VDAC1 and ANT proteins was significantly reduced ($p<0.05$), while VDAC2, VDAC3 and cyclophillin D were not significantly altered at protein level (Fig B). Downregulation of VDAC1 and ANT expression in the aging human heart may underlie the increased predisposition of the atria to injury during stress.

1956-Pos

Ranolazine Reduces Mitochondrial Tyrosine Nitration During Cardiac Ischemia and Reperfusion Injury

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Excess superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) generate peroxynitrite ($OONO^-$) during cardiac ischemia-reperfusion (IR) injury. $NO^{\bullet}$ alone may be cardioprotective whereas $OONO^-$ has deleterious effects. Tyrosine nitration by $OONO^-$ may lead to dysfunctional mitochondrial proteins. Ranolazine (RAN), a slow Na^+ channel blocker and anti-ischemic drug, may also attenuate mitochondrial complex I respiratory activity. We tested if the tyrosine nitration of mitochondrial proteins that occurred during IR was reduced when RAN was given just before ischemia. **Method:** Guinea pig hearts were perfused with Krebs -Ringer solution and subjected to one of six treatments: (i) control (no ischemia), (ii) 30 min global ischemia alone, (iii) 30 min ischemia + 10 min reperfusion, (iv) ischemia reperfusion plus RAN given for 10 min before, but not during ischemia, (v) ischemia plus RAN (no reperfusion), (vi) RAN control perfusion (no ischemia). Mitochondria were isolated immediately after each treatment. Tyrosine nitration was measured by Western blotting using 3nitro-tyrosine (3-NT) antibody. **Result:** RAN markedly improved cardiac function. Two bands positioned at about 25 kDa and 15 kDa were 3-NT immunopositive in all experiment groups. Compared to the control, mitochondria after ischemia reperfusion displayed increased 3-NT immunopositivity at the 25 kDa and 15 kDa positions by approximately 100% and 28%, respectively. Treating hearts with RAN before ischemia reperfusion decreased the 3-NT immunopositive 25 kDa band density to non-ischemia levels and the 15 kDa band density to 10% of the ischemia reperfusion alone level. The nitrated proteins require further identification. **Conclusion:** Cardiac injury increases the tyrosine nitration of selected mitochondrial proteins. Inhibition of complex I may underlie the cardiac injury-induced increase in mitochondrial protein tyrosine nitration. This reduction in mitochondrial protein nitration may correlate with the improved cardiac function we observed previously with RAN.

1957-Pos

Modulation of the Mitochondrial Permeability Transition Pore of Cardiac Myocytes by Inorganic Polyphosphate

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Background: Inorganic polyphosphate (polyP) is a long polymer made of up to several hundred orthophosphates linked together by phosphoanhydride bonds.Previously we found that polyP of rat liver mitochondria participates in formation of a channel with properties similar to the mitochondrial permeability transition pore (mPTP) suggesting a possible role in pathophysiology. The aim of this study was to investigate the role of polyP in the regulation of mitochondrial Ca homeostasis and Ca-induced opening of mPTP in cardiac myocytes. **Methods:** We used primary cultures of adult rabbit ventricular myocytes with enzymatically reduced levels of mitochondrial polyP achieved by adenoviral expression of polyP hydrolyzing enzyme from yeast (scPPX). Cytosolic Ca ($[Ca]_i$), mitochondrial Ca ($[Ca]_m$), mitochondrial membrane potential, and mPTP activity were measured using the fluorescent dyes indo-1, rhod-2, TMRM, or calcein red, respectively. **Results:** 1) No difference was detected in amplitude, rise and decay time of $[Ca]_i$ transients induced by electrical field stimulation (1 Hz) in control and scPPX expressing intact myocytes. 2) In permeabilized cells under conditions of mitochondrial Ca overload, mitochondrial Ca uptake in control cells was followed by fast Ca release which was prevented by the mPTP inhibitor cyclosporine A. The rate of mitochondrial Ca release was significantly slower in scPPX cells. 3) Similar levels of basal mitochondrial membrane potential were observed in both cell types, however Ca-induced mitochondrial membrane depolarization was more pronounced in control cells. 4) Mitochondria of permeabilized myocytes expressing scPPX were less sensitive to Ca-induced mPTP opening as estimated by the kinetics of calcein red release and the degree of Ca-induced mitochondrial membrane depolarization. **Conclusion:** Our data indicate that reducing of the mitochondrial polyP levels decreases Ca-induced opening of the mPTP in cardiac myocytes.

1958-Pos

Hydroxide Ion Channel Controls Uncoupling and Thermogenesis of Brown Fat Mitochondria

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Uncoupling proteins (UCP1- UCP5) are six-transmembrane-domain transport proteins of the inner mitochondrial membrane (IMM). They increase electrical conductance of the IMM, thus dissipating the electrochemical proton gradient across this membrane and uncoupling mitochondrial respiration and ATP synthesis. By controlling mitochondrial membrane potential, UCPs can affect many aspects of mitochondrial function and have been implicated in regulation of body's energy efficiency, reducing fat depositions, thermogenesis, diabetes, and protecting the cell against oxidative damage and ageing. The founding member of the family, UCP1, is specifically expressed in brown adipose tissue (BAT) and is responsible for adaptive thermogenesis mediated by this tissue. Due to its unusually high level of expression, upon activation UCP1 completely uncouples BAT mitochondria and converts the energy of the substrate oxidation into heat. Since UCP1 can dissipate large amounts of energy, it has attracted attention as a potential target to treat obesity. In spite of their physiological and therapeutic significance, the mechanism of operation of uncoupling proteins including their ionic selectivity has long remained unknown due to the lack of direct methods to study their activity in their native membrane environment. Here, by applying the patch-clamp technique to the whole inner membrane of BAT mitochondria and for the first time directly measuring transmembrane currents produced by UCP1, we show that UCP1 is a ligand-gated hydroxide (OH-) ion channel activated by fatty acids. UCP1 is the only hydroxide ion channel reported to date. Thus, BAT thermogenesis involves the outward transport of protons by the electron transport chain along with the outward transport of OH- by UCP1, thereby amounting to cycling of water across the IMM and not to futile cycling of protons as was largely considered before.

1959-Pos

Characterization of an Anion Channel on the Inner Membrane of Heart Mitochondria

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Preconditioning is a powerful form of cardioprotection whereby a brief ischemic episode, or a brief exposure to drugs such as volatile anesthetics, can protect the myocardium from a subsequent prolonged ischemia. It triggers an intracellular signaling cascade that leads to the delay in the opening of the mitochondrial permeability transition pore (mPTP). Depolarization of the inner membrane of mitochondria (IMM) can delay mPTP opening. Ion channels have been identified on the IMM that may play key roles in this depolarization. Yet their molecular identities and detailed electrophysiological characterizations have been elusive. In the present study, we recorded ion channel activities on the IMM isolated from guinea pig hearts. Mitoplasts (mitochondria sans the outer membrane) were formed by incubating mitochondria in a hypotonic buffer. The inside-out configuration of the patch clamp technique was utilized. We have identified a channel with a primary conductance of 109 ± 5 pS (n=9) in equimolar 150 mM KCl. The channel exhibited voltage-dependent behavior, with activity being more prominent at positive membrane potentials. When the 150 mM KCl bath solution that corresponded to the mitochondrial matrix side was replaced with 150 mM K-glutamate, channel activity was abolished. When TEA-Cl substituted for KCl, channel activity was not significantly affected. These results suggested an anion channel permeable to chloride. This was confirmed by DIDS (100 μM), a chloride channel blocker, which abolished channel activity. However, bongkrekic acid (100 nM), a specific inhibitor of the mitochondrial adenine nucleotide translocase, failed to inhibit channel activity. In addition, the presence of 2 mM Mg2+ in the buffer solution, a concentration that blocks IMAC, the inner membrane anion channel, did not prevent channel opening. Experiments are currently underway to further characterize and identify this anion channel on the IMM.

1960-Pos

Upregulation Leads Bcl2 to Behave as a Mitochondrial Decoy Receptor for Bax

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Cytochrome c release, the commitment step of apoptosis, is regulated at the mitochondria through protein-protein interactions between the Bcl2 family proteins. An imbalance of this interaction network due to the upregulation of the proto-oncogene *Bcl2* leads to a resistance to apoptosis and is associated with tumor formation. Bcl2 overexpression inhibits BAX oligomerization and mitochondrial outer membrane (MOM) permeabilization. However, the molecular

mechanisms through which upregulation of Bcl2 affects earlier steps of BAX-mediated apoptosis are not fully understood. We found that BAX insertion into the MOM was the earliest apoptotic step inhibited by Bcl2 overexpression. Paradoxically, we also found that BAX translocation to the mitochondria was not inhibited but rather spontaneously increased in this same genetic context. This increase in mitochondrial associated BAX required a physical interaction between BAX and Bcl2. We therefore propose that, at least when upregulated, Bcl2 behaves as a 'decoy receptor' which sequestrates BAX at the mitochondria but inhibits its insertion into the MOM, committing the cell to survive.
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1961-Pos
Compartmentalization of BCL2 Family Proteins Mediated by Organelle Lipid Membranes
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Cancer is defined by a pronounced inhibition of cell death. The BCL2 family of proteins tightly regulates the delicate balance between life and death. One method of regulation is the compartmentalization of antagonistic members. For example, Bax, a pro-apoptotic member of this family, acts as the penultimate factor in the apoptotic cascade by releasing apoptogenic factors such as Cytochrome C from the mitochondrial lumen. The normally cytosolic protein translocates from one internal compartment to another through an elusive mechanism. Individual organelles are defined not only by function (mediated by specific membrane bound proteins), but by the unique composition of their phospholipid membranes. In this work, we have evaluated the contribution of organelle lipids to the localization of of BCL2 proteins.

1962-Pos
In Search of the Structure of MAC in the Mitochondrial Outer Membrane
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Several groups have tried to determine the structure of the channel (MAC) formed in mitochondrial outer membranes (OM) of apoptotic cells or in synthetic membranes by Bax and related proteins/peptides, using electron microscopy (EM), atomic force microscopy and x-ray diffraction. Here, pore-like structures ~3-10 nm were handpicked from transmission EM images of uranyl-acetate-stained OMs isolated from control and apoptotic (IL3-deprived) FL5.12 cells. These ''candidate pores'' were aligned by correlation procedures, and class averages defined by principal component and K-means analyses. Main differences in the class averages were (1) the presence of one or more dark (stain-filled) pores, and (2) the nature of white (stain excluding) features around the pores. A class average consisting of a single 3-nm pore with pronounced white rim was rotationally averaged and used as a reference for cross-correlation searches of 50 OM images from control and apoptotic cells. Searches using this 3-nm ''donut'' motif and the same motif doubled in size (6-nm ''donut'') yielded thousands of ''hits'' in both control and apoptotic membranes, which were subsequently aligned and classified as before. The predominant stain-filled structures found with both motifs were not circular but elongated (up to ~4x6 nm), extending away from stain-excluding crescent-shaped features. The radial anisotropy ruled out reference bias and was inconsistent with pores formed by rings of evenly spaced protein subunits. We hypothesize that the different classes of structures detected represent stages in formation of MAC as an increasingly large membrane bilayer defect (or ''cleft'') induced by successive aggregation or clustering of Bax/Bak molecules. A progressive assembly mechanism for MAC has been recently suggested by real-time monitoring of MAC conductances in isolated mitochondria by patch clamping (Martinez-Caballero et al. J Biol Chem 284: 12235-45). Supported by NIH grant GM57249.

1963-Pos
Effects of MAC Formation on Mitochondrial Morphology
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Accumulating literature associate mitochondrial dynamics with apoptosis, since regulation of either process has reciprocal effects. These processes seem to converge in formation of the mitochondrial apoptosis induced channel, MAC, which releases cytochrome c and triggers the degradation phase of apoptosis. While Bax and Bak, core components of MAC, were shown to interact with fusion and fission proteins, some studies also suggest proteins from the intermembrane space could leak to the cytosol and further promote mitochondrial fission during apoptosis. The temporal relationship between apoptosis induction, MAC formation and mitochondrial fragmentation was investigated by time lapse microscopy. MAC function was induced through staurosporine treatment and microinjection of tBid or cytochrome c. MAC formation and mitochondrial dynamics under these conditions were monitored in Hela cells (clone 10) that stably express low levels of GFP-Bax and were transiently transfected with a pDsRed-2 plasmid. GFP-Bax relocation to mitochondria only during apoptosis signals MAC formation, while pDsRed-2 expression shows mitochondrial structure as red fluorescence. Treatment with staurosporine and microinjection with tBid or cytochrome c induced relocation of Bax and collapse of the mitochondrial network. The temporal relationship between these two events was further analyzed. Interestingly, pretreatment with iMAC2, a specific MAC blocker, protected against cell death and prevented mitochondrial fragmentation after tBid injection. Our results suggest a link exists between MAC formation and collapse of the mitochondrial network during apoptosis. Supported by NIH grant GM57249.

1964-Pos
Mechanism of the Mitochondrial Cytochrome C Release Wave in Bid-Induced Apoptosis
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Bid, a BH3-only Bcl2 family protein, plays a central role in apoptosis. Bid is cleaved by caspase-8 and other enzymes forming tBid that induce mitochondrial outer membrane (OMM) permeabilization and cytochrome c (cyto-c) release. However a mystery remains how Bid synchronizes the function of a large number of discrete organelles, particularly in mitochondria-rich liver or muscle cells. Here we showed that tBid (0.5-50nM) elicited progressive OMM permeabilization and complete cyto-c release with a dose-dependent lag time and rate in H9c2 cell populations. Once started, the OMM permeabilization was not prevented by tBid washout. In contrast, the dose-response for digitonin-induced OMM permeabilization displayed quantal behavior. In single cell imaging studies, permeabilized H9c2 and primary human cardiac cells transfected with cyto-c-GFP showed complete tBid-induced cyto-c-GFP release closely followed by mitochondrial depolarization. The cyto-c-GFP release started at discrete sites and propagated through the mitochondria with a constant velocity and a relatively stable kinetic of release in each organelle. Similar tBid-induced cyto-c-GFP release wave was documented in intact H9c2 cells transfected with tBid. The waves were not dependent on Ca^{2+}, caspase activation or permeability transition pore opening. However, treatment with MnTMPyP, a ROS scavenger or overexpression of mitochondrial superoxide dismutase suppressed the coordinated cyto-c release and also inhibited tBid-induced cell death. On the other hand, both superoxide and hydrogen peroxide sensitized mitochondria to the tBid-induced permeabilization. Thus, tBid engages a ROS-dependent inter-mitochondrial signaling mechanism for spatial amplification of the apoptotic signal by mitochondrial waves.

1965-Pos
Role of Milton Domains in the Calcium-Dependent Regulation of Mitochondrial Motility
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The mammalian GRIF1 and OIP106, and the drosophila Milton are kinesin-binding proteins that form a complex with the Miro GTPase, an outer mitochondrial membrane EF-hand protein, to support the movement of mitochondria along the microtubules. Our study demonstrates that in H9c2 cells overexpressing OIP106 or GRIF1, the basal motility of mitochondria is increased, whereas the sensitivity to calcium-induced movement inhibition is decreased. To dissect the interaction between Milton, kinesin and Miro, three different Milton constructs were tested: Milton (1-450), the soluble domain of Milton; Milton (750-1116), lacking the kinesin heavy chain binding site and Milton (847-1116) that lacks additional ~100 amino acid presumably containing part of the Miro binding site. Immunohistochemistry revealed that the overexpressed Milton (1-450) was cytoplasmic, whereas the other two Milton constructs showed mitochondrial localization. The basal mitochondrial motility was increased by Milton (750-1116) but was not altered by Milton (847-1116) or Milton (1-450). A plot of mitochondrial motility against slowly rising cytoplasmic [Ca2+] induced by thapsigargin (2μM), shows that overexpression of Milton (750-1116) significantly reduced the calcium sensitivity of mitochondrial motility reminiscent of OIP106 and GRIF1. By contrast, Milton (847-1116) or Milton (1-450) did not have any effect. The thapsigargin-induced cytoplasmic calcium signal was not affected by any of the Milton constructs. These data indicate that