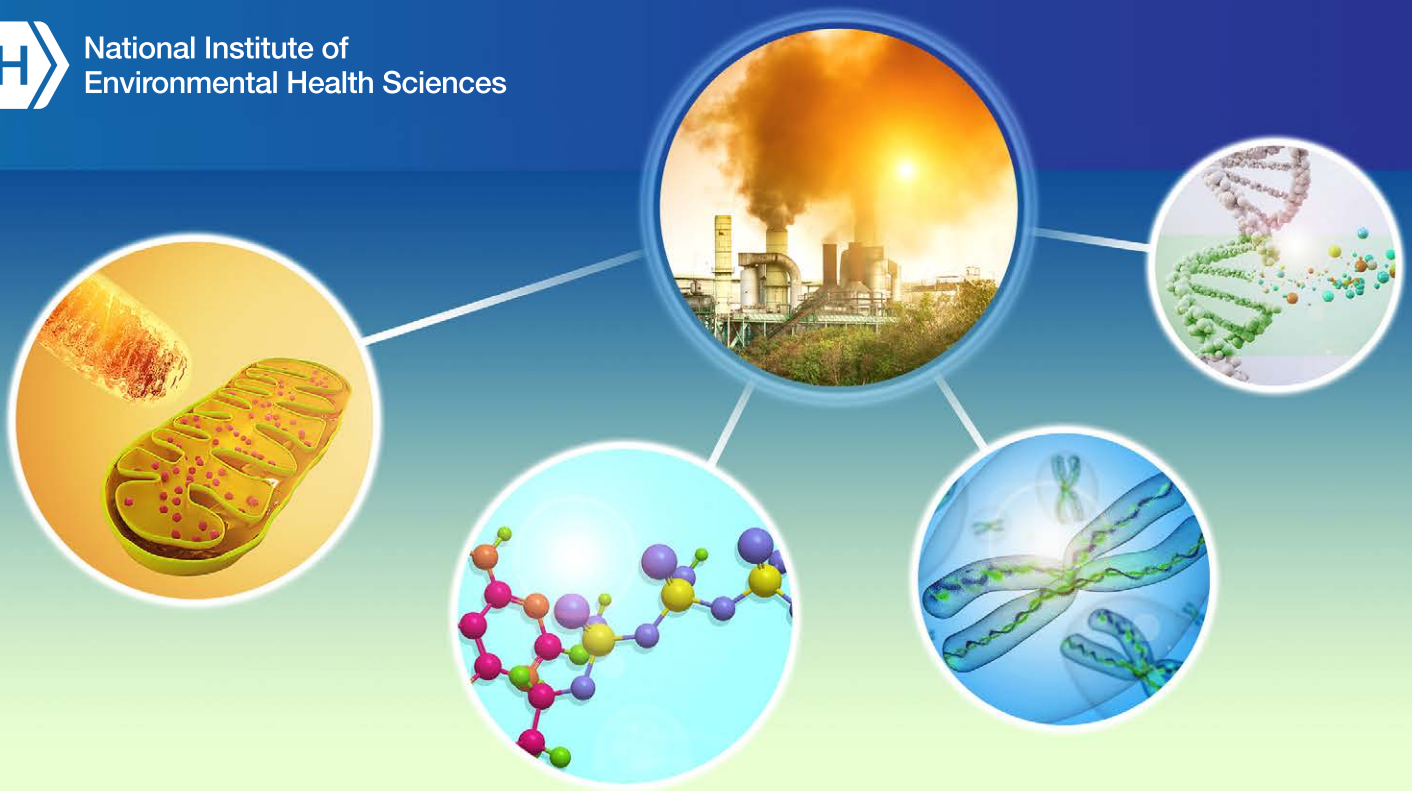
National Institute of
Environmental Health Sciences



New Approaches for Characterizing Mitochondrial Signaling in Response to Environmental Stress Grantee Meeting

Wednesday, November 7, 2018

8:30 a.m. – 5:00 p.m.

NIEHS Building 101, Rodbell Auditorium
Research Triangle Park, N.C.

National Institutes of Health • U.S. Department of Health and Human Services

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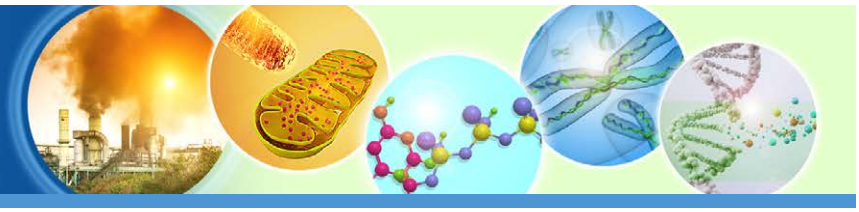
Agenda

National Institute of Environmental Health Sciences



New Approaches for Characterizing Mitochondrial Signaling in Response to Environmental Stress

November 7, 2018

NIEHS Building 101, Rodbell Auditorium • 111 TW Alexander Drive, Research Triangle Park, N.C.

AGENDA

7:30 a.m. **Registration**

8:30 a.m. **Welcome and Introductions**
Rick Woychik, Deputy Director, NIEHS

Grantee Updates – Session One

Moderator: Kimberly McAllister, NIEHS

8:45 a.m. **ROS Driven Mitochondrial-Telomere Dysfunction During Environmental Stress**
Ben Van Houten and Patricia Opresko, University of Pittsburgh

9:15 a.m. **Inducible Mouse Models of Mitochondrial ROS Signaling and Environmental Stress**
Gerald Shadel, Salk Institute for Biological Studies

9:45 a.m. **New Oxidation-Sensing Probes to Evaluate Mitochondrial Dysfunction in Lung Injury**
Cristina Furdui and Bruce King, Wake Forest University

10:15 a.m. **Break**

Grantee Updates – Session Two

Moderator: Fred Tyson, NIEHS

10:30 a.m. **Study of the Mitochondrial-Cellular Response to Environmental Stress by Fluorescence Imaging**
Gyorgy Hajnoczky and Gyorgy Csordas, Thomas Jefferson University

11:00 a.m. **Imaging Heme Based Mitochondrial-Cell Signaling Networks in Cell and Animal Model**
Amit Reddi, Georgia Institute of Technology

11:30 a.m. **RT-SQuARED-M Method to Study the Precise Role of Mitochondria in the Oxidative Damage Caused by Environmental Toxins**
Kasturi Mitra, University of Alabama at Birmingham

Noon **Lunch**

1:15 p.m. **Histone Tails As an Energy Reservoir for Cellular Metabolism**
Raul Mostoslavsky, Massachusetts General Hospital

Grantee Updates – Session Three

Moderator: Dan Shaughnessy, NIEHS

1:45 p.m. **Imaging Mitochondrial Signaling in B-Cells Ectopically Implanted in the Eye**
Carlos Moraes, University of Miami

2:15 p.m. **Irgm1 Reveals New Connections Between Mitochondrial Quality Control and Autoimmunity**
Michael Fessler, Acting Chief, Immunity, Inflammation, and Disease Laboratory, NIEHS

2:45 p.m. **Break**

3:00 p.m. **Mitochondrial Dysfunction in Pathobiology**
John Lemasters, Medical University of South Carolina

3:30 p.m. **Mitochondrial Dynamics in the Regulation of Energy Expenditure and Fuel Preference**
Orian Shirihai, UCLA

4:00 p.m. **Panel Discussion**
Moderator: Janine Santos, NIEHS

5:00 p.m. **Meeting Ends**

Presentation Abstracts

ROS Driven Mitochondrial-Telomere Dysfunction During Environmental Stress

Ben Van Houten, Patty Opresko
University of Pittsburgh

Maintenance of mitochondrial and telomere function are critical for healthy aging, and significant cross-talk occurs between these distinct compartments. Many environmental factors cause mitochondrial dysfunction and subsequent generation of reactive oxygen species (ROS), which have been proposed to induce telomere damage and shortening. The purpose of this project is to test the hypothesis that oxidative damage at telomeres cause mitochondrial dysfunction, and conversely that oxidative damage to mitochondrial DNA cause telomere dysfunction, and that this reciprocal damage contributes to several environmentally-induced human diseases, including neurodegeneration in Parkinson's disease (PD). For this we developed a highly innovative targeted method for causing oxidative damage in either the mitochondria or at telomeric DNA, in the absence of damage to other regions. We find that a mitochondrial-targeted fluorogen-activating-peptide (Mito-FAP) and a malachite green dye analog, which specifically targets singlet oxygen to Complex IV when irradiated with 660 nm light, induces rapid mitochondrial dysfunction in HEK293 cells. By controlling the amount of dye and light exposure we found conditions to reduce mitochondrial respiration to 10% of control within 4 hrs of damage, as measured by Seahorse Bioanalyzer. This singlet oxygen damage to the mitochondrial electron transport chain (ETC) caused a second wave of mitochondria-generated superoxide and loss of Complex I, III, and IV activities. Rho zero cells, lacking respiration, were refractory to the second wave of superoxide production. This persistent mitochondria-induced ROS caused a loss of mitochondrial membrane potential and inhibition of cell growth, without apoptosis. Using a novel ratiometric imaging approach we observed oxidation of Cys in the nucleus due to mitochondrially generated hydrogen peroxide. While COMET assays indicated no overall increase in nuclear damage, we observed telomere loss and fragile telomeres 48 hr after mito-FAP-dye plus light damage. We adapted this system to damage mitochondria in live zebrafish, and find Mito-FAP activation in the CNS can ablate mitochondrial function in 5-day old zebrafish embryos. In parallel experiments, we targeted damage to the telomeres by FAP tagging telomere binding protein TRF1 (FAP-TRF1) and validated this system in tractable cancer cell lines. We demonstrate that activation of the FAP bound dye selectively induces the common oxidative lesion 8-oxoguanine (8-oxoG) exclusively at the telomeres. A single round of 8-oxoG formation at telomeres caused no obvious telomeric or growth defects in the cancer cells. However, we found repeated cycles of telomere damage over 24 days induced several hallmarks of telomere crisis. In stark contrast, a single round of 8-oxoG induction in normal human fibroblast and epithelial cell lines increased cellular senescence and triggered p53 and p21 activation. Having established this system in normal cells with intact checkpoint signaling and stress responses, we are now poised to examine consequences of telomere damage on mitochondria integrity and function. This project builds tools and capacity for examining ROS-mediated mitochondrial cross-talk in response to environmental stressors. Completion of this project will lay the foundation for developing new interventions to better mitigate the negative effects of environmental exposures on telomere and mitochondria function, serving to ameliorate or delay aging-related diseases and pathologies.

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Mitohormesis in Mice via Sustained Basal Activation of Mitochondrial and Antioxidant Signaling

Gerald Shadel
Salk Institute for Biological Studies

Transient mitochondrial stress can promote beneficial physiological responses and longevity, termed "mitohormesis." To interrogate mitohormetic pathways in mammals, we generated mice in which mitochondrial superoxide dismutase 2 (SOD2) can be knocked-down in an inducible and reversible manner (iSOD2-KD). Depleting SOD2 only during embryonic development did not cause post-natal lethality, allowing us to probe adaptive responses to mitochondrial oxidant stress in adult mice. Liver from adapted mice had increased mitochondrial biogenesis and antioxidant gene expression and fewer reactive oxygen species. Gene expression analysis implicated non-canonical activation of the Nrf2 antioxidant and PPARγ -PGC-1α mitochondrial signaling pathways in this response. Transient SOD2 knock-down in embryonic fibroblasts from iSOD2-KD mice also resulted in adaptive mitochondrial changes, enhanced antioxidant capacity, and resistance to a subsequent oxidant challenge. We propose that mitohormesis in response to mitochondrial oxidative stress in mice involves sustained activation of mitochondrial and antioxidant signaling pathways to establish a heightened basal antioxidant state.

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Mitochondria-targeted Probes for Imaging Protein Sulfenylation

Cristina M. Furdui[1], S. Bruce King[2]
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Mitochondrial reactive oxygen species (ROS) are essential regulators of cellular signaling, metabolism and epigenetics underlying the pathophysiology of numerous diseases. Despite the critical function of redox regulation in mitochondria, there are limited methods available to monitor protein oxidation in this key subcellular organelle. We will present recently published data on compounds for imaging sulfenylated proteins in mitochondria: DCP-NEt_2-Coumarin (DCP-NEt_2C) and rhodamine-based DCP-Rho1, and emerging results on the first multi-functional DCP-NEt_2C-Bio with the added capability of enabling pulldown of sulfenylated proteins. Studies are presented on the reactivity of compounds with a model protein sulfenic acid (AhpC-SOH) and mitochondrial localization to identify optimized experimental conditions for labeling, visualization and enrichment of sulfenylated proteins that would be independent of mitochondria membrane potential and not impact mitochondrial function. These probes were applied to image mitochondrial protein sulfenylation under conditions of serum starvation and in a cell culture model of lung cancer exposed to ionizing radiation and silver nanoparticles, agents serving dual functions as environmental stressors and cancer therapeutics.

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Study of the Mitochondrial-Cellular Response to Environmental Stress by Fluorescence Imaging: Developing Novel Tools for Monitoring Mitochondria-ER Local Calcium2and ROS Signaling

György Csordás, György Hajnóczky
Thomas Jefferson University

Environmental agents are associated with altered ROS production, dysregulation of Ca2+ homeostasis and mitochondrial cell death. However, the causative pathways remain largely unknown partly because the role of mitochondria in cell survival is mediated through local communication between mitochondria and other organelles, which have been difficult to directly monitor. Our hypothesis is that stress induced by several environmental agents like arsenic (As) impairs mitochondria-endo/sarcoplasmic reticulum (Mito-ER/SR) functional and structural coupling leading to altered mitochondrial Ca2+ and ROS homeostasis. Our aim was to set up organelle-targeted fluorescent protein based technology to study the effect of environmental agents such as As on Ca2+ and ROS homeostasis, paying special attention to the local Mito-ER/SR communication. We have generated and validated a collection of fluorescent sensors targeted to the Mito-ER interface for measurement of local [Ca2+] and redox. Each construct has been produced as plasmid DNA and selected constructs have already been inserted to a viral vector to quantitatively evaluate the effect of As on the local arrangements of ROS, Ca2+ and organellar structure. In vivo liver imaging of mice treated with 100ppm As in the drinking water for 11 days revealed suppression of the fast cytoplasmic [Ca2+] rise triggered by glucagon and increase in the mitochondrial Ca2+ uptake. In complementing, isolated hepatocyte studies, As-induced a dose and time dependent increase in ROS exposure, which was accompanied by suppression of IP3-induced Ca2+ signaling. The changes were stronger at the Mito-ER interface than in the bulk cytoplasm. Antioxidant pre-treatment abolished the As-induced Ca2+ dysregulation, suggesting that the IP3R desensitization was induced by ROS. Despite the decreased IP3-linked Ca2+ mobilization, As exposure stimulated mitochondrial Ca2+ uptake without any change in the expression of the Ca2+ uniporter components. This might be caused by the increased tightness of the Mito-ER contacts observed after As exposure. In conclusion, our studies have created a novel fluorescence-based toolkit for Mito-ER monitoring and suggest that the As-induced liver injury might be mediated by an early increase in ROS, which causes impaired local Mito-ER structural and Ca2+ communication.

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Imaging Heme Based Mitochondrial-cell Signaling Networks in Cell and Animal Models

Amit Reddi
Georgia Institute of Technology

As mitochondria are central hubs for energy metabolism and formation of intermediate metabolites, they must be able to transmit signals that communicate alterations in its functioning to the nucleus so that cells can remodel metabolism in response. Mitochondrial-nuclear signaling is particularly important when mitochondrial function is compromised by environmental toxicants, including heavy metals. However, **the nature and dynamics of these inter-organelle signals and the mechanisms by which they are transduced are poorly understood**. Given the importance of heme-regulated transcription factors that control diverse cellular processes from energy metabolism to the anti-oxidant stress response to cell proliferation, and the exquisite sensitivity of mitochondrial heme homeostasis to heavy metals, we propose that mitochondrial-derived heme signals the cellular response to heavy metal toxicity through mitochondrial-nuclear retrograde regulation. However, the chemical and genetic tools available to study the transmission of heme-based signals do not exist. In this presentation, I will describe the development and application of a new class of genetically encoded ratiometric fluorescent heme sensors, heme affinity reagents, and proteomics approaches to study the transduction of heme-based signal transduction in response to cellular stress.

RT-SQuARED-M Method to Study the Precise Role of Mitochondria in the Oxidative Damage Caused by Environmental Toxins.

Kasturi Mitra
University of Alabama at Birmingham

Mitochondrial fission and fusion oppose each other and determine the dynamics in mitochondrial structure (mtDynamics). The details of the bidirectional relationship between mitochondrial dynamics and redox status are unclear and the methods to integrate these mitochondrial aspects are lacking. Moreover, it has been hypothesized that mtfission/fusion can create hot-spots for mtROS production. Thus, we developed a microscopy based analytical method of Real Time Simultaneous Quantitative Assessment of the Redox Environment and Dynamics of Mitochondria (RT-SQuARED-M). RTSQuARED-M detects mtFission/Fusion events and mtROS at specific mitochondrial sites (local), in mitochondrial subpopulations within a cell (zonal) and in the total mitochondrial population in a cell (global). We used RT-SQuARED-M to probe if oxidative environmental carcinogens, like TCDD, influence the relationship between mtROS and mtDynamics, and thus modulate the mtROS hotspots. We found that mtFusion events may underlie mtROS hot-spots, and TCDD driven skin carcinogenesis is initiated by alteration of mtDynamics driven mtROS production. TCDD alters DRP1 functionality, and genetic modulation of Drp1 alters the RT-SQuARED-M output to modulate the TCDD effects. Moreover, RTSQuARED- M can be generally used to investigate the quantitative relationship of mtDynamics (fission/fusion) and mtEnergertics (ATP/ROS). Using global RT-SQuARED-M (which we have named Mito-Single Cell-SQuARED or Mito-Since2) we found that tumor initiating cells interconvert between 3 states with distinct mitochondrial dynamics/energetics relationships, where the relationship of mitochondrial dynamics with ATP or redox environment are mutually exclusive. RT-SQuARED-M, is anticipated to become a significant addition in the standard tool kit for studying the basic aspects of mitochondrial biology and also as a translational tool for drug screening or personalized therapies.

Histone Tails as an Energy Reservoir for Cellular Metabolic Functions

Raul Mostoslavsky
Harvard University

Metabolism represents the organic and chemical processes within a cell that are necessary to maintain life. It is also the way that a cell interacts with energy sources, coordinating energy intake, storage and utilization, ultimately allowing proper cellular growth and function. A key molecule in metabolism is Acetyl-CoA, a molecule sitting at the crossroads of multiple metabolic pathways, while also serving as a substrate for histone acetylation, one of the main epigenetic marks in the genome. Recent studies have demonstrated that fluctuations in availability of Acetyl-CoA can directly impinge on levels of histone acetylation, yet whether the reverse is possible remains unknown. In other words, <u>may acetate from histone marks serve as a reservoir to modulate Acetyl-CoA levels in order to sustain metabolic homeostasis? And if so, can changes in the epigenome affect metabolism due to a direct effect on acetate availability?</u> In this context, environmental stresses such as ionizing radiation or hypoxia may directly affect the epigenome, in turn affecting its potential to buffer Acetyl-CoA levels. This hypothesis represents the main focus of this R21/R33 proposal. Specifically, in the first exploratory (R21) phase we have: **1**- Developed a pulse-chase assay using stable-isotope tracing to follow crosstalk between the nucleus and the mitochondria. **2**- Determined the role of the NAD-dependent histone deacetylase SIRT6 in mobilizing acetate from chromatin under conditions of environmental stress. These experiments set the basis to develop the second R33 phase, where we will: **3**- Analyze the role of environmental and nutrient stress in modulating this epigenetics-metabolism crosstalk and **4**- Determine the in vivo significance for the nucleus as an energy reservoir. All together, these experiments should provide direct evidence for a role of the nucleus as a metabolic reservoir, which may point to strategies where modulation of epigenetic marks may serve as a strategy against metabolic diseases and environmental stresses.

Imaging Mitochondrial Signaling in Beta cells Ectopically Implanted in the Eye

Carlos Moraes
University of Miami, Miller School of Medicine

We are establishing imaging techniques and procedures to measure physiological changes in mitochondrial function and signaling. The detection of these signals will allow us monitoring adaptive responses in mitochondria. The model we are using is the insulin secreting beta cell of the mouse pancreatic islet, which can be studied both ex vivo and in vivo. We have developed a procedure where islets can be imaged after being transplanted into the anterior chamber of the mouse eye. The transparent cornea allows for repeated non-invasive in vivo imaging of fluorescent biosensors expressed in beta cells of islet grafts. When streptozotocin-treated diabetic mice are used as transplant recipients, islet grafts not only restore normoglycemia but are also in charge of glucose metabolism. Thus, local imaging readouts can be associated with systemic metabolic readouts. We have also modified existing fluorescent biosensors to study changes in mitochondrial function and signaling in beta cells, including: hydrogen peroxide, ATP, Ca++, NAD+/NADH and pH.

We are using these approaches to study beta cells with defects in different mitochondrial oxidative phosphorylation function, including defects in complex I, III and IV. In addition, we will also determine the effects of the environmental toxins, cadmium and manganese on mitochondrial function using in vivo imaging.

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Irgm1 Reveals New Connections Between Mitochondrial Quality Control and Autoimmunity

Michael Fessler
National Institute of Environmental Health Sciences

Irgm1 is an interferon-γ (IFNγ)-inducible immunity-related GTPase that is thought to support autophagic clearance of both invasive microbes (xenophagy) and defective mitochondria (mitophagy). Recently, we reported that Irgm1-deficient mice have a spontaneous autoimmune exocrinopathy accompanied by increased autoantibodies and induction of IFN-stimulated genes (ISGs) (Azzam et al., JCI Insight 2018). We hypothesized that the spontaneous IFN response observed in Irgm1 deficiency is driven by cGAMP synthase (cGAS)-dependent sensing of uncleared mitochondrial DNA (mtDNA). Here, we show that the abnormal induction of type I IFNs and ISGs in Irgm1-/- macrophages and murine embryonic fibroblasts is associated with defective macro-autophagic flux, decreased mitochondrial membrane potential, and increased cytoplasmic mtDNA. The aberrant cell-autonomous IFN response is normalized upon depletion of cellular mtDNA and following oligomycin/antimycin-enforced mitophagy. Further, silencing or inhibition of the dsDNA sensor cGAS, the adaptor protein stimulator of IFN genes (STING), TANK-binding kinase 1 (TBK1), or IFN regulatory factor 3 (IRF3) also reduced the exaggerated IFN response, together suggesting that a mtDNA-cGAS-STING-IRF3 axis drives the abnormal type I IFN signature of Irgm1-deficient cells. Genetic deletion of the type I IFN receptor rescues the in vivo autoimmune tissue pathology and excess serum autoantibody levels of Irgm1-/- animals, demonstrating the pathogenicity of type I IFN in these animals. Sting deletion was also noted to partially reverse the autoimmune tissue pathology. Taken together, our findings support a model wherein Irgm1-mediated mitochondrial maintenance represses inappropriate mtDNA-dependent activation of autoinflammatory IFN responses. We propose that this fundamental mechanism may play a key role in suppression of type I interferonopathy syndromes.

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Mitochondrial Dysfunction in Pathobiology

John Lemasters
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Mitochondria contribute to pathogenesis in a number of ways. In the mitochondrial permeability transition (MPT), the mitochondrial inner membrane abruptly becomes permeable to solutes up to 1500 Da. Ca^{2+}, oxidative stress, and numerous reactive chemicals induce the MPT, which leads to ATP depletion and consequent necrotic cell killing, as well as mitochondrial swelling causing cytochrome *c* release and apoptosis. The MPT is the penultimate event leading to lethal cell injury after ischemia/reperfusion, drug-induced hepatotoxicity, oxidative stress and other pathologies. When liver is exposed to ethanol *in vivo*, acetaldehyde (AcAld) generation causes hepatic mitochondrial depolarization (mtDepo), which stimulates oxygen consumption. In parallel, mitochondrial voltage dependent anion channels (VDAC) close. Together, VDAC closure and respiratory stimulation promote more rapid oxidation of membrane permeant AcAld by mitochondrial aldehyde dehydrogenase-2. mtDepo also stimulates mitophagy. After chronic ethanol, mitophagic processing becomes impaired, leading to release of mitochondrial damage-associated molecular pattern (mtDAMP) molecules promoting hepatitis and fibrosis. Aldehyde generation in nonalcoholic steatohepatitis and after vinyl chloride exposure may lead to similar events to account for the nearly identical histopathologies of these conditions. In cancer, VDAC closure also occurs. In addition, mitochondrial ATP/ADP exchange appears to change from an electrogenic to a non-electrogenic mechanism. These events increase cytosolic ADP/ATP to promote the Warburg metabolic phenotype of aerobic glycolysis. Overall, mitochondria contribute to pathogenesis by multiple different mechanisms.

Mitochondrial Dynamics in the Regulation of Fuel Preference and Energy Expenditure

Orian Shirihai
University of California, Los Angeles, David Geffen School of Medicine

Mitochondrial fusion and fission allow for the distribution of mitochondrial component across the mitochondria population of the cell. Each mitochondrion on average goes through a cycle of fusion and fission ever 5-30 minutes, allowing matrixes of the entire mitochondrial population to exchange content and reach homogeneity within an hour. Through a similar process, inner membrane components reach homogeneity in less than a day. While homogenization of the mitochondrial population is an integral part of mitochondrial biogenesis, it may prevent the generation of a unique population of mitochondria within the same cellular compartment. We have recently reported that mitochondria surrounding lipid droplets (Peri-Droplet Mitochondria; PDM) maintain a unique proteome which is not equilibrated with the rest of the mitochondria population. We find that the PDM remain on the lipid droplet and do not fuse with the cytosolic mitochondria. Upon activation of lipid consumption by adrenergic stimulation, PDM depart the lipid droplet. We developed a procedure to purify the PDM so that they can be compared to cytosolic mitochondria. We find that PDM have superior capacity to metabolize pyruvate and reduced capacity to oxidize fatty acids, as compared to cytosolic mitochondria. We developed a system where we can measure fatty acid incorporation into triglyceride so that the capacity to build lipid droplets can be studied. Using this approach we find that peri-droplet mitochondria enhance lipid droplet expansion and that this is attributed to mitochondrial ATP Synthesis.

New studies identify conditions that promote mitochondria and lipid droplet interactions and those that remove mitochondria from lipid droplet. Analysis of PDM in mice and human reveals that PDMs can be found in every tissue we have tested thus far, but their isolation is difficult in tissues that store small lipid droplets. We find strong correlation between the occurrence of PDM and diet as well as to insulin sensitivity.

Regulation of Energy Expenditure by Calcium: Adrenergic stimulation of Brown Adipocytes activates mitochondrial energy expenditure. This process is requiring the import of calcium into the mitochondria. Import is balanced with export, to allow for the activation of TCA cycle enzymes. We find that the Na/Ca exchanger plays a key role in calcium export and that activation of energy expenditure is absent in the absence of the exporter. Microscopy reveals that the activation of thermogenesis in cells lacking the Ca exporter lead to permeability transition and cell death. Inhibition of the PTP using Cyclosporine analogues result in complete rescue of brown adipose tissue function in vitro and in vivo.

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Research Triangle Park, N.C.

National Institute of Environmental Health Sciences

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