Mitochondria in Cell Life and Death

3363-Pos Board B518

A Step Forward in Understanding the Mechanism of VDAC Voltage-Gating

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The voltage-dependent anion channel (VDAC) governs the exchange of ions and metabolites between the mitochondria and the rest of the cell. In its open state VDAC exhibits high conductance and selectivity for anions that facilitates the passage of ADP, ATP, and other metabolites. At increased voltages (>30mV) VDAC switches to lower conducting states, termed as ''closed'' states. Closed states are cation-selective and impermeable for ATP. The voltage-induced transition from the open to closed states is referred to as voltage-gating. Although it is well established that VDAC voltage-gating involves large structural rearrangements, the precise molecular mechanism of this process is still under debate. We investigated VDAC voltage-gating by systematically titrating VDAC charge residues and by using thermodynamic and kinetic approaches to study opening and closing of the channel. All the models proposed so far agree that N-terminal region plays a key role in VDAC voltage-gating. According to the original idea, the N-terminal region is a part of a mobile voltage sensor domain, which slides in and out of the channel lumen in response to the applied voltage. The alternative models consider independent movement of the N-terminal region upon gating. In order to test the role of VDAC N-terminal region in voltage-gating, we engineered a double Cys mutant of murine VDAC1 that cross-links the α-helix to the β-strand 11 of the pore wall. The cross-linked VDAC1 reconstituted into planar lipid membranes exhibited typical voltage gating, which suggests that the N-terminal α-helix is located inside the pore of VDAC in the open state and remains associated with the pore wall during voltage gating. Our findings support a model where β-barrel is not rigid but undergoes a conformational change that leads to a partial constriction upon transition to the closed states.

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Novel Mechanism of Mitochondrial Respiration Control through Competition between Hexokinase-2 and Tubulin for VDAC Binding

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The voltage dependent anion channel (VDAC) is involved in regulation of metabolite flux across the mitochondrial outer membrane (MOM). Hexokinse II (HK2) is known to bind the MOM where it phosphorylates glucose into glucose-6-phosphate (G6P). High expression of HXK2 is a common phenotype of many cancers, where its concentration can be 200 times of that in noncancerous cells, and is implicated in the Warburg effect. It is believed that VDAC serves as a HXK2 binding site in the MOM. The 15 amino acid N-terminal sequence of HXK2 is responsible for mitochondrial binding and, when conjugated to TAT (TAT-HK2), binds to mitochondria with higher affinity than native HXK2, causing HXK2 detachment. We have previously found that dimeric tubulin reversibly binds and partially blocks VDAC inhibiting metabolite flux across the MOM. Now we show that this binding can be attenuated by TAT-HXK2 peptide as well as by full length HXK2. We have found that TAT-HXK2 and recombinant full length HXK2 inhibit tubulin blockage of VDAC reconstituted into planar lipid bilayers without altering characteristic channel properties such as single channel conductance and selectivity. Binding of HXK2 to VDAC is verified by the generation of high-frequency excess current noise without channel closure. HXK2 bound to VDAC prevents subsequent tubulin binding, but only when added before tubulin, and inhibits tubulin-induced VDAC blockage in a dose dependent manner. Moreover, G6P, which is known to cause HXK2 detachment from the MOM, fully reverses the inhibition of tubulin-VDAC binding. This suggests that HXK2 detachment from VDAC (and hence the MOM) is caused by a HXK2 conformational change upon G6P binding. Thus we propose a novel mechanism of mitochondrial respiration control in cancer cells through the competition between HXK2 and tubulin for VDAC binding.

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Reprogramming of Mitochondrial Ca^{2+} Handling in MICU1-Deficient HeLa Cells

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Recent studies have revealed MCU as the pore forming domain and MICU1 as a critical Ca^{2+}-sensitive regulator of the mitochondrial Ca^{2+} uniporter. However, the exact role of MICU1 in Ca^{2+} transport remains to be addressed. Our previous studies showed that prolonged down-regulation of MICU1 in HeLa cells (shMICU1) promotes mitochondrial Ca^{2+} uptake at low [Ca^{2+}], which unexpectedly, fails to effectively increase matrix [Ca^{2+}]. To determine the source of discrepancy between the mitochondrial Ca^{2+} uptake and the matrix [Ca^{2+}] phenotypes, first we simultaneously monitored ruthenium red-sensitive clearance of added Ca^{2+} from the cytoplasm and the corresponding matrix [Ca^{2+}] response in permeabilized shMICU1 cells. Under conditions of similar cytoplasmic Ca^{2+} clearance, shMICU1 cells showed a smaller matrix [Ca^{2+}] increase than the control, indicating enhanced buffering of Ca^{2+} in the matrix. Enhanced Ca^{2+} binding in the matrix likely reflects alkalinization and enhanced phosphate transport. To test if upregulation of Ca^{2+} buffering is directly linked to MICU1 depletion, we also assessed mitochondrial Ca^{2+} handling after 72hr silencing of MICU1 (siMICU1). In siMICU1cells both mitochondrial Ca^{2+} uptake and the matrix [Ca^{2+}] rise were effectively stimulated at low Ca^{2+} levels. Thus, upregulation of matrix Ca^{2+} buffering seems to be a component of an adaptive response to sensitization of mitochondrial Ca^{2+} uptake in shMICU1. The adaptive response is likely to be important to attenuate some MICU1-depletion induced cellular impairments that we found to manifest as attenuated mitochondrial ATP production and cell proliferation.

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MICU1-dependent Threshold and Cooperativity of Mitochondrial Ca^{2+} Uptake in the Liver

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Recent studies have revealed MCU as the pore forming domain and MICU1 as a critical Ca^{2+}-sensitive regulator of the mitochondrial Ca^{2+} uniporter. However, the mechanism of the complex Ca^{2+} dependence of the uniporter activity remains elusive. Our previous studies showed that prolonged down-regulation of MICU1 in HeLa cells causes lower threshold and decreased cooperativity of mitochondrial Ca^{2+} uptake. To study the functional significance of the effects of MICU1 we used hepatocytes harvested from the liver of mice exposed to in vivo silencing (4 weeks). Silencing of MICU1 or MCU resulted in >80% decrease in their respective mRNA levels. Silencing of MICU1 caused a left-ward-shifted dose response and decreased cooperativity of mitochondrial Ca^{2+} uptake in both permeabilized and intact hepatocytes. By contrast, silencing of MCU resulted in slower Ca^{2+} uptake in the entire range of Ca^{2+} concentrations without change in threshold. Mitochondrial respiration and cellular ATP content were unaffected in media containing both glycolytic and mitochondrial fuels in either MICU1 or MCU-deficient hepatocytes. However, silencing of MICU1 caused an augmented loss of ATP when the cells were confined to oxidative metabolism and an enhanced sensitivity to mitochondrial Ca^{2+} overload and permeabilization. During stimulation with vasopressin, a Ca^{2+} mobilizing hormone, both MICU1 and MCU-deficient cells displayed an attenuated mitochondrial matrix [Ca^{2+}] increase and stimulation of respiration. Collectively, these results show that keeping the gate of MCU closed by MICU1 at low [Ca^{2+}] is required to maintain healthy mitochondria, and MICU1-mediated control of MCU (cooperativity?) is required to support the propagation of short-lasting calcium spikes and oscillations to the mitochondria and the ensuing physiological stimulation of oxidative metabolism.

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Targeting Mcl-1 and Bak as a Therapeutic Tool to Selectively Induce Apoptosis in Hepaptocelluair Carcinoma

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In this study we seek to identify novel drug targets to induce apoptosis in hepatocellular carcinoma (HCC) cells thus providing opportunities to develop novel treatments to improve the prognosis of liver cancer patients. Several apoptotic pathways are mediated through cleavage of Bid (a BH3 domain-only, pro-apoptotic protein) to produce truncated Bid (tBid). tBid induces apoptosis through induction of outer mitochondrial membrane (OMM) permeabilization by activation of pro-apoptotic Bak that resides in the OMM or cytoplasmic Bax. Due to its localization, Bak can mediate the early phase of the response to tBid. We have recently demonstrated that OMM targeting of Bak and the sensitivity to tBid-induced OMM permeabilization is dependent on the expression of

voltage-dependent anion channel isoform 2 (VDAC2). Here, we show that HCC induced by carcinogenic compounds, aflatoxin or DEN is highly sensitized to tBid-induced OMM permeabilization and cyto c release when compared to normal liver. Expression levels of VDAC2 and Bak were also higher in HCC than in control liver. The role of the VDAC2-Bak pathway in the differential tBid sensitivity was validated by overexpression of VDAC2 in primary hepatocytes. In HCC cells, elevation of Bak was also associated with an increased level of Mcl-1, an inhibitor of Bak. Furthermore, both constitutive and drug-induced genetic deletion of Mcl-1 in mouse embryonic fibroblasts caused sensitization to tBid-induced OMM permeabilization in the absence of a change in Bcl-xL or Bax . Based on these results, we are evaluating the possibility of selective killing of HCC cells by the combination of a Bak activator and an Mcl-1 inhibitor.

3368-Pos Board B523
Which Domain of VDAC2 is Necessary for Bak Insertion to the Outer Mitochondrial Membrane and tBid - Induced Cytochrome C Release?

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VDAC proteins represent a main component of the outer mitochondrial membrane (OMM). The VDAC family is composed of 3 isoforms with more than 70 percent similarity. Although their primary role was known to be in ion and metabolite transport between mitochondria and cytosol, it has been discovered that VDACs are also involved in apoptotic pathways. We have recently found that specifically the VDAC2 isoform is needed for tBid-induced cytochrome c release, due to its role in supporting Bak insertion to the OMM (Roy et al. 2009. EMBO Rep.10:1341-7).

To determine the domain(s) of VDAC2 which supports Bak insertion, VDAC1 and VDAC2 amino acid composition compared and the unique attributes of VDAC2 sequence were considered for mutational analysis. To test functional significance of the changes in VDAC2 or VDAC1, VDAC2-/- MEF cells that are resistant to tBid, were transfected with the mutants and insertion of Bak to the OMM and cytochrome c release were monitored in permeabilized cells by immunoblotting. Previously, we showed that the VDAC2-specific N terminal tail and cysteins are dispensable for tBid-induced OMM permeabilization. To approach the remaining differences four chimeras were prepared: VDAC1 (1-185)VDAC2(198-295), VDAC2(1-12)VDAC1(1-185)VDAC2(198-295), VDAC2(1-188)VDAC1(177-283) and VDAC2(13-188)VDAC1(177-283). These studies revealed that tBid dependent OMM permeabilization is only supported by VDAC2(1-188)VDAC1(177-283) and VDAC2(13-188)VDAC1(177-283), indicating that the first two third of VDAC2 is required and sufficient for Bak targeting to the OMM and tBid induced cytochrome c release.

3369-Pos Board B524
Outer Mitochondrial Membrane Protein Distribution and Function Depend on Mitochondrial Fusion

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Cells and tissues deficient in mitochondrial fusion have been shown to have defects linked to the exchange of inner membrane (IMM) and matrix components, particularly mitochondrial DNA. Outer membrane (OMM) constituents originate in the cytoplasm, thus the role of intermitochondrial transfer of OMM components by fusion remained unexplored. Here we show that fibroblasts lacking the GTPases responsible for OMM fusion, Mfn1/2, have a more heterogeneous distribution of OMM proteins than wild-type cells, and in particular that heterogeneity of pro-apoptotic Bak leads to dysregulation of Bid-dependent apoptotic signaling. Homogeneous distribution of Bak is partially rescued by introduction of Mfn2 into Mfn1/2-/- cells. Furthermore, fusions between mitochondria lacking and containing Bak result in hybrids sensitive to Bid. Proteins with different modes of OMM association display varying degrees of heterogeneity in Mfn1/2 /- cells and different kinetics of transfer during fusion in fusion-competent cells. Efficient coupling of OMM to IMM fusion depends on the presence of both Mfn isoforms and is antagonized by the mitochondrial fission protein, Drp1. Thus, OMM function depends on mitochondrial fusion and is a locus of dysfunction in conditions of fusion deficiency.

3370-Pos Board B525
Mitochondrial Fusion Dynamics in Skeletal Muscle of Healthy and Diseased Rat

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Mitochondria are highly mobile and dynamic organelles in many cell types. However, in the muscle, mitochondria are crammed into the narrow space among the myofilaments. Until now, it remained unclear if mitochondrial fusion occurs in and supports the contractile activity of skeletal muscle (SM). Here we applied mitochondria matrix-targeted DsRed and photoactivable GFP to study mitochondrial fusion in rat FDB SM fibers. In vivo electroporated fibers were imaged by confocal microscopy. When we tagged the mitochondria in ~5% of cell area with twophoton photoactivation of GFP, rapid spreading of 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. Expression of Autosomal Dominant Optical Atrophy-causing mutants of the fusion protein Opa1 or ethanol exposure caused suppression of fusion, which is followed by mitochondrial dysfunction and late onset myopathies. When challenged by repetitive tetanic stimulation, ethanol exposed cells displayed intracellular Ca^{2+} dysregulation, appearing as a fatigue pattern. Ethanol also induced a decrease in Mfn1 protein levels, without significantly altering Mfn2, Opa1 or deltapsi. Depletion of Mfn1 alone was sufficient to suppress mitochondrial fusion in FDB fibers. Fusion inhibition was apparent before any cell dysfunction, suggesting that suppression of fusion is not secondary to other problems in the cells. To directly test the role of Mfn1 in Ca^{2+} regulation, RyR1-transfected control and Mfn1KO MEFs were stimulated with caffeine. Mfn1KO cells showed oscillatory Ca^{2+} transients that decayed faster than that in the control. Thus, fusion dynamically connects skeletal muscle mitochondria and serves as a target mechanism of both mutations and environmental cues to cause impaired excitation-contraction coupling.

3371-Pos Board B526
BCL-2 Proapoptotic Proteins Distribution in U-87 MG Glioma Cells before and after Hypericin Photodynamic Action

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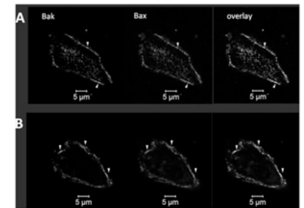
Apoptosis is a key process in the development and maintenance of tissue homeostasis. This process of controlled cell death is tightly regulated by a balance between cell survival and damage signals. We focused our attention towards the intrinsic mitochondrial apoptotic pathway, where Bcl-2 family of proteins plays the major role. We were particularly interested in two pro-apoptotic players Bak and Bax. Here we investigated their role in apoptosis triggered by photodynamic action. We show the localization of Bax and Bak in U-87 MG human glioma cells incubated with photosensitizer hypericin (Hyp) before and after photodynamic action. Apoptotic stimulus by Hyp photodynamic action caused Bax translocation into mitochondria. However our results suggest that under these conditions there are two populations of mitochondria: one which contains Bax and Bak simultaneously, and is almost exclusively localized near the plasma membrane; the other which contains Bax only and is distributed throughout the cell. The different protein content and spatial distribution of these two populations suggest that they can play different roles in response to apoptotic stimuli.



3372-Pos Board B527
Stimulation of Bax Mitochondrial Localization by Bcl-xL

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Cytochrome c release, the commitment step of apoptosis, is regulated at the mitochondria through protein-protein interactions between the Bcl-2 family proteins. An imbalance of this interaction network due to the up-regulation of the proto-oncogenes Bcl-2 and/or Bcl-xL lead to a resistance to apoptosis and is associated with tumor formation. Bcl-xL overexpression act at the level of the mitochondrial outer membrane (MOM) by inhibiting Bax-mediated apoptosis; more particularly MAC formation and cytochrome c release. However, the molecular mechanisms through which Bcl-xL affect earlier steps of Bax-mediated apoptosis are not fully understood. Surprisingly, we found that mitochondrial Bax redistribution and change of conformation were not inhibited but rather spontaneously increased in response of Bcl-xL overexpression. In order to further investigate the molecular mechanisms involved in this