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Abstracts

S15 Cell Physiology, Apoptosis and Mitochondrial Signalling

Lectures

15L.1 Dissecting the BAK-driven outer mitochondrial membrane permeabilization pathway using *in vitro* reconstituted systems

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BAK is a key proapoptotic member of the BCL-2 family, which primarily functions by forming an apoptotic pore in the mitochondrial outer membrane (MOM) allowing for the release of multiple deadly factors into the cytosol. Under healthy conditions, BAK adopts an inactive, "non-permeabilizing" conformation at the MOM. Upon apoptosis triggering, BAK interacts with specific components of the MOM and shifts to an activated "permeabilizing" state. However, the precise molecular pathway by which BAK proapoptotic function is activated remains ill defined, and the biophysical mechanism of pore formation by BAK remains unclear. We examined these issues using a combination of biophysical and biochemical techniques in an *in vitro* reconstituted system consisting of recombinant purified proteins and MOM-like liposomes. Using this minimalistic system we demonstrate that BAK possesses an intrinsic pore-forming activity, which can be unleashed by sequential interaction with selected mitochondrial lipids and proteins. The BAK-driven liposome permeabilization pathway is associated with membrane insertion and oligomerization of the protein, and culminates with formation of a proteolipidic pore which gradually grows in size. We also present information on the topography adopted by BAK at distinct stages along the membrane permeabilization pathway.

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15L.2 Mitochondria in regulation of cell death in cardiovascular and brain pathologies

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Mitochondrial membrane permeabilization has been considered as the central, irreversible event in mitochondrial pathway of apoptosis. Opening of mitochondrial permeability transition pore (MPTP) is one of the means of permeabilization of mitochondrial membranes leading to the release of cytochrome *c* from mitochondria, caspase activation and apoptotic or necrotic cell death. MPTP has been implicated in various pathologies including heart ischemia/reperfusion, beta amyloid-induced neurotoxicity in Alzheimer's disease, etc. In such pathologies, inhibition of MPTP is critical for cardio- and neuroprotection. We have shown that low concentrations of NO may activate signalling pathways involving activation of protein kinase C and protein kinase G (PKG) and leading to increased resistance of mitochondria to opening of MPTP. Similarly, high physiological concentrations of estrogens trigger signalling cascades involving Akt, NO synthase, PKG and rendering mitochondria increased resistance to calcium-induced MPTP and protecting against ischemia-induced apoptosis in the heart. In the contrary to previous beliefs, there is accumulating evidence that mitochondrial cytochrome *c* release does not necessarily lead to cell death. If cells have high ability to reduce cytosolic cytochrome *c*, then caspase activation can be blocked even at post-cytochrome *c* level. We and others have shown that reduced cytochrome *c* is less capable in caspase activation than its oxidized form. Now we demonstrate that cytochrome *c* reducing agents such as TMPD can prevent ischemia-reperfusion-induced caspase activation and apoptosis in perfused heart or in neuronal cell cultures exposed to staurosporin or oligomeric forms of beta amyloid. Mitochondria also posses activities which can oxidize or reduce cytosolic cytochrome *c* and their external cytochrome *c*-reducing activity may depend on cytosolic concentrations of NAD(P)H.

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15L.3 pH Spikes in individual mitochondria

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Mitochondria are multifunctional intracellular organelles involved in energy production, apoptosis, and calcium signalling. The pH within the mitochondrial matrix (pH_{mito}) is an important bioenergetic parameter that has been well studied in isolated mitochondria. How pH_{mito} is regulated in intact living cells is less clear and all *in situ* studies so far report spatially averaged measurements of pH_{mito}. In this study, we use a new pH-sensitive GFP-based fluorescent probe targeted to the mitochondrial matrix, mito-SypHer, to study pH_{mito} homeostasis at the level of single mitochondria. In Hela cells expressing mito-SypHer, we observed that individual mitochondria undergo spontaneous basifications transients, pH spikes. The pH spikes occurred randomly in time and space and had a characteristic profile, with a rapid onset (time to peak 1.6 ± 0.1 sec), a slower decay (t=8.5 ± 0.6 sec), and an average amplitude of 0.38 ± 0.05 pH units. Spikes were not spatially restricted to single mitochondria but were also observed in clusters of interconnected mitochondria, suggesting

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functional coupling. The pH spikes were abrogated by protonophores and their frequency strongly decreased by respiratory chain inhibitors (rotenone: -57%, antimycin: -96%) or by inhibition of the ATP-synthase (oligomycin: -52%). Conversely, inhibition of the adenine nucleotide exchanger (ANT) by actractyloside increased spike frequency by 510%. Normal pH spikes were observed in cells pre-treated with the SERCA-ATPase inhibitor thapsigargin, indicating that the pH elevations did not require calcium release from intracellular stores. Simultaneous ψ_{mt} and pH_{mito} measurements revealed concomitant depolarization and basification transients. Superoxide flashes with similar properties were previously reported in individual mitochondria with a circularly permutated YFP (Wang *et al.*, 2008, *Cell* 134). Since this probe is known to be pH-sensitive, the signals reported as superoxide flashes might have been due to pH spikes. Alternatively, superoxide flashes could generate pH spikes *via* Fenton and dismutation reactions, although we did not detect ROS elevations with the mitochondrial ROS sensor roGFP. In summary, we show that individual mitochondria in intact HeLa cells undergo spontaneous basification transients. The pH spikes are not due to calcium release from stores, but require functional OXPHOS machinery.

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15L.4 Mitochondrial cholesterol and cell death

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Cholesterol is a critical component of biological membranes, which determines their structural and biophsycial properties. Its distribution within membranes is heterogeneous, partitioning in specialized domains called rafts, where modulate signaling pathways. Due to this fundamental role cholesterol levels are highly regulated. Cholesterol distributes to different subcellular compartments by vesicular dependent and independent mechanisms. Compared to plasma membranes, mitochondria are cholesterol-poor organelles, with estimates of 0.5-3% of the total cholesterol pool. While hepatic mitochondrial cholesterol plays an important physiological role such as in the synthesis of bile acids, its accumulation contributes to liver diseases, such as alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC). Mitochondrial cholesterol loading in ASH and NASH models sensitizes hepatocytes to oxidative stress and inflammatory cytokines, contributing to fatty liver disease by a mechanism that involves mitochondrial GSH (mGSH) depletion due to changes in mitochondrial membrane dynamics. mGSH depletion protects cardiolipin from oxidation to peroxidized cardiolipin, which determines mitochondrial membrane permeabilization by proapoptotic bcl-2 family members, such as Bax. Interestingly, mitochondrial cholesterol accumulation also occurs in HCC, which contributes to chemotherapy resistance. However, despite cholesterol loading, HCC cells exhibit unimpaired transport of GSH into mitochondrial matrix due to the overexpression of mGSH carriers, 2-OG and DIC. This maintenance of mGSH prevents cardiolipin peroxidation. Peroxidized cardiolipin, however, overcomes the resistance to mitochondrial membrane permeabilization induced by Bax. These results characterize mitochondrial cholesterol/peroxidized cardiolipin as a rheostat in cell death regulation.

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15L.5 Signalosomes transmit signals from plasma membrane receptors to mitochondria

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When an agonist binds to a plasma membrane receptor, a signaling cascade is triggered that targets intracellular organelles. Mitochondria respond by opening the mitochondrial ATP-sensitive K^+ channel ($mitoK_{ATP}$) and producing ROS for further signaling. Cardioprotection against ischemia- reperfusion (IR) injury is a useful experimental model for probing this process, because these signals reduce infarct size by about 70%. Many receptors produce the cardioprotective response, including Gi protein-coupled receptors (adenosine, acetylcholine, bradykinin, opioids, and phenylephrine), the Na,K-ATPase (ouabain, digitalis), and the L-type Ca^{2+} channel (Ca^{2+}). We have found that the entire signaling cascade is assembled in plasma membrane caveolae, then buds off as a 140 nm signalosome, internalizes, and migrates to mitochondria. The terminal kinases of the cascade phosphorylate a protein on the outer membrane. This leads to activation of an inner membrane PKCε, which opens $mitoK_{ATP}$ by phosphorylation. The signalosomes can be isolated and purified from the perfused heart and displays activity *in vitro*. This allows us to study a signaling unit in its naturally organized state with preserved functionality. Most signalosomes are functionally active within minutes of receptor activation. Interestingly, the adenosine signalosome requires an additional step of ROS activation after internalization, and the adenosine receptor remains active *in vitro*.

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15L.6 The gas pedal of brain mitochondria: glutamate supply for OXPHOS is fully regulated by cytosolic Ca^{2+} *via* activation of aralar

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The regulation of OXPHOS is still not understood in detail. ADP formed by ATP-consuming enzymes activates OXPHOS but in the heart cytosolic ADP is only insignificantly increased *in vivo* during elevated work loads [1] and therefore the parallel stimulation of OXPHOS and work load by cytosolic Ca^{2+} (Ca^{2+}_{cyt}) was assumed [2]. However, activation of dehydrogenases by matrix Ca^{2+} [3] complies only partially with the *in vivo* findings, therefore we hypothesized that other mechanisms should be responsible for mitochondrial activation by Ca^{2+}_{cyt}. We have found recently [4-6] that the glutamate dependent respiration of brain mitochondria can be stimulated by Ca^{2+}_{cyt} due to the activation of aralar [7], the glutamate aspartate carrier ($S_{0.5}$ = 260 nM Ca^{2+}_{free}). Depending on its initial concentration, Ca^{2+} can activate state $3_{glu/mal}$ of brain mitochondria up to