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Regulation of Mitochondrial Motility by Milton-like Proteins, OIP106 and GRIF1

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Regulation of Mitochondrial Motility by Milton-like Proteins, OIP106 and GRIF1

GRIF1 and OIP106 are mammalian homologs of Milton, a kinesin-binding protein that forms a complex with Miro GTPase, an integral outer mitochondrial membrane EF-hand protein. Kinesin, Milton and Miro have been proposed to function together in the movement of mitochondria along the microtubules. We report here the influence of overexpression of OIP106 and GRIF1 on mitochondrial motility and its inhibition by agonist-induced cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) signals in H9c2 cells. HA-tagged OIP106 and GRIF1 were overexpressed at similar level and were localized to the mitochondria as detected by immunocytochemistry. In OIP106 and GRIF1 overexpressing cells, elongated mitochondria with varied degree of aggregation were visualized both in live and in fixed, immunostained samples. Mitochondrial motility at resting Ca^{2+} (< 100nM) was greatly enhanced by OIP106 and to a lesser extent by GRIF1 (26.6± 2.3 and 21± 1.9 motility units, respectively against a control value of 14.9±1.4 motility units). Furthermore, the Ca^{2+}-dependent inhibition of motility during stimulation by vasopressin (100nM) was suppressed by overexpressed OIP106 (40.0±3.2%), whereas GRIF1 (52.1± 4.3%) had no significant effect compared to control (58.0±3.3%). Overexpression of OIP106 or GRIF1 did not alter either basal $[Ca^{2+}]_c$ or agonist-induced $[Ca^{2+}]_c$ levels measured by fura2. Collectively, these data show that both OIP106 and GRIF1 can modulate mitochondrial motility presumably, by promoting the association of mitochondria with the motor proteins. Furthermore, OIP 106 seems to have greater efficacy in the control of mitochondrial movements.

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Mitochondrial localization and function relationship

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Mitochondrial contribution to cell signaling and function relies on the association and local communication of mitochondria with the ER and the plasma membrane (PM). We have recently shown that the local Ca2+ communication between ER and mitochondria is supported by interorganellar tethers, and created synthetic linkers that connected the outer mitochondrial membrane (OMM) to the ER and sensitized mitochondria to ER Ca2+ release. To study the kinetics and short-term effects of the linkage formation, we have now devised fluorescent protein pairs targeted to the OMM and ER or PM containing FKBP12 or FRB domains. Rapamycin causes heterodimerization of these proteins to form OMM-ER or OMM-PM bridges. Confocal imaging of the inducible fluorescent linker pairs revealed increased association of ER or PM-patches with the mitochondria within minutes of rapamycin exposure. The linkage formation between the ER and mitochondria was followed by a decrease in mitochondrial motility (>40% in 15 min) and by sensitization of mitochondria to IP_3 receptor-mediated Ca2+ release. Linkage formation between PM and mitochondria also suppressed mitochondrial motility. The initial kinetics and spatial distribution of linkage formation could be followed via recording FRET between CFP-and YFP-containing linker partners. A longer version of the linker was found to show faster increase of the FRET signal, supporting the idea that the rate of linkage formation positively correlates with the tightness of the ER-mitochondrial contacts. Currently, we are using the FRET kinetics to visualize the areas of close ER-mitochondrial interface and study its relevance in the Ca2+ signal propagation to the mitochondria. Thus, inducible interorganellar linkers provide a tool to assess the distance between organelles in live cells, to establish rapid changes in the subcellular distribution and dynamics of the organelles and to evaluate the ensuing changes in organellar and cell function.

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Bak/bax-dependent Apoptotic Signaling In Vdac2$^{-/-}$ Cells

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Bid, a pro-apoptotic Bcl-2 family protein, upon activation forms truncated Bid (tBid) that binds to the outer mitochondrial membrane (OMM) and engages Bak/Bax-dependent release of cytochrome c (cyto c) and other intermembrane space proteins from mitochondria to the cytosol to induce apoptosis. The voltage-dependent anion channel (VDAC) is the major permeability pathway for metabolites and ions in the OMM but its role in the tBid-induced OMM permeabilization remains controversial. Previously we reported that among the VDAC isoform-specific knockout mouse embryonic fibroblasts (MEFs), only VDAC2$^{-/-}$ MEFs lack tBid induced complete cyto c release and loss of $\Delta\Psi$m. Here we show by single cell fluorescence imaging that permeabilized VDAC2$^{-/-}$ MEFs expressing cyto c-GFP were resistant to tBid (37nM)-induced cyto c-GFP release. Furthermore, by rescuing VDAC2$^{-/-}$ MEFs with VDAC2 the tBid-induced cyto c-GFP release was restored. In addition, tBid adenovirus infection caused less cell death in intact VDAC2$^{-/-}$ MEFs than in wildtype (WT) MEFs. It has been reported that VDAC2 is required for proper targeting of Bak in OMM. Indeed, Bak did not appear in the membrane fraction of VDAC2$^{-/-}$ MEFs. Along this line we show that permeabilized Bak$^{-/-}$ cells were more resistant to tBid induced cyto c release and loss of $\Delta\Psi$m than WT and Bax$^{-/-}$ MEFs. Strikingly, washout of the cytosol further desensitized the Bak$^{-/-}$ MEFs to tBid. Unlike VDAC2$^{-/-}$ MEFs, the Bak$^{-/-}$ MEFs constitutively overexpressed Bax that was primarily localized in the cytosol. However, recombinant Bax (200nM) could induce cyto c release and depolarization in VDAC2$^{-/-}$ MEFs and also supported the tBid-induced cyto c release. Thus, in VDAC2$^{-/-}$ cells Bak does not localize to the mitochondria and fails to interact with tBid and does not allow a compensatory increase in Bax. The combination of these two mechanisms greatly attenuates tBid-induced OMM permeabilization and apoptosis.

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Mitochondrial fusion-fission dynamics during hypoxia/reoxygenation

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Structural, biochemical, and functional abnormalities of mitochondria during hypoxia/reoxygenation (H/R) are widely believed to be important pathogenic factors that underlie cell injury. However, mitochondrial fusion-fission dynamics responses to H/R are unclear. We investigated the effect of H/R and chemical hypoxia evoked by KCN on cellular ATP, $\Delta\Psi_m$, and on mitochondrial morphology and fusion. Sixty min H caused cellular ATP decrease to 71±3%. $\Delta\Psi_m$ showed progressive decrease during H, gradually improved in the first 30 minutes R and decreased again during longer R. Mitochondrial fusion activity decreased to 65% during H and to 58% during R. In addition, anomalous fusion (autofusion and fusion at multiple sites among 2-3 mitochondria) produced donut-shaped mitochondria during R. Cyclosporine A (CSA), an inhibitor of the permeability transition pore (PTP) relieved the fusion inhibition (70%) and prevented donut formation during R. Five mM and 10 mM KCN could induce cellular ATP decrease to 49±6% and 23±2%, and $\Delta\Psi_m$ decrease to 41±5% and 7±2%, respectively. Depolarized mitochondria were associated with donut-formation in 5 mM KCN, and with massive swelling in 10 mM KCN. Fusion activity decreased to 31% in 5 mM KCN and 13% in 10 mM KCN, respectively. Neither H nor KCN evoked cleavage of Opa1, the mitochondrial inner membrane fusion protein. Thus, both physical and chemical H induced respiratory inhibition to gradually lower $\Delta\Psi_m$ and cellular ATP level, and caused fusion inhibition that was not dependent on Opa1 cleavage. This is sharp contrast of the uncoupler-induced fusion inhibition that has been attributed to the rapid $\Delta\Psi_m$ dissipation-induced Opa1 cleavage. A consequence of PTP opening, the matrix swelling seems to be a key to donut formation since this could be evoked by both mastoparan, a potent PTP activator and by valinomycin, a potassium ionophore.

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Governing Respiration: Tubulin's C-Terminus Interaction with VDAC

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Mitochondria have long been known to localize within and move along the tubulin-microtubule network. It was shown that tubulin binds to isolated mitochondria with high-affinity and specifically associates with the mitochondrial voltage-dependent anion channel (VDAC). We found that nanomolar concentrations of dimeric tubulin vastly increase VDAC sensitivity to voltage allowing for VDAC blockage at low transmembrane potentials. Tubulin interaction with VDAC requires the presence of anionic C-terminal tails (CTT) on the intact protein. Tubulin with proteolytically cleaved CTTs does not block the channel. Actin, also an acidic protein but lacking CTTs, does not induce VDAC blockage. Two synthetic peptides with the sequences of mammalian α and β brain tubulin CTT do not induce detectable channel closure up to micromolar concentrations. These results suggest a completely new role for

dimeric tubulin and its charged CTT. We propose a model for tubulin-VDAC channel interaction in which the tubulin CTT penetrates into the channel lumen, potentially reaching through the channel and interacting with a positively charged domain of VDAC. We found that tubulin/VDAC interaction is greatly dependent on the state of VDAC phosphorylation. Remarkably, phosphorylated VDAC is more than an order of magnitude more sensitive to tubulin-induced closure than dephosphorylated VDAC.
Tubulin addition to isolated mitochondria increases Km for ADP and lowers oxygen consumption, most likely by restricting flux of ADP through VDAC. Thus interaction of tubulin CTT with VDAC blocks nucleotide passage into and out of mitochondria, thereby regulating oxidative phosphorylation. Examination of the evolution of CTT sequences in mitochondria-containing cells reveals high conservation of charge and length of the tails. Considering the known conservation of VDAC folding pattern throughout mitochondria-containing eukaryotes, we propose that this interaction is widespread and ancient.

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Spontaneous Oscillations in Mitochondrial Membrane Potential of Cultured Neurons Did Not Correlate With Cytosolic Calcium Concentration
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We used two-photon imaging of the fluorescent dye tetramethyl-rhodamine methyl ester (TMRM) to visualize *spontaneous oscillations* of mitochondrial membrane potential in cultured rat hippocampal neurons. TMRM-loaded cells displayed two distinct forms of spontaneous oscillation when imaged in artificial cerebral spinal fluid (ACSF) at room temperature: fast oscillation of individual mitochondria (flickering) corresponding to periods of depolarization lasting several seconds, and slow oscillation of groups of mitochondria (wave) corresponding to depolarizations lasting 1-3 minutes. Similar types of spontaneous oscillation have been reported previously using isolated mitochondria and cultured cells, although the underlying cause(s) of the oscillations is unclear. In isolated mitochondria, flickering can be triggered by mitochondrial Ca uptake (*Biophys. J.* 87: 3585, 2004) and waves can be induced by local Ca elevation (*Cell* 89: 1145, 1997). We tested whether Ca could exert similar effects in cultured hippocampal neurons by testing cells loaded with both TMRM and a Ca-sensitive fluorophore (fura-2 or fluo-3). Cellular fluorescence was imaged repeatedly every second for up to 60 minutes. Under our conditions (in ACSF at room temperature), neurons displayed spontaneous oscillations in both TMRM and Ca-sensitive dyes, but there was no correlation between these signals. Furthermore, some neurons displayed fast and slow TMRM oscillations without alteration in Ca levels, while others exhibited fast Ca oscillations (lasting 3-8 sec) but no TMRM oscillations. In addition, Ca oscillations were often synchronized in adjacent neurons, whereas TMRM oscillations were not. These results indicate that oscillations in cytosolic calcium levels do not appear to be directly coupled to oscillations in mitochondrial membrane potential in cultured hippocampal neurons. This does not rule out the possibility that Ca might influence mitochondrial oscillations through an indirect action.

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Flavonoid And Low Level Long Wavelength Laser Irradiation Effects Seen In Human T Cells
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The goal of present studies was to investigate human T cells death/survival/proliferation balance in response to low power far-red (FR) and near-infrared (NIR) laser irradiation doses and/or various concentrations of flavonoids Quercetin (QUE) or Epigallocatechin gallate (EGCG). Changes induced in mitochondrial reticulum state in correlation with apoptosis induction were additionally monitored. Peripheral blood derived lympocytes and human T leukemic Jurkat cells were cultured in standard conditions. QUE or EGCG were introduced in the culture media in various concentrations (1 - 150 μM) for various time periods (6 - 136 h). Therapeutic lasers with emission wavelengths in the range 600 - 900 nm were used to expose cells to single irradiation doses of 0.8-1.8 μJ/cell, with irradiation regimes of once per day, or every second day, realizing total irradiation doses of 1-15 μJ/cell. Using appropriate fluorophore-conjugated surface markers (AnnexinV-FITC for dying cells), mitochondrial (JC1 and MitoTracker dyes) and nuclear probes (7-AAD, Hoechst and PI as DNA stains), mitochondrial membrane depolarization / hyperpolarization related apoptosis induction, cell cycle blockade/progression, cell survival/death rates and cell death style choices were followed up by fluorescence/confocal microscopy and flow cytometry. The obtained data reveal significant, laser wavelength, dose, irradiation regime and cell state dependent photobiomodulation of flavonoid effects in human T cells.
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L-arginine and Tetrahydrobiopterin Inhibit Mitochondrial Permeability Transition Pore by Preventing ROS Formation by Mitochondrial Nitric Oxide Synthase
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Background: The functional role of mitochondrial nitric oxide synthase (mtNOS) in heart has remained a matter of debate. **Methods:** We used laser scanning confocal microscopy in combination with fluorescent dyes to characterize mitochondrial NO and ROS production and the permeability transition pore (PTP) activity in permeabilized cat ventricular myocytes. **Results:** Stimulation of mitochondrial calcium uptake resulted in a dose-dependent increase in mitochondrial NO production when L-arginine, a substrate for mtNOS, was present. The potential contribution of the caveolae-located eNOS and SR-targeted nNOS was ruled out based on the fact that disruption of caveolae with methyl-βετα-cyclodextrin or prevention of SR uptake with thapsigargin did not affect calcium-induced NO production. Collapsing the mitochondrial membrane potential, blocking the mitochondrial calcium uniporter and respiratory chain abolished mitochondrial NO production. In the absence of L-arginine, calcium-induced NO production was significantly decreased; however an increased ROS production was observed. Inhibition of mitochondrial arginase (which limits L-arginine availiability) resulted in 50% inhibition of calcium-induced ROS production. Both mitochondrial NO and ROS production were blocked by the nNOS inhibitor (4S)-N-(4-amino-5[aminoethyl]aminopentyl]-N′-nitroguanidine and the calmodulin antagonist W-7, while the eNOS inhibitor L-NIO or the iNOS inhibitor 1400W had no effect. The superoxide dismutase mimetic MnTBAP abolished calcium-induced ROS generation and increased NO production threefold. In the absence of L-arginine, mitochondrial calcium uptake induced opening of the mitochondrial PTP, which was blocked by cyclosporin A, MnTBAP and reversed by L-arginine. The essential mtNOS co-factor tetrahydrobiopterin also inhibited mitochondrial ROS generation and PTP opening at a concentration of 100 μM, while 10 μM tetrahydrobiopterin had no effect. **Conclusion:** Our data demonstrate the importance of L-arginine and tetrahydrobiopterin for the regulation of mitochondrial oxidative stress and modulation of PTP opening by mtNOS.