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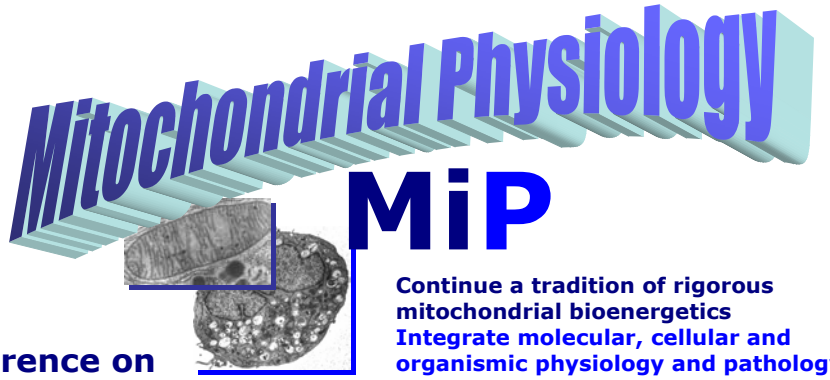

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Mitochondrial Physiology

12-16 Sept. 2003 **Schröcken, Vorarlberg, Austria**

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MIP2003 Abstracts

Editor: **Erich Gnaiger**

Abstracts In addition to abstracts for oral MiP presentations (presenting authors are underlined), abstracts are included as contributions to the discussion, and expressions of interest in future MiP meetings.

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From hypoxia to hyperoxia - mitochondria at physiological oxygen levels

Session 1 Oxygen levels, gradients and heterogeneity in cells and tissue – effects on mitochondrial respiratory control.

1-01. Muscle oxygenation and mitochondrial function *in vivo* studied by ^{31}P MRS and NIRS in patients with vascular disease and in patients with chronic renal failure on haemodialysis.

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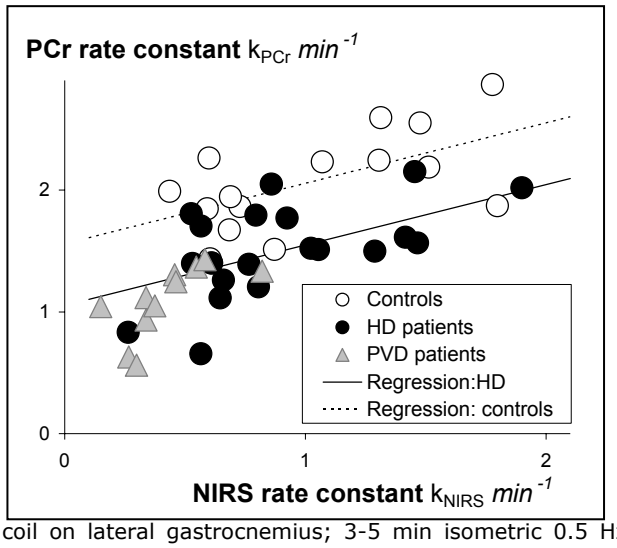
Defects of muscle oxidative ATP production, often multifactorial, occur in many diseases. It is useful to distinguish the contributions of (a) intrinsic mitochondrial loss/dysfunction, and (b) defective O_2 supply/diffusion. Both can be studied invasively, but noninvasive methods have advantages for patient tolerance. ^{31}P magnetic resonance spectroscopy (MRS) studies of post-exercise PCr recovery test overall 'mitochondrial function'. Near-infrared spectroscopy (NIRS) can help to distinguish (a) from (b); however, interpretation is complicated. In exercise, increased NIRS changes despite impaired oxidative ATP synthesis are evidence of defective O_2 supply e.g. in peripheral vascular disease (PVD) [1]. In patients with chronic renal failure on haemodialysis (HD), interpretation is complicated by disagreement about the source of the NIRS signal: if it is largely deoxymyoglobin [2], rather than deoxyhaemoglobin, then it reports mainly cellular PO_2 [2]. This matters more in HD, where impaired intramuscular O_2 diffusion dissociates abnormalities in cellular and capillary PO_2 [3], than in PVD where the vascular defect decreases both. Analysis of NIRS recovery avoids some complications; however, this is the resultant of known O_2 demand (obtainable from PCr recovery rate) and unknown O_2 supply (since parallel blood flow and arteriovenous $[O_2]$ difference data are unobtainable). Furthermore PCr recovery is itself affected by the cellular PO_2 (and also – or perhaps consequently – by cellular acidification due to increased glycolytic ATP synthesis) [1]. Nevertheless, insights may be obtained from the relationship between the PCr recovery rate constant (k_{PCr}, which in the absence of appreciable acidification is arguably proportional to the notional maximum ATP synthesis rate at high [ADP]) [1], and the 'rate constant' of NIRS recovery (not strictly exponential kinetics, but defined analogously as k_{NIRS} = 0.693/(time to half-recovery)). The figure shows this for three groups of male subjects: 23 HD patients (24-71y), 11 PVD patients (57-77 y) and 15 similar-aged controls, studied using a ^{31}P MRS/NIRS calf protocol described elsewhere [1] (in brief, GE 1.5T MR scanner, surface coil on lateral gastrocnemius; 3-5 min isometric 0.5 Hz plantar flexion at 50% & 75% maximum voluntary contraction force and subsequent recovery; simultaneous NIRS acquisition using RunMan CWS-2000). In PVD k_{PCr} and k_{NIRS} are both reduced (by 48±5% and 59±7% respectively, P<0.001): consistent with a pure vascular defect, although excluding some loss/defect of mitochondria will require more detailed analysis at low cell PO_2 [1]. In HD k_{PCr} is reduced by 28±5%, but k_{NIRS} is not abnormal. Is this is to be explained by the O_2-conduction defect [2]? Note that k_{PCr} and k_{NIRS} are near-linearly related in both HD and controls, with a common slope but a significant vertical separation; thus in HD k_{PCr} is reduced both absolutely and in relation to k_{NIRS}. This is probably consistent with O_2-'limitation' in both groups, but also an intrinsic mitochondrial abnormality in HD, for which there are many possible reasons [3]. This is not a physiologically explicit argument, for the reasons given above, but it is borne out by simulation: PCr resynthesis rate and vascular O_2 delivery are modelled as decaying exponentials, and k_{PCr} is a hyperbolic function of cellular PO_2 (which itself has a hyperbolic relationship to NIRS signal). This gives a quasi-exponential NIRS recovery as long as O_2 supply declines more slowly than use, and results in a near-linear relationship between k_{PCr} and k_{NIRS}, as observed. In aerobic exercise to steady state, rate of O_2 supply and use are equal at the end of exercise. Vascular insufficiency is modelled by a shortfall in O_2 supply: this slows NIRS and PCr recovery, resembling the PVD data. Addition of an intrinsic mitochondrial defect (modelled by a low k_{PCr}, additional to the effect of cellular hypoxia) results in a vertical separation between the lines relating k_{PCr} and k_{NIRS} resembling the relationship seen for HD. This is a simple approach to a complex problem, but capable of refinement.



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1-02. Muscle microvascular blood flow distribution during dynamic exercise in humans.

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The elucidation of muscle blood flow distribution patterns during dynamic exercise is important for understanding how the matching of blood flow to local metabolic demand is regulated. Muscle microvascular blood flow distribution was examined in the quadriceps muscles of young, healthy adults (n=6) during dynamic knee extension exercise. Blood flow was measured at rest, 20, 40, 60 watts (W) by near-infrared spectroscopy (NIRS; Hamamatsu NIRO-300) and indocyanine green (ICG) dye at 2 separate locations on each of the Rectus Femoris (RF; mid and distal), Vastus Lateralis (VL; mid and distal) and Vastus Medialis (VM; proximal and distal) muscles. Within individuals, both inter- and intra muscle flow heterogeneity was clearly apparent. However, group averaged (n=6) blood flow was similar between muscle groups across workloads except at 60 W where distal VM flow (172.58±28.61 ml · 100ml^{-1} · min^{-1}) was lower ($P<.05$) than distal VL (253.40±38.06 ml · 100 ml^{-1}·min^{-1}), mid VL (236.20±36.76 ml · 100 ml^{-1}·min^{-1}) and mid RF (225.03±24.71 ml · 100 ml^{-1}·min^{-1}). These results suggest that within the tissue volume measured by NIRS, blood flow is generally similar between different muscles of the quadriceps, with spatial heterogeneity apparent only at high workloads or within individuals. (This study was supported by Concordia University, NSERC, FRSQ and the Danish National Research Foundation).

1-03. Measuring the heterogeneity of oxygen supply to demand in the heart muscle.

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Blood flow to the heart muscle is very heterogeneously distributed, which means that oxygen supply is very heterogeneous. It was unknown whether energy turnover, and therefore oxygen demand, is proportional to local oxygen supply. To measure local energy turnover we developed computer models to analyze carbon-13 NMR spectra taken from tissue biopsies after 5.5 min infusion of carbon-13 labeled substrate. One of our models incorporates 132 differential equations for isotope distribution via the TCA cycle to predict the isotopic isomer distribution found in glutamate after transamination. Monte Carlo simulation showed that from the 9 distinct carbon-13 multiplets measurable in glutamate after 5.5 min infusion of carbon-13 enriched acetate, six metabolic parameters can be quantitated, among others the TCA cycle flux. The method has been applied to study the spatial heterogeneity of TCA cycle flux, and therefore of oxygen consumption, in the heart in anesthetized pigs. In the normal heart we find substantial heterogeneity of myocardial oxygen consumption and blood flow, despite uniform cardiac anatomy and biophysical model predictions of homogeneous work performance. The standard deviation of true spatial heterogeneity is almost 50% of the mean myocardial oxygen consumption value. Myocardial blood flow measured with radioactively labeled microspheres reveals a similar heterogeneity. However, local oxygen consumption in 1 mL voxels is strongly correlated with local blood flow and therefore with oxygen supply, with correlation coefficients of about 0.7, which keeps the spread of the oxygen supply to demand ratio limited in the normal heart. During coronary stenosis blood flow is reduced very heterogeneously, independent of the baseline blood flow level. Oxygen consumption tends to be reduced in proportion to the flow reduction, but nevertheless the correlation between local blood flow and oxygen consumption diminishes rapidly, leading to greater heterogeneity of the oxygen supply to demand ratio. The voxels with high energy turnover are expected to be especially vulnerable to ischemic tissue damage during coronary occlusion, explaining the often patchy patterns of infarct damage in the heart.

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1-04. Mitochondrial respiratory control can compensate for slow intracellular diffusion of oxygen in rat cardiomyocytes.

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Diffusion of oxygen from the sarcolemma to mitochondrial innermembrane is the final step in the oxygen cascade. At physiological extracellular oxygen concentrations, myoglobin in the cytoplasm of cardiac myocytes facilitates intracellular diffusion of oxygen. However, this step may still be a factor limiting mitochondrial oxidative phosphorylation when mitochondrial oxygen demand is moderately increased. This was demonstrated in single cardiomyocytes isolated from the rat in which myoglobin oxygen saturation (S_{Mb}) was visualized with a subcellular spatial resolution (0.2 μm) [1]. When the quiescent cardiomyocyte was superfused with a 3% oxygen gas at room temperature, S_{Mb} was uniform within the cell. In contrast, when mitochondrial respiration was increased by ~9 times using an uncoupler of oxidative phosphorylation (1 μM CCCP), quite steep radial S_{Mb} gradients was demonstrated from

the sarcolemma to the center of the cell (the hypoxic cell core). Fluorometry of mitochondrial NADH with the same spatial resolution demonstrated intracellular heterogeneities of mitochondrial metabolism that mirror image the radial S_{Mb} gradients [2]. Thus, in CCCP treated single cardiomyocytes, intracellular oxygen diffusion certainly restricted oxidative phosphorylation in mitochondria locating near the center of cell (the anoxic core). Then, to examine the role of mitochondrial intrinsic regulatory mechanism (i.e. the respiratory control), mitochondrial oxygen demand was elevated by electrically pacing the cell at 37°C (with 1 mM extracellular Ca^{2+}), instead of the uncoupler. Surprisingly, despite that the radial S_{Mb} gradients of the same magnitude were produced, mitochondrial NADH fluorescence in the cell core was comparable to that near the sarcolemma [3]. From these results, we conclude that, at moderately increased oxygen demand, the rate of oxygen diffusion in the intracellular space may limit oxygen delivery to mitochondria, particularly those locating near the cell core, but mitochondrial respiratory control may compensate for such slow intracellular diffusion and sustain oxidative metabolism in mitochondria even in oxygen depleted hypoxic core.

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1-05. Mitochondrial oxygen kinetics and heterogeneity of oxygen in tissues – from cytochrome *c* oxidase to muscle power output.

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Heterogeneity of intracellular oxygen pressure in tissues stems from intracellular oxygen gradients, mainly from the microcirculation giving rise to intercellular heterogeneity of oxygen supply, and from cell-to-cell differences in respiratory activity (recruitment). Such oxygen heterogeneity is more pronounced in pathological states, e.g. ischemia-reperfusion. While biochemical studies of reaction kinetics at varying substrate concentrations generally aim at homogeneity of substrates in the test tube, the physiological interpretation must consider the effects of substrate heterogeneity on the overall response of metabolic flux. In many cases, however, only tissue-averages of substrate concentrations are known. The present study investigates (1) the validity of extrapolating results on oxygen kinetics of isolated mitochondria measured by high-resolution respirometry (OROBOROS *Oxygraph* [1]) to muscle tissue *in vivo* [2], (2) the effect of oxygen heterogeneity on the relation between tissue oxygen consumption rate and average intracellular tissue p_{O2}, and (3) the possible role of myoglobin in the modulation of intracellular oxygen heterogeneity and hence tissue oxygen kinetics.

Hyperbolic oxygen kinetics in isolated mitochondria contrasts with a critical oxygen pressure in tissues. The oxygen pressure, p_{50}, at which mitochondrial respiration is reduced to 50 % of maximum rate, varies with turnover rate of cytochrome *c* oxidase, which depends on metabolic activation state and excess capacity of cytochrome *c* oxidase [1,3]. It is obvious that extracellular p_{O2} is a poor determinant of cell respiration if intracellular oxygen gradients are significant. An intracellular diffusion models predicts a hyperbolic oxygen dependence of respiration on external oxygen pressure, in agreement with results in isolated cells and muscle fibers [4]. Surprisingly, the effect of oxygen heterogeneity on tissue respiratory kinetics has been largely ignored. Bell-shaped intercellular oxygen frequency distributions in tissues lead to a sigmoidal oxygen dependence. The oxygen affinity of isolated mitochondria [3] is significantly higher compared to the dependence of muscle tissue respiration on average myoglobin-reported oxygen pressure [2]. This difference is quantitatively resolved by a simulation model of oxygen heterogeneity. In contrast to enzyme-substrate compartmentation, heterogeneity of substrate versus enzyme distribution shifts the kinetic response curve *in vivo* to the right of the kinetics measured in the homogenous *in vitro* system. In the extreme, temporary shut-down of regional tissue oxygen supply leads to linear oxygen conformance [4] in relation to average intracellular tissue oxygenation.

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Session 2 Cytochrome *c* oxidase, oxygen kinetics and NO.

2-01. Pumping steps in the cytochrome *c* oxidase catalytic cycle.

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Cytochrome *c* oxidase is one of the energy generators of the respiratory chain of mitochondria and many bacteria, which catalyses the reduction of oxygen to water and uses free energy released in this reaction to drive proton translocation across a biological membrane

and thereby to generate proton motive force [1]. The catalytic cycle consists of an *oxidative* phase, where the enzyme is oxidised by O_2, and a *reductive* phase where it is reduced before binding the next O_2 molecule. Identification of the partial reactions that are associated with proton translocation is a prerequisite for elucidating the molecular mechanism, but has remained controversial [2]. Measurements of the electric potential generation and the quantity of protons translocated across the membrane during different steps of the catalytic cycle starting from resting oxidized enzyme and freshly re-oxidized cytochrome *c* oxidase brought us to the conclusion that resting oxidized enzyme is incompetent in proton translocation and has to be activated by primary reduction [3]. The delivery of the first two electrons into the binuclear site of oxidized resting cytochrome c oxidase, and the reaction with oxygen, are required for the activation of the enzyme and are not coupled to proton translocation. In the activated enzyme each electron transfer step from cytochrome c to the binuclear catalytic site in the presence of oxygen is coupled to translocation of one proton across the energy-transducing membrane. The results presented establish how proton translocation is distributed in the catalytic cycle, and also suggest how the efficiency of the proton pump may be regulated *in vivo*.

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2-02. Regulation of mitochondrial membrane potential and ROS production via ATP/ADP-interaction and reversible phosphorylation of cytochrome *c* oxidase.

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In bovine heart cytochrome c oxidase 7 high-affinity binding sites for ADP or ATP have been identified. At high ATP/ADP ratios in the mitochondrial matrix an allosteric ATP-inhibition of ascorbate respiration was found, due to binding of nucleotides to subunit IV. This ATP-inhibition is only found with the cAMP-dependent phosphorylated enzyme (at the cytosolic side of subunit I), and is abolished by dephosphorylation by a calcium-activated intermembrane protein phosphatase [1]. The inhibition of respiration at high matrix ATP/ADP-ratios represents a new feed-back inhibition of oxidative phosphorylation (second mechanism of respiratory control) [2], and is postulated to keep the mitochondrial membrane potential (deltapsi) at low values (< 140 mV), due to saturation of the mitochondrial ATP synthase at 100-120 mV [3]. Abolition of the allosteric ATP-inhibition by diiodo-L-thyronine (T2) [4], low concentrations of palmitate, or calcium-activated dephosphorylation of cytochrome c oxidase, would increase the mitochondrial membrane potential to high values (140-200 mV), which were shown to induce the production of ROS (reactive oxygen species). We propose that in vivo stress results in abolition of the allosteric ATP-inhibition of cytochrome c oxidase, increase of mitochondrial deltapsi, and the production of ROS, which could cause cell apoptosis and multiple degenerative diseases. A large number of recent publications have demonstrated a transient increase of mitochondrial deltapsi (hyperpolarization) by various compounds and parameters which induce apoptosis [5].

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2-03. Three new tissue specific isoforms of cytochrome c oxidase in mammals.

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Mammalian cytochrome c oxidase (COX) is composed of 13 subunits per monomer and has been crystallized as a dimer. In addition to the 3 largest, mitochondrial encoded subunits, which contain the 4 catalytic redox centers, the mammalian enzyme contains 10 nuclear encoded subunits. COX contains skeletal muscle/non-skeletal muscle-specific isoforms for subunits VIa, VIIa, and VIII. These isoforms, which have been known for more than two decades, are encoded by individual genes and expressed in a developmental manner.

Recently, we have found three additional COX subunit isoforms in mammals including humans, which are encoded by individual genes and share similar exon-intron organization with their paralogues. The first isoform is for subunit IV (COX IV-2). It is lung-specific, strongly expressed in smooth muscle, and induced after birth [1]. Ubiquitously expressed paralogue COX subunit IV-1 has been shown to be a key regulatory subunit. It senses the ATP/ADP ratio and adjusts COX activity to physiological energy demand. The presence of the COX IV-2 isoform might be related to adaptations to high substrate oxygen concentrations as present in lung. The yeast COX subunit Va and Vb isoforms, which are believed to be homologous to mammalian subunit IV, are

regulated by the oxygen concentration at the transcriptional level, and provide COX with about four-fold increased electron-flow kinetics under hypoxia. Interestingly, at the gene level *COX4-2* is regulated by a novel oxygen-sensing element and is induced under hypoxia *in vivo* and *in vitro*.

Second, we found a new isoform of subunit VIb [2], which links the two COX holoenzymes into a dimeric form at the cytosolic side. Northern analysis with a variety of rat tissues, including the female gonads, demonstrated exclusive expression of VIb-2 in testes. *In situ* hybridisations with human, rat, and mouse testes sections revealed VIb-2 transcripts in all testicular cell types. Within the seminiferous tubules in humans, the paralogous isoform VIb-1 shows stronger signals in the periphery compared to the lumen.

Finally, we have found a third isoform for the smallest nuclear encoded COX subunit VIII (COX VIII-3) in human, lemur, rat, and mouse, which localizes to the mitochondria [3].

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2-04. Reversible and irreversible alterations to mitochondrial cytochrome oxidase K_m for oxygen by nitrigen oxides.

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Mitochondrial cytochrome oxidase is competitively and *reversibly* inhibited by inhibitors that bind to ferrous haem, such as carbon monoxide and nitric oxide. In the case of nitric oxide, nanomolar levels inhibit cytochrome oxidase by competing with oxygen at the enzyme's haem-copper active site [1]. This raises the K_m for cellular respiration into the physiological range. This effect is readily reversible and may be a physiological control mechanism [2]. Here we show that a number of *in vitro* and in *vivo* conditions result in an *irreversible* increase in the oxygen K_m [3].These include: treatment of the purified enzyme with peroxynitrite or high (μM) level of nitric oxide; treatment of the endothelial-derived cell line, b.End5, with NO; activation of astrocytes by cytokines; reperfusion injury in the gerbil brain. Studies of cell respiration that fail to vary the oxygen concentration systematically are therefore likely to significantly underestimate the degree of irreversible damage to cytochrome oxidase.

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2-05. Nitric oxide inhibition of mitochondrial respiration and sensitisation to hypoxia.

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Nitric oxide (NO) inhibits mitochondrial respiration in two ways: (a) rapid, potent and reversible inhibition of cytochrome oxidase by NO itself, and (b) inactivation of multiple components by NO derivatives: peroxynitrite or S-nitrosothiols. We have shown that a wide range of cells expressing the inducible isoform of NO synthase (iNOS) inhibit their own respiration and that of co-incubated cells via the reversible NO inhibition of cytochrome oxidase. The NO inhibition of cytochrome oxidase is competitive with oxygen, causing a dramatic increase in the apparent K_M of respiration for oxygen. The presence of 50 nM NO raises the K_M of respiration for oxygen from well below 1 μM to 30 μM O_2 i.e. into the physiological range for O_2, making respiration oxygen limited. Thus NO may regulate respiration, but also potentially sensitises cells to hypoxia. We have shown that NO from iNOS in inflammatory-activated rat aorta reversibly inhibits aortic respiration, and makes the respiration rate proportional to oxygen level over the physiological range. This causes a considerable sensitisation of the aorta to hypoxia-induced cell death. 4 hours of 5% O_2 hypoxia caused no significant necrosis of the vessel wall in the absence of NO or iNOS, but caused extensive necrosis in the presence of an NO donor or iNOS expression. This sensitisation to hypoxia may be important in a wide range of inflammatory, infectious, ischaemic, cancerous and degenerative pathologies where iNOS is expressed.

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2-06. Inhibition of cytochrome *c* oxidase by NO: study of the reaction mechanism in Keilin-Hartree particles, mytochondria and SH-SY5Y neuroblastoma cells.

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The inhibition of cytochrome c oxidase (CcOX) by nitric oxide (NO), previously studied with the purified enzyme [1] has been investigated in Keilin Hartree particles, coupled mitochondria and human neuroblastoma cells [2]. Using polarographic techniques we have observed that in the presence of NO, respiration is promptly inhibited. Following the recovery of respiration, raised by removal of free NO with excess HbO_2 , two different inhibition pathways have been identified. The two pathways are due to the formation of two different adducts in the reaction of CcOX with NO, namely the nitrite- or the nitrosyl- derivative [3]. Interestingly from the pathophisiological view point, in the presence of the nitrosil adduct a more severe and long lasting inhibition of respiration is observed. In addition this species is light sensitive, thus easily detectable. We observed that recovery of respiration is influenced by the concentration of reductants at the level of complex IV. The nitrite adduct is accumulated at low reductant concentration while the nitrosilated predominates at the higher concentrations. These different conditions have been tested using KH particles, state 3 and 4 mitochondria and cultured cells by increasing TMPD concentration. The results obtained suggest that the same mechanism of inhibition of respiration by NO described for the purified enzyme operates under more complex integration levels (KH, mitochondria and cells). We propose that the two inhibition pathways are differently populated depending on the rate of electron transfer to CcOX, governed by the concentration of reduced cytochrome c.

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2-07. Mitochondria in S-nitrosothiol-induced cell death.

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Nitric oxide (NO) is cytotoxic, and has been implicated in a wide range of inflammatory, ischaemic and neurodegenerative diseases, as well as in host defence against pathogens. Mitochondria may be involved in NO-induced cell death, as NO has three relevant actions on mitochondria: NO inhibits mitochondrial respiration; NO stimulates the production of oxidants by mitochondria, including superoxide, hydrogen peroxide and peroxynitrite; and all these oxidants (as well as S-nitrosothiols but not NO itself) can stimulate mitochondrial permeability transition (MPT). NO inhibits mitochondrial respiration in two different ways: (1) acutely and reversibly due to NO inhibition of cytochrome oxidase in competition with oxygen and (2) more slowly due to peroxynitrite or S-nitrosothiols inactivating mitochondrial respiratory complexes, mainly complex I [1, 2]. In many pathological situations there is also a large increase in cellular calcium which may have damaging effects on mitochondria such as activation of MPT or inhibition of the respiratory chain. We investigated whether NO itself or synergistically with elevated concentrations of calcium can cause irreversible damage to mitochondria and whether NO or S-nitrosothiols can induce mitochondria-mediated apoptosis in perfused heart.

We found that NO and calcium synergistically stimulated production of peroxynitrite by mitochondria and inhibited mitochondrial respiration with pyruvate (but not succinate) as substrate. NO plus calcium-induced supression of mitochondrial respiration was partially caused by inhibition of complex I (due to S-nitrosation or Fe-nitrosylation) and partially by the loss of cytochrome c from mitochondria [2].

We also found [3] that perfusion of rat hearts with a physiological S-nitrosothiol GSNO at 0.4-1.0 mM concentrations for just 10 min caused the release of cytochrome c from mitochondria, inhibition of mitochondrial respiratory chain and caspase activation. Inhibited respiratory chain activity was restored when exogenous cytochrome c was added to mitochondria, indicating that respiratory inhibition was caused by lack of cytochrome c in mitochondria. Release of cytochrome c, respiratory inhibition and caspase activation were prevented when hearts were pre-perfused with cyclosporin A, suggesting that MTP was involved. In contrast, perfusion of the hearts with 'pure' NO donor DETA/NO, releasing similar levels of NO to the GSNO, had no measurable effect on the heart. These data suggest that S-nitrosothiols (but not NO) are potent inducers of apoptosis in the heart and that S-nitrosothiol-induced apoptosis is mediated by MTP.

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2-08. Role of mitochondrial nitric-oxide synthase.

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The production of nitric oxide ($NO^{\cdot}$) by mitochondria is catalyzed by a constitutive, mitochondrial nitric-oxide synthase (mtNOS). This enzyme has the same cofactor and substrate requirements as other constitutive nitric-oxide synthases. Its occurrence was demonstrated in various mitochondrial preparations (intact, purified mitochondria, permeabilized mitochondria, mitoplasts, submitochondrial particles) from different organs (liver, heart) and species (rat, pig, dog) by various techniques (immunoblotting of submitochondrial fractions, immunohistochemistry). Given that this production may establish the basis for a novel regulatory pathway of energy metabolism, oxygen consumption, and oxygen free radicals production, mtNOS had to be biochemically characterized to provide basis for its regulation. mtNOS was identified as bNOS-alpha by various methods (mass spectrometry of proteolytic fragments, amino acid analysis, MW, pI and analysis of PCR fragments), excluding it as a novel isoform or other splice variants. Distribution of mtNOS transcript indicated ist occurrence in liver, brain, heart, muscle, kidney, lung, testis, and spleen. In contrast to bNOS, mtNOS has two posttranslational modifications: acylation with myristic acid and phosphorylation at the *C*-terminus. The former modification is a reversible and posttranslational process, which may serve for subcellular targeting or membrane anchoring. The latter modification could be linked to enzymatic regulation. Endogenous NO**.** reversibly inhibits oxygen consumption and ATP synthesis by competitive inhibition of cytochrome oxidase. The increased *K*m of cytochrome oxidase for oxygen and the steady-state reduction of the electron chain carriers provided experimental evidence for the direct interaction of this oxidase with endogenous $NO^{\cdot}$. The relative increase of H_2O_2 production by $NO^{\cdot}$-producing mitochondria suggests that this protein utilizes $NO^{\cdot}$ as an alternative substrate, thus preventing the full reduction of the respiratory chain and the subsequent burst of oxygen radicals. Finally, modulators of cytochrome oxidase (the irreversible step in oxidative phosphorylation) had been proposed during the last 40 years. Nitric oxide is the first molecule that fulfills this role (it is a competitive inhibitor, produced at a fair rate near the target site) extending the oxygen gradient to tissues.

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2-09. The existence and significance of a mitochondrial nitrite reductase.

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Nitric monoxide (NO) exerts a great variety of physiological functions. L-Arginine supplies amino groups which are transformed to NO in various NO-synthase-active isoenzyme complexes. NO-synthesis is stimulated under various conditions causing accumulation of stable NO-metabolites in the tissue. The major oxidation product found is nitrite. Elevated nitrite levels were reported to exist in a variety of diseases including HIV, reperfusion injury and hypovolemic shock. Denitrifying bacteria such as Paracoccus denitrificans have a membrane bound set of cytochromes (cyt cd_1, cyt bc) which were shown to be involved in nitrite reduction activities. Mammalian mitochondria have similar cytochromes which form part of the respiratory chain. Like in bacteria quinols are used as reductants of these types of cytochromes. The observation of one-e^- divergence from this redox-couple to external dioxygen made us to study whether this site of the respiratory chain may also recycle nitrite back to ist bioactive form NO. Thus, the aim of the present study was to confirm the existence of a reductive pathway which reestablishes the existence of the bioregulator NO from its main metabolite NO_2^-. Our results show that respiring mitochondria readily reduce added nitrite to NO which was made visible by nitrosylation of deoxyhemoglobin. The adduct gives characteristic triplet-ESR-signals. Using inhibitors of the respiratory chain for chemical sequestration of respiratory segments we were able to identify the site where nitrite is reduced. The results confirm the ubiquinone/cyt bc_1 couple as the reductant site where nitrite is recycled. The high affinity of NO to the heme-iron of cytochrome oxidase causes an impairment of mitochondrial energy-linked respiration. „Nitrate tolerance" of angina pectoris patients using NO-donors may be explained by the existence of mitochondrial nitrite-reductase.

2-10. Monitoring the effects of NO on cytochrome redox states in mitochondria of intact cells by multi-wavelength visible light spectroscopy.

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We have developed an optical system based on visible light spectroscopy for the continuous study of changes in the redox states of mitochondrial cytochromes in intact mammalian cells. Cells are suspended in a closed incubation chamber in which oxygen (O_2) and nitric oxide (NO) concentrations are monitored simultaneously with light intensity spectra in the visible region (490-650 nm). The system monitors redox changes during cellular respiration by using a multi-wavelength least-squares algorithm to fit the absorption spectra of cytochromes b_H, *c* and aa_3 to changes in the measured attenuation spectrum. Since we have determined the optical pathlength through the cells these changes can be expressed as absolute concentration changes. We demonstrate the validity of the system by monitoring the action of known respiratory chain inhibitors on cytochrome redox states in cultured cell lines. NO is known to modulate respiration through the reversible inhibition of complex IV, shown previously in mitochondria using visible light spectroscopy [1]. We have closely examined the O_2 concentrations at which reduction of the cytochromes occur when exogenous NO is added and following the inhibition of NO synthase. We note that there is an early reduction of cytochromes *c* and aa_3, whilst O_2 consumption and the redox state of cytochrome b_H in complex III are maintained. This effect, also observed by others studying O_2 consumption and cytochrome *c* redox state alone [2], appears to be enhanced in the presence of NO and diminished when NO synthase is inhibited. Since complex III is known to be one of the main sites of reactive oxygen species (ROS) production [3], we have further investigated this effect by studying the time course of ROS generation.

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Session 3: Respiratory and functional control in mitochondria and cells. Oxygen and excess capacity.

3-01. Mitochondrial gene expression during oxygen deprivation: Control of transcription and message stability.

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When exposed to anoxia, embryos of the brine shrimp *Artemia franciscana* undergo a profound metabolic downregulation characterized by steep drops in intracellular pH (pH_i) and ATP levels [1]. The metabolic depression is sufficient to permit survival for several years without oxygen at room temperature [2]. Transcriptional arrest in both nuclear and mitochondrial compartments is rapid (minutes to hours). Use of isolated mitochondria to study transcriptional responses to anoxia offers advantages: the localized nature of transcript initiation, processing, and degradation, all of which may be followed *in organello*. In response to anoxic incubation of embryos for 4 h followed by anoxic mitochondrial isolation and anoxic assay at pH 6.4, transcription rate is depressed by 89% relative to controls (normoxia, pH 7.8) [3]. Under normoxia, the incubation at low pH (6.4) reduced transcription by 74%. Ribonuclease protection assays showed that new initiation was markedly reduced at low pH. DNA footprinting of putative transcriptional promoters revealed proteins at regular intervals upstream of the 12S rRNA in the control region, which previously had been indirectly inferred to contain promoters for H-strand transcription. However, our hypothesis that initiation is reduced at low pH because of a change in DNA binding by mitochondrial transcription factors was not confirmed. We propose that regulation of initiation may be mediated by covalent modification or by protein-protein interactions not detected by footprinting.

Considering the near absence of biosynthesis of mitochondrial mRNA under anoxia, one might predict that mRNA stability would be extended. Using dot blots of total mitochondrial RNA, we show during *in organello* incubations that both O_2 deprivation and acidic pH (pH 6.4) elicit increases in half-lives of selected mitochondrial transcripts on the order of 5-10 fold or more, relative to normoxic controls at pH 7.8. Polyadenylation of these transcripts was measured under the same incubation conditions using a RT-PCR based assay. The results demonstrate that low pH and anoxia promote significant deadenylation of the stabilized transcripts in several cases, measured either as change over time in the amount of polyadenylation within a given size class of poly(A) tail, or as the total amount of polyadenylation at the endpoint of the incubation. This study is the first direct demonstration that for a metazoan mitochondrion, polyadenylation is associated with destabilized mRNA. This pattern has also been demonstrated in bacteria, chloroplasts and plant mitochondria and may indicate a conserved mechanism for regulating message half-life that differs from the paradigm for eukaryotic cytoplasm, where increased mRNA stability is associated with polyadenylation. (Supported by grants from the NSF and DARPA)

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3-02. Complexes of the respiratory chain: functional capacity in eukaryotic cell cultures. Mechanism and regulation of proton pumping.

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An overview on the relative functional capacities of respiratory complexes I, III and IV and their regulatory features in isolated mammalian mitochondria and in murine and human cell cultures is presented.

Three aspects will , in particular, be dealt with:

1. Functional capacities of the respiratory complexes in different tissues in the course of differentiation and aging.
2. The mechanism and physiological role of slips in the proton pump of respiratory complexes in relation to the energy balance and thermogenesis.
3. Regulation by the cAMP cascade of cellular respiration.
4. Genetic disorders in the assembly and activity of complex I in human disease.

3-03. Mitochondrial threshold effects.

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The study of mitochondrial diseases has revealed a dramatic variability in the phenotypic presentation of mitochondrial genetic defects. To understand this variability, different authors have studied energy metabolism in transmitochondrial cells lines carrying different proportions of various pathogenic mutations in mitochondrial DNA. The same kind of experiments were performed on isolated mitochondria or on tissue biopsies taken from patients with a mitochondrial disease. The results have shown that in most cases, the phenotypic manifestation of the genetic defect occurs only when a threshold level is passed, and this phenomenon has been named the *"Phenotypic Threshold Effect"*. Subsequently, several authors showed that it was possible to inhibit considerably the activity of a respiratory chain complex, up to a critical value, without affecting the rate of mitochondrial respiration or ATP synthesis. This phenomenon was called the *"Biochemical Threshold Effect".* More recently, the quantitative analysis of the effect of different mitochondrial DNA mutations on the rate of mitochondrial protein synthesis has revealed the existence of a *"Translationnal Threshold Effect"*. In this review the different mitochondrial *"Threshold Effects"* are discussed along with their molecular bases, and the role they play in the presentation of mitochondrial diseases.

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3-04. Hypoxia changes the isoform transcription pattern of cytochrome *c* oxidase subunit IV in rat astrocytes.

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Astrocytes, the most abundant cell type in the brain, play a central role in the regulation of cerebral energy metabolism. They tightly couple their energy production to neuronal activity by being located at a key position between capillaries, transporting oxygen and glucose, and neurons, consuming the energy metabolites provided by them. The resulting differential energetic requirements in astrocytes compared with neurons point to different regulatory mechanisms of oxidative energy metabolism in those two brain cell types.

Cytochrome c oxidase (COX), engaged in oxidative energy metabolism, catalyses the transfer of electrons from ferrocytochrome c to oxygen. Besides the catalytic core, the mammalian enzyme is composed of ten regulatory, nuclear encoded subunits. One of them, COX subunit IV, plays an important role in adjusting energy production to energetic demands through an allosteric inhibition of COX activity at high energy level, i.e. high ATP/ADP ratio [1]. Isoforms of this subunit (IV-1 and IV-2) occur in yeast, tuna fish, and mammals. COX subunit IV-1 is ubiquitously transcribed in all mammalian tissues including brain, while isoform IV-2 shows high transcription levels in adult and fetal lung, as well as fetal muscle [2], thus showing tissue specific and developmentally controlled expression.

Recently, we have found that COX IV-1 isoform is not uniformly expressed in rat brain tissue, but in a rather cell type specific manner: while astrocytes transcribe almost exclusively COX IV-1, a neuronal cell type, namely cerebellar granule cells, shows also strong transcription of COX IV-2.

Investigating the influence of substrate availability, i.e. oxygen, upon mitochondrial energy metabolism in rat astrocytes, we observed a distinct transcription pattern for COX IV-1 and IV-2 under normoxic compared

with hypoxic conditions. So far, yeast has been the only organism known to express two isoforms (Va and Vb), homologous to the mammalian subunit IV, in dependence on oxygen concentration [3]. We can show, that astrocytes switch the transcription pattern from COX IV-1 isoform under normoxic conditions to COX IV-2 under hypoxia.

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3-05. Decreased oxygen affinity of cytochrome *c* oxidase in patients with *SURF1* mutations.

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The respiratory cascade of human organism is characterized by a major drop of the oxygen tension (pO_2) from 20 kPa of the inspired air to a level of less than 1 kPa in some tissues. Even in these conditions of „tissue hypoxia" the sufficient rate of respiration is ensured thanks to the remarkable oxygen affinity of cytochrome *c* oxidase (COX) (1,2). Here we present the first analysis of COX oxygen affinity in human pathology – namely in fibroblasts of patients suffering from Leigh syndrome caused by mutations in the *SURF1* gene. These mutations prevent synthesis of the Surf1 protein necessary for the assembly of COX and result in severe COX deficiency (3) associated with changes in COX structure and both in electron- and proton-transport properties (4).

The oxygen affinity in cultured fibroblasts harbouring *SURF1* mutations was evaluated using high-resolution respirometry and expressed as p_{50} (pO_2 at half-maximal respiration rate). The measurements were performed in two experimental settings – respiration of intact coupled cells with endogenous substrates and unrestricted oxidation of exogenous succinate in digitonin-permeabilised cells after FCCP uncoupling. In intact cells, the p_{50} in patient fibroblasts was 2-fold elevated (0.041 to 0.083 kPa). Under the latter conditions of maximal respiratory rate, the increase was even 3-fold (0.037 to 0.12 kPa). Such decreased oxygen affinity may lead to limitations of respiratory rate in patient cells in tissue hypoxia resulting in impaired energy provision. We hypothesize that low oxygen affinity of COX might be an important etiopathogenic mechanism triggered by *SURF1* mutations.

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General MiP topics

Session 4: Reactive oxygen species and uncoupling proteins.

4-01. Is cell respiration a permanent source of reactive oxygen species?

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Reactive oxygen species (ROS) are recognized to be involved in an ever increasing number of diseases. Mitochondria are assumed to be the major intracellular source of ROS. It is generally accepted in the literature that 1-4% of oxygen consumed by mitochondria is regularly released as superoxide radical ($O_2^{\bullet-}$) into the cell [1]. The high cellular quantity of these organelles, the existence of various one-electron carriers and the high turnover rate of oxygen favor mitochondrial respiration as a cellular source of oxygen activation. However, conflicting results on the role of these organelles as a physiological / pathophysiological ROS generator exist in the literature depending on mitochondrial source, preparation procedure, detection systems and experimental conditions. We, therefore, critically re-evaluated frequently applied detection methods for $O_2^{\bullet-}$ with an enzymatic model system and intact isolated rat heart mitochondria. These $O_2^{\bullet-}$ detection systems may lead to unreliable results due to unspecific autoxidative processes, direct interaction with the mitochondrial respiratory chain and the presence of superoxide dismutase in the mitochondrial matrix. Looking for a more reliable method for mitochondrial ROS

formation we decided to measure the more stable and membrane-permeable dismutation product of $O_2^{\bullet-}$, H_2O_2. A non-invasive procedure was applied for mitochondrial H_2O_2 detection in order to avoid any interaction of mitochondria with the detection system and *vice versa* [2]. Mitochondrial ROS formation unequivocally could be demonstrated only in the presence of antimycin A. In the absence of this mitochondrial complex III inhibitor we were unable to observe mitochondrial H_2O_2 release irrespective of the methods applied, the state of respiration established (state IV, state III, uncoupled respiration), and substrates used for respiration (complex I, complex II substrates) [3]. However, our studies do not exclude that mitochondrial ROS formation can occur under certain pathophysiological conditions.

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4-02. Characterization of superoxide production by mitochondrial complex I.

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It is believed that production of superoxide and other reactive oxygen species by mitochondria contributes to a variety of pathological disorders and aging. In contrast to complex III, little is known about the mechanism of superoxide production by complex I. In order to gain understanding of this process, rat skeletal muscle mitochondria were incubated under a variety of conditions and the rate of superoxide production was assessed by measuring the hydrogen peroxide production rate. We found that the largest rate of superoxide production was in mitochondria respiring on succinate in the absence of rotenone, this confirms earlier work for other tissues that superoxide production from complex I is relatively large under conditions of reverse electron transport [1-3]. Our findings support the conclusion from studies using brain and heart muscle mitochondria that the production of superoxide is exquisitely sensitive to protonmotive force (Δp) [1, 3]. Furthermore, we have discriminated between the electrical ($\Delta\psi$) and chemical (ΔpH) components of Δp and found that superoxide production is almost entirely dependent on the ΔpH component. This result strongly suggests that the mechanism of superoxide production by reverse electron transport is closely linked to the mechanism of proton pumping by complex I. We found that the superoxide production rate from complex I with pyruvate and malate as respiratory substrates was less than 5% of the rate from complex I with succinate as substrate. This was not due to significant differences in Δ□ or ΔpH between forward and reverse electron transport. Whilst the reason for the marked asymmetry of superoxide production by complex I remains unclear, it is not simply explainable in terms of $\Delta\psi$ and ΔpH.

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4-03. Mitochondrial glycerophosphate dehydrogenase - a new site of ROS generation.

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Mitochondrial glycerophosphate dehydrogenase (mGPDH) is an enzyme with extremely high variations of its expression in mammalian tissues. Activity of mGPDH differs up to 100-times, when liver and brown fat mitochondria are compared [1]. In KCN- or antimycin A-inhibited brown adipose tissue mitochondria we have found pronounced glycerophosphate (GP)-dependent peroxide generation that is directly linked to the mGPDH function. We also observed that the rate of the GP-dependent hydrogen peroxide generation could be five-fold increased by one electron acceptor ferricyanide. This activating effect is linked only to GP-dependent and not to succinate- or NADH-dependent peroxide generation. This indicates that mGPDH is less protected against electron leak evidently due to the absence of Coenzyme Q-binding protein in mGPDH enzyme complex [2].

As shown by combined spectrophotometric and oxygen uptake measurements, the rate of ferricyanide-activated hydrogen peroxide generation decreased in parallel to reduction of added ferricyanide. At increasing ferricyanide concentrations both, the rate of hydrogen peroxide generation and FeCN/O ratio decreased.

GP-dependent ROS production can be detected also in liver mitochondria, in which mGPDH biogenesis was hormonally stimulated, or as in human placental mitochondria, where the mGPDH activity is relatively high. Destruction of the mitochondrial membranes by freezing thawing, connected with the release of endogenous cytochrome *c*, increased the rate of hydrogen peroxide production. Addition of cytochrome *c* to disrupted, and also to the intact mitochondria decreased GP-dependent ROS production. Similar inhibitory effect had also Coenzyme Q. We may thus speculate that mGPDH could be a potential risk for the cell metabolism and therefore its expression is highly reduced in most animal tissues. However, hormonal activation of mGPDH biogenesis could be also considered as a useful regulatory device, because some recent data suggest that cellular ROS production can selectively and reversibly inhibit the activity of various mitochondrial enzymes [3].

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4-04. Characterization of oxygen consuming reactions in hematopoietic stem cells: evidence of the occurrence of NAD(P)H oxidase activity.

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Despite of the growing interest in the field of stem cell research, promising important advance in the basic understanding of cell differentiation as well as in the cell-based therapeutic clinical application [1], a biochemical metabolic characterisation of this unique cell type is lacking. This study was aimed to partly fill such gap focusing attention on the terminal oxidative metabolism. The cell type chosen was human haematopoietic stem cell (HSC) mobilised from bone marrow by cytokine (G-CSF) treatment and collected from peripheral blood [2]. The protocol of cell isolation based on positive immuno-selection by a monoclonal antibody raised to CD34 antigen (a specific surface marker of HSCs) resulted in never less than 99 % of phenotipically homogeneous cell population. The main results obtained by an extensive analysis carried out on such HSC samples can be summarised as follow:

- Polarographic measurements of endogenous respiration of whole cells revealed a CN-sensitive oxygen consumption rate of about 0.05 nmoles O_2/min/10^6cells, which was indicative of a very low mitochondrial oxidative metabolism when compared with that of other cell types.
- Confocal microscopy imaging of HSCs, confirmed the presence of a few but functional Δp-generating mitochondria (detected by uncoupler-sensitive mito-tracker); an extensive and quantitative analysis of the cell population revealed an inverse correlation between the mitochondrial content and the intensity of the signal attributable to the CD34 stem cell marker. In addition, analysis of the mitochondrial intracellular network of other cell types allowed to relate and compare the respiratory activity to the mitochondrial area.
- The small amount of mitochondrial respiratory complexes in HSCs was verified by differential spectrophotometric analysis on whole cell lysate and by blue-native 2D SDS-PAGE analysis of the oxidative phosphorylation complexes in isolated mitoplasts.
- A re-examination of the CN-insensitive endogenous respiration, amounting to about 50 % of the overall endogenous respiration, revealed that this was completely abolished by DPI (a specific inhibitor of flavoenzymes) and sensitive to externally added catalase and/or superoxide dismutase, suggesting the involvement of a NAD(P)H-oxidase-like activity converting O_2 to $O_2^{\cdot-}$. This was verified by the occurrence of *ter*-butyl isotiocyanide shiftable absorbance peaks at 425 and 558 nm, indicative of the presence of cytochrome b_{558}, the NAD(P)H oxidase prosthetic group.
- Reverse-PCR amplification of total RNA cell extracts followed by sequencing showed the expression in HSCs of membrane bound and cytosolic subunits of the NAD(P)H oxidase (gp91^{phox}-NOX2, p22^{phox}, p67^{phox}, p47^{phox}). Furthermore cross-immunoprecipitation analysis revealed the occurrence of an assembled complex and the phosphorylation state of the p47 cytosolic subunit.

Taken all together these results show that the mitochondrial oxidative phosphorylation capacity of the CD34+ hematopoietic stem cell is very low when compared with that of other cell types. This could be a consequence of the low energy demand of the G_0-phase in which the resting HSC rely. The very low tension of O_2 of the bone marrow stromal micro-environment (stem cell niche), could also be a factor conditioning the expression of the oxidative phosphorylation system. It is noteworthy, however, the presence of mitochondria able to locally generate and maintain a transmembrane potential as shown by the confocal microscopy analysis. This was particularly evident in a sub-population of the CD34+ cells apparently expressing lower level of the surface marker (whose progressive lost is indicative of commitment), suggesting a role of the mitochondrial oxidative metabolism in the early stage of HSCs differentiation.

The novelty emerged from this study is the discovery of the presence of a NAD(P)H oxidase activity in HSCs (never reported before). Although both the catalytic and regulatory subunits of the NAD(P)H oxidase system are expressed and assembled, the activity, measured as DPI-sensitive oxygen consumption rate, is much lower then that of macrophagic cells where the NAD(P)H oxidase serves as a powerful oxygen producing bactericide system. Low active isoforms of the macrophagic NAD(P)H oxidase have, however, been reported in other non macrophagic cells and it has been suggested their involvement in oxygen radical-mediated intracellular signalling [3]. It is tempting to suggest that following activation by external stimuli, the HSC NAD(P)H oxidase might be involved in a ROS-mediated intracellular signalling leading (or contributing) to cell differentiation. The nature of the external stimuli, the type of oxygen reactive species, the targets of the activated intracellular system are under investigation to validate our hypothesis.

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4-05. Control of mitochondrial superoxide production by UCP3.

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The recent discovery that superoxide activates mitochondrial uncoupling proteins has led to new proposals for the as yet undefined physiological function of the UCP1 homologues [1]. Using the activity of the citric acid cycle enzyme, aconitase, as a sensitive measure of matrix $O_2^{\cdot-}$ [2] we have been able to demonstrate that inhibition of UCP3 in isolated skeletal muscle mitochondria causes an increase in mitochondrial matrix ROS. The addition of 500 μM GDP to inhibit UCP3 decreases aconitase activity by 25% when succinate is used as a respiratory substrate in the presence of rotenone. Furthermore, this inhibition also occurs with other purine but not pyrimidine nucleoside di- and tri-phosphates, in common with known properties of UCPs [3]. The IC50 for GDP-sensitive aconitase inhibition is 46 μM. However, this effect does not require the presence of fatty acids as it occurs both in the presence and absence of bovine serum albumin. Conclusive evidence that the increase in matrix ROS is mediated via UCP3 inhibition comes from the absence of any GDP-sensitive aconitase damage in skeletal muscle isolated from UCP3 -/- mice. Overall, these findings support a role for UCP3 in antioxidant protection of mitochondria.

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4-06. Understanding the mechanism by which the lipid peroxidation product 4-hydroxy-2-nonenal induces mitochondrial uncoupling through the adenine nucleotide translocase and uncoupling proteins.

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Oxidative stress, associated with mitochondrial dysfunction and aging, can cause peroxidation of membrane phospholipids to generate reactive aldehyde species. 4-Hydroxy-2-nonenal (HNE) is a major product of lipid peroxidation and is considered to be an important mediator of the toxic effects elicited by oxidative stress. However, HNE at low, non-toxic concentrations can also modulate various cellular processes including signal transduction, gene expression and cell proliferation [1].

Recently, it has been demonstrated that HNE (and structurally related compounds) uncouples mitochondrial respiration and that the observed uncoupling by HNE is mediated through the uncoupling proteins (UCP1, UCP2 and UCP3) and the adenine nucleotide translocase (ANT) [2]. Superoxide also causes UCP-mediated uncoupling, probably via lipid peroxidation and HNE production [3]. Mild uncoupling of mitochondrial respiration reduces the production of reactive oxygen species, which in turn decreases the production of HNE, suggesting a possible physiological role for HNE in a negative feed-back loop which protects against damage by oxidative stress.

The mechanism(s) by which HNE interacts with these proteins to cause uncoupling is unknown. However it is known that HNE is able to form adducts with various other cellular proteins and it is thought that these addition reactions form the basis for the observed physiological effects of HNE [1]. A potential first step in identifying the mechanism of HNE-induced uncoupling is to determine if HNE forms adducts with UCPs and/or the ANT under the conditions where uncoupling is observed. We provide evidence that the ANT is selectively modified by HNE and that the modification of the ANT is completely inhibited by carboxyatractylate.

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4-07. Recombinant and natural usefulness of uncoupling proteins.

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Uncoupling proteins (UCP) are members of the large family of anion carriers of the mitochondrial inner membrane. The archetype is the mammalian UCP1 expressed exclusively in brown adipose tissue where it transports protons and induces uncoupling of mitochondrial respiration hence promoting energy expenditure and thermogenesis. Two closely related UCPs are known in mammals, UCP2 and UCP3. In other phyla birds, fishes, invertebrates, plants, UCPs have been described too. There is an existing consensus that all UCPs operate on the same basis, initially

demonstrated with UCP1: uncoupling is induced by "fatty acids" and is inhibited by nucleotides (GDP being the more selective). If this consensus remains true, there remains however an important difference between UCP1 and the others (at least in mammals): UCP1 is expressed at very high level in brown adipose tissue mitochonria, accounting for few percent of membrane proteins, whereas UCP2 and UCP3 considerably less abundant (two orders of magnitude for UCP2), which would limit greatly their bioenergetical importance.

Genetic manipulation of UCPs genes in mice produced contrasting results: ablation of UCP1 results in cold intolerance (as predicted), inactivation of UCP2 or UCP3 failed to produce a "bioenergetic" phenotype: no cold intolerance, no obesity, no mitochondriopathy. Modified parameters in UCP2 knock out were an increased ROS production in macrophages, and improved insulin secretion in response to glucose. Increasing the expression of UCPs has been shown to be beneficial with respect to obesity, or oxidative stress related pathologies.

We have generated transgenic mice expressing the UCP1 in muscle and heart . The study of isolated normoxic perfused hearts showed that an amount of UCP1 as large as that found in brown adipose tissue does not change significantly the relationship between oxygen consumption and work output. This demonstrates that presence of a UCP acting as UCP1 does has no influence on energy expenditure at least when mitochondria produce ATP. This may be explained by inhibition of the protein by endogenous nucleotides and may be also by the membrane potential dependence of the UCP1 activity. Ischemia-reperfusion period induces the activity of UCP1 in heart of transgenic mice. This activity is accompanied by protection of the heart from the deleterious consequences of the ischemia-reperfusion period. This very likely results from the effect of UCP1 on mitochondrial membrane potential. Comparison of two transgenic lines with different expression levels of UCP1 in heart indicates that protection is dependent on a high level of expression of the UCP1.

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4-08. UCP2 function in the reconstituted system and isolated mitochondria.

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Reconstituted human mitochondrial uncoupling protein1 UCP2 expressed in yeast served us as a standard for further studies of UCP2 in mitochondria. We identified2 that namely some polyunsatu-rated fatty acids (PUFAs), omega-6 all-cis-8,11,14-eicosatrienoic, all-cis-6,9,12-octadecatrienoic acids, (the most potent in upregulation of UCP2 expression via PPARbeta) and omega-3 PUFA, cis-5,8,11,14,17-eicosapentaenoic and docosahexaenoic acid are the best activators of UCP2 protonophoric activity, most probably by FA cycling mechanism1. FAs are essentially required for UCP1 function3 and this is assumed for UCP2 as well. UCP2-dependent H+ translocation activated by all tested FAs was inhibited by purine nucleotides with decreasing affinity2: ADP > ATP ~ GTP > GDP >> AMP. Also 3H-GTP (3H-ATP) binding to isolated E.coli-(Kd ~5 μM) or yeast-expressed UCP2 (Kd ~1.5 μM) has demonstrated that purine nucleotides must interact with UCP2 also in vivo2. We have further used the number of 3H-GTP high affinity (Kd <0.4 μM) binding sites as a measure for the amount of UCP2 (dimer) content in mitochondria of various tissues (in pmols/mg protein): 182 in lung mitochondria2; ~100 in brain (may include UCP4,5), 74 in kidney2; 28 in skeletal muscle2(may include UCP3); and ~20 in liver mitochondria2; all compared to 800 in brown adipose tissue mitochondria, representing mostly UCP1. Knowing that Vmax values for UCP2 and UCP1 are comparable2, we predict that maximum uncoupling in un-stimulated liver mitochondria can reach ~1.5 mV in potential decrease, a value hardly measurable. On the contrary, when rats were injected with lipopolysaccharide (LPS), 3H-GTP binding sites increased up to 60 pmols per mg protein, which corresponded to ~3.5-times increase in UCP2 mRNA as quantified by RT-PCR on a LightCycler. Liver mitochondria from LPS-stimulated rats exhibited H2O2 production (estimated by scopoletin oxidation in the presence of horseradish peroxidase and FAs) which was highly enhanced by GDP, whereas no GDP sensitivity was found in mitochondria from un-stimulated control rats. Thus probably a small (predicted ~4.5 mV) decrease of membrane potential in liver mitochondria from LPS-stimulated rats is amplified into a large drop in reactive oxygen species (ROS) production as a consequence of UCP2-mediated uncoupling. The studied phenomenon of LPS-stimulation of UCP2 expression in liver thus reflects a systemic response to the simulated bacterial infection which does recruit UCP2 molecules. In macrophages, including those resident in liver (Kupffer cells), UCP2 is decreased upon LPS stimulation1, which results in increasing of their ROS. During infection the whole body ROS content is elevated due to macrophage attack in which ROS play an important role. The described activation of UCP2 expression in hepatocytes could serve as an adaptation re-setting homeostasis of reactive oxygen species back to low values. (The project was supported by the grants of Grant Agency of the Czech Republic, No. 301/02/1215; and by the Internal Grant Agency of the Academy of Sciences of the Czech Republic No. A5011106.)

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4-09. Skeletal muscle mitochondria from continuously shivering cold-acclimated UCP1-ablated mice: no resultant uncoupling.

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Mice lacking uncoupling protein 1 (UCP1) were originally observed to be extremely cold sensitive [1]. However, our previous studies have shown that even these UCP1-ablated mice can develop cold tolerance through long-term preacclimation to 18°C. The mechanism of heat production in UCP1(-/-) mice is adaptive skeletal muscle-derived shivering thermogenesis [2]. Improved heat production in preacclimated UCP1(-/-) mice could be through an enhanced training status of skeletal muscle: an increased ability to shiver. Alternatively, skeletal muscle has been proposed as being responsible for a non-brown adipose tissue derived nonshivering thermogenesis mediated by UCP3 [3]. Therefore, we decided to investigate skeletal muscle from UCP1(-/-) mice as a model of continuous shivering, as well as a possible role of UCP3 in adaptive changes of skeletal muscle upon cold exposure.

The effect of cold acclimation on oxidative phosphorylation and UCP3 activity were compared in skeletal muscle mitochondria isolated from the hind limbs of wild-type and UCP1-ablated mice acclimated at 24°C (control) or acclimated at 18°C for 4 weeks with the following 4 weeks at 4°C ("cold" groups). The amount of skeletal muscle mitochondria increased by 61 % in cold-acclimated UCP1(-/-) mice, compared to cold-acclimated wild-type mice. However, oxygen consumption in State 3 and in Uncoupled State decreased by 18 – 20 % in these mitochondria oxidizing pyruvate. Cold acclimation improved fatty acid oxidation in both strains of mice, independently of UCP1 ablation. No difference between the two strains was found in the rate of oligomycin-insensitive oxygen consumption in mitochondria isolated from animals acclimated to different temperatures, demonstrating that cold acclimation has no effect on proton leak in skeletal muscle.

To explore whether UCP3 mediates adaptive changes in skeletal muscle from UCP1- ablated mice, we also analysed possible effects of the inhibitor of UCPs, GDP, on mitochondrial thermogenesis. However, there was no effect of GDP on basal respiration or on fatty acid-induced respiration; similarly, the sensitivity to free fatty acids was unchanged. Thus, there was no evidence for a higher UCP3 activity in muscle mitochondria from UCP1-ablated mice than in wild-type or for any induction of UCP3 activity by cold acclimation.

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4-10. Possible involment of AMPK in obesity resistance induced by respiratory uncoupling in white fat.

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A regulatable proton leak in mitochondria may be a general mechanism for controlling metabolic rates, energy balance and thermogenesis. The mechanism of uncoupled thermogenesis is well characterized in mitochondria of mammalian brown fat, where it depends on protonophoric function of uncoupling protein 1 (UCP1). The mechanism by which respiratory uncoupling may reduce accumulation of fat can be analyzed in transgenic mice in which the UCP1 gene is driven by the fat-specific aP2 promoter to achieve enhanced expression in both brown and white fat. These mice with aP2-*Ucp1* transgene are resistant to obesity. This resistance reflects a lower accumulation of triacylglycerols in white adipose tissue, namely in adipocytes from subcutaneous fat depots. Transgenic UCP1 could decrease mitochondrial membrane potential, elevate oxygen consumption[2] and reduce ATP/ADP ratio in adipocytes. The transgene also induced mitochondrial biogenesis, probably by way of induction of transcription factor NRF-1 (nuclear respiratory factor-1)[3]. A strong diminution of fatty acid synthesis, accompanied by down-regulation of expression of genes for FAS (fatty acid synthase) and ACC (acetyl-CoA carboxylase), was found in white fat[4]. The lipolytic effect of noradrenaline was lowered in white adipocytes of transgenic animals. Likewise expression of HSL (hormone-sensitive lipase) was down regulated in subcutaneous fat. On basis of the above findings we have suggested that AMPK might be involved in the marked changes of lipid metabolism in the transgenic mice, resulting in obesity resistance. To test this idea, expression of PPAR-γ (Real-Time Quantitative PCR), ATP and AMP contents (HPLC) and *AMPK* activity in adipose tissue of control and transgenic mice were estimated. Expression of the gene for PPAR-γ was depressed by the transgene in subcutaneous fat (2.95 $\pm$0.42 vs. 1.18 $\pm$0.12 A.U.). ATP/AMP ratio in adipocytes from subcutaneous fat of transgenic animals was significantly lower than in control mice (7.11 +/- 0.34 vs. 5.31 +/- 0.26). Western blot analysis using polyclonal antibodies to phospho-AMPKα (Cell Signaling, USA) and specific peptide phosphorylation assay have been performed for sample extracts from control and transgenic animals and result will be presented on our poster. All the effect of transgenic UCP1 on biochemical characteristics of white fat of aP2-*Ucp1* mice are in accordance with the activation of AMPK. We hypothesize that UCPs modulate lipid metabolism in adipocytes *via* AMPK.

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4-A01. Communication between the mitochondrial thioredoxin system and 2-oxo acid dehydrogenase complexes involves thiyl radicals of the complex-bound dihydrolipoate and reactive oxygen species.

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The 2-oxo acid dehydrogenase complexes occupy key branch points in the mitochondrial metabolism: involvement of pyruvate into the citric acid cycle by the pyruvate dehydrogenase complex; oxidation of 2-oxoglutarate by the 2-oxoglutarate dehydrogenase complex, which is the rate-limiting step of the cycle; and oxidation of the branch chain 2-oxo acids by the branch chain 2-oxo acid dehydrogenase complex, which is an important regulatory point of aminoacid metabolism. The complexes are self-assembled into symmetrical structures from multiple copies of their three component enzymes, resulting in dimensions comparable to intercristae space [1]. In a multistep process involving a number of cofactors (thiamine diphosphate, lipoic acid, CoA, FAD and NAD^+) and sequential action of 2-oxo acid dehydrogenase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3), the complexes split a carbon-carbon bond of a 2-oxo acid, preserving its energy in acyl-CoA and NADH. Reduced activities of the complexes are involved in pathophysiology of thiamine deficiency and many neurodegenerative diseases [2], which highlights essential role of these reactions in mitochondria. Our objective is to understand the mechanisms of cross-talks between the complexes and other mitochondrial pathways, which may underlie signal transduction through the complexes. Product inhibition of the 2-oxo acid dehydrogenase complexes by NADH and acyl-CoA represents such regulation at the level of metabolite concentration. Phosphorylation/dephosphorylation of the pyruvate and branch chain 2-oxo acid dehydrogenases is involved in hormonal signal transduction. However, the 2-oxoglutarate dehydrogenase is not regulated by phosphorylation. In contrast, we found that the 2-oxoglutarate dehydrogenase complex is extremely responsive to the redox-dependent regulation provided by thioredoxin and the lipoic acid residues compartmentalised within the complex [3]. Thioredoxin scavenges the thiyl radicals of the complex-bound dihydrolipoate, thus increasing catalytic performance of the 2-oxo acid dehydrogenase complexes in a wide range of their substrate concentrations. However, a side reaction of the reactive oxygen species (ROS) production by the complexes is increased by thioredoxin as well. Biological significance of the latter reaction catalyzed by the pyruvate and branched chain 2-oxo acid dehydrogenase complexes is limited because under the substrate ratio stimulating ROS production these complexes are phosphorylated, i.e. inactive. However, the 2-oxoglutarate dehydrogenase complex may be a significant source of reactive oxygen species in mitochondria, and this activity of the complex is under control of thioredoxin. While thioredoxin protection of the complex is necessary for the normal function of the citric acid cycle, the accompanying accumulation of ROS may be deleterious if the ROS scavenging potential of mitochodria is compromised. Mitochondrial thioredoxin system, including thioredoxin, thioredoxin reductase, thioredoxin peroxidase and NADPH, is a known participant of ROS scavenging. Thus, our data point to the intimate link between the rate-limiting step of the citric acid cycle, ROS generation by the 2-oxoglutarate dehydrogenase complex and thioredoxin-dependent pathways. A disbalance in this cross-talk may input to the mitochondria-dependent cellular death cascade, which is supported by association of neurodegeneration with a decreased activity of the 2-oxoglutarate dehydrogenase complex.

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4-A02. Superoxide, not only oxygen, in inhaled air is necessary for life.

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Conventional wisdom holds that superoxide (ROS) is the main carrier of negative charge in ionized air. We investigated the effect of inhalation of air that was ionized by an electroefflivial generator of negative air ions (mostly superoxide) on the generation of H_2O_2 in rat heart and brain mitochondria. Negative air ions (NAI) in inhaled air has been traditionaly considered essential for life, such that a total absence of NAI is fatal and NAI deficiency induces variuos disorders. NAI are present in clean, rural air and their level is substantially lower in polluted, urban air as well as in isolated spaces. A. L. Tchiujevsky created an electrical generator of NAI in order to prevent health problems that result from their deficiency. We have shown that ~ fMsuperoxide and ~ηM H_2O_2 are formed in solutions under the influence of the Tchijevsky Lustre ionizer (DIOD, Moscow) [1]. We have found that NAI stimulate the processes of ROS transformation, such as the activation of SOD in blood and the generation of H_2O_2 in heart mitochondria [2,3]. We study the effect of NAI (ROS) inhalation on formation of H_2O_2 in mitochondria of rat brain. Brain is more directly supplied with air through both lungs and nose. The

classic system of H_2O_2 generation in mitochondria was used with addition of succinate and antimycin. Glutamate was included to increase the rate of respiration. H_2O_2 formation in heart mitochondria was observed at 26° C, while in brain mitochondria it was detected only at 37° C. A similar response to inhalation of ROS was observed in mitochondria of both tissues, i.e. increase in H_2O_2 formation per 60-70% and in rate of ADP phosphorylation per 50-60%. NAI are absorbed in airways and cannot penetrate into tissues. We suggest that external ROS initiates the signal action for the generation of internal ROS in tissues by transduction through the ROS-generating systems, most likely through NADPH-oxidase of the receptors in airways and blood vessels. Our data show that the inhalation of external ROS stimulates formation of internal ROS. The level of stimulated formation of H_2O_2 is within the physiological range, it does not impaire, but rather stimulates oxidative phosphorylation. Elevation of H_2O_2 level in tissues up to 1μM also regulates other processes. Particularly, we observed that the influence of ROS on the mitochondria in homogenate increases the restoration of blocks of mitochondrial-reticular network, therefore, incubation media without ROS fails to reproduce completely the physiological properties of mitochondria.

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Session 5: Mitochondrial pathologies and ageing.

5-01. Respiration and energy coupling in OXPHOS complexes assembly defects.

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Human mitochondrial diseases due to disorders of oxidative phosphorylation (OXPHOS) enzymes constitute a heterogeneous group of diseases caused by mutations either in mitochondrial or nuclear DNA with predominant affection of tissues with high energy demands. They range from severe and often fatal encephalomyopathies of early infancy and childhood to a milder disorders of adults and a variety of senescence degenerative diseases. During the last decade, increasing number of mitochondrial disorders have been found to result from mutations in nuclear genes that encode either OXPHOS subunits, or specific assembly proteins and other components of mitochondrial biogenetic machinery (1). Among the most severe belong the frequent specific defects of cytochrome c oxidase (COX) presenting as subacute necrotising encephalomyopathy (Leigh syndrome) that are caused by mutations in the *SURF1* gene, encoding the Surf1 protein essential for COX assembly. Our studies of Surf1p-deficient fibroblast (2) showed that different *SURF1* mutations cause pronounced decrease of the normal-size COX complexes, significant accumulation of incomplete COX assemblies of 90-120 kDa and alteration of OXPHOS supracomplexes. In contrast to 70-90 % decrease of COX activity observed by spectrophotometric assay, oxygen consumption analysis in whole cells revealed only a small (<30%) decrease of COX activity that was completely inhibited by a mild detergent. In patient fibroblasts the ADP-stimulated respiration was 50% decreased and TMRM cytofluorometry showed a significant decrease of mitochondrial membrane potential □□m in state 4, and increased sensitivity to FCCP. Decreased steady state levels of □□m have been also found in COX deficient cells harbouring heteroplasmic A8344G mtDNA mutation in $tRNA^{Lys}$ (3) while increased □□m appears to be associated with different defects of mitochondrial ATP synthase (4). We conclude that the absence of the Surf1 protein leads to the formation of incomplete COX complexes, which *in situ* maintain rather high electron-transport activity, while their H^+-pumping is impaired. Enzyme inactivation by the detergent in patient cells indicates instability of incomplete COX assemblies.

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5-02. Does modulation of mitochondrial respiration state affect yeast lifespan?

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The mitochondrial electron transport chain, necessary for oxidative phosphorylation, is also

the main intracellular source of reactive oxygen species (ROS). The flux rate of electrons through mitochondrial electron transport chain carriers has a profound effect on the rate of generation of mitochondrial ROS - high flux (state 3 respiration; defined in vivo as high respiratory state value RSV 1) minimizes mitochondrial ROS, whereas low flux (state 4 respiration; low in vivo RSV) leads to higher mitochondrial ROS. In view of the documented inverse correlation between yeast chronological life span and oxidative damage 2, we hypothesized that manipulation of respiration state to increase the flux rate of electrons will extend the chronological life span of yeast. Our strategy was to express in yeast a mitochondrial enzyme alternative oxidase (AOX) that is not naturally present in *S. cerevisiae* but common in most other fungi, as well as plants and protists. Alternative oxidase, which catalyses oxygen reduction by accep-tance of electrons from ubiquinone, allows electron transport that is not coupled to oxidative phosphorylation. Hence its presence creates state-3-like electron flux rates through ubiquinone, regardless of the respiratory state of the main electron transport chain. By this mechanism, alternative oxidase is believed to minimize the amount of ROS produced by mitochondria. Our results show that in comparison to the AOX-negative control strain, expression of AOX is correlated with a) increased chronological life spans of two *S. cerevisiae* strains examined so far; b) improved RSV of the main (cyanide-sensitive) electron transport chain; c) less rapid decrease of RSV with age; and d) increased resistance to 50 °C heat induced cell death (known to be mediated by mitochondrial production of ROS 3). These results support the hypothesis that interventions designed to maintain a high RSV extend yeast chronological life span, most likely as a result of decreased production of mitochondrial ROS. Future experiments will include direct assessments of mitochondrial ROS and oxidative damage under these experimental conditions. (This work is supported by the U.S. Veterans Administration and NIH P01AG20641.)

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5-03. Respiration and coupling of oxidative phosphorylation in proliferating, growth arrested, and senescent primary human fibroblasts.

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Human cells in primary culture have a finite lifespan, a phenomenon termed „replicative senescence". After about 50 population doublings, cells stop proliferation and arrest irreversibly in the G_1 phase of the cell cycle. Cellular energy metabolism is an important aspect of aging, as shown by life span extension through caloric restriction. Analysis of the glycolytic pathway in young and old cells revealed age-associated changes in the activity of several enzymes. Staining cells with the oxidant-sensitive dye dihydrorhodamine showed that senescent fibroblasts exhibit oxidative stress, a possible consequence of metabolic imbalance. Based on these results, we wanted to know whether mitochondrial function is impaired in senescent cells.

Mitochondrial respiratory function was analyzed by high-resolution respirometry with the OROBOROS *Oxygraph* [1]. The experimental regime started with routine respiration, followed by inhibition of ATP synthase with oligomycin, and uncoupling by stepwise titration of FCCP. Finally, respiration was inhibited by sequential addition of rotenone and antimycin A [2]. Respiration per cell was highly increased in old fibroblasts, owing to increased mitochondrial content (citrate synthase activity) in line with an increase in cell size. Normalization of respiratory parameters by citrate synthase activity diminished several differences obtained when expressing results per cell number. The capacity of the respiratory chain, reflected by uncoupled respiration per citrate synthase, is unchanged in old and young fibroblasts. Oligomycin-inhibited respiration, however, was significantly increased in senescent cells. Further, senescent cells exhibit a slightly decreased uncoupling control ratio, and a decreased ratio between uncoupled respiration and oligomycin-inhibited respiration. This indicates a lower coupling state of mitochondria in senescent fibroblasts. Additionally, we performed series of control experiments using young fibroblasts arrested in G_0 by contact inhibition. Comparing these cells with senescent cells, the difference in the coupling state is much more striking than between proliferating and senescent cellls. These results indicate that there is no loss of mitochondrial respiratory capacity in senescent fibroblasts. The coupling state is lower in old cells compared to young ones, which might be a consequence of oxidative stress [3]. Interestingly, G_1 arrested young fibroblasts exhibit a very high coupling state, a phenomenon which warrants further study.

Partial uncoupling, as observed in senescent fibroblasts, leads to a diminished oxidative ATP production or a compensatory increase in electron transport. Our titration regime applied to intact cells provides the basis for distinguishing between these alternatives. For this purpose, we define the phosphorylation respiratory control ratio, RCR_P = (R-4o)/3u, where R, 4o and 3u are the routine, oligomycin-inhibited and uncoupled respiratory rates, respectively. Despite differences of the respiratory control ratio, $RCR_{3u/4o}$, in the range of 4 to 11 in young and senescent fibroblasts, the RCR_P was constant (0.17 to 0.19), indicating that by a compensatory increase of routine respiration, a fixed proportion (18 %) of respiratory capacity was maintained for coupled oxidative phosphorylation in senescent and young fibroblasts.

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5-04. Probing energy metabolism of cells by measurements of oxygen consumption and by fluorometric determination of mitochondrial membrane potential.

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An important factor influencing mitochondrial activity is cell density. In a variety of cell cultures cell density has been revealed as a major factor determining respiratory activity. In secondary fibroblasts, 3T3 cells, human proximal and distal tubule epithelial cells, HaCaT cells and to a clearly minor extent in SV40 transformed 3T3 cells oxygen consumption decreased with increasing cell density. This represented a general decrease of energy requirements because lactate production, ATP-content, NAD-content and NAD-redox potential also were downregulated and mitochondria could extensively be stimulated by uncoupling agents like CCCP.

In addition, secondary cell cultures showed significant cell type-specific differences in their oxygen consumption regardless at which density the cultures were compared. With increasing oxygen demand these were: Human umbilical vein endothelial cells (HUVECs) < mouse skin fibroblasts < human distal renal tubule cells < human proximal tubule cells < chicken cardiomyocytes.

A third factor influencing respiratory activity is ageing. Isolated mitochondria and whole living cells have been studied by using a specific dye (DASPMI: dimethylaminostyryl-methylpyridiniumiodine), staining selectively mitochondria in living cells. DASPMI is reacting to an increase of mitochondrial membrane potential with an increased fluorescence. This was demonstrated e.g. with mitochondria isolated from pigeon hearts. DASPMI-stained mitochondria showed an upregulation of fluorescence when incubated with substrates, ATP or O_2. In contrast inhibitors of the respiratory chain or oxygen depletion caused a decrease in the fluorescence reflecting clearly the state of mitochondrial membrane potential. Using DASPMI an almost homogenous mitochondrial population in ageing and in H_2O_2 – treated HUVECs could be divided into two subpopulations with different membrane potential.

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5-05. Effect of IL-1β treatment on respiration of human peritoneal mesothelial cells. A combined test of cell membrane permeabilization and mitochondrial function.

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The peritoneal mesothelium acts as a bioactive cellular membrane regulating serosal responses to injury, infection and neoplastic diseases. Inflammation of the serosal surfaces induces an "activated" mesothelial cell phenotype. In the present study we simulated activation of cultured human peritoneal mesothelial cells (HPMC, isolated from human omentum majus) by treatment with the pro-inflammatory cytokine interleukin-1beta (IL-1β). Respiratory activity of suspended cells was analysed by high-resolution respirometry (OROBOROS Oxygraph) to assess changes in respiratory capacity and coupling of oxidative phosphorylation in activated HPMC. Respiration of cells in RPMI culture medium was low, owing to a high degree of plasma membrane permeabilisation in suspended cells and respiratory defects induced by exposure of mitochondria to high Ca^{2+} concentrations [1]. RPMI, therefore, was replaced by mitochondrial respiration medium (MiR05 [2]).

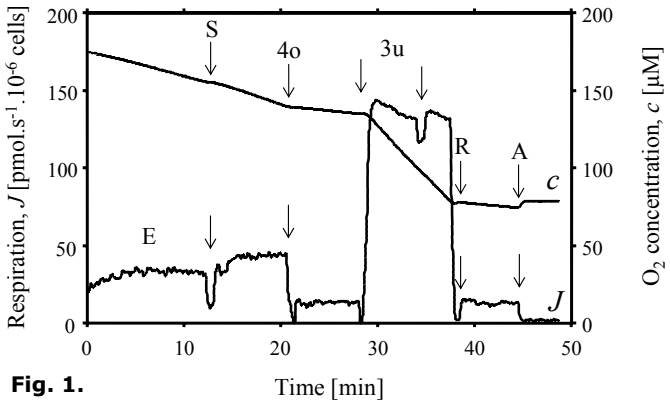

Fig. 1.

In a combined test for plasma membrane integrity and mitochondrial function [3], the respirometric titration regime included endogenous respiration, E, addition of succinate and ADP, S, oligomycin (state 4o), FCCP (state 3u), rotenone, R, and antimycin A, A (Fig. 1; *c* is the trace for oxygen concentration, *J* is the trace for respiration). Endogenous respiration of controls in MiR05 was 45.1 ± 6.4 pmol·s^{-1}·10^{-6} cells. The stimulatory effect of succinate and ADP on respiration is related to cell membrane permeabilisation as (S-E)/(3u-E). Similarly, in the presence of succinate, the relative progressive inhibition by rotenone and antimycin A, (R-A)/(3u-A), is an index of cell membrane permeabilisation. The viability of 80 % derived from these two respiratory indices was consistent with independent data from CASY cell viability analysis.

After IL-1β activation for 48 hours, respiratory capacity declined (P<0.05) without affecting cell viability. The respiratory adenylate control ratio ($RCR_{3u/4o}$; state 3u/4o ratio of respiration) remained unchanged after IL-1β treatment at 11.1 ± 0.6, indicating tight coupling of oxidative phosphorylation and integrity of the inner mitochondrial membrane. Treatment of HPMC with IL-1β resulted in a decrease of CS activity (P < 0.05) and increase of LDH activity (P<0.05). The present data demonstrate that activation of peritoneal mesothelial cells with IL-1β is associated with a decrease of oxidative phosphorylation and mitochondrial content that appears to be compensated by an increase in glycolytic capacity.

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5-06. Cytochrome *c* release by intrinsic Bax in brain and kidney mitochondria is regulated through the oligomeric state of creatine kinase.

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Localisation of cytochrome c in the contact sites: The distribution of cytochrome c and the octamer of mitochondrial creatine kinase were studied in contact site and cristae fractions of rat kidney mitochondria. It was observed that factors increasing or decreasing contact sites such as dextran or glycerol led also to a related change of cytochrome c content and creatine kinase activity in the contact site fractions. Cytochrome c and the octamer of mitochondrial creatine kinase both bind to cardiolipin encircling the adenine nucleotide translocator (ANT). Thus, factors specifically changing the structure of the ANT and presumably the orientation of cardiolipin were found to influence the distribution of cytochrome c and creatine kinase in the contact sites [1]. The c-conformation of ANT, induced by atractyloside, resulted in high concentration of cytochrome c and creatine kinase activity in the contact sites, whereas the ANT m-conformation caused by bongkrekate was followed by a shift of cytochrome c and creatine kinase activity towards the cristae.

The Bax dimer is a component of the creatine kinase ANT complex: The ANT complex between cytochrome c and creatine kinase octamer persisted after dissolving brain or kidney membranes by Triton X-100 and could be precipitated by Bax specific antibodies. Alternatively the complex was isolated from the Triton extract by anion exchange chromatography through binding of creatine kinase [2]. Both methods revealed that a Bax dimer and the outer membrane pore protein were components of the creatine kinase ANT complex.

Function of the intrinsic Bax in cytochrome c release: After reconstitution of the complex in phosphatidyl choline, cholesterol vesicles we studied the release of the intrinsic cytochrome c. Addition of external Bax was ineffective, whereas cytochrome c was liberated upon dissociation of the octamer structure of creatine kinase. The cytochrome release following the creatine kinase dissociation was inhibited by Bcl-2, indicating the involvement of the complex integrated Bax in this process.

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5-07. Diabetes induces metabolic adaptations in rat liver mitochondria: role of coenzyme Q and cardiolipin contents.

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Several studies have been carried out to evaluate the alterations in mitochondrial functions of diabetic rats. However, results are sometimes controversial, since experimental conditions diverge, including age and strain of used animals. The purpose of this study was to evaluate the metabolic modifications in liver mitochondria, both in the presence of severe (STZ-treated rats) and mild hyperglycaemia (GK rats), when compared with control animals of similar age. Moreover, metabolic alterations were evaluated also at initial and advanced stages of the disease. We observed that both models of diabetes (type 1 and type 2) presented a decreased susceptibility of liver mitochondria to the induction of permeability transition (MPT). Apparently, there is a positive correlation between the severity of diabetes mellitus (and duration of the disease) and the decline in the susceptibility to MPT induction. We also found that liver mitochondria isolated from diabetic rats presented some metabolic adaptations, such as an increase in coenzyme Q and cardiolipin contents, that can be responsible for the observed decrease in the susceptibility to MPTP opening.

5-08. Mitochondria as the prime target of anthracycline cardiotoxicity - molecular damage of cardiac mitochondrial creatine kinase.

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Anthracyclines are efficient anti-cancer drugs, but their use is limited due to serious cardiotoxic side effects. This cardiotoxicity is still poorly understood, but impairment of mitochondrial functions like respiration and generation of high-energy phosphates seems to

play a key role. We have shown in a comprehensive *in vitro* study that clinically relevant concentrations of anthracyclines preferentially damage the cardiac isoform of mitochondrial creatine kinase (MtCK) as compared to the ubiquitous isoform found e.g. in brain [1]. Our results show that anthracyclines act through competitive binding to mitochondrial membranes and oxidation of susceptible cysteines [1]. This has negative effects on three essential properties of MtCK: membrane binding, octameric state and enzymatic activity [2]. Similar oxidative modification of MtCK has been observed with peroxynitrite [3] that contributes to MtCK damage during ischemia/reperfusion [4]. Peroxynitrite also affects much more the cardiac/muscle MtCK isoform. To confirm our hypothesis that anthracycline-induced damage of MtCK-linked "high energy" phosphoryl transfer is causally linked to drug cardiotoxicity, we currently conduct experiments with isolated mitochondria, cardiomyocytes and perfused heart. First results obtained by respirometry and electron microscopy show that MtCK-dependent mitochondrial properties like creatine-stimulated respiration and mitochondrial ultrastucture are impaired by anthracyclines at clinically relevant concentrations, with heart mitochondria being more susceptible than those isolated from brain. (This research has been supported by Schweizerische Herzstiftung, Wolfermann-Nägeli Stiftung, Swiss Cancer League and a Swiss National Science Foundation MHV Subsidy to M.T.-S.)

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5-09. Pathogenic mtDNA mutations in cancer samples.

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Mitochondrial involvement in carcinogenesis, considered since the work of Warburg has recently been supported by numerous findings (reviewed in [1]). We have analyzed mtDNA variation in various cancer samples, comparing them with normal tissue controls, and identified mutations and polymorphisms, both known and novel, in mitochondrial tRNA, rRNA and protein genes. Most remarkably, in a colon cancer sample we have found the A3243G mutation in the homoplasmic state. The A3243G MELAS mutation is one of the best characterized mtDNA defects and is always manifested clinically as a severe mitochondrial disease. On the molecular level this mutation is known to affect tRNA-Leu (UUR) aminoacylation and the wobble modification in the anticodon. Cells harbouring more than 95% of A3243G mtDNA exhibit a severe respiratory-deficient phenotype. They have significantly lowered oxygen consumption rates and electron transfer activities, lowered ATP/ADP ratio and energy charge and show signs of increased oxidative damage [2]. Accordingly, this mutation has never been found in homoplasmic state in living tissue. The A3243G mutation is therefore likely to contribute to a respiratory deficient, highly glycolytic and therefore hypoxia-resistant phenotype, which would confer selective advantage to an expanding tumor [3]. Our results are therefore consistent with the hypothesis that changes in mtDNA leading to a respiratory defect are an important factor in generation and progression of cancers.

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5-A01. Threshold expression of respiratory chain defects in mitochondrial cytopathies.

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Despite a better characterization of the genetic defects leading to mitochondrial disorders, their pathogenetic mechanisms are for the most part, not understood. Particularly, the relationship between the

presence of a defect in a given OXPHOS complex, and the occurrence of specific clinical signs (phenotype) remains problematic. We have analysed the relationship between the presence of single defects in the activity of respiratory chain complexes and mitochondrial respiration in muscle biopsies from 50 individuals screened for mitochondrial diseases. Our results demonstrate the first direct evidences for the existence of a "biochemical threshold effect" that directly intervenes in the clinical expression of respiratory chain defects in human muscle. It is characterized by the fact that an activity defect in a respiratory chain complex must exceed a critical value before a decrease in mitochondrial respiration can be observed. We discuss the implications of this biochemical threshold effect *in vivo* for the diagnosis and the therapy of mitochondrial diseases.

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5-A02. Mobilization of the adenine nucleotide translocator: molecular bases of the biochemical threshold effect observed in mitochondrial diseases?

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The existence of a biochemical threshold effect in the metabolic expression of respiratory chain deficiencies has considerable implications for the understanding of mitochondrial bioenergetics and the study of mitochondrial diseases. However, the molecular bases of this phenomenon remain unclear. We report here a new mechanism to explain this threshold effect, based on a reserve of enzymes not initially participating in the respiratory rate that can be activated either to respond to a flux increase, or to compensate for a defect induced by a mutation. We show that this mobilization occurs through (i) the assembly of inactive adenine nucleotide translocator isoform 1 (ANT1) subunits into oligomeric active carriers, or (ii) conformational changes in the ANT1 in a PTP-like structure. We discuss how these transitions are sensitive to the steady-state of OXPHOS functioning, or tissue, and analyze their consequences on the threshold effect.

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5-A03. Mitochondria as mutators.

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We have tested whether mitochondrial dysfunction impacts on the genetic stability of the nuclear genome. Using *Saccharomyces cerevisiae* as a model organism, we analyzed the consequences of disrupting mitochondrial function on genetic stability of the nuclear genome. In wild type yeast exposed to mitochondrial respiratory chain inhibitors or mutant yeast lacking the entire mitochondrial genome (rho^0) or yeast with a mitochondrial mutation (rho^-), we tested the instability of the nuclear genome by measuring the frequency of canavanine resistant colonies. The *CAN1* gene of *S. cerevisiae* encodes a transmembrane amino acid transporter that renders the cell sensitive to lethal arginine analogue, canavanine. Any inactivating mutation in this gene results in a canavanine resistant phenotype (CAN^R). We calculated the frequency of canavanine resistant colonies as a measure of spontaneous nuclear mutational events in rho^0 and rho^- strains. We found that, compared to the wild type cells, nuclear mutational events were significantly higher in both rho^0 and rho^- strains. Likewise, inhibition of electron transport by antimycin and other agents in wild type cells resulted in increase frequency of mutation in nuclear genome. Our studies have also revealed that *REV1*, *REV3* or *REV7* gene products, implicated in error-prone translesion DNA synthesis, mediate the genetic instability of the nuclear genome arising as a result of mitochondrial dysfunction. Our results described here in yeast model provide a direct link between mitochondrial dysfunction and genetic instability, which has important implications in human cancer and aging.

5-A04. Comparision of tissue respiration changes in myocardium and skeletal muscle after ^{137}Cs incorporation.

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The growth of cardiovascular diseases (e.g. ischemic heart disease – in 3,5 times) is observed in regions after the Chernobyl disaster [1, 2]. Radionuclide of ^{137}Cs is known to be a main dose-forming radionuclide, and influence the energy metabolism of the muscular tissues. The parameters of tissue respiration of the myocardium and sceletas muscles slices of Wistar rats were studied with Clark electrode [3]. The rats were fed during 2 month with the products contaminated with ^{137}Cs radionuclide. The groups of animals with specific activity of incorporated ^{137}Cs from 1,500, 17,000, 30,000 and 60,000 Bq/kg were formed. The doses were 270,

1,940, 3,500, and 7,400 μGr respectively. The decrease in the respiratory activity of myocardium were found when the animals were fed till incorporation level from 1,500 Bq/kg to 17,000 Bq/kg. And ^{137}Cs accumulation level 30,000–60,000 Bq/kg led to stimulation of respiratory activity, and increase of sensitivity of oxidative phosphorilation system of myocardium to 2,4-dinitrophenol was found. In skeletal muscles significant increase of respiratory activity was observed at 17,000–60,000 Bq/kg specific activity. No changes were found in oxidation phosphorilation system. These phenomena were considered in terms of cesium action as a source of radiation and potassium antagonist [3], and differences in oxidative phosphorilation conditions in myocardium and skeletal muscles.

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Session 6: Mitochondrial structure and function.

6-01. Intracellular restrictions of diffusion and metabolic channeling of ADP in normal and diseased cardiac muscle cells.

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Recent studies have revealed the structural and functional interactions between mitochondria, myofibrils and sarcoplasmic reticulum in cardiac cells. From quantitative analysis of confocal images, it is clear that mitochondria are precisely organized in the heart muscle cells with the very small variation in the distances between mitochondria. In this highly organized intracellular environment, direct channeling of adenosine phosphates between organelles and via energy transfer networks has been identified (1,2,3). It can be shown using mathematical models, that such channeling indicates limitation of diffusion of adenosine phosphates in cardiac cells due to very specific intracellular structural organization (3). This diffusion limitation exists in healthy cardiac cells and disappears after short treatment by trypsin or after ischemia-reperfusion cycle. Intriguingly, ischemic preconditioning of the heart before ischemia-reperfusion cycle was able to preserve diffusion limitations of adenosine phosphates. It has been hypothesized on the basis of these results that in oxidative muscle cells the ATPases and mitochondria are organized into functional complexes, termed as the intracellular energetic units, ICEUs (1). According to our data, these complexes can be destroyed by trypsin treatment or ischemia-reperfusion cycle in the heart without ischemic preconditioning. However, how such complexes are formed, is still not clear and is the subject of active research.

Here, we analyzed the possible role of two principally different modes of restriction distribution for adenosine phosphates: (a) the uniform diffusion restriction and (b) the localized diffusion limitation in the vicinity of mitochondria. According to our analysis, the measurements of the respiration rate as a function of exogenous and endogenous ADP including channeling of endogeneous ADP as revealed by use of competitive pyruvate kinase and phosphoenolpyruvate system, can be reproduced by the mathematical model regardless to diffusion restriction distribution used. However, the same is not true for measured stabilization of oxygen consumption rate after addition of 2mM ATP or ADP as well as buildup of ADP concentration in the medium after addition of ATP. Our analysis revealed that only the second mechanism considered - localization of diffusion restrictions - is able to account for the experimental data. These localized restrictions are the result of structural organization of the cell and the basis for functional coupling between ATPases and mitochondria in the cardiac muscle cells. As long as functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase is accounted for in the model, the metabolic stability of the heart in the wide range of workload changes can be reproduced within structural unit (ICEU) of the heart muscle cell.

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6-02. Potassium cycling across the inner membrane of rat liver mitochondria is initiated by nonesterified long-chain fatty acids.

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CoA-thioesters of fatty acids are important physiological sources for feeding electrons to the

mitochondrial respiratory chain. However, unesterified fatty acids can also change the mitochondrial oxygen uptake by (i) permeabilization of the inner membrane to protons (uncoupling), (ii) initiation of an ATP-hydrolyzing futile cycle (consisting of activation/hydrolysis of fatty acids/CoA-thioesters) and (iii) by inactivation ADP/ATP exchange across the inner membrane as well as the electron flow in the respiratory chain. Here we propose a novel mechanism for fatty acid action in mitochondria, namely, that fatty acids stimulate oxygen uptake due to the onset of an energy-dissipating K^+ cycling across the inner membrane. The proposed mechanism is based on the following findings. (1) Similarly to the bivalent cation ionophore A23187, free fatty acids stimulate large-scale swelling of mitochondria suspended in sligthly alkaline KCl medium, which is paralleled by a release of endogenous Mg^{2+} [1]. (2) In energized mitochondria fatty acids induce a large-scale swelling followed by a spontaneous contraction [2]. (3) The contraction disappears when respiration is blocked by antimycin A or when mitochondria are pretreated with quinine (an inhibitor of the K^+ /H^+ antiporter) [2]. (4) The swelling phase is accompanied by a decrease of the transmembrane potential ($\Delta\psi$) and an increase of oxygen uptake, whereas the contraction is followed by an increase of $\Delta\psi$ and a decrease in oxygen uptake [2]. Depletion of mitochondria from Mg^{2+} is known to unmask silent ion-conducting systems embedded in the inner membrane (reviewed in [3]). Consequently, there are good reasons to postulate that the swelling phase is due to operation of the K^+ uniporter plus that of the inner membrane anion channel (IMAC), whereas the contraction phase results from the energy-driven K^+ extrusion mediated by the K^+/H^+-antiporter. A simultaneous operation of the K^+ uniporter and the K^+/H^+ antiporter generates the energy-dissipating K^+ cycling.

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6-03. Ryanodine receptor in mitochondria of excitable cells: Physiological and pathological relevance.

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Mitochondrial Ca^{2+} uptake plays an essential role in the regulation of numerous cellular processes including energy metabolism and cytosolic Ca^{2+} homeostasis. Dysfunction of these Ca^{2+}-regulated processes leads to the development of diseases such as cardiomyopathy, diabetes, and neurodegeneration. Despite extensive study, there is little information regarding the molecular identity of mitochondrial Ca^{2+} uptake mechanisms (Gunter et al, 1994). Our laboratories have recently discovered a ryanodine receptor (RyR) localized in the inner mitochondrial membrane of cardiac muscle cells. This mitochondrial RyR (mRyR) serves as a rapid mode of Ca^{2+}-induced Ca^{2+} uptake mechanism in cardiac muscle cells (Beutner et al, 2001).

Herein, we address five questions regarding the physiological and pathological significance of mRyR: 1) Which RyR isoform functions as the mRyR? 2) What is the distribution of mRyR in various tissues? 3) Is mRyR subjected to redox regulation? 4) What are the functional implications of mitochondrial Ca^{2+} uptake through the mRyR? 5) What changes may possibly occur to the mRyR in cardiomyopathy?

Using a multidisciplinary approach that includes Western blot analysis with RyR subtype specific antibodies, pharmacology of [^{3}H]ryanodine binding, and transgenic RyR1 knockout mice, we show that the mRyR in the rat and mouse heart muscle cells is similar to the subtype expressed dominantly in skeletal muscle sarcoplasmic reticulum (RyR1). The mRyR exists only in excitable cells such as cardiac muscle and neurons but not in non-excitable cells such as liver and glial cells. Reducing agents decrease the binding whereas oxidizing agents increase the binding of ryanodine for mRyR. Moreover, Ca^{2+} influx through the mRyR increases oxygen consumption. In RyR1 knockout mice, Ca^{2+}-activated oxygen consumption is diminished and is attended by a significant reduction of adenine nucleotide translocase protein levels. Finally, in the cardiomyopathic hamster, the mRyR protein level is increased but the uptake of Ca^{2+} is reduced secondary to depolarization of mitochondrial potential.

Our results have broad implications by demonstrating that mRyRs transduce the transient increases in cytosolic Ca^{2+} concentrations to a mitochondrial Ca^{2+} signal to trigger oxidative ATP synthesis in excitable cells. Disruption of this signal cascade may contribute to the energy failure in diseases such as cardiomyopathy.

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6-04. Intracristal helical filaments.

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Helical filaments can be observed in the intermembrane compartment of isolated mitochondria from rat liver by electron microscopy. Similar filaments have been described in renal, liver and neuronal mitochondria of other mammals and in invertebrates [1, 2]. No

information is available regarding the molecular composition of the intracristal helical filaments. In this study we have purified the intracristal helical filaments to homogeneity and analysed their molecular composition by MALDI TOF mass spectrometry.

Following gradient centrifugation of a glutaraldehyde-stabilized intermembrane protein extract from rat liver mitochondria, helical filaments appeared in a fraction that was homogeneous under electron microscopy. Intracristal helical filaments are right-handed structures with a diameter of about 16 nm, a pitch of 14 nm and a length of several hundred nanometers, and the molecular weight is estimated to be from 6 to 10 MDa. A rabbit antiserum was raised against the filament-containing fraction. 2D electrophoresis followed by Western blotting revealed six spots with molecular weights of 75, 55, 50, 37 and 22-21 kDa respectively. N-terminal sequencing and MALDI TOF mass spectrometry of tryptic digests of these six spots showed that they contained, respectively, kinesin, catalase, glutamic acid dehydrogenase, rhodanese and serine β-lactamase. It is unlikely that kinesin, a microtubular motor protein, is a component of the intracristal helical filaments, because it is located on the cytosolic surface of mitochondria. Catalase is considered to be a peroxisomal protein. The glutamic acid dehydrogenase and rhodanese are located in the mitochondrial matrix and they take part in the degradation of amino acids and the detoxification of cyanide and cyanogenic agents, respectively. Serine β-lactamase is associated with the mitochondrial ribosome [3], and it belongs to the bacterial superfamily of peptidoglycan synthesizing and hydrolising proteins and shows a close sequence similarity to the bacterial class C β-lactamases. Our results, obtained by affinity chromatography and immunogold-EM, suggest that serine β-lactamase is a component of the intracristal helical filaments.

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6-05. Mitochondrial cubic membrane.

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Cubic membranes are symmetric, periodic structures that occur in numerous cell types from all kingdoms and in virtually any membrane-bound cell organelles. Cubic membranes are based on highly curved surfaces that are mathematically analogous to periodic minimal surfaces used in describing both crystalline and liquid crystalline materials at a variety of length scales. So far three types of cubic membrane morphologies - gyroid (G), double diamond (D) and primitive (P) - have been identified. Through computer simulation of Transmission Electron Microscopy (TEM) and EM tomography, cubic membranes which appear as zigzag patterns in TEM micrographs have been identified.

Our current work is focused on cubic membrane structure. We have studied the cubic membrane structures in two membrane-bound organelles of unicellular microorganisms: the chloroplasts of green algae (*Zygnema* [1]*)* and the mitochondria of starved amoeba (*Chaos carolinensis* [2]) (Figure 1); and in ischemic Mongrel dogs' myocardial mitochondria (unpublished data). Our recent data obtained with amoeba *Chaos* strongly suggest that cubic transition of mitochondrial cristae (upon starvation) plays a protective role against reactive oxygen species and may thus be a cellular response to oxidative stress. Transition of the heart mitochondrial crisate into zigzag forms (cubic membrane) in response to acute ischemic insult may represent the earliest myocardial cell adaptation to increased work demand due to acute ischemic stress.

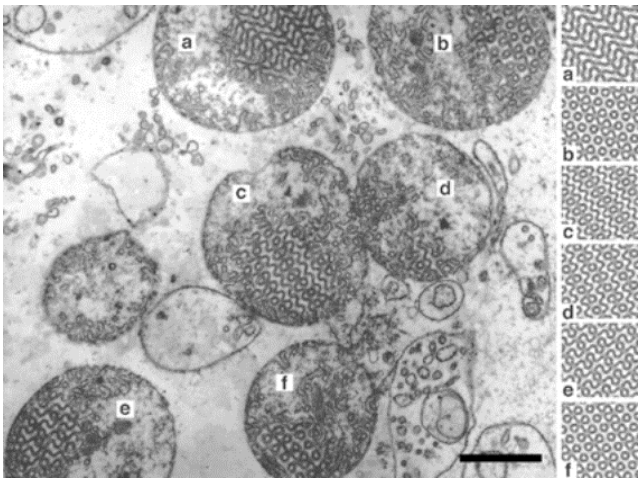

Figure 1: A typical TEM overview shows several mitochondria in amoeba *Chaos carolinensis* after 10 days of starvation (lack of supply with food organisms). Bar = 1 μm. The complex patterns in each of the mitochondria labeled a–f are simply different views of the same type of 3-D structure. This is illustrated by the simulated TEM projections (labeled a–f) on the right. From Ref 1.

In our presentation we will address our latest result of morphological characterization and biochemical analysis of the mitochondrial cubic membrane in different living models that are currently undertaken in our laboratory.

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6-06. Mitochondrial control of calcium ions influx into electrically non-excitable cells.

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A decrease in the mitochondrial inner membrane potential leads to a marked inhibition of Ca^{2+} influx through the plasma membrane calcium channels (CRAC channels) into Jurkat T cell suspended in a solution of pH 7.2 [1,2], and it is less pronounced at higher pH [3]. This effect results from the extracellular pH-dependent sensitivity of CRAC to intracellular Ca^{2+}. In non-treated cells the excess of Ca^{2+} accumulated in the vicinity of the plasma membrane is buffered by respiring mitochondria. Mitochondrial uncoupling results in $[Ca^{2+}]_c$ build up in sub-plasma membrane compartment which exerts a feed-back inhibitory effect on CRAC. This inhibition is expressed as 7-fold decrease of the affinity of CRAC for extracellular Ca^{2+} in cells suspended in a solution of pH 7.2 and is rather negligible when extracellular pH is 7.8. In cells suspended in a pH 7.8 buffered saline solution mitochondrial uncoupling, which prevents the accumulation of cytosolic calcium in the mitochondrial matrix, leads to a significantly faster recovery of $[Ca^{2+}]_c$ after calcium transients due to opening of CRAC. This effect is not observed when the pH of extracellular milieu is adjusted to pH 7.2 probably because of the strong feed-back inhibition of calcium entry. We propose a functional model of CRAC in Jurkat cells which may explain this phenomenon. We also propose physiological consequences of the discussed phenomenon.

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6-07. Effects of the extramitochondrial ADP on the permeability transition pore of mouse liver mitochondria.

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Mitochondrial adenine nucleotide translocator (ANT) changes between two conformations in which the ADP/ATP-binding site is either on the matrix side of the inner membrane (m-state) or on the cytoplasmic side (c-state). Bongkrekic acid (BA) binds at the m-state of ANT (low affinity state) and inhibits the mitochondrial permeability transition pore (PTP). Carboxyatractylate (CAT) and atractylate bind the c-state of ANT (high affinity state) and stimulates the opening of PTP. It was suggested that the c-state conformation is required for PTP opening. Matrix adenine nucleotides decrease the sensitivity of the PTP to Ca^{2+} by binding to the ANT. We established that under condition of matrix adenine nucleotide depletion the Ca^{2+}-dependent opening of the PTP by CAT could be reversibly diminished by added extramitochondrial ADP. Following pretreatment of mouse liver mitochondria with 50 μM CAT and 100 μM Ca^{2+}, the activity PTP increased, but addition of 5 mM ADP inhibited the swelling of mitochondria. Extramitochondrial Ca^{2+} concentration measured with Calcium-Green 5N evidenced that 5 mM ADP did not remarkably decrease the free Ca^{2+}. Release of Ca^{2+} from loaded mitochondria was stopped after addition of 5 mM ADP. Measuring of the mitochondrial respiration in the presence of ANT inhibitor evidenced that CAT addition caused a remarkable decrease in the maximum amount of calcium ions, which can be accumulated by mitochondria. Addition of 5 mM ADP after 50 μM CAT did not change the respiration, but increased the mitochondrial capacity for Ca^{2+} at more than 5 times. Similar results were obtained in the presence of BA 5 μM. BA increased the stability of mitochondrial membrane, but not at that extent as ADP. BA abolished the action about of 0.2-1.0 mM ADP, but can not induce swelling of mitochondria in the presence of 5mM. We conclude, that the outer side of inner mitochondrial membrane has low affinity sensors for ADP, modifying the activity of PTP.

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6-08. Rapid suppression of mitochondrial permeability transition by methylglyoxal.

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Methylglyoxal (MG) (pyruvaldehyde) is a reactive carbonyl compound produced in glycolysis. MG can form covalent adducts on proteins resulting in advanced glycation end products that may alter protein function. Here we report that MG covalently modifies the mitochondrial permeability transition pore (PTP), a high-conductance channel involved in the signal transduction of cell death processes. Incubation of isolated mitochondria with MG for a short period of time (5 min), followed by removal of excess free MG, prevented both ganglioside GD3- and Ca^{2+}-induced PTP opening and the ensuing membrane depolarization, swelling and cytochrome c release. Under these conditions MG did not significantly interfere with mitochondrial substrate transport, respiration or oxidative phosphorylation. The suppression of permeability transition was reversible following extended

incubation in MG-free medium. Of the 29 physiological carbonyl and dicarbonyl compounds tested only MG and its analogue glyoxal were able to specifically alter the behavior of the PTP. These findings demonstrate that MG rapidly modifies the PTP covalently and stabilizes the PTP in the closed conformation. This is probably due to the formation of an imidazolone-adduct on an arginine residue involved in the control of PTP conformation. We deduce that the permeability transition constitutes a potentially important physiological target of MG.

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6-09. Mitochondrial creatine transporter: a fairytale.

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Creatine (Cr) plays a key role in cellular energy metabolism and is found at high concentrations in metabolically active cells such as skeletal muscle and neurons. These, and a variety of other cells, take up Cr from the extra cellular fluid by a high affinity Na^+/Cl^- - dependent creatine transporter (CrT). Mutations in the *crt* gene, found in several patients, lead to severe retardation of speech and mental development, accompanied by the absence of Cr in the brain [1].

In order to characterize CrT protein(s) on a biochemical level, antibodies were raised against synthetic peptides derived from the N- and C-terminal cDNA sequences of the putative CrT-1 protein. In total homogenates of various tissues, both antibodies, directed against these different epitopes, recognize the same two major polypetides on Western blots with apparent Mr of 70 and 55 kDa [2,3]. The C-terminal CrT antibody (α-CrT_{COOH}) immunologically reacts with proteins located at the inner membrane of mitochondria as determined by immuno-electron microscopy, as well as by subfractionation of mitochondria. Cr-uptake experiments with isolated mitochondria showed these organelles were able to transport Cr via a sulfhydryl-reagent-sensitive transporter that could be blocked by anti-CrT antibodies when the outer mitochondrial membrane was permeabilized. We concluded that mitochondria are able to specifically take-up Cr from the cytosol, via a low-affinity CrT, and that the above polypeptides would likely represent mitochondrial CrT(s) [3]. However, by mass spectrometry techniques, the immunologically reactive proteins, detected by our anti-CrT antibodies, were identified as E2 components of the α-keto acid dehydrogenase multi enzyme complexes, namely pyruvate dehydrogenase (PDH), branched chain keto acid dehydrogenase (BC-KADH) and α-ketoglutarate dehydrogenase (α-KGDH). The E2 components of PDH are membrane associated, whilst it would be expected that a mitochondrial CrT would be a trans-membrane protein. Results of phase partitioning by Triton X-114, as well as washing of mitochondrial membranes at basic pH, support that these immunologically cross-reactive proteins are as expected for E2 components, that is membrane associated rather than trans-membrane [4]. On the other hand, the fact that mitochondrial Cr uptake into intact mitoplast could be blocked by our α-CrT_{COOH} antibodies, indicate that our antisera contain antibodies reactive to genuine CrT. This is also supported by results from plasma membrane vesicles isolated from human and rat skeletal muscle, where both 55 and 70 kDa polypeptides disappeared and a single polypeptide with an apparent electrophoretic mobility of ~ 65 kDa was enriched.

Due to the fact that all anti-CrT antibodies that were independently prepared by several laboratories seem to cross-react with non-CrT polypeptides, specifically with E2 components of mitochondrial dehydrogenases, further research is required to characterise on a biochemical / biophysical level the CrT polypeptides, e.g. to determine whether the ~ 65 kDa polypeptide is indeed a bona-fide CrT and to identify the mitochondrial transporter that is able to facilitate Cr-uptake into these organelles. Therefore, the anti-CrT antibodies available so far should only be used with these precautions in mind. This holds especially true for quantitation of CrT polypeptides by Western blots, e.g. when trying to answer whether CrT's are up- or down-regulated by certain experimental interventions or under pathological conditions.

As there was no mitochondrial creatine transporter protein found, the creatine uptake was studied again. After mitochondrial volume measurements, mitochondria appeared not to enrich creatine: the creatine concentration in mitochondria increased significantly, yet remained at ~30 % of the external creatine concentration. Pulse chaise experiments showed that creatine was migrating freely into and out of mitochondria. This indicates that there is no active creatine uptake system within mitochondria.

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6-A01. Dynamic regulation of mitochondrial oxidative phosphorylation from the cytosol in the heart muscle.

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Creatine kinase (CK) and glycolysis are important bioenergetic buffers processes in the cardiac muscle cell. While the role of compartmentalized creatine kinase in energy transfer has been investigated, the role of glycolysis in the dynamic regulation of the mitochondria was not well defined. By measuring the response time of mitochondrial oxygen consumption to dynamic workload jumps (t_{mito}) in isolated rabbit hearts, we studied the effect of inhibiting energetic systems (CK and/or glycolysis) on transcytosolic signal transduction that couples cytosolic ATP hydrolysis to activation of oxidative phosphorylation. Groups of perfused rabbit hearts were exposed to 15 min of: (a) 0.4 mM iodoacetamide (IA, n=6) to block CK (CK activity <3% of control), (b) 0.3 mM iodoacetic acid (IAA, n=5) to inhibit glycolysis (GAPDH activity <3% of control), or (c) control (n=7). Pre-treatment t_{mito} was similar across groups at 4.3 ± 0.3 seconds (mean±SEM). No change in t_{mito} was observed in control hearts, however in IAA and IA treated hearts, t_{mito} decreased by 15 ± 3% and 40 ± 5% respectively, (P<0.05 vs control), indicating quicker energy supply-demand coupling in the absence of ADP/ATP buffering by CK or glycolysis. The faster response times in IAA and IA groups were independent of the size of the work load jump, and the increase in myocardial oxygen consumption during work load steps was unaffected by CK or glycolytic blockade. These results demonstrate that buffering of phosphate metabolites by glycolysis in the cytosol contributes appreciably to slower mitochondrial activation and may enhance contractile efficiency during increased cardiac work loads. Glycolysis may therefore play a similar role as creatine kinase in mediating the dynamic control of the mitochondria in heart muscle.

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6-A02. The mitochondrial genome of the pigeon louse, *Campanulotes bidentatus compar* (Insecta: Phthiraptera) is highly rearranged.

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The mitochondrial genomes of metazoans usually have 37 genes: 22 for tRNAs, 13 for proteins and two for ribosomal RNA subunits. The arrangement of genes in the mitochondrial genomes of metazoans is generally conserved within phyla [1]. However, Shao *et al* [2] discovered that the wallaby louse from the Amblycera has a highly rearranged genome relative to the ancestor of insects. The mitochondrial genome of the pigeon louse, *Campanulotes bidentatus compar*, a species from the Ischnocera, has been partially sequenced to determine the gene arrangement of lice from this lineage. Results indicate that the mitochondrial genome of this louse is also highly rearranged. A particularly intriguing translocation was the movement of the two ribosomal RNA genes away from one another and from the minority strand to the majority strand. This arrangement questions the accepted model of transcription of mitochondrial genomes. At present our focus is on the process of how such extraordinary gene rearrangements evolved. For example, the rate of gene rearrangements in lice and insects was found to be correlated with the rate of nucleotide substitution [3]. This high substitution rate is hypothesised to be a result of the failure of repair mechanisms to maintain nucleotide integrity. Additional hypotheses that question the accepted mechanisms of gene inversions and rearrangements will also be discussed. *Campanulotes bidentatus compar* and other lice will provide models with which to test these hypotheses.

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