautophagy provides eukaryotes with an adaptive response to nutrient deprivation that prolongs survival. Constitutive $InsP_3R$ Ca^{2+} signaling is required for macroautophagy suppression in cells in nutrient-replete media. In its absence, cells become metabolically compromised due to diminished mitochondrial Ca^{2+} uptake. Mitochondrial uptake of $InsP_3R$ released Ca^{2+} is fundamentally required to provide optimal bioenergetics by providing sufficient reducing equivalents to support oxidative phosphorylation. Absence of this Ca^{2+} transfer results in enhanced phosphorylation of pyruvate dehydrogenase and activation of AMPK, which activates pro-survival macroautophagy. Thus, constitutive $InsP_3R$ Ca^{2+} release to mitochondria is an essential cellular process that is required for efficient mitochondrial respiration and maintenance of normal cell bioenergetics.

41. Pathophysiology of the Mitochondrial Permeability Transition. **PAOLO BERNARDI**, *Department of Biomedical Sciences, University of Padova, Italy*

The mitochondrial permeability transition (PT) is a Ca^{2+}-dependent increase of mitochondrial inner membrane permeability to solutes with molecular masses up to about 1,500 Da. Its occurrence is always accompanied by depolarization and Ca^{2+} release, while onset of matrix swelling, depletion of matrix pyridine nucleotides, outer membrane rupture and release of intermembrane proteins including cytochrome *c* depend on the open time. The PT is due to the reversible opening of a high-conductance, voltage-dependent channel in the inner mitochondrial membrane, the PT pore (PTP). In spite of many efforts, its molecular identity remains unknown. I shall cover the essential aspects of PTP pathophysiology, with specific emphasis on the role of the outer membrane and the TSPO (formerly known as peripheral benzodiazepine receptor), on the mechanism of action of cyclosporin A, and on the consequences of PTP opening. From this analysis the PTP emerges as an evolutionarily conserved pathway involved in Ca^{2+} homeostasis, and as a viable target for therapeutic intervention in cancer and degenerative diseases.

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42. Deficit in Mitochondrial Control of Calcium Signaling During Excitation-Contraction Coupling Contributes to Neuromuscular Degeneration in ALS. JIANXUN YI,[1] CHANGLING MA,[1] YAN LI,[1] EDUARDO RÍOS,[1] NOAH WEISLEDE,[2] JIANJIE MA,[2] and JINGSONG ZHOU,[1] *[1]Rush University, Chicago; [2]UMDNJ-Robert Wood Johnson Medical School, Piscataway*

Mitochondrial Ca^{2+} uptake regulates metabolism and synthesis of ATP to meet demands of muscle contraction. Whether mitochondrial Ca^{2+} uptake modifies Ca^{2+} signaling in skeletal muscle during excitation-contraction (E-C) coupling remains an open question. While studies show that mitochondria in skeletal muscle may take up Ca^{2+} during contraction, it is not known whether altered mitochondrial Ca^{2+} uptake can play a role in pathophysiological conditions. Our previous study using the G93A amyotrophic lateral sclerosis (ALS) mouse model shows that muscle fibers display defective mitochondria with loss of the inner membrane potential in fiber segments. The finding of localized mitochondrial defects in ALS fibers presents a unique opportunity to test whether changes in mitochondrial function can affect intracellular Ca^{2+} signaling, as Ca^{2+} release activity can be compared in regions with normal or defective mitochondria in the same fiber. By loading muscle fibers with TMRE (a probe of mitochondrial potential) and fluo-4 (a Ca^{2+} indicator), simultaneous measurements of mitochondrial function and Ca^{2+} transients were made under patch-clamping condition. We found that fiber segments with defective mitochondria displayed ~10% greater Ca^{2+} transients elicited by voltage. These regional differences in Ca^{2+} transients were abolished by the application of BAPTA, a fast Ca^{2+} chelator that reduces mitochondrial Ca^{2+} uptake. Using a mitochondrion-targeted Ca^{2+} biosensor (mt11-YC3.6) in ALS fibers, we monitored the dynamic change of Ca^{2+} inside mitochondria during voltage-induced Ca^{2+} release and detected a reduced Ca^{2+} uptake by mitochondria in the fiber segment with depolarized mitochondria, which mirrors the elevated Ca^{2+} transients in the cytosol. Our study constitutes a direct demonstration of the importance of mitochondria in shaping the cytosolic Ca^{2+} signaling in skeletal muscle during E-C coupling. Malfunction of mitochondrial control of Ca^{2+} signaling may contribute to neuromuscular degeneration in ALS.

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43. Cardioprotection by Cyclosporin A: Mitochondrial Permeability Transition Pore Inhibition or Enhanced Cell Volume Regulation? ROBERTO J. DIAZ, KELLY FERNANDEZ, KORDAN HARVEY, ALINA HINEK, TANEYA HOSSAIN, and GREGORY J. WILSON, *Division of Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, Canada*

Cardiomyocyte death under conditions of ischemia/reperfusion (I/R) is thought to be importantly affected by the open/closed state of the mitochondrial permeability transition pore (MPTP), modulated in part through cyclophilin D (CypD) to inhibit MPTP opening. We have shown that ischemic preconditioning (IPC) enhances cell volume regulation in cardiomyocytes, triggering Cl^- efflux, and that blocking cell volume regulation during IPC by inhibiting Cl^- or K^+ transport across the cell membrane blocks the protection of IPC. To determine the effects of CsA on Cl^- transport, we treated 24-h cultured rabbit cardiomyocytes with 0.2 μM CsA, a concentration known to produce protection in isolated rabbit cardiomyocytes. We measured the effect of CsA on cardiomyocyte Cl^- transport across the sarcolemma using the Cl^- indicator SPQ, a technique we have previously reported (Diaz et al. 2010. *Cardiovasc. Res.* 87:545–551). Treatment of 24-h cultured rabbit cardiomyocytes with 0.2 μM CsA+DMSO 0.04% vol/vol ($n = 54$ cells) triggered an increase ($P < 0.0001$) in SPQ fluorescence equating to an efflux of 38.8 ± 4.7 mM (mean ± SEM) Cl^- after 20 min (10-min CsA+DMSO treatment and 10-min washout), as compared to DMSO control (3.2 ± 2.5, $n = 36$). This CsA+DMSO effect on Cl^- transport was blocked by Cl^- channel inhibition with 50 μM IAA-94 (18.1 ± 2.5, $P = 0.0131$ vs. CsA+DMSO, $P = 0.158$ vs. DMSO control, $n = 35$). The large Cl^- efflux by CsA, equivalent to that caused by IPC or pharmacological preconditioning, suggests CsA protects against cardiomyocyte I/R necrosis at least in part through a volume regulatory mechanism, and not simply by acting directly on the MPTP through CypD.

44. Toxicity and Antioxidant Capacity of Resveratrol on Liver and Brain Mitochondria—Are There Gender-Dependent Effects? A.C. MOREIRA,[1,2] A.M. SILVA,[2] M.S. SANTOS,[2,3] and V.A. SARDAO,[2] [1]*Doctoral Programme in Experimental Biology and Biomedicine,* [2]*Center for Neuroscience and Cell Biology, and* [3]*Department of Life Sciences, University of Coimbra, Portugal*

Resveratrol (3,5,4'–trihydroxy-trans stilbene) is a stilbenoid naturally produced by several plants. Highly present in the skin of red grapes, and consequently in red wine, resveratrol is commonly recognized by its beneficial properties, including anti-diabetic, anti-viral, anti-inflammatory and cardio- and neuroprotective. Despite its beneficial properties, the toxic effects of this natural compound are still largely unknown. Since mitochondria are essential to support the energy-dependent regulation of several cell functions, the objective of this study was to evaluate resveratrol toxicity on isolated mitochondria from rat brain and liver and explore if gender impacts the toxicity.

Mitochondria were isolated from male and female Wistar-Han rats. Mitochondrial oxygen consumption and $\Delta\Psi$ were measured using an oxygen electrode and a TPP^+ electrode, respectively. Antioxidant properties were evaluated by measuring inhibition of hydroperoxide production by the mitochondrial respiratory chain and TBARS production. Superoxide dismutase (SOD) activity was assessed spectrophotometrically at 550 nm.

Resveratrol decreases state 3 respiration in brain and liver mitochondria, for both genders. Increased repolarization lag phase after ADP phosphorylation was observed in liver mitochondria isolated from both genders. However, alterations were only observed in male brain mitochondria. In all groups, resveratrol decreased mitochondrial lipid peroxidation induced by ADP-Fe. H_2O_2 production by brain mitochondria was increased by the presence of resveratrol in preparations from male's liver. However, by following Complex I-linked NADH oxidation, we observed that resveratrol did not show a direct effect on that mitochondrial complex. Preliminary results demonstrated that SOD enzymatic activity was modulated by resveratrol with an increase inVmax.

The data obtained shows that resveratrol, despite acting as antioxidant in some oxidative stress models, disturbs mitochondrial hydroperoxide production not due to direct effects on Complex I, but probably through the modulation of superoxide dismutase activity.

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45. Higher Propensity to Mitochondrial Oscillations and Arrhythmias in Type 1 Diabetic Heart. M.A. AON,[1] P. JOUDREY,[2] B. O'ROURKE,[1] S. CORTASSA,[1] N. PAOLOCCI,[1] and F.G. AKAR,[2] [1]*Johns Hopkins University, School of Medicine, Division of Cardiology, Baltimore, MD;* [2]*Cardiovascular Research Center, Division of Cardiology, Mount Sinai School of Medicine, New York, NY*

Led by the overarching hypothesis that the redox environment is decisive for proper heart function, and that mitochondria play a prominent role in dictating its own as well as the cardiomyocyte redox status through reactive oxygen species (ROS) levels, we investigated whether mitochondrial dysfunction exists at the onset of a streptozotocin (STZ)-induced type 1 DM in guinea pig (GP). The decisive importance of the intracellular and mitochondrial redox balances is epitomized by the fact that elevated ROS levels increase the propensity to mitochondrial membrane potential ($\Delta\Psi_m$) depolarization

followed by whole cell mitochondrial oscillations and cellular and myocardial dysfunction setting the stage for lethal arrhythmias in hearts subjected to ischemia-reperfusion injury.

We tested baseline NAD(P)H and GSH levels in cardiomyocytes and mitochondria isolated from sham, STZ-treated, and STZ plus insulin-treated GPs. Isolated mitochondria exhibited more oxidized levels of both NAD(P)H and GSH like cardiomyocytes from STZ-treated GPs under normal glucose conditions (10 mM). Also, complex II and IV from the respiratory chain appear to be significantly impaired in GPs subjected to STZ-treatment. Very importantly, these redox and energetic impairment conditions in STZ-treated GPs correlated with a higher susceptibility to the triggering of laser-flash-induced whole cell mitochondrial oscillations in isolated diabetic cardiomyocytes. Moreover, hearts from STZ-treated GPs clearly exhibit larger $\Delta\Psi_m$ heterogeneity that strongly correlates with higher arrhythmia scores when analyzed by high resolution optical mapping. Furthermore, insulin treatment of STZ-treated animals, or preincubation of cardiomyocytes from diabetic heart with 3 mM of the cell permeable ethyl ester form of GSH prevented the increase in propensity to flash-induced mitochondrial oscillations.

These results represent important evidence in favor of the causal role of mitochondrial dysfunction on the diabetic heart dysfunction, and that both energetic and redox aspects of mitochondrial and cardiomyocyte function appear to be severely compromised in the diabetic heart.

46. Time Course of Liver Mitochondrial Function During Septic Shock by Cecal Ligation and Puncture in Rat (CLP). P. EYENGA,[1] D. ROUSSEL,[2] S.S. SHEU,[3] C. NEGRIER[1], and J.P. VIALE,[1] *[1]EA 4171 Hemostase Sepsis et Inflammation, Universite Claude Bernard, Lyon 1, F-69008, France; [2]Physiologie Cellulaire et Moleculaire, UMR 5123, Villeurbanne F-69100, France; [3]Department of Translational Medicine, Thomas Jefferson University, Philadelphia, PA*

Introduction: Mitochondrial damage and dysfunction are thought to play an important role in the pathogenesis of sepsis-induced organ failures. Brealey clinical study was link skeletal muscle ATP depletion to severity and poor outcome of septic shock patients. In previous experimental study, we found a preserve of mitochondrial function and inner membrane permeability (submitted to Crit Care). Thus, we hypothesized that the mitochondrial uncoupling occurring in the CLP model of septic shock is time dependence.

The present study examined the time course of liver mitochondrial function, and the mechanism of ATP wastage in the late period of septic shock.

Material and method: 36 adults male wistars rats were assigned to receive cecal ligation and double puncture by 19 gauge needle (CLP sepsis) (half animals) or sham operation (control group). Plasma lactic acid, ALT, nitrite/nitrate, vitamin E, levels and liver mitochondrial variables, including , respiratory activity, ATP production in the steady state, and hydrogen peroxide production, were assessed at various time points (6, 24, and 36) h after surgery. mRNA gene expression of uncoupling protein 2 (UCP 2) was quantify in the liver.

Measurements and Main Results: In the CLP group's hypotension was associated with an increased in plasma lactic acid, ALT and decrease of plasma albumin. Increase in plasma nitrite/nitrate, and decrease in vitamin E was also observed. 24 h after CLP, we noted that mitochondrial cytochrome-C oxidase activity, oxygen consumption, ATP synthesis are decreased. Mitochondrial uncoupling of ATP synthesis to oxygen consumption occurring at 36 h after CLP.

A persistent increased in the mitochondrial state 4 respiration from 24 h, associated to upregulation of mRNA gene expression of UCP 2, and increased in mitochondria hydrogen peroxide production was noted in the same group's.

Conclusion: The late septic shock comes along of a decrease in mitochondrial ATP and oxygen fluxes, associated with the increased in inner membrane permeability as showed by increased in state 4 and upregulation of UCP 2.

47. Mitofusin 2 Promotes Renal Epithelial Cell Survival After Metabolic Stress. JONATHAN M. GALL,[1] ZHIYONG WANG,[1] MARC LIESA,[2] ANTHONY MOLINA,[2] ANDREA HAVASI,[1] JOHN H. SCHWARTZ,[1] ORIAN SHIRIHAI,[2] STEVEN C. BORKAN,[1] and RAMON G.B. BONEGIO,[1] *[1]Renal Section, Boston Medical Center, Boston, MA; [2]The Obesity Center, Boston Medical Center, Boston, MA*

The role of mitofusin 2 (MFN2), a key regulator of mitochondrial morphology and function in the renal stress response is unknown. To assess its role, the MFN2 floxed gene was conditionally deleted in the kidney of mice (MFN2 cKO) by Pax2 promoter driven Cre expression (Pax2Cre). MFN2 cKO caused severe mitochondrial fragmentation in renal epithelial cells, a site critical for normal organ and tubular function. In newborn cKO pups, renal histology, organ or tubular function did not differ from littermate Cre-negative pups. Furthermore, profound organelle fragmentation caused by MFN2 deficiency did not significantly alter baseline oxygen consumption or increase apoptosis in proximal tubule cells in primary culture. In contrast, ATP depletion, a stress that resembles acute ischemia, resulted in greater injury to the mitochondrial outer membrane and an 80% increase in apoptosis in MFN2 deficient *vs.* control cells. Despite similar stress-induced Bax 6A7 epitope exposure, MFN2 deficiency significantly increased mitochondrial Bax accumulation associated with greater release of both apoptosis

inducing factor and cytochrome c. These data show that mitochondrial fragmentation caused by MFN2 deficiency plays a permissive role during renal development but is critical for renal epithelial cell survival under stress. Despite equivalent Bax activation, MFN2 deficiency increases renal cell injury partly by increasing the accumulation of Bax, a primary cause of stress-induced outer membrane injury and apoptosis. Interventions directed at mitochondrial remodeling may preserve renal function by inhibiting stress-induced apoptosis of renal epithelial cells.

48. Mitochondria-Related Gene Expression Changes Are Associated with Fatigue in Patients with Non-metastatic Prostate Cancer Receiving External Beam Radiation Therapy. CHAO-PIN HSIAO,[1] DAN WANG,[1] ARADHANA KAUSHAL,[2] and LEOREY SALIGAN,[1] *[1]National Institute of Nursing Research and [2]National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892*

Background: To describe relationships between mitochondria-related gene expression changes and fatigue in prostate cancer patients receiving external beam radiation therapy (EBRT).

Methods: A prospective, exploratory, and repeated measures design was used. Self-report questionnaires and peripheral whole blood samples were collected at 7 time points. The Human Mitochondria RT^2 Profiler™ PCR Array was utilized to identify differential regulation of genes involved in mitochondrial biogenesis and function.

Results: There were significant changes in fatigue scores (P = .02–.04) and mitochondria-related gene expression (P = .00–.05) over time compared to baseline. Mean fatigue score was 1.66 (SD = 1.66) at baseline, 3.06 (SD =1.95) at midpoint of EBRT, 2.98 (SD = 2.20) at the end of EBRT, and 2.64 (SD = 2.56) at 4 wk after EBRT. 11 genes related to mitochondrial function and structure were differentially expressed over time during EBRT. Three of the 11 genes (*BCL2L1*, *FIS1*, and *SLC25A37)* were >2.5-fold up-regulated, while 8/11 genes were >2-fold-down-regulated (*AIFM2*, *BCL2*, *IMMP2L*, *MIPEP*, *MSTO1*, *NEFL*, *SLC25A23*, and *SLC25A4*). Eight of 11 genes were significantly associated with fatigue scores (P < .00–.04). Using pathway analysis, the network pathways of combined observations from four time points were depicted.

Conclusion: This study provides evidence that 11 genes related to mitochondrial function not only were significantly expressed during EBRT but also 8 of the 11 genes were significantly related to the changes in fatigue reported by prostate cancer patients during EBRT. These findings provide the identification of possible pathways, early biomarkers, and novel intervention targets of cancer treatment-related fatigue.

49. The Influence of Diet in Rat Hepatic Mitochondria Physiology and Membrane Composition: Rapeseed Oil As a Case Study. J.P. MONTEIRO,[1] C. PEREIRA,[1] E. MACIEL,[2] I. BALDEIRAS,[3] F. PEIXOTO,[4] M.R. DOMINGUES,[2] A.S. JURADO,[1] and P.J. OLIVEIRA,[1] *[1]Center for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal; [2]Mass Spectrometry Centre, QOPNA, Department of Chemistry, University of Aveiro, Portugal; [3]Laboratory of Neurochemistry, Department of Neurology, Coimbra University Hospital, Portugal; [4]CECAV–Centre of Animal Sciences and Veterinary/Chemistry Department, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

It is now evident that the diet is related with physiological changes occurring in organisms. However, still a large avenue remains unopened regarding the role of diet on mitochondrial physiology. In the present work, we investigated the effects of a rapeseed oil-based diet on rat mitochondrial bioenergetics and membrane composition. Wistar rats were fed either a modified or a custom rodent diet, with differences between groups assessed at different times (11, 22, and 33 d). Considerable differences on mitochondrial membrane composition were noted, either regarding the relative composition in terms of the fatty acids present (with a decrease in the saturated to unsaturated ratio observed with the modified diet), either regarding phospholipid class proportions (an increase in the PC:PE ratio under the same conditions). Mass spectrometry showed significant differences on the major species of cardiolipin present, with predictable increased incorporation of oleic acid as a result of exposure to the modified diet. Rapeseed oil diet decreased membrane fluidity, but only on the outer regions of the membrane bilayer, as determined by the use of DPA-PH as probe. Regarding mitochondrial physiology, rats subjected to our modified diet have decreased hepatic mitochondrial state 3 respiration, which becomes evident after 22 d, and are more susceptible to Ca^{2+}-induced transition pore induction. Regarding differences on oxidative stress indicators, the rapeseed oil-enriched diet seemed to promote a decrease in hydroperoxide production by the respiratory chain, but no differences in protein carbonyl groups were detected. In conclusion, the results indicate that a rapeseed diet causes alterations on mitochondrial bioenergetics, which may result from membrane remodeling. Such alterations may impact not only energy supply to the cell, but also the resistance of the hepatocyte to drug-induced dysfunction, as mediated by mitochondria.

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50. Mitochondrial Dysfunction and Neuronal Injury Mediated by Voltage-Gated Calcium Channels. N.B.

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Excessive calcium influx, triggering mitochondria-mediated death signaling, is an essential step in glutamate excitotoxicity. Although it is clear that injurious glutamate-dependent calcium influx occurs mainly through NMDA receptors, there is a much uncertainty concerning the contributions of other potential calcium loading pathways such as voltage-gated calcium channels (VGCCs). We have found that in the majority of neurons in hippocampal and cortical cultures, maximal L-type VGCC activation induces much lower calcium elevations than toxic NMDA receptor activation. Consequently, and consistent with the "calcium load" hypothesis of excitotoxicity, few depolarization-activated neurons exhibit calcium deregulation, mitochondrial dysfunction and cell death. However, in a small subset of neurons, VGCC activation evoked stronger calcium elevations, approaching those induced by toxic NMDA. These neurons are characterized by elevated expression of VGCCs and enhanced voltage-gated calcium currents, mitochondrial depolarization and cell death. Preventing VGCC-dependent mitochondrial calcium loading (with FCCP) resulted in stronger cytoplasmic calcium elevations, while conversely, facilitating mitochondrial calcium accumulation (with CGP 37157) accelerated mitochondrial depolarization. Both these observations further implicate VGCC activation in mitochondria-mediated cell death. Addition of extracellular zinc facilitated VGCC-dependent mitochondrial calcium overload and dysfunction. We conclude that there are several disease-relevant scenarios in which VGCC-mediated amplification of mitochondrial calcium loading may well contribute to neuronal degeneration.

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*A Bioenergetic Etiology for Complex Diseases: Interaction between Mitochondrial Genetics and the Epigenome. **DOUGLAS C. WALLACE**, *Center for Mitochondrial and Epigenomic Medicine, Children's Hospital of Philadelphia, and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104*

Bioenergetic dysfunction is being found to be a common factor in a wide range of rare and common diseases. Rare diseases associated with more severe mutations in nuclear DNA (nDNA) and/or mitochondrial DNA (mtDNA) encoded bioenergetic genes now constitute a well established class of diseases. These mitochondrial diseases encompass phenotypes ranging from lethal childhood Leigh syndrome to adult-onset diabetes mellitus and deafness. Because bioenergetics requires the interaction of over a thousand nDNA genes as well as the mtDNA oxidative phosphorylation genes, the inheritance patterns of mitochondrial diseases can be highly complex, thus explaining why the medical community was slow to appreciate the prevalence and importance of these diseases.

Characterization of the genetics, biochemistry, and physiology of mitochondrial diseases has produced concepts that are applicable to a wide spectrum of metabolic and degenerative diseases, cancer, and aging. The importance of bioenergetic dysfunction in the common diseases initially escaped the attention of the Western medical community because Western medical thinking was based on a predominately anatomical perspective of disease and a Mendelian perspective of genetics. These traditional conceptual frameworks led to the exhaustive search for nDNA encoded genetic variants that account for the common diseases, e.g., common diseases must be caused by common nDNA variants. Regrettably, these studies failed to identify the anticipated nDNA variants.

A major clue as to why the search for common disease variants in the nDNA was unproductive comes from the fact that the environment pays a major role in the etiology of the common diseases. For most organisms, the most important factor in the environment is the availability and utilization of calories (energy). Anatomy only becomes relevant to energy exploitation at the species level where differences in anatomy permit utilization of different sources of energy, e.g., the different beak shapes of Darwin's finches. However, since all of the individuals within a species such as *Homo sapiens* exploit the same categories of calories, their anatomy is constant. What is important within a species is regional and seasonal differences in the types, abundance, and demands of the available calories. Adjusting to regional bioenergetic differences requires stable changes in cellular energetics occurring over thousands of years. This is most readily achieved by the accumulation of functional variants in the mtDNA bioenergetic genes, since the mtDNA has a high mutation rate and the most deleterious mtDNA mutations are eliminated before fertilization via an intra-ovarian selective system. Cyclic seasonal changes are most readily addressed by changes in gene expression, since mutations are irreversible. These cyclic changes are achieved by alterations in the epigenome which permits coordinate expression of the hundreds of nDNA-encoded bioenergetic genes. Epigenomic regulation of bioenergetic genes is cued to the energy environment through protein modifications mediated by high energy cellular intermediates (ATP, acetyl-CoA, S-adenosyl-methionine, reactive oxygen species, and redox state) which fluctuate depending on the availability of environmental calories. Thus, the common variants that predispose to common diseases are predominantly due to alterations in the mtDNA and the epigenome, not the nDNA.

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