

Session 6: Respiration, Coupling, Permeability Transition, and UCPs



6-01. The effect of high-fat feeding on intramuscular lipid and lipid peroxidation levels in UCP3-ablated mice.

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Uncoupling protein-3 (UCP3) has been suggested to be involved in the protection against reactive oxygen species (ROS) and ROS-related compounds, such as lipid peroxides. We, specifically, hypothesized that UCP3 is an exporter of fatty acid anions to prevent the accumulation of fatty acids which otherwise are prone to lipid peroxidation by ROS, a feature especially important in situations of lipid oversupply, such as high-fat feeding. The aim of the present study therefore, was to study effect of UCP3 ablation on intramuscular lipid peroxide levels and high-fat diet induced alterations in muscle lipid metabolism.

UCP3-ablated mice indeed showed ~3-fold higher levels of intramuscular lipid peroxides upon standard chow feeding, compared to their wild-type littermates. Remarkably however, this difference was no longer apparent upon the high-fat diet. The latter finding was accompanied by the finding that, upon HF feeding, intramuscular triacylglycerol (IMTG) levels were ~50% lower in UCP3^{-/-} mice, in comparison to the UCP3^{+/+} animals. Oxidative capacity, measured as succinate dehydrogenase (SDH) activity was however similar between UCP3^{-/-} and UCP3^{+/+} mice. Thus, increased oxidative capacity cannot account for these differences.

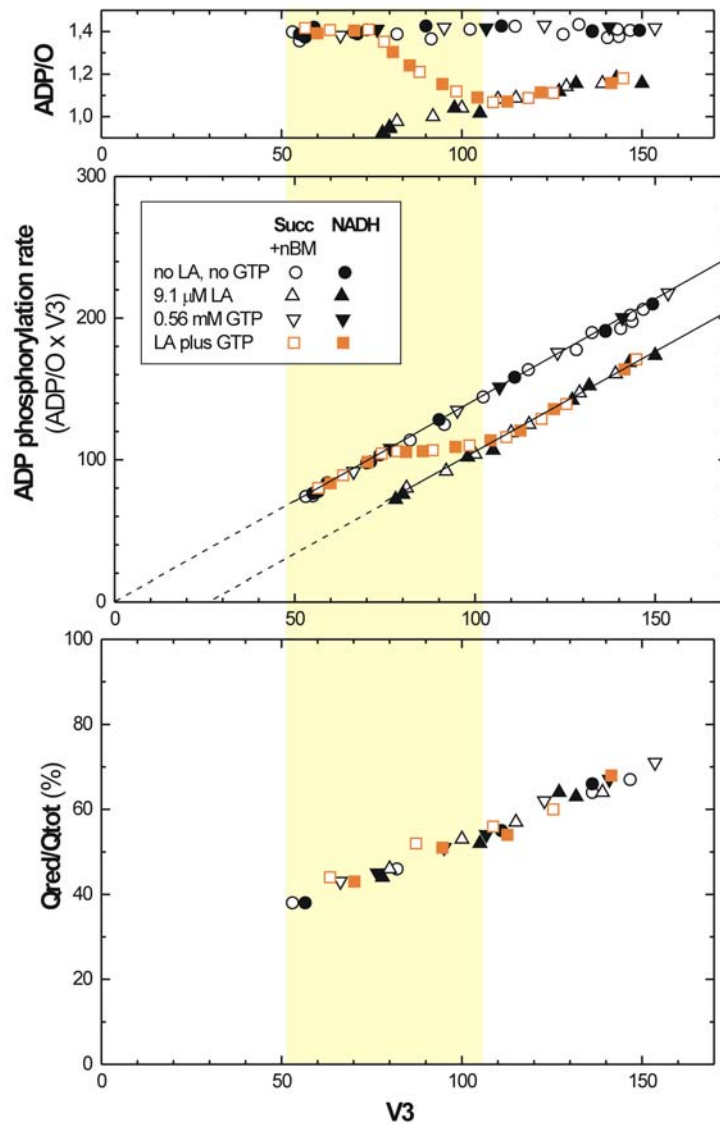
Increased lipid peroxide levels in UCP3-ablated mice supports a role for UCP3 in protecting mitochondria against ROS-induced damage. Upon high-fat feeding, however, other metabolic adaptations seem to be able to protect skeletal muscle from fatty acid accumulation.

6-02. Inhibition of *Acanthamoeba castellanii* uncoupling protein activity by GTP depends on the redox state of quinone.

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The mitochondrial respiratory chain of amoeba *Acanthamoeba castellanii*, a non-photosynthesizing amoeboid protozoon, like that of plant mitochondria, possesses energy-dissipating pathways like a cyanide-resistant alternative oxidase, non-phosphorylating rotenone-insensitive internal and external NADH dehydrogenases, and a free fatty acid-activated, purine nucleotide-inhibited uncoupling protein. In isolated *A. castellanii* mitochondria respiring in state 3 with external NADH or succinate, the linoleic acid (LA)-induced purine nucleotide-sensitive uncoupling protein activity is able to uncouple oxidative phosphorylation. The LA-induced uncoupling can be inhibited by a purine nucleotide (GTP) when quinone (Q) is sufficiently oxidized, indicating that in *A. castellanii* mitochondria respiring in state 3, the sensitivity of uncoupling protein activity to GTP depends on the redox state of the membranous Q (Q_{red}/Q_{tot}) [1]. Namely, the inhibition of the LA-induced uncoupling by GTP is not observed in uninhibited state 3 respiration as well as in state 3 respiration progressively inhibited by complex III



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inhibitors, i.e., when the rate of quinol (QH₂)-oxidizing pathway is decreased. On the contrary, the progressive decrease of state 3 respiration by declining respiratory substrate availability (by succinate uptake limitation or by decreasing external NADH concentration), i.e., when the rate of Q-reducing pathways is decreased, progressively leads to a full inhibitory effect of GTP (Fig. 1). Moreover, in *A. castellanii* mitochondria isolated from cold-treated cells, where a higher uncoupling protein activity is observed, the inhibition of the LA-induced proton leak by GTP is revealed for the same low value of the Q reduction level. These results are in agreement with those obtained for rat skeletal muscle [2] and potato tuber [3] mitochondria.

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2. Jarmuszkiewicz W, Navet R, Alberici LC, Douette P, Sluse-Goffart C, Sluse

6-03. Elucidation of the mechanism of mitochondrial uncoupling protein function using a synthetic glycolipid (glucose-O- ω -palmitate).

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Mitochondrial uncoupling protein 1 (UCP1) is a 32kDa protein situated in the inner membrane of mitochondria from brown adipose tissue (BAT) of mammals and human infants. The protein uncouples ATP synthesis from oxygen consumption by catalysing the dissipation of the proton electrochemical gradient across the mitochondrial inner membrane. UCP1 function is the basis of non-shivering thermogenesis generated by BAT under conditions of cold exposure [1]. Long chain fatty acids, such as palmitate are known to increase proton leak through UCP1, however the mechanism is unclear. Two main models for the mechanism of action of UCP1 have been proposed: (i) that UCP1 acts as a proton conduit across the mitochondrial inner membrane and importantly that

fatty acids act as cofactors/activators providing an additional carboxyl group at a key intra-membrane site [2] and (ii) that protonated fatty acids freely flip across the mitochondrial inner membrane and uncouple the mitochondria, and that UCP1 act as a 'flippase', translocating the fatty acid anions back across the bilayer leaflets of the inner membrane [3]. In order to distinguish between the two models, we synthesised glucose-*O*- ω -palmitate [4], which theoretically cannot flip across the inner membrane but can provide the carboxyl group for catalysis of proton translocation. Glucose-*O*- ω -palmitate was shown to be 93 % pure, by $^1\text{H-NMR}$, and stable even under acidic conditions. We show that glucose-*O*- ω -palmitate cannot facilitate UCP1 function, either in liposomes containing reconstituted native UCP1 or in BAT mitochondria. Our data do not support the activation model but lend weight to the 'flippase' model of UCP function.

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6-04. Functional and proteomic impacts of variations of the expression of uncoupling proteins in living cells.

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Uncoupling proteins (UCPs) are consumers of the proton electrochemical gradient built up by the respiratory chain and in this way they compete with the processes of oxidative phosphorylation, including ADP and inorganic phosphate import and ATP synthesis. In some way, their (over)expression in a cell can be considered as a stress at the energetic level. Indeed, in front of a decrease in the oxidative phosphorylation yield (uncoupling due to the presence and the activity of UCPs), cells do not remain neutral and react by promoting their general metabolic capacity. This concept will be illustrated at the functional and proteomic levels, by recombinant UCP1 from BAT expressed in a UCP-free cell i.e. the yeast *Saccharomyces cerevisiae*. UCP1 activity is stimulated by free fatty acids and inhibited by purine nucleotides. Here we investigated how active and regulated recombinant UCP1 expressed in yeast at ~ 1 and ~ 10 $\mu\text{g}/\text{mg}$ of total mitochondrial proteins induced changes in the mitochondrial proteome and in oxygen free radicals production. Using two-dimensional differential in-gel electrophoresis (2D-DIGE), we found that most of the proteins involved in the response to ectopically expressed UCP1 are related namely to energy metabolism. We also quantified the cellular H_2O_2 release in the absence or in the presence of UCP1. Our results suggest that UCP1 has a dual influence on free radicals generation. On one side, FFA-activated UCP1 was able to decrease the superoxide anion production, demonstrating that a decrease in the generation of reactive oxygen species is an obligatory outcome of UCP1 activity even in a heterologous context. On the other side, an increase in UCP1 content was concomitant with an increase in the basal release of superoxide anion by mitochondria as a side-consequence of the overall increase in oxidative metabolism. Some pathologies and exogenous stresses that are accompanied by an increase in the expression of tissue-specific UCPs will also be analysed at the level of the mitochondrial proteome.

6-05. The permeabilisation of mitochondria and bilayer phospholipid membranes by palmitic acid and Ca^{2+} .

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Palmitic acid (PAL) has recently been found to be a physiological activator of apoptosis [1]. PAL induced the opening of a cyclosporin A (CsA)-insensitive pore in mitochondria [2]. In the present study we compared the properties of this pore with the well-known CsA-sensitive mitochondrial permeability transition pore (PTP). Also the contribution of the PAL/ Ca^{2+} -activated pore in the release of cytochrome c from mitochondria was studied.

We found that the PAL/ Ca^{2+} -induced CsA-insensitive swelling of mitochondria was not affected by the ADP - an inhibitor of PTP opening - nor by openers of this pore (inorganic phosphate, atractyloside). However, this swelling was inhibited by physiological concentration ATP ($[I]_{50} = 1.3 \text{ mM}$), which is a less effective inhibitor of PTP. This action of ATP occurs from the outside of the inner mitochondrial membrane. Earlier we found that PAL bound Ca^{2+} with high affinity [3]. Palmitoleic acid and 2-bromopalmitic acid, which have no such high Ca^{2+} affinity, failed to induce the pore opening. These results are in agreement with our data on the effects of this fatty acid on the Ca^{2+} -dependent permeability of artificial lipid membranes (BLM and liposomes). Based on these series of experiments we conclude that the formation of the PAL-induced pore is connected with ability of PAL to form a complex with Ca^{2+} in membrane.

The PAL-induced pore is short-lived and closes spontaneously. We have shown that this is accompanied by recovery of the membrane potential of the inner mitochondrial membrane. Opening of PAL/ Ca^{2+} -activated, short-lived pores results in the CsA-insensitive release of cytochrome c from mitochondria. The addition of cytochrome c to mitochondria promoted fast recovery of mitochondrial membrane potential after depolarization induced by opening of PAL/ Ca^{2+} -activated pores, but not in case of opening of PTP. These results suggest that in addition to the PTP there are PAL/ Ca^{2+} -activated pores in mitochondria. This pore is thus likely to be a trigger of PAL-induced apoptosis and can be related to some pathologies, e.g. myocardial ischemia. We found that heaviness of myocardial infarction of ischemic patients correlates directly with the content of PAL in human blood serum.

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6-06. Mitochondria are sensitive to carcinogenic chemicals.

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It is well known that under fast proliferation cells tend to use high glycolysis and low respiration. Examples of this are embryonic cells (of low differentiation) and cells *in vitro*. Fast proliferating cells prefer glucose, although non-fermentable (respiration dependent) substrates are available too. Cancer cells show strikingly high glycolysis while their respiration is low or disturbed (1). Cancer cells are generally of low stage differentiation. Cells of high degree differentiation usually show high respiration.

The associations above lead to a question: is there a causal relationship between e. g. the degree of differentiation and respiration? Or between proliferation rate and rate of glycolysis? In embryonic cells the glycolysis / respiration ratio undoubtedly is *regulated*, by some *normal* physiological mechanism (i.e. it is not caused by "lack of oxygen " as may be in some cases of cancer).

We found that mitochondria dependent functions were relatively sensitive to toxic effects of various carcinogenic chemicals (2). We assessed the growth inhibitory effects of the chemicals on the yeast *Saccharomyces cerevisiae* plated on fermentable *versus* non-fermentable medium (glucose *versus* glycerol as the sole carbon source). On non-fermentable (respiratory) medium, concentrations of carcinogens inhibiting growth were much lower than on fermentable medium. The difference was up to ten-fold. Among the carcinogens tested were thioacetamide, 4-nitroquinoline-N-oxide, adriamycine, ethionine, thiourea, 2-naphthylamine, benzidine and cadmium. At the same time mitochondrial DNA frequently showed *petite* mutations.

Experiments on the regulation of the *respiration/glycolysis* ratio showed that *petite* mutants may be incapable of utilizing galactose to support growth although the wild type parent strain grows on that substrate; this mitochondrial sugar utilization factor needs a renewed interest.

One also noticed how well *petite* strains grow on glucose medium. In shaking culture (wild type strain) the cell mass (biomass) obtained from high glucose medium (under glucose repression (catabolite repression) was *compared* to the cell mass obtained from growth under non-fermentable conditions (with glycerol as the sole carbon source). The amount of carbon source used was measured, i.e. the diminishing of the carbon source in the medium. In other words, for a given biomass (amount) obtained under these two different growth conditions the amount of substrate was known.

It is of interest to compare the calculated amount of ATP *theoretically obtainable* from the amount of substrate used. In case of the respiratory culture, this calculated figure was 2.5 – 3.0 times higher than for culture under glucose repressed condition. This leads to the next question: could the cell cycle be more expensive energy-wise when the mitochondria are at work and the cell cycle is much longer?

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**6-07. Low abundance of UCP2 (UCP3, UCP4, UCP5) is sufficient for attenuation of mitochondrial ROS production.**

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Owing to low expression levels (with exception of UCP1 in brown fat), thermogenic function of mitochondrial uncoupling proteins is rather marginal and the most relevant function seems to be attenuation of reactive oxygen species (ROS) formation in

mitochondria resulting from a weak uncoupling [1-3]. To document this view, we analyzed absolute amounts of transcripts for UCP2, UCP3, UCP4, and UCP5 by quantitative RT-PCR on a LightCycler (Roche). There was a 300-fold difference in UCP2 mRNA levels in rat tissues with the maximum level found in spleen (ten times exceeding levels of a house keeping gene GAPDH), and with the minimum level found in the brain. Six times more UCP2 transcript than UCP3 transcript was quantified in the rat heart; in contrast, 10 times more UCP3 than UCP2 transcript was found in rat skeletal muscle. In the rat heart, UCP4 mRNA levels were by one order of magnitude lower than in the brain. UCP4 and UCP5 transcripts were 10 times more abundant in the mouse brain than the UCP2 transcript. Very low UCP5 transcript levels were indicated in rat skeletal muscle and heart.

We attempted to quantify the amount of UCP2 protein from the number of $^3\text{H-GTP}$ high affinity binding sites in mitochondria of mouse tissues with subtracted background evaluated in mitochondria of UCP2 KO mouse. Mitochondrial H_2O_2 production in selected mouse tissues was quantified in the presence and absence of fatty acids and nucleotides using Amplex Red fluorescence in the presence of horse raddish peroxidase. Results were correlated with the assumed amounts of UCPn. Also ectopic expression of UCP2 in *S.cerevisiae* yeast gave weak uncoupling. We conclude that the UCPn amounts in mitochondria of various tissues are sufficient for attenuation of mitochondrial ROS production.

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6-08. No evidence for superoxide or 4-hydroxy-nonenal activation of uncoupling protein 1 (UCP1) as elucidated from studies of SOD2-overexpressing or UCP1-ablated mice.

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In brown-fat mitochondria, UCP1 is activated by fatty acids in a way that is compatible with thermogenesis being regulated physiologically through a simple kinetically competitive interaction between fatty acids and purine nucleotides [1]. However, it has been suggested that in addition to fatty acids, other cofactors are required for activation of UCPs (including UCP1), namely superoxide [2] and lipid peroxidation products, e.g. 4-hydroxy-nonenal (HNE) [3]. Artificial superoxide-generated systems were used in these studies, and it has not been clear whether physiologically regulated superoxide levels would activate UCP1.

We have created P1 artificial chromosome transgenic mice expressing the human mitochondrial superoxide dismutase 2 (SOD2) and thus generated mice with a physiologically controlled augmentation of SOD2 expression, leading to increased SOD2 enzyme activities and lowered superoxide levels. Our study of brown-fat mitochondria isolated from these SOD2 transgenic mice showed that UCP1 activity was not altered.

As a further examination of the ability of ROS and ROS-derived lipid peroxide to activate UCP1, we utilised the availability of UCP1-ablated mice to identify the possible UCP1-dependent effect of proposed UCP1 activators (particularly HNE), by dissecting out the possible UCP1-independent effects. No UCP1-dependent HNE effect was found, but

HNE changed basal proton leak characteristics independently of UCP1 in a way that might be explained by direct permeabilization of the mitochondrial membrane. There was no increase in HNE adducts in brown-fat mitochondria isolated from UCP1(-/-) mice, when adapted to room temperature or to the cold. The absence of oxidative damage in UCP1(-/-) mitochondria was not due to enhanced activity of antioxidative enzymes.

Thus, all evidence obtained in this study indicates that superoxide and 4-hydroxy-nonenal have no physiological role in regulation of UCP1 activity.

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6-09. The metabolic phenotype of HT-29 human colon cancer cells is normalized by low intracellular folate levels.

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Animal intervention trials suggest that folate deficiency can have an inhibitory effect on the progression of established colonic tumor cells. An increased glycolysis, conversion of glucose to lactate for ATP production, is a major characteristic of tumor cells, including colon tumors. Recent studies have shown that inhibition of glycolysis is an effective strategy to kill cancer cells. Our study was performed to investigate whether differences in folate status could effect energy metabolism of HT-29 human colon cancer cells. Therefore, HT-29 cells were grown in different types and concentrations of folate; synthetic folic acid (10 or 100 ng/ml pteroylglutamic acid; PGA) or natural folate (10 or 100 ng/ml 5-methyl-tetrahydrofolate; MTHF). The results of our studies demonstrate that long-term culture of HT-29 colon cancer cells in low levels of PGA (10 ng/ml), that leads to low intracellular levels of tetrahydrofolate and 5-methyl-tetrahydrofolate, reduces the ATP content and lactate production of these cells compared to long-term culture in either 100 ng/ml PGA or 10-100 ng/ml MTHF. The decrease in glycolysis was accompanied with an increase in mitochondrial mass as well as mitochondrial oxygen consumption. These results indicate that low intracellular folate levels (folate deficiency) can revert the metabolic phenotype of HT-29 colon cancer cells towards a phenotype characteristic for normal cells. This observation is further supported by the fact that also the growth rate of HT-29 colon cancer cells is diminished under these conditions. Gene expression studies using DNA microarrays were performed in HT-29 cells cultured in different forms and concentrations of folate to reveal mechanistic details of the glycolytic switch. HT-29 cells cultured in low intracellular folate levels clearly show a different genomic profile. Although no clear effects on mitochondrial OXPHOS genes were found, our results point to a shift in gluconeogenesis.

6-10. Mitochondrial permeability transition in an invertebrate: Absence of a calcium-regulated pore in the face of profound Ca²⁺ storage.

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Mitochondria are contributors to both the energetic processes necessary for life and to signaling events leading to death of eukaryotic cells. When mammalian mitochondria are exposed to high calcium concentrations in the presence of the co-activator P_i, especially if

accompanied by oxidative stress and adenine nucleotide depletion, a large swelling, uncoupling of respiration and release of cytochrome c (cyt-c) can be observed. These phenomena are due to a sudden increase in permeability of the inner mitochondrial membrane to solutes with a molecular weight up to approximately 1500 Da, a phenomenon known as the mitochondrial permeability transition (MPT). The MPT is mediated by a multi-protein complex, which can be defined as a voltage-dependent, cyclosporine A sensitive and calcium activated inner membrane pore (MPTP). Activation of the MPTP *in vivo* in response to hypoxic and oxidative stress leads to necrotic and apoptotic cell death [1].

Embryos of the brine shrimp *A. franciscana* are exceptional in their ability to tolerate anoxia at room temperature for years [2] and to maintain viability under conditions that are known to open the MPTP in mammalian species thereby leading to cyt-c release and cell death [3]. Minimum molecular constituents of the regulated MPTP in mammals are believed to be the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) and cyclophilin D. Western blot analysis revealed that mitochondria from *A. franciscana* possess all three required components [4]. As expected, when measured with a Ca^{2+} -dependent fluorescent probe, rat liver mitochondria release matrix calcium upon addition of $\geq 100 \mu\text{M}$ extra-mitochondrial Ca^{2+} , (indicating MPTP opening), whereas brine shrimp mitochondria continue to take up extra-mitochondrial Ca^{2+} and do not release internal stores even up to 1.0 mM exogenously-added Ca^{2+} (no MPTP opening). Furthermore, swelling of *A. franciscana* mitochondria in response to added Ca^{2+} was not observed (nor was cyt-c release), in contrast to the rapid swelling of rat mitochondria after addition of $100 \mu\text{M}$ Ca^{2+} as monitored by optical absorbance at $\lambda = 520 \text{ nm}$. Several other inducers of the mammalian MPTP, such as atractyloside, mastoparan or phenylarsine oxide were also ineffective on *A. franciscana* mitochondria, thereby confirming the absence of a regulated MPTP. Only the thiol-reactive compound HgCl_2 induced a permeability transition. However, size exclusion studies with polyethylene glycols revealed an exclusion limit of 540 Da, indicating that the permeability transition was not mediated by the classical MPTP (exclusion limit 1500 Da) [4].

In mammals, the release of cyt-c stimulates caspase-dependent apoptosis by binding to Apaf-1. This process leads to formation of the apoptosome, which is composed of cyt-c, caspase 9, Apaf-1, and ATP (or dATP). A downstream target of the apoptosome is pro-caspase 3, and when cleaved it is transformed to the executor caspase, caspase 3 [5]. However, the involvement of cyt-c in apoptotic signaling of higher invertebrates is controversial. Low levels of caspase 9- and caspase 3-like activities can be detected in cell free extracts of *A. franciscana* embryos when measured by cleavage of the fluorogenic substrates Z-DEVD-R110 and Z-LEHD-R110. However, in contrast to experiments with cell free extracts of human hepatoma cells (C3A), adding cyt-c to extracts from *A. franciscana* embryos fails to elevate caspase 3 activity. Possible involvement of other mitochondrial apoptosis inducing factors such as AIF, HtrA2/OMI or endonuclease G in apoptotic signaling in *A. franciscana* needs further investigation.

Based on our *in vitro* experiments, a role for cyt-c in the apoptosis signaling of *A. franciscana* is questionable at this point. However, the absence of a functional MPTP in *A. franciscana* mitochondria likely contributes to prolonged anoxia tolerance in this species by avoiding energetic catastrophe during recovery. Furthermore, the striking capacity for calcium uptake by these mitochondria may provide protection against calcium overload in the cytoplasm during oxygen limitation. We speculate that the absence of the MPTP may be a general feature of invertebrates and that the lower hypoxia tolerance of mammals may be explained in part by the evolution of the regulated MPTP.

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6-11. Single channel properties of the mitochondrial poly-3-hydroxybutyrate/ Ca^{2+} /polyphosphate complex.

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Although mitochondrial ion transport has been studied in great detail, and it is proven that its regulation can affect a number of crucial physiological and pathological processes, very little is known about the molecular identity of mitochondrial ion channels. On the other hand, it is known that mitochondrial membrane contains non proteinaceous complex composed of poly-3-hydroxybutyrate/ Ca^{2+} /polyphosphate (PHB/ Ca^{2+} /polyP) [1]. A similar complex can assemble in the membrane of *E.coli* when calcium is present in the incubation medium. Such a complex of bacterial origin is known to form cation selective ion-channels of ~ 100 pS when reconstituted into black lipid bilayers [2].

Recently we have isolated mitochondrial PHB/ Ca^{2+} /polyP and studied the properties of single channels that are observed on reconstitution of the complex into black lipid bilayers [3]. The mitochondrial PHB/ Ca^{2+} /polyP complex can form large, multi-state ion channels. In symmetric 150 mM KCl, the maximal conductance of the channel ranged from 350 pS to 750 pS. For voltages more than ± 60 mV, conductance fluctuated in the range of 50–200 pS. In the presence of a 1:3 gradient of KCl, at pH = 7.4, selectivity periodically switched between different states ranging from weakly anion-selective ($V_{\text{rev}} \sim 15$ mV) to ideally cation-selective ($V_{\text{rev}} \sim 29$ mV), without a significant change in its conductance. Overall, single channel properties observed in our experiments were very similar to the properties of the permeability transition pore seen in patch clamp experiments of the native mitochondrial membranes.

Channel detection frequency was increased when isolation was performed using mitochondria pre-incubated with calcium, in comparison to mitochondria treated with EDTA. The amount of polyP in the mitochondrial membrane fraction of calcium treated mitochondria is ~ 3 times higher than for EDTA-treated mitochondria, which suggests an increased concentration of the PHB/ Ca^{2+} /polyP complex. This observation is consistent with the idea that formation and incorporation of PHB/ Ca^{2+} /polyP complex into mitochondrial membrane can be induced by elevated concentration of calcium. Such a mechanism could underlie the calcium induced permeability transition of the mitochondrial inner membrane.

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6-12. Hepatitis C virus core protein binds to mitochondria and induces Ca²⁺ uptake, ROS production, complex I inhibition, and permeability transition.

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Hepatitis C virus replicates in hepatocytes and produces a chronic liver disease associated with oxidative stress and excess apoptosis of hepatocytes. The HCV core protein is present in endoplasmic reticulum and our lab has previously demonstrated that it also localizes to mitochondria, increases mitochondrial ROS production, and sensitizes cells to oxidant-induced apoptosis.[1,2]. The aim of this study was to examine the mechanisms by which HCV core protein affects mitochondrial function. Liver mitochondria were isolated from control and HCV transgenic (TG) mice expressing the viral proteins core, E1, E2 and p7 in the liver. Core protein was concentrated in the mitochondrial fraction and proteinase K digestion studies demonstrated its presence on the mitochondrial outer membrane. Functional abnormalities of mitochondria were also present. There was an oxidation of the mitochondrial glutathione pool that decreased GSH by 40% without changing the total content of GSH+GSSG. Mitochondria derived from transgenic liver had reduced activity of complex I NADH oxidase activity compared to that of normal liver (56.7±1.1 vs. 44.4±3.0 nmol/min mg protein, $P<0.01$) but normal activity of complex III. Complex I dependent ROS production was also increased. Incubation of control mitochondria in vitro with recombinant core protein also caused an oxidation of the mitochondrial GSH pool and a selective inhibition of complex I. In addition, core protein increased Ca²⁺ uptake into isolated mitochondria, measured by Rhod-2 fluorescence, and sensitized mitochondria to oxidant-induced membrane permeability transition. HCV core protein also sensitized hepatoma cells to oxidant-induced mitochondrial depolarization and cell death and this sensitization could be reversed by either antioxidants (N-acetylcysteine) or intracellular Ca²⁺ chelation (BAPTA-AM). In conclusion, these studies demonstrate that HCV core protein is a mitochondrially active protein that increases mitochondrial ROS production and sensitizes cells to oxidant-induced cell death. The results support a model in which core protein localizes to the mitochondrial outer membrane where it increases Ca²⁺ uptake. Increased mitochondrial Ca²⁺ results in an increase in ROS production, oxidation of the intramitochondrial glutathione pool, and inhibition of complex I by transglutathionylation. The resulting mitochondrial effects may contribute to liver injury and oxidative stress seen in chronic hepatitis C.

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6-13. Respiratory coupling control ratio and respiratory capacity in cultured fibroblasts and myeloid progenitor cells. Effects of experimental cell density.

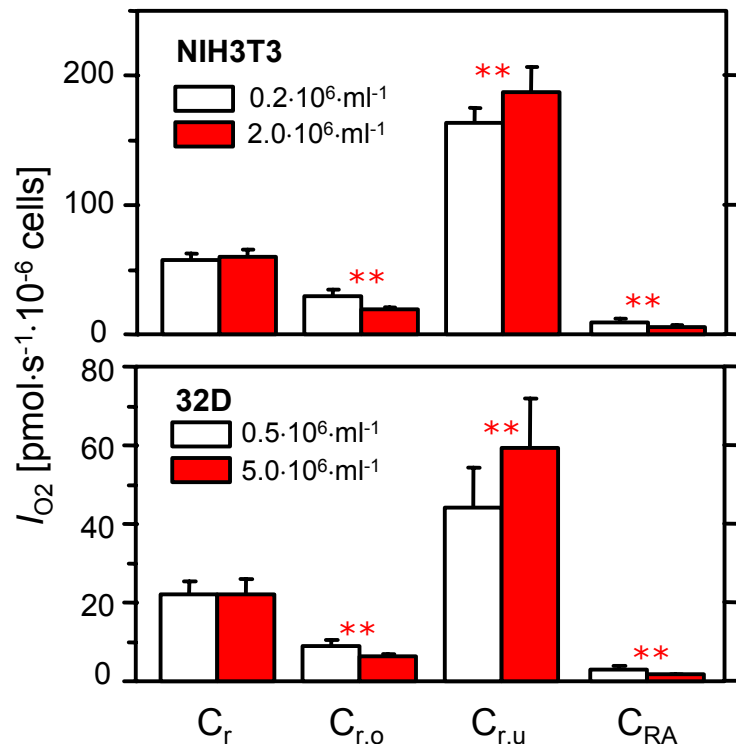
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The uncoupling control ratio (UCR) is a sensitive index for the integrity of mitochondrial function in cells. The UCR is the respiratory ratio of uncoupled to physiologically controlled states, when intact cells are incubated in culture medium (routine respiration) or in the absence of exogenous energy substrates (endogenous respiration). Even in healthy control cells, large differences of UCR values are reported, with high UCR typically ranging from 2.5 to 3 at experimental cell densities of 0.2 to 1·10⁶ ml⁻¹ [1-3]. In contrast, UCR values of 1.1 to 1.9 are reported by other groups using experimental cell densities >3·10⁶ ml⁻¹ [4,5]. Hypothetically, cell-cell signalling may exert a density-dependent effect on routine respiration in the physiologically controlled state, whereas removing respiratory control by uncoupling should eliminate such potential density-related effects.

We measured respiratory activities of intact NIH3T3 fibroblasts (adherent) and mouse pro-myeloid 32D (suspension) cells, each at two cell densities, using the OROBOROS Oxygraph-2k for high resolution respirometry. Cells were grown to a standardized density. Cells from each culture flask (N=12 for each cell type) were suspended at low and high experimental densities (0.2 and 2.0·10⁶·ml⁻¹ for NIH3T3; 0.5 and 5.0·10⁶·ml⁻¹ for 32D). After recording routine respiration of cells suspended in 2 ml culture medium, ATP-synthase was inhibited by oligomycin (2 µg·ml⁻¹). Increasing the oligomycin concentration had no effect on respiration. Subsequently, stepwise FCCP titration was performed up to the optimum FCCP concentration required for maximum stimulation of respiration. Optimum FCCP was 6.3 ± 1.4 and 7.8 ± 2.5 µM at low cell density, and decreased significantly at high density by a factor of 1.4 and 1.6 in NIH3T3 and 32D, respectively. Finally, respiration was inhibited by 0.5 µM rotenone and 2.5 µM antimycin A. Respiratory oxygen flux was corrected on-line for instrumental background (DatLab 4), which is a standard procedure in high-resolution respirometry [1] and is particularly important at low cell densities, to eliminate corresponding methodological artefacts. Data were analysed by a paired t-test and presented as means ± SD.

Routine respiration in culture medium was 58.7 ± 5.4 and 22.1 ± 3.5 pmol·s⁻¹·10⁻⁶ cells in fibroblasts and 32D, respectively, independent of cell density. Uncoupling by FCCP stimulated respiration of fibroblasts 3.2 ± 0.17 fold and 2.9 ± 0.22 fold above routine levels at high and low cell density. An even more pronounced effect of cell density was observed in 32D cells (UCR was 2.7 ± 0.4 and 2.0 ± 0.4 at high and low density). Uncoupled respiration, therefore, decreased significantly by 13 % and 26 % in fibroblasts and 32D at low density. In contrast, respiration inhibited by oligomycin



increased significantly at low density in both cell types (Fig. 1). The respiratory control ratio (uncoupled to oligomycin-inhibited respiration, $RCR_{u/o}$) decreased two-fold at low cell density, from 9.5 to 5.7 in NIH3T3 and from 10.0 to 5.0 in 32D cells. This significant change of the $RCR_{u/o}$ at constant routine respiration was in direct contrast to hypothetical expectations on the effects of cell density. These density-related effects were comparable in magnitude to changes in $RCR_{u/o}$ reported in the context of oxidative stress [2], senescence [3], or cell cycle arrest [3].

The divergent effect of experimental cell density on respiration in the two consecutively induced metabolic states ($C_{r,o}$ and $C_{r,u}$) rules out experimental artefacts related to the oxygen measuring system and to potential errors in cell counting. Further control experiments were performed on digitonin-permeabilized 32D cells, which were suspended at the two different densities in mitochondrial medium MiR05. The optimum digitonin concentration was adjusted to cell density, and maximum respiration was measured with succinate/rotenone and 3 mM ADP. Cytochrome c had no effect and FCCP stimulated respiration by only 10 % above state 3. As expected, oxygen flow (respiration per million cells) was identical in permeabilized cells at low and high cell density.

Respiration inhibited by rotenone+ antimycin A (C_{RA}) was 9.7 ± 2.8 and 3.0 ± 0.7 $\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ cells in intact NIH3T3 and 32D measured at low cell densities at an oxygen concentration of 80 μM in culture medium. After permeabilization of cells with digitonin in mitochondrial respiration medium MiR05, respiration inhibited by rotenone+antimycin A was significantly lower than in intact cells (3.1 ± 1.5 and 1.1 ± 0.6 $\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ in the two cell types). The difference provides a minimum estimate of non-mitochondrial respiration in intact cells, amounting to 10 % of routine respiration in both cell types, which increases at high oxygen concentration [6]. At high cell density, the rotenone+antimycin A inhibited respiration was significantly lower in NIH3T3 and 32D cells, measured at 80 μM oxygen in culture medium, possibly indicating a correspondingly decreased ROS production at high protein concentration. Subtraction of the non-mitochondrial component from total respiration at different cell densities did not remove the density effect in the oligomycin-inhibited or uncoupled states. Permeabilized cells had identical rotenone+antimycin A inhibited respiration at high and low cell density.

Our results point to the importance of application of comparable cell densities in respiratory studies. Though our data clearly show that cell density affects UCR, this does not explain low UCR values reported in the literature with the use of high experimental cell density.

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6-14. Brain mitochondrial production of reactive oxygen species is increased by calcium-induced permeability transition.

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Non-physiological increases in mitochondrial production of reactive oxygen species (ROS) are considered to be essential in the pathogenesis of several acute and chronic neurodegenerative diseases. Pathological calcium fluxes in neuronal cell death has been extensively investigated, but the role of calcium in mitochondrial ROS production is

currently unresolved and both increases and decreases of the detection of ROS by calcium-loading in mitochondria have been reported [1,2].

In the present study, we demonstrate that the production of ROS, as detected by Amplex Red oxidation in isolated rat brain mitochondria respiring on complex I substrates, is increased by calcium if the mitochondria undergo permeability transition (mPT) and large amplitude swelling. In contrast, the detection of ROS was decreased by the same dose of calcium if the mPT was blocked with cyclosporin A and its cofactor ADP. Unspecific permeabilization by the ionophore alamethicin produced a similar increase of ROS. Alamethicin permeabilization and mPT induced an immediate loss of NAD(P)H fluorescence and membrane potential, diminished respiration, release of cytochrome *c* and reduced levels of GSH. The ROS production following permeabilization was dependent on availability of respiratory substrates, and improved accessibility of electron donors increased the O₂ utilization and ROS detection.

The mPT dependent increase of ROS can likely be attributed to both the loss of endogenous oxidant scavenging systems and escape of cytochrome *c*. However, challenging mitochondria with 1.4 and 8 μmol/mg calcium produced a similar extensive level of swelling but the latter dose led to a substantially greater ROS burst and to a significant inhibition of mitochondrial O₂ consumption following permeabilization. Thus, calcium overload combined with mPT is suggested to directly affect redox centers in the respiratory complexes. As the calcium-triggered ROS generation is dependent on inner membrane permeabilization it may be subject to pharmacological modulation of the mPT.

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6-15A. Changes of the mitochondrial function by the new fluorine-containing K_{ATP} channel openers.

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Pharmacological ATP-sensitive potassium (K_{ATP}) channels activation is associated with cardioprotection and can simulate certain aspects of ischemic preconditioning [1], but protective mechanism of K_{ATP} channels openers has not been clarified yet. Recent experiments suggest that cardioprotection by hypoxic preconditioning or exposure to the ATP-dependent K⁺ channel opener increases mitochondrial resistance to oxidative injury [2], but Hanley et al described K_{ATP} channel-independent targets of diazoxide and have proposed that pharmacological preconditioning may be related to partial inhibition of respiratory chain complexes [3]. The question arose whether new fluorine-containing analogues of diazoxide and the potential mitochondrial K_{ATP} channel openers [4] affect the mitochondrial respiratory chain and what is the mechanism of their protective action. We have investigated the effect of DiazoFm and DiazoFp (new fluorine-containing analogues of diazoxide which have been synthesized by Prof. Yagupolskii LM), upon the mitochondrial function and especially upon the oxidative phosphorylation.

For this purpose we have used polarographic methods of oxygen consumption analysis and biochemical methods.

We have shown that the ability to inhibit ADP-stimulated respiration by DiazoFm (30 μM) and DiazoFp (30 μM) in concentrations 2.5- and 1.4-fold lower than those for diazoxide (30 μM) using succinate as a substrate. These effects were observed in the absence of K⁺ in the medium. The fluorine-containing K_{ATP} channel openers did not change the activity of succinate dehydrogenase significantly (by -7 %) compared to diazoxide (by -27 %). In other experiments it was established that DiazoFp activated ADP-stimulated respiration using 2- oxoglutarate as a substrate of oxidation (by -34 %). All

K_{ATP} openers investigated showed an uncoupling effect irrespective of substrates used. This effect was more expressed when using succinate as a substrate and is abolished by the application of 5-hydroxydecanoate (200 μM), an inhibitor of the mitochondrial K_{ATP} channels. We have also shown that DiazoFm prevented the Ca²⁺- dependent depression of oxidative phosphorylation.

Our investigations indicate that fluorine-containing K_{ATP} channel openers do not significantly affect the mitochondrial respiratory chain, especially the complex II. The uncoupling effect and decrease of depression of oxidative phosphorylation by Ca²⁺ can be considered a protective mechanism of action of the new fluorine-containing K_{ATP} channel openers.

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