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Cardiolipin Reorganization and Phase Transition Induced by Dynamin-Related Protein 1 Facilitates Mitochondrial Membrane Fission

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Cardiolipin (CL) is a unique, dimeric phospholipid essential for mitochondrial dynamics in eukaryotic cells. Dynamin-related protein 1 (Drp1), a member of the dynamin superfamily of large GTPases, maintains the balance of mitochondrial division and fusion by rapidly catalyzing mitochondrial fission. Although recent studies have indicated a role for CL in stimulating Drp1 self-assembly as well as GTPase activity on the mitochondrial surface, the exact mechanism by which CL functions in membrane fission remains unclear. Here we use a variety of fluorescence spectroscopic and imaging approaches, together with model membranes, to demonstrate that Drp1 and CL function cooperatively in effecting membrane fission in three distinct steps: (i) Drp1's preferential association with unconstrained, fluid-phase CL molecules located at a high spatial density in the membrane bilayer, (ii) CL's reorganization in concert with Drp1 self-assembly, and (iii) CL's rapid phase transition from a lamellar, bilayer structure to an inverted hexagonal, non-bilayer configuration in the presence of Drp1 and GTP, resulting in the creation of localized membrane constrictions that are primed for fission. We propose that Drp1 thus catalyzes mitochondrial division.

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VDAC3 Forms Typical Voltage-Gated, Anion-Selective, and Tubulin-Sensitive Channels

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The Voltage Dependent Anion Channel (VDAC) forms the major pathway for metabolites across the mitochondrial outer membrane (MOM). Mammalian mitochondria have three isoforms of this protein: VDAC1, VDAC2, and VDAC3 which may play different roles in the regulation of mitochondrial functions. VDAC1 is the most studied isoform, since it is the major protein of the MOM in most cells, followed by VDAC2. Both, VDAC1 and VDAC2 reconstituted into planar membranes form voltage-dependent anion-selective channels whose conductance is modulated by dimeric tubulin. As counterpart, the poor channel-forming ability of VDAC3 together with its low proficiency in restoring normal growth in pore-less yeast led to hypothesize that this isoform does not form channels and could be just a modulator of the other two isoforms. We developed an improved protocol of purification and refolding of mouse recombinant VDAC3 that allowed us to fully characterize channels formed by VDAC3 reconstituted in planar lipid membranes and to compare its channel properties with those of VDAC1 and VDAC2. We observed the typical VDAC channel behavior of VDAC3, such as high conductive open state (3.5-4.1 nS in 1M KCl) and multiple low conductive states at potentials $\geq \pm 40$mV, selectivity for anions with $P_{Cl^-}/P_{K^+} = 1.7$, and typical voltage-gating. This suggests a structural homology of VDAC3 with the other two isoforms. Interestingly, the most distinct characteristic of VDAC3 is its significantly lower sensitivity to the blockage by tubulin, which would lead to an increased permeability of this channel to ATP and other metabolites. This might explain why the down-regulation of VDAC3 expression among the three VDAC isoforms in some cancer cells causes the most drastic decrease in their mitochondrial membrane potential, or why VDAC3 is predominantly expressed in the short-term high energy demanding cells, such as spermatozoa.

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Channeling of Mitochondrial Energy in Cardiac and Cancer Cells by the Metabolically-Dependent Outer Membrane Potential

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Mitochondria are the main source of energy in eukaryotic non-proliferating aerobic cells. In cancer cells, significant part of the energy metabolism is supported by the aerobic glycolysis. Oxidative phosphorylation metabolites cross the mitochondrial outer membrane through the voltage dependent anion channel (VDAC). The possibility of the voltage-gating functioning of VDAC under physiological conditions and proposed mechanisms of generation of the outer membrane potential (OMP) require further study. In this work, we present a computational thermodynamic analysis of a possible generation of OMP by the creatine kinase(CK)-VDAC and VDAC-hexokinase(HK) complexes, as well as by their intermembrane complexes with the adenine nucleotide translocator (ANT). The CK-VDAC and VDAC-HK complexes function as direct steady-state voltage generators, using the free energy of kinase reactions, while the ANT-CK-VDAC and ANT-VDAC-HK complexes allow the application of a part of the sum of the corresponding "kinase voltage" and the inner membrane potential to the outer membrane. The developed computational models demonstrate a high probability of generation of the metabolically-dependent OMP with magnitudes high enough to close free VDACs. We suggest that in case of cardiac cells, the CK-VDAC-mediated channeling of mitochondrial energy, earlier postulated by Saks and Wallimann, leads to the metabolically-dependent generation of a high OMP during systole, thus causing electrical closure of free VDACs and essentially avoiding the mitochondrial ATP usage by non-contraction system consumers. In case of cancer cells, that have a very high percentage of hexokinase attached to mitochondria, the electrical closure of free VDACs by OMP allows the preferential VDAC-HK-mediated channeling of the mitochondrial ATP to initiate the aerobic glycolysis by an anti-turbo mode. (Research grants #111852128625 and #520154531565, Colciencias).

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Alpha-Synuclein Blocks VDAC Suggesting Mechanism of Mitochondrial Regulation and Toxicity in Parkinson Disease

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Alpha-synuclein (α-syn), an intrinsically disordered neuronal protein, is implicated in the etiology of Parkinson disease (PD) and a number of other neurodegenerative dementias. Though recent research demonstrates the involvement of α-syn in a variety of mitochondrial dysfunction in neurodegeneration, the molecular mechanism of α-syn toxicity and its effect on neuronal mitochondria remain vague. We demonstrate a functional interaction between α-syn and the voltage-dependent anion channel (VDAC), the major conduit for ATP and other bioenergetics metabolites in the mitochondrial outer membrane. We found that at nanomolar concentrations, the full-length α-syn, its 45 amino acid C-terminal truncated mutant, as well as β- and α-synuclein isoforms induce reversible and highly voltage-dependent blockage of VDAC reconstituted into planar lipid bilayers. Binding parameters varied with each isoform, revealing the key role of the negatively charged C-terminal of synucleins in blocking a positively charged pore of VDAC. Synuclein-blocked states of VDAC differ in ionic selectivity, implying the existence of several conformation states of synuclein molecules in the channel pore. We propose a model of α-syn interaction with VDAC, in which the negatively charged C-terminus of α-syn enters the net-positive channel pore, providing a steric block for ATP flux. Experiments with a yeast strain deficient in VDAC1 demonstrate that α-syn toxicity in yeast depends on VDAC, revealing α-syn interaction with the channel in living cells. Thus, our findings show the long-sought physiological and pathophysiological roles for monomeric α-syn, which reconcile previous observations of various synuclein effects on mitochondrial bioenergetics.

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Mitochondrial DNA: The Heart of the Matter

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Cardiac conduction defects and cardiomyopathy are commonly reported in patients with inborn errors of metabolism harboring pathogenic mutations in either mitochondrial or nuclear DNA (mtDNA and nDNA, respectively). We hypothesized that the pathophysiology of inborn errors of metabolism varies due to synergistic heterozygosity between the two genomes. We examined a 13-generation Mennonite pedigree with autosomal recessive cardiomyopathy due a mutation in the adenine nucleotide translocator-1 (ANT1). Substantial variability in the progression of heart disease segregated with maternal lineage, and the severity of cardiomyopathy correlated with the mtDNA haplogroups (Strauss, et al 2013). To determine the causative nature of this correlation, we examined the influence of inherited mtDNA mutations on ANT1-cardiomyopathy in the mouse. We introduced homoplasmic mtDNA ND6 or COI missense mutations into the mouse female germ line, generating mice with complex I or IV deficiency, respectively, and analyzed Ant1-dependent cardiomyopathy on the different mtDNA backgrounds. On wt mtDNA background, the Ant1-/- mice developed a distinctive concentric dilated cardiomyopathy, characterized by substantial myocardial hypertrophy and ventricular dilation. Both COI and ND6 mtDNA mutations accelerated Ant1-/- age-dependent cardiomyopathy, as evidenced by ultrastructrural abnormalities, bioenergetic defects, mtROS production, sensitized mitochondrial permeability transition, increased mtDNA damage, and heart failure, which ultimately

attenuated the lifespan of Ant1-deficient mice. Our results prove mtDNA dictates the penetrance of age-related cardiomyopathy and mammalian lifespan. Therefore, therapeutics that most effectively preserve mitochondrial DNA and bioenergetics will provide the most promise for healthy aging.

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Activation of the Mitochondrial Permeability Transition Pore Leads to the Increase in Amount of C-Subunit of ATP Synthase Associated with Channel-Forming Complex of Polyhydroxybutyrate and Inorganic Polyphosphate

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Low permeability of the mitochondrial inner membrane is critical for maintaining the mitochondrial electrochemical potential - the driving force for ATP production. Acute stress conditions, lead to the increase in the mitochondrial inner membrane permeability due to the opening of the permeability transition pore (PTP). PTP allows free movement of ions and small molecules leading to mitochondrial depolarization, ATP depletion and cell death. Recent studies suggest that C-subunit of the mitochondrial ATP synthase plays a central role in PTP. Previous work in our laboratory showed that mitochondria contain non-protein complex composed of polyhydroxybutyrate, inorganic polyphosphate and calcium that forms an ion channel with properties resembling PTP. Here we explore the possibility of interactions between these non-protein components and C-subunit during the induction of PTP.

To induce PTP,isolated energized mitochondria were treated with calcium. Control mitochondria were treated with calcium either in the presence of ruthenium red, inhibitor of calcium uptake or Cyclosporin A, inhibitor of PTP. This was followed by a water-free chloroform extraction of channel forming fraction of PTP. Components of the extract were analyzed using immunoblot analysis. We found significantly increased amount of C-subunit associated with channel forming fraction extracted from mitochondria with activated PTP. In contrast, C-subunit was not detectable in the extract when Ruthenium Red was present and significantly decreased in the presence of Cyclosporin A or in the absence of calcium.

These results show that C-subunit is likely an interacting partner of the pore-forming complex of polyphosphate, calcium and polyhydroxybutyrate. We hypothesize that fully functional PTP requires calcium-induced formation of the pore made of both the complexed polymers and the c-subunit of ATP synthase

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Small-Molecule PKD Inhibitor Prevents Mitochondrial Fragmentation and Dysfunction during Gq-Protein Coupled Receptor Stimulation in Cardiac Cells

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Regulation of mitochondrial morphology and dynamics is crucial for the maintenance of various cellular functions in cardiomyocytes. Abnormal mitochondrial morphologies concomitant with mitochondrial dysfunction are frequently observed both in human heart failure (HF) and in animal HF models. However, it is still unclear which cardiac signaling pathways regulate mitochondrial morphology and function under pathophysiological conditions. Recent reports suggest that G_q-protein coupled receptor (G_qPCR) signaling pathways are critical for the development and progression of HF. Therefore, we hypothesize that G_qPCR stimulation induces mitochondrial fragmentation and dysfunction, which initiates cardiomyocyte death. We found that protein kinase D (PKD) activated by G_qPCR signaling was translocated to outer mitochondrial membrane (OMM) observed by Western blot analysis of cytosolic and mitochondria-enriched fractionated proteins and by live cell imaging of fluorescence resonance energy transfer (FRET). We also found that G_qPCR-mediated PKD activation induced mitochondrial fragmentation, leading to increased reactive oxygen species (ROS) generation as well as increased mitochondrial permeability transition pore (mPTP) opening, which initiates apoptotic signaling activation and cardiomyocyte death. These morphological and functional changes in cardiac mitochondria were mediated via PKD-dependent phosphorylation of mitochondrial fission protein, Dynamin-Like Protein 1 (DLP1) at S637. Moreover, pretreatment with a novel potent PKD inhibitor CRT0066101 effectively inhibited G_qPCR-mediated PKD translocation to OMM, DLP1 phosphorylation at S637, mitochondrial fragmentation, ROS generation and mPTP activation. In conclusion, we demonstrate that G_qPCR stimulation induces mitochondrial fragmentation and dysfunction through PKD-dependent phosphorylation of DLP1 at S637, which likely contributes to cardiomyocyte injury. Thus, small-molecule PKD inhibitor may become a novel and potent therapeutic for preventing cardiac cell injury and death during HF.

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EPR Data Support the Existence of a Symmetric BH3-in-Groove Homodimer in Oligomeric BAK

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The BAX or BAK oligomeric pore formation in the mitochondrial outer membrane is a critical step in apoptosis, yet their structures are not clearly understood. Czabotar *et al.* (Cell 2013, **152**, 519) reported a crystal structure of a water-soluble tetramer of the BAX fragment (helices α2-α5) fused to the green-fluorescent protein (GFP), in which the α2-α3 extended helices and the α5 helix, respectively, were juxtaposed to each other, in an anti-parallel orientation, forming a symmetric "BH3-in-groove homodimer (BGH)." We have constructed a GFP-BAK fusion protein using the α2-α5 helices of mouse BAK, designated as GFP-BAKα2-α5, which also forms a soluble tetramer. To determine whether the BGH exists in the BAK oligomers in the membrane or not, we spin labeled the C-terminal hexahistidine-tagged soluble form of mouse BAK (helices α1-α8) at residues 84, 122, 128 and 135 and the corresponding residues in GFP-BAKα2-α5. We then compared the continuous wave (CW) EPR spectra of the spin-labeled residues from the tetrameric GFP-BAKα2-α5 with those from the oligomeric BAK in membrane. Spin labeled residue 122R1, located in the loop interconnecting helices α4 and α5 in the homology model of the GFP-BAKα2-α5 tetramer, displayed a mobile lineshape. The corresponding residue in the oligomeric BAK also had a remarkably similar lineshape, indicating that the two residues are in similar structural environments. Residues 84R1, 128R1 and 135R1, located at the anti-parallel helical interfaces in the BGH also had remarkably similar immobile lineshapes both in the GFP-BAKα2-α5 tetramer and in the oligomeric BAK in membrane, further strengthening the above conclusion. The intra-dimer distances between 84R1 spin label pairs in the GFP-BAKα2-α5 and the oligomeric BAK, determined by the double electron electron resonance (DEER) method, also support this interpretation.

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Modulation of Membrane Interactions of Anti-Apoptotic Regulator Bcl-xL by Lipids

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The Bcl-2 family of proteins (e.g., pro-apoptotic Bax and anti-apoptotic Bcl-xL) regulates the mitochondrial outer membrane permeabilization during the early stages of apoptosis. The prevalent Embedded Together Model of Bcl-2 action suggests that the membrane environment is critical for their proper functional interactions, consistent with the increasing evidence of lipids being involved in the regulation of apoptotic response. In this study, we apply a collection of fluorescence-based methods to investigate the effect of various lipids on the pH-triggered membrane interactions of Bcl-xL. The initial membrane association was studied using a FRET assay with donor-labeled Bcl-xL and acceptor-labeled vesicles, while the insertion/refolding of Bcl-xL into the membrane was monitored using the environment-sensitive probe NBD selectively attached in the middle of hydrophobic helix α6. Our results demonstrate that the lipid composition affects the pH-dependence of both initial membrane association and subsequent insertion/refolding of Bcl-xL. We found that a linear correlation exists between the membrane surface potential created by anionic lipids and the pKa of membrane binding, suggesting that the initial step is controlled by an electrostatic mechanism. The effect of lipids on the membrane insertion/refolding step is more complex and appears to be influenced by the size of the lipid headgroup. The kinetics of both the membrane association and membrane insertion/refolding is affected by the presence of non-bilayer forming lipids commonly found in mitochondria. While the presence of phosphatidylethanolamine accelerated the process, addition of lysophosphatidylcholine had the opposite effect, suggesting that mechanical properties of the bilayer also play a role. Taken together our results indicate that lipids can modulate the membrane interactions of Bcl-xL in multiple ways, providing an additional regulatory mechanism that ensures proper control of a complex cascade of apoptotic reactions leading to cell death or survival. NIHGM-069783, Fulbright-CONICYT, BRTP.

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Binding of Pro-Apoptotic Protein Bax to Cytoprotective UDCA and TUDCA

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Hydrophobic bile acids, such as deoxycholic acid (DCA), strongly induce apoptosis in both hepatic and non-hepatic cells while hydrophilic bile acids,