

Session 3: Signalling To and From the Mitochondria.

I. Nitric Oxide and Calcium



3-01. Interactions of nitric oxide with mitochondrial cytochrome oxidase: a complete kinetic model requires binding to both haem and copper.

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The nitric oxide signalling pathway is classically mediated by activation of soluble guanylate cyclase. However, in 1994 it was additionally shown that mitochondrial oxygen consumption by cytochrome oxidase is reversibly inhibited by NO in a manner apparently competitive with the oxygen tension [1]. Inhibition of mitochondrial respiration by NO at cytochrome oxidase has since been implicated in a wide range of physiological processes, [2-3] including redox signalling, oxygen sparing and brain blood flow-metabolism coupling. It was initially suggested that NO inhibited by binding to the ferrous haem a_3 oxygen binding site. However, in 1997 we demonstrated that NO could also interact with an oxidised copper centre in the enzyme (Cu_B) that does not bind oxygen [4]. A recent paper has attempted to explain all the extant literature data within a simple one-site competitive model [5]. Here we report experimental and modelling studies that demonstrate that such a model is inconsistent with NO interactions with the enzyme. Although NO inhibition is always oxygen sensitive, the sensitivity is not consistent with a pure competitive interaction. Non-competitive interactions at a second (Cu_B) site are required and these effects predominate at low oxygen consumption rates and high pO_2 . We describe a complete kinetic model of NO inhibition of cytochrome oxidase which, given the cellular NO, O_2 and oxygen consumption rates, describes not only the expected degree of inhibition of the enzyme, but also the nature of the inhibited state. Surprisingly at low NO concentrations (<50 nM) the copper interaction is preferred over the haem even at relatively low oxygen tensions (20 μ M). Nitric oxide:copper reactions are therefore crucial in maintaining NO control of mitochondria throughout the *in vivo* range of mitochondrial oxygen consumption rates and oxygen tensions.

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3-02. Differential sensitivity of cytochrome c oxidase and guanylate cyclase to endogenous nitric oxide at physiological oxygen concentrations.

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Nitric oxide (NO) stimulates cGMP production by binding to the haem iron of soluble guanylate cyclase. NO also binds to the binuclear center of cytochrome c oxidase reversibly inhibiting mitochondrial oxygen consumption in competition with oxygen [1,2]. Recent work suggests that guanylate cyclase is significantly more sensitive to being activated by NO than cytochrome c oxidase is to being inhibited at physiological p_{O_2} [3]. The aim of this study was to determine the sensitivity of both enzymes to endogenously produced NO at physiological oxygen concentrations (30 $\mu\text{M O}_2$). We used HEK 293 cells transfected with the inducible isoform of the NO synthase gene under the control of a tetracycline-inducible promoter. In this system, NO is generated inside the cells at different levels for extended periods of time. The amount of NO produced was within the physiopathological range (up to 1.3 μM). NO-stimulated cGMP production was measured at 30 $\mu\text{M O}_2$ in hypoxic chambers, while mitochondrial oxygen consumption was determined by high-resolution respirometry (OROBOROS Oxygraph-2k). Under physiological conditions of p_{O_2} , the NO concentration giving half-maximal activation (EC_{50}) of guanylate cyclase was around 3 nM, whereas that required to achieve 50 % inhibition of respiration (IC_{50}) was determined to be 160 nM from a value of 14 $\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ cells for controls. These data show that, in our system, the IC_{50} of cytochrome c oxidase is more than 50-fold higher than the EC_{50} of soluble guanylate cyclase to endogenous NO, thus confirming previous studies using NO donors which suggest that the latter enzyme is more sensitive than mitochondrial respiration to NO at physiological oxygen pressure.

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3-03. Crosstalk between mitochondria and ER: nitric oxide and calcium.

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Studies with isolated mitochondria are performed at artificially high p_{O_2} (220 to 250 μM oxygen), although this condition is hyperoxic for these organelles. It was the aim of this study to evaluate the effect of hypoxia (20-30 μM) on the calcium-dependent activation of 2-oxoglutarate dehydrogenase (or 2-ketoglutarate dehydrogenase; OGDH) and mitochondrial nitric-oxide synthase (mtNOS). Mitochondria had a P/O value 15% higher in hypoxia than that in normoxia, indicating that oxidative phosphorylation and electron transfer were more efficiently coupled, whereas the intramitochondrial free calcium concentrations were higher (2-3-fold) at lower p_{O_2} . These increases were abrogated by ruthenium red indicating that the higher uptake via the calcium uniporter was involved in this process. Based on the difference on $K_{0.5}$ for calcium for mtNOS and Krebs cycle (for oxoglutarate dehydrogenase 0.16 μM and mtNOS ~ 1 μM), mitochondria can produce nitric oxide at relatively "high calcium" microdomains. Nitric oxide, by binding to cytochrome oxidase in competition with

oxygen, decreases the rate of oxygen consumption. This condition is highly beneficial for the following reasons: i, these mitochondria are still able to produce ATP and support calcium clearance; ii, it prevents the accumulation of ROS by slowing the rate of oxygen consumption (hence ROS production); iii, the onset of anoxia is delayed, allowing oxygen to diffuse back to these sites, thereby ameliorating the oxygen gradient between regions of high and low calcium concentration. In this way, oxygen depletion at the latter sites is prevented. This, in turn, assures continued aerobic metabolism by activating the calcium-dependent dehydrogenases.



3-04. Agonist induced mitochondrial ATP production critically depends on the trans-mitochondrial Ca²⁺ flow rate.

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Cell stimulation with an IP₃ generating agonist rapidly evokes increases in cytosolic free Ca²⁺ ([Ca²⁺]_{cyto}) which are partially transferred into the lumen of mitochondria in many different cell types. Recent studies have suggested that such an elevation of the mitochondrial Ca²⁺ concentration ([Ca²⁺]_{mito}) is crucial to stimulate mitochondrial oxidative phosphorylation. Supposable targets regulated by [Ca²⁺]_{mito} are intra-mitochondrial dehydrogenases and metabolite carriers in the mitochondrial inner membrane. Eventually, Ca²⁺ triggered enhancement of these activities leads to increased levels of adenosinetriphosphate (ATP) [1]. Ca²⁺ induced mitochondrial ATP production requires first of all sufficient Ca²⁺ uptake into mitochondria, which is ensured by the negative membrane potential across the mitochondrial inner membrane of energized mitochondria and a positioning of mitochondrial Ca²⁺ uptake channel(s) close to sites of Ca²⁺ release and Ca²⁺ entry. Recent studies have suggested that mitochondrial uptake of entering Ca²⁺ is essential to maintain Ca²⁺ inhibited capacitative Ca²⁺ entry (CCE) [2]. Thus, most of the Ca²⁺ that enters the cell is immediately taken up by subplasmalemmal energized mitochondria. However, the contribution of CCE to the activation of mitochondrial ATP production is not known. Surprisingly, it has been found that upon cell stimulation with an IP₃ generating agonist, [Ca²⁺]_{mito} increases transiently despite a sustained elevation of [Ca²⁺]_{cyto}. Recently we showed that in endothelial cells the transient nature of [Ca²⁺]_{mito} elevation crucially depends on the activity of the mitochondrial Na⁺/Ca²⁺-exchanger (NCX_{mito}), extracellular Na⁺ and the activity of sarcoplasmic reticulum Ca²⁺ ATP-ase (SERCA) [3]. Utilizing mitochondrial targeted firefly luciferase (Luc-mt) as a reliable sensor of changes in ATP levels, the described mitochondrial Ca²⁺ cycling could be compared to that of mitochondrial ATP production upon cell stimulation with an IP₃ generating agonist. Agonist induced mitochondrial oxidative phosphorylation crucially depended on the presence of extracellular Ca²⁺, while just IP₃ mediated transfer of Ca²⁺ from the ER to mitochondria was insufficient to stimulate mitochondrial ATP biosynthesis. Surprisingly, if the mitochondrial Ca²⁺ signal was prolonged by either removal of extra-cellular Na⁺ or by an inhibition of NCX_{mito} with CGP 37157, mitochondrial ATP production in response to the agonist was diminished. In line with these findings, SERCA inhibition during cell stimulation, which also impairs mitochondrial Ca²⁺ extrusion, abolished the agonist induced increase of mitochondrial ATP, too. These findings demonstrate that the agonist induced mitochondrial ATP biosynthesis is impaired if mitochondria are forced to elevate [Ca²⁺]_{mito} during stimulation with an IP₃ generating agonist, indicating that mitochondrial Ca²⁺ clearance contributes to agonist induced mitochondrial ATP production. Therefore it is tempting to speculate, that mitochondrial ATP production rather benefits from the mitochondrial Ca²⁺ turnover than from sustained mitochondrial Ca²⁺ accumulation upon cell stimulation.

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3-05. Molecular structure of the contact sites formed between mitochondria and ER. Evidence for the PTP role in mitochondrial Ca^{2+} uptake.

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The contacts between mitochondria and endoplasmic reticulum (ER) play an important function in cell metabolism – they secure a direct calcium transmission from ER to the mitochondria. Upon opening of the inositol 1,4,5-triphosphate (IP3)-gated channels of the ER, the mitochondrial surface becomes exposed to a higher concentration of Ca^{2+} than that in the bulk cytosol. This enables the uptake of calcium by mitochondria *via* low affinity calcium uniporter [1]. In this study we tried to find answers to the following three questions: How do the contacts between mitochondria and the ER look like? Is the mitochondrial permeability transition pore (PTP) located in the proximity of these contacts? Is PTP the main target for calcium originating from the ER during stimulation of the cell.

For this purpose we studied protein components of mitochondria-associated membranes (MAM fraction) which can be a "junction bridge" between ER and mitochondria. Then, we investigated the roles of the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane and of the adenine nucleotide translocase (ANT), components of the PTP, in modulating mitochondrial calcium response. We overexpressed VDAC and three isoforms of ANT (ANT-1, ANT-2, ANT-3) in HeLa cells and analyzed calcium homeostasis, mitochondrial membrane potential and structure of mitochondria and the endoplasmic reticulum (ER). In our a paper [2] we have proposed that VDAC is a key determinant of Ca^{2+} permeability at ER-mitochondria contacts and is thus responsible for exposing calcium uniporter of the inner mitochondrial membrane to the large [Ca^{2+}] gradients needed for rapidly accumulating Ca^{2+} in mitochondria upon cell stimulation. Based on this and on our recent results, we propose that VDAC increases calcium permeability of the ER-mitochondria contact sites. On the other hand, we observe large, cyclosporin A sensitive, reduction of mitochondrial calcium uptake in ANT-1 and ANT-3 transfected cells. The cytosolic calcium response after agonist stimulation is as in control cells. Moreover, overexpression of ANT-1 and ANT-3 (but not ANT-2) induces mitochondrial fragmentation. This and other our results could clarify the role of a larger complex including VDAC, ANT, cyclophilin D, the peripheral benzodiazepine receptor and members of the Bcl-2 family in the interaction between mitochondria and ER, and the possibility that some of these proteins are a part of the molecular machinery docking mitochondria to Ca^{2+} stores.

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3-06. Calcium activation of the malate-aspartate shuttle in tissues expressing distinct isoforms of the mitochondrial aspartate-glutamate carrier.

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Aralar and citrin are the human isoforms of the aspartate-glutamate carrier (AGC) involved in the NADH Malate-Aspartate shuttle (MAS) for the transfer of reducing equivalents from cytosol to mitochondria [1-4]. Aralar and citrin have several EF-hands motifs in a long amino terminal extension that faces the intermembrane space. We have tested a possible role for extra mitochondrial calcium in activation of MAS, as the step catalyzed by the AGC is the only one irreversible of that pathway. To this end, we have measured the calcium sensitivity of the shuttle reconstituted in isolated mitochondria, in the presence of ruthenium red to inhibit the calcium uniporter. Aralar and citrin have different expression patterns [5], and we have studied the kinetics of calcium activation in tissues with only one isoform. Mitochondria of tissues which express only aralar (namely brain and muscle) exhibit a calcium activation of MAS of 3 fold, with an $S_{0.5}$ of about 340-230 nM, whereas in liver, where only citrin is present, the increase of activity due to calcium was lower (1.5-fold) with a $S_{0.5}$ of 100-140 nM. In the heart, where both isoforms are coexpressed, calcium activation is also observed ($S_{0.5}$ 230 nM, 3.5-fold activation), and appears to be due to aralar since it disappears completely in Aralar-deficient mice.

So far, calcium signalling in mitochondria is believed to take place mainly through calcium uptake in mitochondria across the calcium uniporter (CU), followed by activation of three dehydrogenases (pyruvate, isocitrate and α -ketoglutarate) of the mitochondrial matrix that results in an increased production of mitochondrial NADH. Calcium uptake through CU requires relatively high calcium concentrations [6], higher than those activating MAS. In conclusion, MAS is activated by calcium concentrations smaller than those activating the CU-mitochondrial dehydrogenase pathway, specially through aralar as AGC. Therefore, it is an adequate mechanism to transduce small cytosolic calcium signals to the mitochondria. On the other hand, the very low $S_{0.5}$ for calcium in liver mitochondria in which citrin is the only AGC suggests that MAS would be constitutively activated at the normal resting $[Ca^{2+}]_i$.

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3-07. Subplasmalemmal mitochondria modulate the activity of plasma membrane Ca²⁺ extrusion machinery.

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Mitochondria actively participate to the cellular calcium homeostasis and modulate the pattern of agonist-induced calcium signals by their ability to sequester and release calcium. In particular during agonist stimulation, the Ca²⁺ released from the ER is taken up by mitochondria and subsequently returned back to the ER, thus preventing its depletion [1]. As well, the Ca²⁺ that enters the cell transit through mitochondria toward the ER, short-circuiting the cytosol [2]. The calcium fluxes between mitochondria and the plasma membrane or the ER are difficult to study because, in most mammalian cells, mitochondria assemble into a dynamic network constantly remodeled by fusion and fission reactions. To study this dynamic Ca²⁺ connexion, we overexpressed a protein of the mitochondrial fission machinery, hFis1, and measured Ca²⁺ within organelles with GFP-based Ca²⁺ sensors. hFis1 overexpression induces a rapid fragmentation of mitochondria which then cluster around the nucleus, leaving subplasmalemmal ER regions devoid of mitochondria. We previously showed that this remodeling did not impair the ability of mitochondria to accumulate the calcium released from the ER in HeLa cells [3]. The cytosolic Ca²⁺ signals elicited by histamine were unaltered as long as Ca²⁺ was present in the extracellular medium, but were significantly blunted when Ca²⁺ was removed from the medium. Upon Ca²⁺ withdrawal, the free ER Ca²⁺ concentration, [Ca²⁺]_{ER}, decreased rapidly and cells with fragmented mitochondria were unable to respond to repetitive stimulations. The loss of stored Ca²⁺ was due to an increased activity of the extrusion machinery (Ca²⁺-ATPase and/or Na⁺/Ca²⁺ exchanger) and was associated with an increased influx of Ca²⁺ and Mn²⁺ across Ca²⁺ entry channels. The increased Ca²⁺ influx compensated the loss of stored Ca²⁺, and brief Ca²⁺ additions between successive agonist stimulations fully corrected subsequent histamine responses. We proposed that the lack of subplasmalemmal mitochondria disrupts the transfer of Ca²⁺ from plasma membrane channels to the ER, and that the resulting increase in subplasmalemmal [Ca²⁺] enhances Ca²⁺ extrusion mechanisms.

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3-08. Mitochondrial ATP regulation at fertilization.

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In mammalian oocytes, attachment of the sperm to the oocyte induces drastic changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) consisting of a single relatively long lasting (~5 min) rise in [Ca²⁺]_i followed by short repetitive transients of [Ca²⁺]_i lasting several hours. These changes in [Ca²⁺]_i at fertilization are called Ca²⁺ oscillations and appear to be a prerequisite for the normal development of embryos. Delayed fertilization (postovulatory aging) of oocytes significantly affects both embryonic development and Ca²⁺ oscillations [1,2]. Because Ca²⁺ oscillations depend on Ca²⁺ release and reuptake in the endoplasmic reticulum (ER) and the latter relies upon ATP availability in the ER, we

undertook the present study to address the role of intracellular ATP regulation at fertilization in the delayed fertilized mouse oocyte. Measurement of $[Ca^{2+}]_i$ was conducted using fluorescent dye fura-PE3 while intracellular ATP concentration ($[ATP]_i$) was continuously assessed in single oocytes from changes in intracellular free Mg^{2+} concentration measured by Mg^{2+} sensitive dye magnesium green (MgG). At fertilization, MgG fluorescence was transiently increased concomitant with the first transient elevation of $[Ca^{2+}]_i$ indicating a relative decrease in $[ATP]_i$. In the fresh oocyte (oocytes recovered from the oviduct 12.5 hrs after hCG injection), it was quickly followed by a significant decrease below the baseline indicating a relative increase in $[ATP]_i$. In contrast, in the aged oocytes (oocytes recovered from the oviduct 18.5 hrs after hCG injection), such a decrease in MgG fluorescence was not observed. In the separate experiment, ATP content in the fresh and aged oocytes was determined *in vitro* by the luciferin-luciferase assay. Intracellular ATP contents measured *in vitro* were comparable in the unfertilized fresh and aged oocytes. Intracellular ATP content at 5 hrs after fertilization was increased in the both oocytes, where the fresh oocyte showed a significantly higher value than the aged oocyte. From these results, we conclude that fertilization shifts the set point of intracellular ATP regulation in the fresh oocyte so that abrupt increases in ATP utilizations at fertilization are effectively buffered. In contrast, the aged mouse oocytes failed to readjust the level of intracellular ATP at fertilization. Relative deficiencies of ATP at fertilization may lead to the altered Ca^{2+} oscillations pattern and poor developmental potency commonly noted in the aged oocyte [3].

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3-09. Mitochondrial nitrogen radical synthesis by a NOS-independent mechanism.

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Mitochondria are suspected to produce nitric oxide (NO) and reactive nitrogen species (RNS), which regulate the action of the respiratory chain. However, the existence of a distinct mitochondrial NO synthase enzyme (mtNOS) is debated and the mechanism by which mitochondria produce RNS is unclear [1]. We hypothesized that not mtNOS, but the respiratory chain enzymes are responsible for RNS production. Diaminofluorescein (DAF) was applied for the assessment of RNS production in isolated mouse brain, heart, and liver mitochondria and also in a cultured neuroblastoma cell line. Fluorescence was detected by confocal microscopy and flow cytometry. Respiring mitochondria produced reactive nitrogen species, which were inhibited by catalysts of peroxynitrite decomposition. Mitochondria from different regions had varying morphology, but their DAF fluorescence was similar. Withdrawal of arginine and calcium or the application of nitric oxide synthase inhibitors failed to decrease DAF fluorescence. In contrast, disrupting the integrity of the organelles or withdrawing respiratory substrates markedly reduced RNS production. Inhibition of Complex I abolished the DAF signal, which was restored in the presence of Complex II substrates. Inhibition of the respiratory complexes downstream from the ubiquinone cycle or even dissipating the proton gradient had no effect on DAF fluorescence indicating that the redox state of ubiquinone significantly

affects the reaction. We conclude that mitochondria from brain, heart and liver are capable of significant RNS production via the respiratory chain rather than through an arginine-dependent mitochondrial nitric oxide synthase.

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3-10. Minocycline does not inhibit calcium-induced mitochondrial permeability transition.

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Minocycline, a semisynthetic tetracycline, has been shown to be neuroprotective in certain models of ischemic and neurodegenerative disease and has potential for clinical use. It has recently been suggested that minocycline acts through direct inhibition of calcium-induced mitochondrial permeability transition (mPT) [1,2]. One striking feature of the key studies performed on isolated brain (and liver) mitochondria is the extraordinarily high concentration/dose of minocycline needed to prevent the mitochondrial release of proteins following various triggering events (e.g. calcium, tBID and oxidants) as compared with experiments utilizing cell culture systems. These findings have likely triggered the tendency to use higher and higher doses of minocycline in animal models, e.g. 180 mg/kg i.p. in a recent study on spinal cord injury [3].

In the present study we revisited the conclusion that minocycline directly prevents the induction of mPT and release of pro-apoptotic proteins in brain-derived rodent mitochondria. The objectives were to investigate the effect of a wide concentration/dose range of minocycline on calcium-induced mPT under both energized and de-energized conditions, calcium and tBID-induced release of cytochrome *c* as well as evaluate the effect of minocycline on normal mitochondrial respiratory function and calcium transport.

We demonstrate that minocycline at high concentrations interfere with mitochondrial respiration and calcium transport. Minocycline at relevant concentrations does not inhibit permeability transition or cytochrome *c* release following a calcium insult when mitochondria are respiring on malate and glutamate. These results were confirmed in a de-energized model where induction of mPT is independent of mitochondrial respiratory capacity, thereby allowing a very wide range of minocycline concentrations to be tested (up to 250 μ M, 10 μ mol/mg mitochondrial protein).

We conclude that minocycline does not inhibit mitochondrial permeability transition. Minocycline may prove to be a beneficial neuroprotective agent but its mechanisms of action are still open for discussion and care must be taken to choose relevant doses in animal experiments that can be translated to safe human clinical use.

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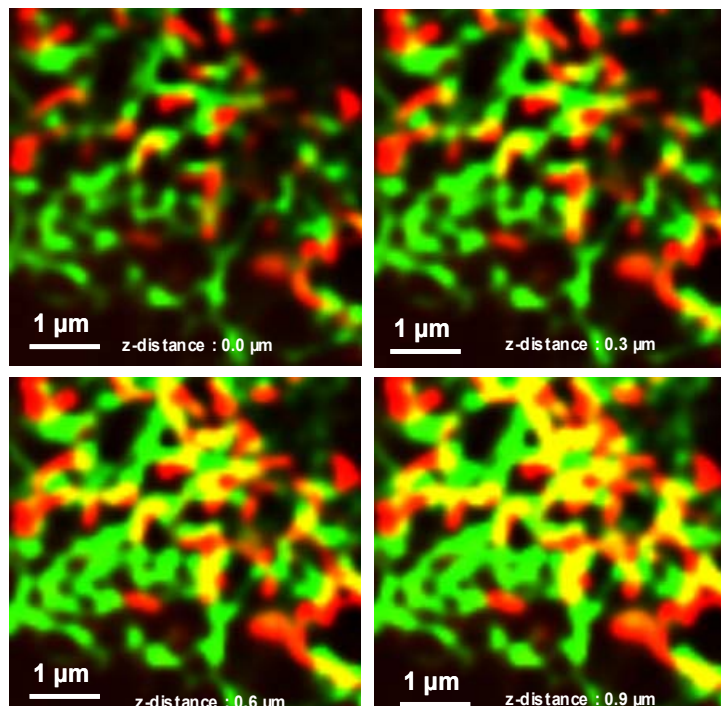
3-11. The ER Ca²⁺-pumps and the mitochondrial Na⁺/Ca²⁺-exchange function in a close relationship of interdependency.

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Intracellular Ca²⁺ controls a remarkable number of signalling pathways within cells. The question is how signalling specificity is maintained with the use of such a promiscuous messenger. In many cells spatial and temporal Ca²⁺ gradients are dynamically generated and thought to play crucial roles in achieving signalling specificity [1]. Growing evidence suggests that local Ca²⁺ communication between the endoplasmic reticulum (ER) and mitochondria is of utmost importance for the formation, maintenance and control of specific local Ca²⁺ microdomains within a cell. However, the molecular mechanisms of Ca²⁺ signal transmission between these organelles are only fractionally decoded and thus still subject of acute research [2]. Recently we showed that

mitochondria deliver extra-cellular Ca²⁺ towards the ER Ca²⁺ pumps (SERCAs) during cell stimulation with an IP₃-generating agonist [3]. Thereby mitochondria seem to imbibe Ca²⁺ close to Ca²⁺ entry channels at the plasma membrane and subsequently release the absorbed Ca²⁺ via the mitochondrial Na⁺/Ca²⁺-exchanger (NCX_{mito}). Inhibition of NCX_{mito} using the benzothiazepin derivate CGP 37157 prevented complete Ca²⁺ refilling of the ER during cell stimulation. In addition, SERCA inhibition during cell stimulation with an IP₃-generating agonist increased mitochondrial Ca²⁺ concentration ([Ca²⁺]_{mito}) at once. This effect was clearly attenuated by NCX_{mito} inhibition indicating that the mitochondrial Ca²⁺ elevation upon SERCA inhibition is accomplished by the NCX_{mito} working in reversed



Images were taken using an array confocal laser scanning microscope (ACLSM). Endothelial cells (Ea.hy926) expressing YC4-ER and mtDsRed (Mitochondria in red, ER in green) were analysed. The orange colour indicates possible contact sites between both organelles. For the colour picture, see www.mitophysiology.org.

In addition this result implicates that the ER Ca²⁺ pumps function in a close relationship of interdependency to the NCX_{mito}. Thus it is tempting to speculate, that both Ca²⁺ transporters located at different organelles are also physically linked (see figure) at least during cell stimulation. Such a coupling of these proteins might be accomplished by yet unknown scaffolding-, anchoring- or adaptor-proteins and may explain the observed functional interdependency.

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3-12. Mitochondrial abnormalities in a PC12 cell model of Alzheimer's disease.

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Mitochondrial dysfunction plays an important role in the pathogenesis of Alzheimer's Disease (AD) and other neurodegenerative disorders. The histopathological hallmarks of AD are extracellular senile plaques, consisting mainly of amyloid beta (A β) peptide, and intracellular neurofibrillary tangles (NFT), consisting of hyperphosphorylated tau protein. Other characteristics are oxidative stress, diminished brain metabolism, reduced synaptic density, chronic inflammation and neuronal loss in selected brain regions. Today it is still unknown, whether intracellular or extracellular A β is responsible for the oxidative stress-induced neurotoxic effects on mitochondria [1]. It also might be possible that an altered distribution of APP in subcellular compartments plays a crucial role for enhanced oxidative stress in the AD brain.

We investigated the chronic effects of the Swedish double mutation in the beta amyloid precursor protein (APP) (APPsw) and human wildtype APP (APPwt) on mitochondrial function and acute effects of supplementary stress (A β_{1-42} , ER and oxidative stress) on mitochondrial calcium ([Ca²⁺]_m) and mitochondrial membrane potential ($\Delta\psi_m$) in PC12 cells. We determined the APP expression, A β production and secretion into the extracellular compartment under basal conditions. APPwt and APPsw PC12 cells express the same amount of APP, but A β production and secretion is 3-5 fold increased in APPsw PC12 cells due to the mutation [2]. Then, we investigated the chronic effects of APP and A β on mitochondria and found no differences on basal [Ca²⁺]_m. But the APPsw cells exhibit a significant reduced $\Delta\psi_m$ compared to wt and vector PC12 cells. We also found reduced ATP levels and reduced cytochrome c oxidase activity. Acute stimulation with secondary insults showed only small effects on [Ca²⁺]_m, but distinct changes in $\Delta\psi_m$ especially on APPsw cells. Only stimulation with Thapsigargin led to a significant enhanced calcium uptake into the mitochondria in APPsw cells. Our findings support the hypothesis that APP or A β might affect the mitochondrial calcium homeostasis and energy metabolism in a way, which enhanced the vulnerability of the cells for oxidative stress and other secondary insults during AD.

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3-13. Selenium modifies calcium homeostasis in cells undergoing metabolic and genetic mitochondrial stress.

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Calcium signalling plays an important role in various physiological and pathological processes. Mitochondria could transiently accumulate an appreciable amount of calcium and thereby affect cellular calcium homeostasis [1,2]. Many mitochondrial diseases can modify cellular Ca²⁺ fluxes.

We have studied intracellular calcium signals in following osteosarcoma cell lines: 1. wild type, 2. depleted of mtDNA (Rho0), 3. 98 % heteroplasmy NARP mutant, 4. wild type starved. We measured basal and activated cytosolic calcium levels, an organization of mitochondria within the cell, the mitochondrial membrane potential, and ATP content of the cell.

We have found that in Rho0 cells mitochondrial stress causes disruption of mitochondrial filaments, decrease of mitochondrial membrane potential and increase in basal cytosolic calcium concentration. In starved cells we have observed reduced responses to thapsigargin, SARCA inhibitor. It seems that in these cells ROS production was also altered.

Oxidative stress and imbalance between free radical generation and detoxification may play a pivotal role in the pathogenesis of many mitochondrial diseases. Mitochondrial produced superoxide is a major cause of cellular oxidative damage. Therefore, we have investigated how selenium, a potent antioxidant, influences the osteosarcoma cells under genetic and metabolic stress conditions. Moreover, selenium is an essential component of several enzymes and has been linked to regulatory functions in cell growth, cytotoxicity, and transformation possibly involving redox regulation. We have investigated effects of selenium on calcium signaling in thapsigargin and CCCP treated cells. We have found that selenium reduces calcium release from intracellular stores but causes higher basal cytosolic Ca^{2+} . These effects were highly dependent on selenium concentration.

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3-14A. Effects of Amyloid beta peptides on mitochondrial function in glial cells in culture: mitochondria as targets of calcium and oxidative stress.

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Amyloid beta ($A\beta$) peptides accumulate in the CNS in Alzheimer's Disease. We have used digital fluorescence imaging techniques to examine the effects of $A\beta$ peptides in mixed cultures of neurons and astrocytes from rat or mouse hippocampus. The $A\beta$ caused sporadic increases in intracellular calcium concentration in astrocytes while neurons remained quiescent [1]. $A\beta$ also increased the rate of generation of free radical species in astrocytes but not neurons [2]. The increase in ROS generation was attributable to the calcium dependent activation of an NADPH oxidase expressed in the astrocytes. Western blots and immunofluorescence studies showed that the astrocytes express gp91^{phox}, p67^{phox} and p40^{phox}, the membrane and soluble subunits of the phagocytic NADPH oxidase. We also found that mitochondria undergo complex depolarisations in the astrocytes in response to the ROS generation by the oxidase. This raises the more general question concerning the consequences of ROS generation for the mitochondria of cells that generate large amounts of ROS 'professionally'. In the astrocytes, $A\beta$ peptides caused a slowly progressive dissipation of mitochondrial potential. This was dependent on ROS generation by the oxidase - it was blocked by antioxidants, by inhibitors of the oxidase (apocynin, DPI, AEBSF) and was absent in cells cultured from gp91^{phox} knockout transgenic mice [3]. The loss of potential was also prevented or reversed by the addition of additional mitochondrial substrates to all complexes of the chain - by glutamate, methyl succinate or pyruvate, strongly suggesting that the depolarisation must be due to impaired mitochondrial substrate supply. Superimposed on the slow progressive loss of potential were abrupt transient depolarisations. These could be very large, lasting 10s of seconds or even a few minutes. Most such events were fully reversible, although some led to cell death and lysis. Simultaneous measurements of mitochondrial potential and intracellular calcium showed that these events invariably accompanied a calcium transient and they were abolished in the absence of calcium. They were also abolished by antioxidants, by inhibitors of the NADPH oxidase, in cells from the gp91^{phox} knockout mice, and by cyclosporin A, none of which altered the calcium signal. Therefore, the oxidant stress generated by activation of

the oxidase sensitises mitochondria to calcium signals causing openings of the mitochondrial permeability transition pore which may be transient and reversible.

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