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isoforms predominantly localized in the mitochondria and were absent or minimally present in the sarcoplasmic reticulum, whereas others abundantly distributed in both cellular compartments. Additionally, select HAX-1 proteins enhanced cell survival following exposure to H2O2, similar to the prototypical variant I, while others interestingly promoted cell death. To better understand the functions of the different HAX-1 isoforms in modulating cell survival and death, current experiments are under way to examine by quantitative RT-PCR their expression profile during homeostasis, after stress induction, and in disease conditions.

212-Pos Board B12
Nogo-A Knockdown Inhibits Hypoxia/Reoxygenation-Induced Activation of Mitochondrial-Dependent Apoptosis in Cardiomyocytes
Jason P. Sarkey.
Nogo-A has been well-characterized as a potent inhibitor of axonal regeneration and plasticity in the central nervous system, however the role of Nogo-A in non-nervous tissues is essentially unknown. In this study, Nogo-A expression was shown to be significantly increased in left ventricular tissue from human patients with DCM and from patients who have experienced an ischemic event. Nogo-A expression was clearly associated with cardiomyocytes in culture and was localized predominantly in the endoplasmic reticulum. In agreement with the findings from human tissue, Nogo-A expression was significantly increased in cultured cardiomyocytes subjected to hypoxia/reoxygenation. Knockdown of Nogo-A in cardiomyocytes markedly attenuated hypoxia/reoxygenation-induced apoptosis, as indicated by the significant reduction of DNA fragmentation, phosphatidylserine translocation, and caspase-3 cleavage, by a mechanism involving the preservation of mitochondrial membrane potential, the inhibition of ROS accumulation, and the inhibition of cytochrome c release. Together, these data indicate that knockdown of Nogo-A in cardiomyocytes may serve as a novel therapeutic strategy in the treatment of ischemic/hypoxic injury.

213-Pos Board B13
Real-Time Dynamics of Ca^{2+}, Phosphatidylserine, Caspase-3/7, and Morphological Changes in Apoptosis: Retinal Ganglion Cells Under Elevated Pressure
Siyuan Lu, Jae Kyoo Lee, Anupam Madhukar.
Quantitative information on the dynamics of multiple molecular processes in individual live cells under controlled stress is central to the understanding of the cell behavior of interest and the establishment of reliable models. We report on the dynamics of the apoptosis regulator intracellular Ca^{2+}, surface marker phosphatidylserine (PS), effector caspase-3/7, and morphological changes examined simultaneously in individual transformed retinal gangling cells undergoing apoptosis at elevated hydrostatic pressure. A custom-designed imaging platform that allows long-term real-time imaging of morphological and molecular-level physiological changes in large numbers of live cells (beyond the field-of-view of typical microscopy) under controlled pressure is employed.[1] Intracellular Ca^{2+} elevation and PS translocation to the outer leaflet of the plasma membrane at the early stages (typically <5 hours after the onset of 100 mmHg pressure) followed by gradual caspase-3/7 activation at late stages (typically >5 hours) is found. The data reveal a strong temporal correlation between the Ca^{2+} elevation, PS translocation, and morphological changes (neurite retraction and soma shrinkage) in the vast majority of the cells. This suggests that Ca^{2+} is likely responsible for the onset of PS translocation and apoptotic morphological changes. Moreover, the data show a significant cell-to-cell variation in the onset of caspase-3/7 activation, an inevitable consequence of the stochastic nature of the underlying biochemical reactions not captured by conventional assays based on population-averaged cellular responses. This study demonstrates that the approach of simultaneously imaging multiple intracellular events in large numbers of live cells provides statistically significant data to enable refinements and testing of models of signaling pathways, here apoptosis. [1] Lee JK, Lu S, Madhukar A (2010) Real-time Dynamics of Ca^{2+}, Caspase-3/7, and Morphological Changes in Retinal Ganglion Cell Apoptosis under Elevated Pressure. *PLoS ONE*, In Press.

214-Pos Board B14
Biophysical Basis for Specificity of Action of Human Isoforms of Secretory Phospholipase A_2 During Cell Death
Jennifer Nelson, Amanda M. Berbert, Elizabeth Gibbons, Katalyn R. Pickett, Michael Streeter, Ashley O. Warcup, Celestine H.-Y. Yeung, Allan M. Judd, John D. Bell.
The hydrolytic activity of secretory phospholipase A_2 ($sPLA_2$) toward mammalian cells depends on the health of the cell. During the process of programmed cell death, changes occur in the cell membrane that render it susceptible to hydrolytic attack. Various isozymes of $sPLA_2$ respond differently to cell death depending on the timing and origin of the process. This study used flow cytometry to classify subpopulations of S49 lymphoma cells during several modes of programmed death depending on their vulnerability to $sPLA_2$ and identify the basis for isozyme specificity. Most death stimuli (thapsigargin, dexamethasone, actinomycin D, paclitaxel, and methotrexate) caused a reduction in membrane lipid-neighbor interactions detected as increased binding of the fluorescent dye merocyanine 540 and modest permeability to a vital stain, propidium iodide. In each of these cases, all $sPLA_2$ isozymes tested (snake venom and human groups IIa, V, and X) displayed enhanced ability to hydrolyze the cell membrane. In contrast, cells exposed to a calcium ionophore showed the increase in merocyanine 540 binding without accompanying permeability to propidium iodide. Under these conditions, only the snake venom and human group X enzymes hydrolyzed cells that were dying. Lastly, the human group IIa enzyme, although most active of the isoforms tested toward anionic artificial bilayers, hydrolyzed dying cells at a rate that was only 1/100 that of the other isozymes. These results suggested that each of these human isozymes fills different physiological roles in responding to cell death and provides probable molecular explanations for the distinctions.

Mitochondria in Cell Life and Death

215-Pos Board B15
Interaction of Creatine Kinase and Nucleoside Diphosphate Kinase with Mitochondrial Cardiolipin Membranes: Differences in Mechanism and in the Effect on Enzyme Catalysis
Malgorzata Tokarska-Schlattner, Marie-Lise Lacombe, **Uwe Schlattner**.
Mitochondrial isoforms of creatine kinase (MtCK) and nucleoside diphosphate kinase (NDPK-D) have critical functions in bioenergetics, membrane topology and organelle morphology with roles in human health and disease. X-ray structural analysis, electron microscopy, surface plasmon resonance (SPR) and scanning calorimetry revealed that both kinases form large oligomers that bind to and cross-link mitochondrial membranes via anionic phospholipids, mainly cardiolipin; at least MtCK can also induce cardiolipin-rich membrane domains. First we used surface plasmon resonance combined with thermodynamic analysis to study kinase/cardiolipin interaction. The two kinases differed in their membrane binding mechanism: (i) NDPK-D showed monophasic binding due to electrostatic interactions of a triad of basic amino acids, while binding of MtCK was biphasic, with only the main component depending on electrostatic interactions of C-terminal basic amino acids. (ii) Rising temperature increased cardiolipin affinity of MtCK, in particular in the second binding component, but not of NDPK-D, indicating hydrophobic interactions in case of MtCK. (iv) Kinase/membrane interaction occurred to be an entropy-driven binding process, in particular for MtCK, possibly due to charge neutralization, release of bound water, and effects on membrane order. Second, we studied the effect of membrane-association on kinase enzyme activity. While basic cardiolipin had no effect on MtCK, NDPK-D was strongly inhibited. This inhibition was relieved by doxorubicin that strongly competes for cardiolipin binding. We propose a model for MtCK and NDPK-D interaction with cardiolipin-containing lipid membranes. For NDPK-D, a single phase, purely electrostatic binding would lead to a partial shielding of the enzymes' active sites and thus catalytic inhibition. For MtCK, a two-phase binding model of rapid electrostatic docking and slower anchoring via hydrophobic stretches is proposed, which does not affect the active sites.

216-Pos Board B16
Probing VDAC Voltage-Gating Mechanism by pH: Functional and Structural Implications
Oscar Teijido Hermida, Matthew T. Eddy, Robert G. Griffin,
Sergey M. Bezrukov, Tatiana K. Rostovtseva.
VDAC controls fluxes of ATP/ADP and other respiratory substrates across mitochondrial outer membrane by using its characteristic ability to switch or ''gate'' between the so-called ''open'' and ''closed'' states. While most metabolites go freely through a unique open state, the closed states are virtually impermeable to ATP and ADP. Therefore, unveiling molecular mechanisms of VDAC gating is important in our understanding of mitochondrial respiration and metabolism in health and pathology. Available crystal structure of VDAC solved to the atomic level of resolution does not provide data on VDAC gating mechanism in spite of a number of different models that have been proposed. Although effects of pH on VDAC gating have been shown previously, here we further explore this approach by performing functional and structural studies on VDAC at extremely low pH. In our experiments with VDAC reconstituted into planar lipid membranes, voltage-gating is drastically increased as pH decreases from 7.4 to 3.0. Interestingly, the effect of pH on gating is fully reversible, i.e., gating returns to the initial behavior after returning

pH back to 7.4. Further, we explore the effects of pH on channel selectivity and protein-protein interaction of VDAC with dimeric tubulin that is known to block VDAC pore with nanomolar efficiency. To address structural rearrangements upon gating, we also use magic angle spinning NMR to study conformational changes in recombinant human VDAC1 as a function of pH ranging from 3 to 11. Under these conditions we observe reversible changes in chemical shifts upon changing the pH from 7 to 4. These observations support functional results of VDAC voltage-gating at different pH and complement the data with site-specific information about residues affected by pH changes. The mechanism of VDAC gating and its relevance to in vivo situation are discussed.

217-Pos Board B17

Membrane Lipid Composition Regulates Tubulin-VDAC Interaction

Tatiana K. Rostovtseva, Michael Weinrich, Meng-Yang Chen, Kely L. Sheldon, Sergey M. Bezrukov.

Accumulating evidence suggests that lipids play an important role in mitochondrial function, dynamics, and morphology and in the permeabilization of the mitochondria outer membrane (MOM) that initiates apoptosis. Recently we have found that dimeric αβ-tubulin, a subunit of microtubules, regulates mitochondrial respiration by directly blocking the VDAC pore (Rostovtseva et al., PNAS 2008). Here, we show that the mechanism of tubulin-VDAC interaction is complex and greatly depends on membrane lipid composition. The on-rate of tubulin-VDAC binding varies up to 100-fold depending on the particular lipid used for bilayer formation but is independent of the charge of lipid headgroups, and the presence of cholesterol. The off-rate of tubulin-VDAC binding does not depend on lipid content. The gramicidin A (gA) channel was used to probe the effect of tubulin on lipid bilayer mechanics. We found that 30 nM of tubulin increased gA channel lifetime 10-fold in DOPE bilayers but did not affect it in DOPC. Tubulin effects on gA were significantly reduced in low salt, suggesting a hydrophobic interaction. Using confocal fluorescence microscopy we observed tubulin binding to the membranes of giant unilamellar vesicles (GUVs) made from DOPC and DOPC/DOPE directly. We found that adsorption of the fluorescently labeled dimeric tubulin (Tubulin-HiLyte488) on the GUV membranes requires the presence of DOPE. We did not observe tubulin binding to GUVs made from pure DOPC. We propose that prior to tubulin's characteristic blockage of VDAC by permeation of tubulin's C-terminal tail into the channel lumen, there is an additional, non rate-limiting step whereby tubulin first binds to the membrane. Our findings suggest a new regulatory role of mitochondrial lipids in control of MOM permeability and hence, mitochondrial respiration, through tuning of VDAC sensitivity to blockage by tubulin.

218-Pos Board B18

Tubulin-VDAC Interaction: Salt Dependence of Conductance and Reversal Potential

Maria Queralt-Martin, Tatiana K. Rostovtseva, Vicente M. Aguilella.

The recently reported blocking of mitochondrial voltage-dependent anion channel (VDAC) by tubulin [1] has raised new questions about the role of tubulin C terminal tails (CTT) in the low conductance states exhibited by VDAC channel. The voltage sensitive reversible channel closure observed suggests that tubulin is more than an enhancer of VDAC gating. However, there is no conclusive evidence about the nature of the channel interaction with tubulin tails. Experiments have also revealed that VDAC-tubulin interaction is also influenced by the lipid composition of the membrane hosting the channel. While the duration of tubulin induced closure seems to be independent of salt concentration, the opposite is true with VDAC open time between successive blockages. The change in conductance between open and close states is also concentration dependent. By performing single channel conductance measurements as well as reversal potential measurements (in open and closed states) we analyze the importance of electrostatic interactions between VDAC ionizable residues and the CTT negative charge in the binding of tubulin. The anion selectivity of VDAC in its open state is turned into cation selectivity under tubulin blockage. The recently published 3D structure of VDAC [2] seems fully compatible with the partial penetration of alpha or beta tubulin tails into the channel lumen.

[1] T. K. Rostovtseva et al. PNAS, 105 (2008) 18746.
[2] S. Hiller et al. Trends Biochem. Sci., 35 (2010) 514.

219-Pos Board B19

Reduced Tyrosine Nitration of VDAC and Decreased Apoptosis by Mitochondria-Directed Therapy After Cardiac Ischemia Reperfusion in Isolated Hearts

Meiying Yang, David F. Stowe, Ashish K. Gadicherla, Basam Wakim, James S. Heisner, Amadou K.S. Camara.

Superoxide ($O_2^{\bullet -}$) produced during cardiac ischemia-reperfusion (IR) injury reacts with nitric oxide to form peroxynitrite ($ONOO^-$). $ONOO^-$ induces protein tyrosine nitration (tyr^N) that causes protein structural alteration and dysfunction. The mitochondrial voltage-dependent anion channel (VDAC) plays an important role in regulating the metabolic and energetic functions of mitochondria and contributes to mitochondrial-mediated apoptosis. It is not known if VDAC is nitrated by $ONOO^-$ during IR or how this modification might compromise cardiac function after IR. Because of the importance of VDAC modification, we hypothesized that the clinically used anti-anginal drug ranolazine (RAN), which also reduces cardiac IR injury, does so via a mitochondrial mechanism, i.e., in part by decreasing VDAC tyr^N. To test this, isolated guinea pig hearts were perfused with KR buffer for 40 min (time control, TC), or for 30 min of ischemia plus 10 min of reperfusion, with or without 10 μM RAN infused before ischemia. Mitochondria were isolated at the end of each treatment. VDAC tyr^N was determined by IP with anti-nitrotyrosine antibody (NTab), followed by Western blotting (WB) with anti-VDAC antibody. The effect of RAN on VDAC tyr^N was also examined. Cytochrome *c* release was checked as the marker for apoptosis. We found that enhanced VDAC tyr^N was increased by 108% after IR vs. TC and cytochrome *c* was higher in the cytosol after IR than after TC. RAN treatment decreased VDAC tyr^N by 31%, while decreasing cytochrome *c* release by 38%, compared to IR. These results indicate that VDAC tyr^N and a concomitant increase in cytochrome *c* release occur during IR injury, and importantly, that cardioprotection by RAN occurs in part by reducing VDAC tyr^N, which may impede activation of apoptotic pathways during IR injury.

220-Pos Board B20

A Mitochondrial ATP-Sensitive Potassium Channel from the ROMK Family

D. Brian Foster, Alice Ho, Anders Garlid, Jasma Rucker, Marjan Gucek, Robert Cole, Keith Garlid, Brian O'Rourke.

Mitochondrial potassium modulates mitochondrial 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 employing repeated fractionation at the organellar, protein, and peptide levels. Briefly, 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 prior to 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%. Subsequent bioinformatic analysis using TargetP and PSORT detected a mitochondrial localization sequence near the N-terminus of ROMK. Mitochondrial localization was confirmed in neonatal rat ventricular myocytes (NRVM) transiently transfected with truncation mutants of human ROMK isoforms fused with green fluorescence protein (GFP) or V5-tag at the C-terminus of the channel. Imaging by 2-photon microscopy showed that predicted targeting presequences confer colocalization of GFP with tetramethylrhodamine methyl ester (TMRM) staining of the mitochondria. Furthermore, 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. Reverse-Transcription/Polymerase Chain Reaction (RT-PCR) identified 3 isoforms (ROMK1, ROMK2 &ROMK6) in the adult rat hearts and NRVMs. The findings support an isoform of ROMK as a candidate for the pore-forming subunit of $mitoK_{ATP}$.

221-Pos Board B21

MAC Function Triggers a Bax/Bak Dependent Bystander Effect

Pablo M. Peixoto, Jennifer K. Lue, Shin-Young Ryu, Kathleen W. Kinnally.

Collateral spread of apoptosis to nearby cells is referred to as the bystander effect - a process that is integral to tissue homeostasis and a challenge in anti-cancer therapies. In many systems, apoptosis relies on permeabilization of the mitochondrial outer membrane to factors like cytochrome c and Smac/DIABLO. This permeabilization occurs via formation of a mitochondrial apoptosis-induced channel, MAC, and was mimicked here by single-cell microinjection of cytochrome c in Xenopus embryos. Waves of apoptosis were observed in vivo from the injected to the neighboring cells. This finding indicates that a death signal generated downstream of cytochrome c release diffused to neighboring cells and ultimately killed the animals. The role of MAC in bystander effects was then assessed in mouse embryonic fibroblasts that did or did not express its main components Bax and/or Bak. Exogenous expression of GFP-Bax triggered permeabilization of the outer membrane