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MiP*summer* 2008

2nd MiP*summer* School on
Mitochondrial Respiratory Physiology
12-18 July 2008 Schröcken, Vorarlberg, Austria

Programme and Abstracts

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Program overview

Saturday, 12. July

Afternoon	Registration at Hotel Mohnenfluh, check-in at Hotel Mohnenfluh and Hotel Tannberg
18:30	Welcome reception and dinner at Hotel Mohnehnfluh

Sunday, 13. July – Thursday, 17. July

07:30-08:45	Breakfast
09:00-12:45	Morning lectures: Gemeindesaal Schröcken (Lecture Hall) Lectures, with coffee break.
13:00	Break: Lunch; alpine walks and talks.
15:00 or 16:00	Workshops; Poster flash presentations and poster sessions; Special interest sessions; Discussions, with coffee break.
19:00	Dinner

Tuesday, 15. July
12:00 (in case of favourable weather conditions): **MiP*summer* Walk** to the cheese and wine reception at the *Alpmuseum uf m Tannberg*; refreshment in the lake Körebersee or at Hotel Körbersee; **MiP*summer* Party.**

Thursday, 17. July
21:00 After dinner MiP*summer* Lecture by **Guy C Brown** (Cambrdige, UK) Mitochondria and the future of death.

Friday., 18. July Departure in the very early morning.
See you at EBEC 2008*, Dublin, Ireland* - www.tcd.ie/Biochemistry/ebec2008/

MiP*summer* 2008

The programme of the 2nd MiP*summer* School on "Mitochondrial Respiratory Phyisology" provides a balance between introductions into the basic concepts, advanced methodological and scientific approaches, and specific applications, with a focus on mitochondrial respiratory function linked to the general theme of MiP with basic scientific and biomedical perspectives.

1 - Sunday, 13. July

Mitochondrial respiratory capacity and respiratory control.

Chair: Vilma Borutaite (LT), Steven C Hand (US)

1-01L 09:00-10:30 **Erich Gnaiger** (Innsbruck, AT) Mitochondrial respiratory control: Electron transport system, oxidative phosphorylation and leak – *ETS*, *OXPHOS* and *LEAK*. *Poster 1-01L*.

10:30 Coffee

1-02L 11:00-12:30 **Chuck Hoppel** (Cleveland, USA) Substrates used in studies of OXPHOS. An application example: The effect of developmental age on mitochondrial function in human skeletal muscle.

13:00 Lunch

Chair: Dominique Votion (BE), Erich Gnaiger (AT)

1-03L 15:00-16:30 **Rodrigue Rossignol** (Bordeaux, FR) Ultrastructure of the mitochondrion and its bearing on function and bioenergetics.

Flash presentations of posters:

1-04P 16:30-16:45 **Estelle Hirzel** (Basel, CH) Mitochondrial targeting in adipocytes using antioxidant tetrapeptides. *Communicated by Vilma Borutaite.*

1-05P 16:45-17:00 **Leandro S. da Costa** (Rio de Janeiro, BR) Mitochondrial dysfunction in neuroblastoma cells infected with sindbis virus. *Communicated by Vilma Borutaite.*

1-06P 17:00-17:15 **René G Feichtinger** (Salzburg, AT) Decrease of aerobic mitochondrial energy metabolism in paediatric solid tumors. *Communicated by Steven C Hand.*

1-07P 17:15-17:30 **Franz A. Zimmermann** (Salzburg, AT) Deficiency of complex I of the respiratory chain in oncocytic tumors. *Communicated by Chuck Hoppel.*

17:30-18:45 Coffee and Posters

19:00 Dinner at Hotel Mohnenfluh

21:15 MiP*board* Meeting: MiP2009 and beyond (Hotel Tannberg)

2 - Monday, 14. July

Membrane potential, coupling, substrates and respiratory control

Chair: Hélène Lemieux (AT), Guy C Brown (UK)

2-01L 09:00-10:30 **Vilma Borutaite** (Kaunas, LT) Mitochondrial membrane potential: why and how to measure.

10:30 Coffee

2-02P 11:00-11:15 **Patrick Subarsky** (Innsbruck, AT) Regulation of oxidative phosphorylation in response to graded uncoupling towards the limit of electron transport capacity. *Poster 2-02L Communicated by Guy C Brown*.

2-03L 11:15-12:00 **Dominique-Marie Votion** (Liége, BE) Mitochondrial respiration in the equine muscle with high-resolution respirometry: Race horses and a rare myopathy.

2-04L 12:00-12:45 **Erich Gnaiger** (Innsbruck, AT) How are respiratory control ratios interpreted? – Concepts and applications. *Poster 2-04L*.

13:00 Lunch

Chair: Vilma Borutaite (LT), Charles Hoppel (US)

Flash presentations of posters:

2-05P 16:00-16:15 **Olga Panasiuk** (Kiev, UA) Different sensitivities of two mitochondrial subpopulations to calcium-induced injury. *Communicated by Chuck Hoppel.*

2-06P 16:15-16:30 **José Lumini-Oliveira** (Coimbra, PT) Effects of endurance treadmill training on skeletal muscle mitochondrial respiratory function of STZ-treated rats. *Communicated by Chuck Hoppel.*

2-07P 16:30-16:45 **Liat Shachnai** (Rehovot, IL) MTCH2/MIMP: A novel regulator of mitochondrial function. *Communicated by Vilma Borutaite.*

16:45–17:45 Coffee and Posters

17:45-18:45 **Special interest groups**

19:00 Dinner at Hotel Mohnenfluh

21:15-22:15 **Special interest groups**

3 - Tuesday, 15. July

Gas interactions with mitochondria

Chair: Dominique Votion (BE), Anthony Hickey (NZ)

3-01L 09:00-10:30 **Guy C Brown** (Cambridge, UK) Nitric oxide and other gas interactions with mitochondria.

10:30-10:45 **Questions and suggestions by participants**

10:45-11:45 Coffee and Posters / Special interest groups

In case of favourable weather conditions:

12:00 **MiP*summer* Walk** to the cheese and wine reception at the *Alpmuseum uf m Tannberg*; refreshment in the lake Körebersee or at Hotel Körbersee.

Chair: Robert Boushel (DK), Steven C Hand (US)

3-02L 17:15-18:00 **Erich Gnaiger** (Innsbruck, AT) Who needs a thermodynamics background in bioenergetics and mitochondrial physiology?

3-03D 18:00-18:45 **Mario Fasching** (Innsbruck, AT) Simultaneous high-resolution measurement of pH and oxygen Flux with the Oxygraph-2k (instrument demo).

19:00 Dinner at Hotel Mohnenfluh.

4 - Wednesday, 16. July

Oxygen kinetics of mitochondrial respiration and spectrophotometry

Chair: Vilma Borutaite (LT), Guy C Brown (UK)

4-01L 09:00-09:45 **Francesca Scandurra** (Innsbruck, AT) Cellular respiration under hypoxia: Mitochondrial oxygen kinetics at steady-state oxygen supply and aerobic-anaerobic transitions.

4-02D 09:45-11:00 **Natascha Sommer** and **Thomas Derfuss** (Giessen, DE) Simultaneous remission spectrophotometry and high-resolution respirometry. Respiratory control by oxygen and redox states of mitochondrial cytochromes in cells.

11:00 Coffee and Posters

4-03L 11:45-12:45 **Erich Gnaiger** (Innsbruck, AT) Competitive and undcompetitive inhibition of cytochrome *c* oxidase by NO in living cells.

13:00 Lunch

Heart and skeletal muscle mitochondria

Chair: Natascha Sommer (DE), Steven C Hand (US)

4-04L 15:00-15:30 **Chuck Hoppel** (Cleveland, US) Ischemia-reperfusion injury in heart mitochondria.

4-05L 15:30-16:00 **Hélène Lemieux** (Innsbruck, AT) Mitochondrial dysfunction in pathologies of the human heart and comparison with mitochondrial respiratory control in the mouse heart.

Flash presentations of posters:

4-06P 16:00-16:15 **Julie H Rennison** (Cleveland, US) High fat fed heart failure animals have enhanced mitochondrial function and acyl-CoA dehydrogenase activities. *Communicated by Erich Gnaiger.*

4-07P 16:15-16:30 **Antonina V Pustovidko** (Moscow, RU) Uncoupling effect of lauryl sulfate on mitochondria. *Communicated by Erich Gnaiger.*

4-08P **Tímea Kurdiová** (Bratislava, SK) Measurement of the mitochondrial respiration capacity in permeabilized muscle fibers from cold adapted mice. *Communicated by Erich Gnaiger.*

16:30-17:45 Coffee and Posters

Chair: Dominique Votion (BE), Charles Hoppel (US)

4-09L 17:45-18:45 **Anthony J.R. Hickey** (Auckland, NZ) Depressed endogenous mitochondrial respiration stimulates elevated cardiac superoxide production in spontaneously hypertensive rats.

19:00 Dinner

21:00 **MiP*society* Party**

5 - Thursday, 17. July

Mitochondrial molecular physiology

Chair: Francesca Scandurra (AT), Guy C Brown (UK)

5-01L 09:00-10:30 **Steven C Hand** (Baton Rouge, US) Mitochondria and apoptosis: Energy-limited states and signaling for cell death.

11:00 Coffee

5-02L 11:30-12:45 **Charles Hoppel** (Cleveland, US) Integrated mitochondrial function and the clinical utility of polarographic analysis.

13:00 Lunch

Chair: Charles Hoppel (US), Erich Gnaiger (AT)

5-03L 15:00-16:00 **Steven C Hand** (Baton Rouge, US) Mitochondrial targeting of protein import.

Flash presentations of posters:

5-04P 16:00-16:15 **Ana Paula P. da Silva** (Rio de Janeiro, BR) Differential inhibition of energy-producing pathways of hepg-2 cells by 3-bromopyruvate. *Communicated by Steven C Hand.*

5-05P 16:15-16:30 **Elisa Sottotetti** (Bologna, IT) PKA stimulation protects transformed cells from apoptosis induced by glucose depletion, by re-activating mitochondrial activity. *Communicated by Erich Gnaiger.*

5-06P 16:30-16:45 **Alexandra Latini** (Florianopolis, BR) Seleno compounds prevents the brain energy impairment induced by methylmercury poisoning in adult mice. *Communicated by Guy C Brown.*

5-07P 16:45-17:00 **Praturi Gopalakrishna** (Hyderabad, IN) Periodontitis: Role of oxidative phosphorylation. *Communicated by Guy C Brown.*

17:00-17:45 Coffee and Posters

17:45-18:30 **MiP*summer* Feedback**

19:00 MiP*summer* Dinner at Hotel Mohnenfluh

5-08S 21:15 **MiP*summer* Special Lecture** (*in the lecture hall*)
Guy C Brown (Cambridge, UK) **Mitochondria and the future of death.**
Guy Brown is the author of 'The Living End. The future of death, aging and immortality'. Macmillan (2007).

MiP*summer* Abstracts

Day 1: Mitochondrial respiratory capacity and respiratory control.

1-01L **Mitochondrial respiratory control: Electron transport system, oxidative phosphorylation and leak – *ETS*, *OXPHOS* and *LEAK*.** Flux control of oxidative phosphorylation and convergent electron transport system studied in intact and permeabilized cells.

Erich Gnaiger

Medical University of Innsbruck, Dept. General and Transplant Surgery, D. Swarovski Research Laboratory, A-6020 Innsbruck, Austria. – erich.gnaiger@i-med.ac.at

Oxidative phosphorylation (*OXPHOS*) is a key element of bioenergetics, extensively studied to resolve mechanisms of energy transduction and respiratory control in the electron transport system (*ETS*). Electron transport capacity is quantified as oxygen consumption in uncoupled mitochondria or cells (*ETS;* State *E*). In contrast, maximum ADP-stimulated respiration is a measure of *OXPHOS* capacity (*P*; State 3). *P/E* ratios yield an index of *OXPHOS* limitation by the phorsphorylation system.

Coupled *OXPHOS* flux was 0.50±0.09 of *ETS* capacity in permeabilized NIH3T3 fibroblasts respiring on glutamate+malate+succinate (GMS), reflecting control of the phosphorylation system over *OXPHOS* in this human cell line [1,2]. Electrons flow to oxygen from Complex I or II with three or two coupling sites. Compared to *ETS* capacity in intact cells [3], conventional State 3 respiration in permeabilized cells was only 0.38±0.06 with ADP and glutamate+malate. *ETS* capacities were identical in intact and permeabilized uncoupled cells, however, with convergent electron flow to the Q-junction from glutamate+malate+succinate through Complexes I and II (CI+II e-input [1]). Convergent CI+II e-input provides the relevant basis for quantifying enzymatic thresholds and excess capacities of individual steps of *OXPHOS*, and for evaluation of mitochondrial defects. Convergent CI+II e-input corresponds to operation of the tricarboxylic acid cycle and mitochondrial substrate supply *in vivo* and yields novel insights into the physiological diversity of mitochondria from various tissues (compare various OXPHOS control analyses presented at MiP*summer* 2008). Multiple substrate-uncoupler-inhibitor titration protocols and advanced OXPHOS flux control analysis extend the diagnostic potential of mitochondrial physiology in health and disease.

This abstract will be presented at: EBEC 2008 Dublin, Ireland; 19-24 July 2008.

Keywords: Electron transport system, flux control ratio, uncoupling, leak, respiratory control ratio, multiple substrate-uncoupler-inhibitor tirations, Q-junction, fibroblasts.

1. Gnaiger E, ed (2007) *Mitochondrial Pathways and Respiratory Control.* OROBOROS MiPNet Publications, Innsbruck: 96 pp.
2. Naimi A, Garedew A, Troppmair J, Boushel R, Gnaiger E (2005) Limitation of aerobic metabolism by the phosphorylation system and mitochondrial respiratory capacity of fibroblasts *in vivo*. The coupled reference state and reinterpretation of the uncoupling control ratio. *Mitochondr. Physiol. Network* 10.9: 55-57. www.mitophysiology.org/index.php?naimia.
3. Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: *Mitochondrial Dysfunction in Drug-Induced Toxicity* (Dykens JA, Will Y, eds) John Wiley (in press).

1-02L **Substrates used in studies of OXPHOS. An application example: The effect of developmental age on mitochondrial function in human skeletal muscle.**

Chuck Hoppel

Case Western Reserve University School of Medicine, Department of Pharmacology, Center for Mitochondrial Diseases, Cleveland, Ohio, USA. - charles.hoppel@case.edu

1-03L **Ultrastructure of the mitochondrion and its bearing on function and bioenergetics.**

Benard G, Rodrigue Rossignol

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The recently ascertained network and dynamic organization of the mitochondrion, as well as the demonstration of energy proteins and metabolites subcompartmentalization, have led to a reconsideration of the relationships between organellar form and function. In particular, the impact of mitochondrial morphological changes on bioenergetics is inseparable. Several observations indicate that mitochondrial energy production may be controlled by structural rearrangements of the organelle both interiorly and globally, including the remodeling of cristae morphology and elongation or fragmentation of the tubular network organization, respectively. These changes are mediated by fusion or fission reactions in response to physiological signals that remain unidentified. They lead to important changes in the internal diffusion of energy metabolites, the sequestration and conduction of the electric membrane potential (Delta Psi), and possibly the delivery of newly synthesized ATP to various cellular areas. Moreover, the physiological or even pathological context also determines the morphology of the mitochondrion, suggesting a tight and mutual control between mitochondrial form and bioenergetics. In this presentation, we delve into the link between mitochondrial structure and energy metabolism.

1-04P **Mitochondrial targeting in adipocytes using antioxidant tetrapeptides.**

Estelle Hirzel[1], Peter Lindinger[1], Peter W Schiller[2], Alex N Eberle[1]

[1]University Hospital and Children Hospital Basel, Department of Biomedicine, University of Basel, Switzerland; - estelle.hirzel@unibas.ch; [2]Clinical Research Institute of Montreal, Quebec, Canada.

Overweight and obesity represent a rapidly growing threat to the health in many countries. Obesity-related co-morbidities include coronary heart disease, hypertension and stroke, type 2 diabetes mellitus and dyslipidaemia [1]. The obesity-related elevations of fatty acids cause oxidative stress due to increased mitochondrial uncoupling and β-oxidation, leading to elevated production of reactive oxygen species (ROS) [2]. ROS might cause a decrease in mitochondrial function, thus exacerbating insulin resistance [3]. Szeto-Schiller peptides (SS-peptides) are tetrapeptides which are taken up and concentrated in the inner mitochondrial membrane >1000-fold [4]. They have been shown to reduce intracellular ROS, cell death and mitochondrial depolarization caused by *t*-butylhydroperoxide (*t*BHP) in neuronal cells lines [5].

As a model for fat tissue, primary human bone marrow-derived mesenchymal stem cells (hBM-MSC) will be differentiated into adipocytes *in vitro*. The influence of different SS-peptides on ATP production, mitochondrial membrane potential, intracellular ROS levels and lipolysis will be tested on cells from healthy, obese and diabetic donors.

This study will provide insights into the effects of SS-peptides on mitochondrial function and their therapeutic potential in obesity.

1. Obesity (2000): preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ. Tech. Rep. Ser. 894: 1-253.
2. Wojtczak L, Schonfeld P (1993) Effect of fatty acids on energy coupling processes in mitochondria. *Biochim. Biophys. Acta* 1183: 41-57.
3. Qatanani M, Lazar MA (2007) Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes Dev*. 21: 1443-1455.
4. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, Szeto HH (2004) Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. *J. Biol. Chem.* 279: 34682-34690.
5. Zhao K, Luo G, Giannelli S, Szeto HH (2005) Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Biochem. Pharmacol.* 70: 1796-1806.

1-05P **Mitochondrial dysfunction in neuroblastoma cells infected with sindbis virus.**

Leandro S. da Costa[1], Nívea Dias Amoêdo[1], Franklin David Runjanek[1], Antonio Galina[1], Andrea T. Da Poian[1], Tatiana El-Bacha[1]

[1]*Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Brazil. - lcosta@bioqmed.ufrj.br*

In this study we demonstrate for the first time that Sindbis Virus (SV) infection induces important alterations in the respiratory parameters of neuroblastoma cells, Neuro2A. Oxygen consumption was measured in intact cells using high-resolution respirometry (OROBOROS Oxygraph-2k). Our results show that infected cells present a 45 % decrease in *ROUTINE* respiration (n=5; P<0.05) and a 38 % decrease in FCCP-induced maximum electron transport system capacity (*ETS*; n=5; P<0.05) when compared to mock-infected cells. Additionally, SV-infected cells show a significant decrease (P<0.05) in oligomycin-inhibited *LEAK* respiration (mean ± SE; n=5; 18.6 ± 1.3 for SV-infected and 32.4 ± 5.6 for mock-infected cells), and a significantly increase (P<0.05) in respiratory control ratio [(RCR) mean ± SE; n=5; 2.02 ± 0.06 for SV-infected and 1.68 ± 0.13 for mock-infected cells]. The decrease in *LEAK* respiration and the increase in RCR suggest mitochondrial coupling and a decrease in proton leak induced by SV-infection possibly as a compensatory mechanism for the decrease in *ROUTINE* respiration and maximum *ETS* capacity. Since we also found that SV-infection significantly increase by two-fold the K_m of hexokinase for glucose, the mitochondrial coupling found in infected cells may also be important to compensate for a possible decrease in glycolytic flux. We propose that bioenergetic alterations of Neuro2A cells are early signs of cell death and may be involved in the pathophysiology of encephalitis observed in SV-infection.

1-06P **Decrease of aerobic mitochondrial energy metabolism in paediatric solid tumors.**

René G Feichtinger[1], JA Mayr[1], F Zimmermann[1], N Jones[1], FH Schilling[2], P Kogner[3], W Sperl[1], B Kofler[1]

[1]*University Hospital Salzburg, Paracelsus Medical University, Müllner- Hauptstrasse 48, 5020 Salzburg, AUSTRIA,* [2]*Olga Hospital, Stuttgart, GERMANY,* [3]*Childhood Cancer Research Unit, Karolinska, SWEDEN. - r.feichtinger@salk.at*

Neuroblastomas (NB) and Wilms tumors (WT) are the two most frequent extra-cranial solid tumors in children. Neuroblastomas originate from the neural crest, whereas Wilms tumors arise from the embryonal kidney. A shift in cellular energy production from oxidative phosphorylation (OXPHOS) to anaerobic glycolysis, called Warburg effect, is a fundamental property of cancer. Succinate dehydrogenase (Complex II) gene mutations are associated with carcinogenesis in pheochromocytoma, another tumor of the neural crest.

The aim of the present study was to determine specific alterations of OXPHOS in 600g supernatants of human NB (n=15) and WT tissues (n=9) by spectrophotometric measurement of the enzymatic activity of citrate synthase (CS), Complex I, Complex II, Complex III, Complex IV (CI, CII, CIII, CIV) and ATP synthase. Compared to non-malignant tissues the activity of citrate synthase, a pace-maker enzyme of the Krebs cycle, was not altered in NB. The enzymatic activities of control kidneys (K), NB and WT are given as Units / g protein (mean ± SD): CS: K: 112±29, NB: 118±29, WT: 75±46; CI: K: 43±9, NB: 10±6, WT 16±11; CII: K: 128±45, NB: 15±16, WT: 24±15; CIII: K: 161±36, NB: 106±36, WT 159±134; CIV: K: 99±34, NB: 28±12, WT: 36±22; ATP synthase: K: 40±17, NB: 9±7, WT: 19±10.

When normalized to CS activity and then compared to control values, we found residual activities of CI (22 %), CII (7 %), CIII (69 %), CIV (27 %) and ATP synthase (19 %) in NB tissues, compared to normal kidney cortex tissue. Similar to NB, WT showed a residual enzyme activity of citrate synthase (70 %), CI (31 %), CII (19 %), CIII (78 %), CIV (39 %) and ATP synthase (48 %).

The reduction of the activity of all complexes of the respiratory chain in NB and WT indicates that in these tumors loss of respiration is not related to a defect of a single enzyme as shown for renal oncocytomas and hereditary pheochromocytomas. Thus an

upstream effector (e.g. VHL, HIF-1α), controlling the overall respiration [1,2] seems to cause the downregulation of OXPHOS in NB and WT.

This work was supported by the Salzburger Kinderkrebshilfe.

1. Godinot C et al (2004) A new role for the von Hippel Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis. *Carcinogenesis* 26: 531-539.
2. Semenza GL (2007) HIF-1 mediates the Warburg effect in clear cell renal carcinoma. *J. Bioenerg. Biomembr.* 39: 231-234.

1-07P Deficiency of complex I of the respiratory chain in oncocytic tumors.

Franz A. Zimmermann,[1] Johannes A. Mayr,[1] Rene Feichtinger,[1] Daniel Neureiter,[2] Beate Alinger,[2] Nikolaus Schmeller,[3] Christian Kögler,[4] Manfred Ratschek,[4] Wolfgang Sperl,[1] Barbara Kofler[1]

[1]Department of Pediatrics, [2]Department of Pathology, [3]Department of Urology, University Hospital Salzburg, Paracelsus Medical University, Austria; and [4]Institute of Pathology, Medical University of Graz, Austria.- f.zimmermann@salk.at

Many solid tumors exhibit a shift in energy metabolism from aerobic oxidation in the mitochondria to anaerobic glycolysis, which was first observed by Otto Warburg more than 80 years ago. Mutations in the mitochondrial enzymes fumarate hydratase and succinate dehydrogenase have been identified in different types of tumors, demonstrating a link between mitochondrial energy metabolism and tumorigenesis. Oncocytomas are tumors characterized by the accumulation of a high number of mitochondria. In order to elucidate the cause of the mitochondrial alterations in oncocytomas, we investigated the activities of respiratory chain enzymes, screened for mitochondrial DNA (mtDNA) mutations and performed by immunohistochemical stainings of oncocytic tumors.

We showed that enzymatic activity of respiratory complex I was undetectable or extremely reduced in renal oncocytomas (n=15), and assembled complex I was lacking as shown of by Blue Native gel electrophoresis. Sequence analysis of mitochondrially encoded subunits of complex I showed pathogenic mutations in ND1, ND4 and ND5 genes in 10/15 renal oncocytoma samples, most of them were frame-shift mutations.

Recently, also an association of mtDNA mutations with oncocytic thyroid tumors has been published [1], showing in 26 % of the cases disruptive mutations in mitochondrially encoded complex I genes. By immunohistochemical staining, we observed a lack of complex I in renal oncocytomas as well as in oncocytic thyroid tumors, while complex V and porin were considerably up-regulated.

In summary, renal oncocytomas and oncocytic thyroid tumors seem to be characterized by a complete loss of complex I and a compensatory upregulation of mitochondria. Thus oncocytic tumors can be regarded as a mitochondrial disease, mostly caused by somatic mutations of the mitochondrial DNA.

This work was supported by the "Children's Cancer Foundation Salzburg", and the "Vereinigung zur Förderung der pädiatrischen Forschung und Fortbildung Salzburg".

1. Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, Ghelli A, Moretti M, Betts CM, Martinelli GN, Ceroni AR, Curcio F, Carelli V, Rugolo M, Tallini G, Romeo G (2007) Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors. *Proc. Natl. Acad. Sci. USA* 104: 9001-9006.

Day 2: Mitochondrial membrane potential, coupling, substrates and respiratory control

2-01L Mitochondrial membrane potential: why and how to measure.

Vilma Borutaite

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2-02P Regulation of oxidative phosphorylation in response to graded uncoupling towards the limit of electron transport capacity.

Patrick Subarsky, Erich Gnaiger

Dept. of General & Transplant Surgery, D. Swarovski Research Laboratory, Innsbruck Medical University, Austria. - patrick.subarsky@oroboros.at

Mitochondrial oxygen consumption is divided between the support of ADP phosphorylation and *LEAK* (*L*, including proton leak through the inner membrane and proton slip in the respiratory complexes). The aim of our study was to determine the distribution of oxygen consumption between the two processes in intact cells (32D, myeloblast-like), using high-resolution respirometry. Electron transport capacity (*E*) was defined as the maximum respiration under conditions of optimal FCCP concentration [1] (74±13 pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells in the absence of oligomycin). Maximum *E* in the presence of optimal FCCP concentration and oligomycin, E_{Omy}, was 0.84±0.04 of *E* without oligomycin. *ROUTINE* respiration (*R*) under physiological control of routine ATP demand was 0.39 of *E*, whereas the oligomycin-inhibited *LEAK* respiration was 0.11 of E_{Omy}. As a model of uncoupling stress, *LEAK* was increased by step-wise titration of FCCP. *LEAK* and the corresponding stress-induced increase in *ROUTINE* respiration were measured and the contribution to coupled respiration (net*R*) was calculated as *R-L*. Our results were compared with two contrasting uncoupling-response models: (i) Full compensation for increased proton leak would result in complete maintenance of phosphorylating activity indicated by an unchanged net*R* up to the limit of electron transport capacity. Only then would net*R* diminish as *LEAK* further increases towards the maximum value of *E* in the fully uncoupled state. (ii) Competition between phosphorylating activity and proton leak would result in net*R* declining linearly towards zero as *LEAK* increases towards maximum. We observed compensation for increased proton leak only at very mild uncoupling, but competition at increasing FCCP concentrations. The results extending into the range of strong uncoupling are in contrast to the effect of mild uncoupling in senescent fibroblasts where an increase in routine respiration fully compensates for the increased *LEAK* respiration [2].

This is an extended abstract which will be presented at EBEC 2008 Dublin, Ireland; 19-24 July 2008. Poster 2L-02 Communicated by Guy C Brown.

1. Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: *Mitochondrial Dysfunction in Drug-Induced Toxicity* (Dykens JA, Will Y, eds) John Wiley (in press).
2. Hütter E, Renner K, Pfister G, Stöckl P, Jansen-Dürr P, Gnaiger E (2004) Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts. *Biochem. J.* 380: 919-928.

2-03L Study of the mitochondrial respiration in the equine muscle with high-resolution respirometry: Feasibility, preliminary results and potential applications.

Dominique-Marie Votion[1a,b], Hélène Lemieux[2], Ange Mouithys-Mickalad[3], Didier Serteyn[1a], Erich Gnaiger[2]

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Equines have been bred since time in memoriam to a large variety of uses of high specificity. Regardless of their intended use, horses are able to perform physical activities at a level that surpasses other animals of similar body size. The athletic potential of an individual relates to oxygen (O_2) transport and utilisation[1]. As regard to the maximal oxygen consumption (VO_{2max}) which is considered as an index of exercise's capacity[2], the horse is an amazing athletic animal. For example, when expressed on a mass specific basis, race horses have values for VO_{2max} *twice* those of elite human athletes[3,4]. During high-intensity exercise, the large VO_{2max} is achieved as a result of remarkable cardiopulmonary adaptations. It might be expected that the respiratory capacity of equine muscle would be also proportionately higher than the one of human.

Measurements of the maximal respiratory capacity of the equine skeletal muscle are scarce[5]. However, it has always been the ambition of physiologists to determine athletic suitability for specific disciplines and to predict athletic performance.

Up to now, cellular energetics of muscles has been based upon the histochemical and biochemical analyses of large biopsy samples[6] (200 to 300 mg of muscle). Given the little practicality of performing muscle biopsy in performance horses, the technique has been limited to scientific protocols or to the evaluation of horses with suspected myopathy[7].

High-resolution respirometry offers the opportunity to perform bioenergetics' studies with a minimal amount of sample using permeabilised tissue[8]. This technique precludes the use of time-consuming preparation of isolated mitochondria and enables the study of mitochondrial function within a preserved intramitochondrial environment. We aimed at studying the feasibility of high-resolution respirometry for investigating the mitochondrial respiratory function of the equine muscles with permeabilised fibres obtained by microsampling.

Microbiopsies were performed in the *triceps brachii* of 3 horses with a 14 G biopsy needle that enables the sampling of 20 to 40 mg of muscle. The specimens were prepared according to the Oroboros Oxygraph-2k manual. From 1 to 4 mg wet weight of sample were put in Oxygraph-2k chambers filled with media warmed at 37 °C in presence of malate plus glutamate. Oxygen was injected into the chambers in order to reach a 500 µM O_2 concentration. Then, the experiment starts with the measurement of *State 2* respiration (*LEAK*) after steady-state respiratory flux. *State 3* of respiration (*OXPHOS*) was initiated by adding ADP in excess. Integrity of mitochondria was estimated by assaying the mixture with Cyt *c*. A further increase of respiration was stimulated by adding succinate. After that, uncoupling of oxidative phosphorylation by stepwise titration of FCCP was used to obtain the maximal stimulation of flux (*ETS* capacity). Finally, the respiration was inhibited by the addition of rotenone (inhibition of complex I) and antimycin A (inhibition of complex III). All measurements were performed at steady-state.

A single biopsy enabled to perform at least 4 titration protocols that was completed within 1.5 hour. The O_2 concentration remained above 240 nmol/ml for all the procedure which may of importance to avoid O_2 dependence of results. Integrity of mitochondrial function in samples preserved for 4 days (at 4 °C in BIOPS) did not demonstrate any alteration thus enabling to delay analysis (and open many perspectives such as sampling during field trials). These preliminary results demonstrated the feasibility of studying mitochondrial respiration in the equine muscle based on microsamples which may, with no doubt, be performed on performance horses without consequence (no scar, no pain, no sedation required). It is now necessary to evaluate the variability between runs, to determine reference ranges taking into account all the parameters that may influence results (age, gender, breed, level of training etc.) and to compare mitochondrial physiology with the one of other athletic and non athletic species.

Such a database will offer the possibility to study the relationship between mitochondrial function and parameters of athletic capacity as well as to the pathophysiological mechanism underlying equine myopathic disorders.

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2-04L How are respiratory control ratios interpreted? Concepts and applications.

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Respiratory steady states have been defined by Chance and Williams (1955) according to a protocol for oxygraphic experiments with isolated mitochondria, for studies of mitochondrial respiratory control [1]. As simple as they may appear, measurements of respiratory States (2-3-4) and respiratory control ratios (RCR) need to be well designed and concepts to be clarified.

In the absence of ADP, respiration compensates mainly for proton leak (*LEAK* respiration*, L*), thus maintaining a high mitochondrial membrane potential. The *L/E* flux control ratio was 0.10±0.01 SD (*N*=5) in fibroblast mitochondria respiring on GMS. At a *L/E* ratio of 0.10, the *LEAK* respiration is 10 % of *ETS* capacity, indicating tight coupling of mitochondria in permeabilized cells. The *L/E* ratio (0.09±0.02 SD; *N*=18) was identical in intact cells, evaluated by inhibition by oligomycin (*L*) and stimulation by uncoupler (*E*). Conventionally, ADP stimulation is expressed by the respiratory control ratio (RCR = State 3/State 4), which is frequently used as an index of coupling for diagnosis of mitochondrial defects. Compared to the *L/E* ratio of 0.10, the State 4/State 3 (*L/P*) ratio with glutamate+malate was 0.25 (RCR=4.0). RCR yields a severe underestimation of coupling, since the *L/P* ratio relates *LEAK* respiration to *OXPHOS* (rather than maximum *ETS*) capacity. The RCR is useful only in the limiting case when the *P/E* ratio is 1.0 and *ETS* capacity is not limited by substrate supply or *ETS* defects [2].

With proper expression of normalized respiratory fluxes (the inverse of the conventional RCR), a linear relationship exists between P/O ratios and 'RCR' [3]. Changes of the 'RCR' may not merely be caused by uncoupling, but frequently are caused by alterations of catalytic OXPHOS capacities, including the phosphorylation system. Interpretation of 'RCR', therefore, is complicated in cases of multiple mitochondrial defects. Experimental examples will be presented on measurements of P/O ratios [3] and respiratory and uncoupling control ratios [4-6], with discussion and critical evaluation of results.

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2-04P **Application: Early impairment of mitochondrial function in apoptosis and protection by overexpression of the antiapoptotic factor *v*-Raf in a mouse pro-myeloid cell line.**

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The cell's ability to respond to extrinsic stimuli, including signals leading to cell death, depends on adequate energy supply. Increasing evidence suggests that signaling pathways not only control cell proliferation and survival but also cellular energy production. Removal of growth factor interleukin 3 (IL-3) in the mouse pro-myeloid 32D cell line results in growth arrest and subsequent apoptosis, which is delayed through overexpression of *v*-Raf, the oncogenic form of the antiapoptotic factor C-Raf (439 cells [1]). High-resolution respirometry was used to study the effect of *v*-Raf on mitochondrial energy transformation. An early time point (8 h) was selected, before the 32D cells deprived of IL-3 become irreversibly committed to death [2]. Viability, mitochondrial content (shown by citrate synthase activity) and glycolytic capacity (shown by lactate dehydrogenase activity) remained unchanged after IL-3 withdrawal in 32D and 439 cells. *ROUTINE* respiration in intact cells [3] was suppressed significantly following removal of the growth factor in 32D cells. This was prevented by *v*-Raf overexpression. Permeabilized cells were used for diagnosis of specific mitochondrial defects before the onset of apoptosis. Electron transport capacity (*ETS*) was identical in intact and permeabilized cells. Significantly impaired capacities for Complex I, Complexes I+II, and Complex IV respiration were observed before any marker of apoptosis could be detected, whereas Complex II respiration remained unchanged. No stimulation of respiration by cytochrome *c* nor uncoupling occurred (compare [4]). Our results demonstrate that IL-3 withdrawal severely compromised mitochondrial respiratory function. The defects are suppressible by *v*-Raf overexpression. Our results point towards a direct link between the key mitogenic and survival kinase C-Raf and mitochondrial energy homeostasis.

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2-05P **Different sensitivities of two mitochondrial subpopulations to calcium-induced injury.**

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Previously, it was found that ω-3 PUFA are protective against myocardial injury after ischaemia-reperfusion. As mitochondria are involved in pathological changes associated with ischaemia-reperfusion injury in the heart, one of the mechanisms of this protection may be altered mitochondrial function. It is also known that within heart muscle, there are two subfractions of mitochondria located in different regions of the cell [1] – subsarcolemmal and interfibrillar mitochondria (SSM and IFM, respectively). These subfractions possess different properties and functions. But the role of mitochondrial functional heterogeneity under physiological and, in particular, pathophysiological conditions, remains to be elucidated.

The registration of mitochondrial permability transition pore (MPT) is a commonly used approach to estimate sensitivity of mitochondria to ischaemia-reperfusion injury, thus the purposes of this study were to determine possible differences in Ca^{2+}-induced swelling between SS and IFM fractions under control conditions, the effect of feeding of rats with ω-3 PUFA on magnitude and kinetics of Ca^{2+}-induced mitochondrial swelling in SS and IF

mitochondrial fraction, possible differences in ω-3 PUFA-evoked alterations in susceptibility to Ca^{2+} between SS and IF mitochondrial fractions.

Two groups of rats were used: the control and the experimental one (CG, EG); the latter was fed with ω-3 PUFA (0.1 ml/100g of epadol containing 45 % ω-3 PUFA). Different mitochondrial fractions were isolated as described priviously [1]. Changes in mitochondrial matrix volumes were measured by the light-scattering technique as swelling of mitochondria is accompanied by a decrease in the absorption (A). SS and IF mitochondria were treated with 10^{-4} M $CaCl_2$ at 5 min. Here it was shown that time–courses of swelling were comparable in both mitochondrial fractions and there was no difference in Ca^{2+}–induced swelling in the two mitochondrial fractions from untreated rats. For a detailed kinetic analysis, typical swelling kinetics was quantified by measuring the maximal rate of the decrase in A (V_{max}) with and without Ca^{2+} addition and also the time required to reach V_{max} [2]. And in CG of rats there was a significant difference between the mitochondrial fractions within these parameters only for the time to reach V_{max} in Ca^{2+} solution (for IFM this parameter was lower).

In the SSM fraction from CG and EG rats, there was no difference in the time course of swelling in Ca^{2+}- free solution, but after Ca^{2+} addition SSM from EG rats showed a decreased sensitivity to Ca^{2+}. However, IFM from EG showed a much less pronounced decrease in A as in Ca^{2+} significantly attenuated V_{max} of decrease in A in Ca^{2+}-free conditions and after Ca^{2+} addition. These results demonstate that IFM from EG is more sensitive to a protective effect of ω-3 PUFA feeding than SSM. IFM had a decreased decline in A and lower V_{max} in Ca^{2+} free than in Ca^{2+} solution.

In summary the present study indicates that IF and SS mitochondrial fractions do not differ in their susceptibility to Ca^{2+}-induced swelling. Feeding of rats with ω-3 PUFA protected both mitochondrial fractions against Ca^{2+}-evoked swelling and the protective effect of ω-3 PUFA is noticeably more pronounced for the IF than SS mitochondrial fraction.

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2-06P Effects of endurance treadmill training on skeletal muscle mitochondrial respiratory function of STZ-treated rats.

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Diabetes mellitus is a group of metabolic diseases that are responsible for the decreased quality of life of millions of persons all over the world. Some studies have reported that hyperglycaemia conditions can lead to defects in mitochondrial biochemistry, structure and function [1]. Regular exercise undertaken for several weeks has been reported to improve mitochondrial function in skeletal muscle by modulating some defense mechanisms [2]. However, it is not yet known to what extent these protective effects occur in skeletal mitochondria from streptozotocin (STZ)-treated rats. Therefore, the purpose of the present work was to analyse the effects of chronic exercise on skeletal muscle mitochondrial function in diabetic rats.

24 adult male Wistar rats were randomly divided into four groups (n=6/group) sedentary (S), STZ (50 mg/kg, i.p. for 18 wks), endurance trained (14 wk treadmill running, 60 min/day, 25m/min - T), and trained STZ (TSTZ). Isolated mitochondria from *gastrocnemius* muscle were examined for *in vitro* assessment of oxygen consumption and TPP^+ electrode-based transmembrane potential ($\Delta\Psi$) parameters using both complex I and II-linked substrates (malate/pyruvate and succinate plus rotenone, respectively).

STZ treatment induced respiratory dysfunction reflected by a decreased respiratory control ratio - RCR (with malate+pyruvate) and in increased state 4 respiratory activity (with both substrates). Mitochondria from trained STZ animals demonstrated lower state

4 (with both substrates), higher RCR (with malate+pyruvate), state 3 (with succinate+rotenone) and energization ΔΨ (with malate+pyruvate) when compared with sedentary STZ group. Endurance treadmill training *per se* increased the RCR and decreased lag phase with both substrates, and increased the ΔΨ without exogenous substrate addition.

In conclusion, long term STZ treatment-induced diabetes resulted in defects on mitochondrial respiratory coupling, which are reversed by endurance treadmill training. Moreover, important adaptive responses of skeletal muscle mitochondria were seen as a consequence of training in healthy control animals.

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2-07P MTCH2/MIMP: A novel regulator of mitochondrial function.

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Apoptosis is a crucial process for proper embryonic development and maintenance of tissue homoeostasis. In the extrinsic death pathway, apoptosis is initiated through activation of the TNF/Fas receptors. Activation of the TNF/Fas receptor results in the cleavage of pro-apoptotic BID into truncated BID (tBID), which translocates to the mitochondria to induce mitochondrial outer membrane permeabilization (MOMP) resulting in the release of apoptogenic factors such as cytochrome *c*. However, the mechanism by which tBID triggers MOMP is largely unknown. Mitochondria carrier homolog 2 (MTCH2)/Met-induced mitochondrial protein (MIMP) was identified in our lab as part of a complex with tBID in cells signaled to die by TNFalpha. MTCH2/MIMP is a novel and previously uncharacterized 33-kDa protein, which is related to members of the mitochondrial carrier protein family. We have revealed that knocking out MTCH2/MIMP in mice results in embryonic lethality and analysis of timed pregnancies revealed that MTCH2/MIMP-/- animals are not viable beyond E7.5. Recently we have generated MTCH2/MIMP liver specific knockout (LKO) mice. Isolated liver mitochondria from the LKO mice show elevated respiration on succinate compared to the control suggesting that MTCH2/MIMP may play a role in regulating the OXPHOS pathway. Our current goals are to continue to use the LKO mice to determine the exact function of MTCH2/MIMP in-vivo.

Day 3: Gas interactions with mitochondria

3-01L Nitric oxide and other gas interactions with mitochondria.

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Nitric oxide (NO) has at least 3 actions on mitochondria that may promote or inhibit cell death:

1. NO inhibits cytochrome oxidase in competition with oxygen, causing a dramatic increase in the apparent K_m of respiration for oxygen, and potentially sensitising cells to hypoxia. We find that NO from nNOS sensitises neurons in culture to hypoxia-induced death, via NO inhibition of neuronal cytochrome *c* oxidase, raising the oxygen requirement for cellular respiration.

2. NO or S-nitrosothiols can inactivate mitochondrial complex I, and this can be reversed by light or DTT, suggesting S-nitrosylation of the complex. We find that this inactivation causes a large increase in oxidant production by mitochondria, which is reversed by light or DTT.

3. NO or its derivatives can induce or inhibit mitochondrial permeability transition. During ischaemia and/or reperfusion of the heart, the mitochondria undergo permeability transition, which results in apoptosis and/or necrosis of the myocytes. We find that pre-perfusion of the heart with NO donors blocks both apoptosis and necrosis, and this is mediated by cGMP and PKG. Permeability transition in isolated mitochondria can be blocked by cGMP and PKG.

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3-02L Do we need a background in thermodynamics for bioenergetics and mitochondrial physiology?

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A fundamental understanding of thermodynamic principles is useful for analysis of mitochondrial energy transformations and efficiencies of generation and utilization of the proton motive force. Electrochemical membrane potentials are maintained in the spacially heterogenous system of a mitochondrial suspension or within cells, and these gradients (forces) are generated by coupling transmembrane fluxes (flow of substance transported per unit area; vector quantities) to chemical reactions (flow of substance transformed per unit volume; scalar quantities). Chemical reactions are most conveniently described as processes taking place in a closed homogenous system, and the most fundamental thermodynamic and kinetic parameters (e.g. the equilibrium constant or the enthalpy of reaction) are described with reference to the equilibrium state. The question, therefore, is frequently raised as to the applicability of 'equilibrium thermodynamics' to non-equilibrium living systems. Even linearity between thermodynamic forces and fluxes is generally restricted to the (non-physiological) near-equilibrium range. May we introduce mitochondrial physiology, therefore, without consideration of thermodynamics? Which information is lost, if we confuse the energetic efficiency of ATP production (power output per power input [1]) with a mere chemical flux ratio (H^+/O_2 or ATP/O_2 ratio)?

Cross-disciplinary application of thermodynamic concepts is made difficult by an unbelievably inconsistent and confusing terminology in the interface between classical thermodynamics [2] and irreversible thermodynamics [3]. It is a scientific curiosity that Linus Pauling (and similarly by Max Perutz) accused Erwin Schrödinger [4] (who published a book on thermodynamics [5]) of not understanding *entropy* in that his '*thermodynamics is vague and superficial to an extent that should not be tolerated even in a popular lecture*' [6]. If such eminent Nobel laureates in chemistry and physics battle with thermodynamics, perhaps we should better stay away from it?

It may be helpful and of practical use to consider some of the fundamental principles for describing the dynamics of open versus closed systems, define intensive quantities (forces) versus size-specific quantities (e.g. fluxes, for example per mass) and extensive quantities (e.g. flows, per system). Such fundamental concepts provide a guide for analysis of respirometric experiments with cultured cells versus isolated mitochondria, as a simple start and introduction to advanced analysis of vectorial (spacially oriented) versus scalar fluxes and flows, distinction of flux-force versus flux-pressure relationships, and consideration of metabolic power and efficiency [1].

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3-03D Simultaneous high-resolution measurement of pH and oxygen flux with the Oxygraph-2k.

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Mulitsensor applications, combining the measurement of oxygen flux with additional analytical parameters are becoming increasingly important in high-resolution respirometry. Many relevant parameters (e.g NO, H_2O_2) pose severe analytical problems in their own right. However, the precise measurement of additional parameters while simultaneously maintaining high-resolution performance for the oxygen measurement creates additional problems and can be a difficult task even for analytical methods very well established otherwise (e.g. pH). The monitoring of acid production is relevant particularly for integration of aerobic and anaerobic metabolism in cultured cells. This is comparable to the approach of combined calorespirometry [1], but separation of these two contributions to total energy metabolism may be resolved at higher resolution with simultaneous oxygen and pH measurement. High quality pH electrodes are readily available, but they have to be integrated into the oxygraph chamber without creating significant oxygen leaks or using incompatible materials that would prohibit high-resolution oxygen measurement. The more complicated geometry of a stopper incorporating a pH electrode makes removal of air bubbles difficult. For the same reason new concepts for recording instrumental backgrounds have to be applied, avoiding intermittent creation of gas phases. Electronic connection of the pH electrode to the multisensor port of the Oxygraph-2k is straightforward. Bracketing calibration with commercial calibration buffers can be done using the Datlab software.

To apply pH measurements to biological systems, some important points should be considered. Like for all potentiometric methods, the output signal is linear with the logarithm of concentration, i.e. at two different pH values the same change in pH value corresponds to different changes in proton concentration. To convert slopes of pH to metabolic fluxes of acidification, the pH values must therefore be converted to actual proton concentrations. Media for biological applications have generally a high buffering capacity. However, buffering dampes the signal recorded by the pH electrode. Therefore, special week buffers should be applied with well defined buffering capacity. The velocity of acid production of the biological sample is calculated from the slope of the measured (free) H^+ activity (concentration) over time and taking into account the buffering capacity. Preferentially, a pH-stat approach is applied, using a feedback control to keep the pH value constant by injecting base into the chamber via the titration-injection micropump (TIP-2k). The acid production per unit of time is then calculated from the rate of base injection, independent of a detailed knowledge of the buffering capacity of the system.

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Day 4: Oxygen kinetics of mitochondrial respiration and spectrophotometry

4-01L Cellular respiration under hypoxia: Mitochondrial oxygen kinetics at steady-state oxygen supply and aerobic-anaerobic transitions.

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When oxygen supply to tissues is restricted and becomes limiting, mitochondrial respiration declines in the transition to hypoxia and aerobic ATP production is compromised. Accurate information on oxygen kinetics of mitochondrial respiration is required to assess the bioenergetic consequences of intracellular oxygen levels and for modelling oxygen gradients in the cell. Recent methodological advances enabled us to address the question, whether mitochondrial respiratory responses to hypoxia differ under conditions of dynamic aerobic-anaerobic transitions compared to steady-state conditions of limiting oxygen supply.

A murine cell line (parental hematopoietic 32D cells) was chosen as a model, since these cells grow in suspension and are small, such that intracellular oxygen gradients remain below the limit of detection [1]. Mitochondrial oxygen kinetics in response to continuously declining oxygen levels was measured at 37 °C in the closed chambers of the Oxygraph-2k (Oroboros Instruments, Austria [2]). After a brief period of anoxia, experiments were continued by monitoring the oxygen dependence of respiration at constant levels of hypoxia in the range of 0.15 to 0.5 of oxygen-saturated respiratory flux. Continuous oxygen supply was maintained over periods of 5 to 20 min by injection of air-saturated incubation medium into the stirred respiration chamber, using an electronically controlled titration-injection-micropump [2,3]. The development of a feedback control system made possible the extension of steady-state studies to higher oxygen levels, maintaining defined windows of oxygen concentration which supported 0.5 to 0.9 of oxygen-saturated flux.

Respiration of intact cells was maintained at 0.95 of oxygen-saturated *ROUTINE* activity at an oxygen pressure of 1.0 kPa (10 µM O_2). Aerobic-anaerobic transitions followed precisely monophasic hyperbolic kinetics in the low-oxygen range <1.1 kPa. The oxygen pressure or concentration supporting half-maximum flux (p_{50} or c_{50}) varied in the range of 0.03 to 0.07 kPa (0.3 to 0.7 µM) as a linear function of *ROUTINE* respiratory activity (10 to 25 pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells), in agreement with the original kinetic model for cytochrome *c* oxidase by Chance [4]. Based on the hyperbolic parameters obtained from aerobic-anaerobic transitions, accurate predictions were possible for mitochondrial respiratory control at hypoxic steady-states extending into the nanomolar range. This is the first quantitative and direct evaluation of closed-chamber oxygen kinetics by an open-system steady-state approach. The low p_{50} agrees with isolated mitochondria [3]. These results exclude the quantitative significance of intracellular oxygen gradients in the small cells, and indicate that oxygen may limit mitochondrial respiration slightly (to 90 % of capacity) under physiological conditions. Under conditions of hypoxia routinely considered in studies of oxygen sensing and signaling, respiration is even less limited by oxygen.

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4-02L Respiratory control by oxygen and redox states of mitochondrial cytochromes in living cells.

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Simultaneous determination of respiratory redox states and rates has been essential for contributing to our understanding of respiratory control in isolated mitochondria. To extend these concepts to intact cells, increased sensitivity is required in a combined detection system. In a closed chamber containing 5-10 Mio cells/ml (such as fibroblasts or promyeloid cells 32D) it takes about 60 s for the transition from 10 µM O_2 to zero oxygen. This generates high demands on time resolution of measuring redox changes of various cytochromes of the electron transport system. Conventional gas/aqueous phase systems applied to maintain low oxygen levels constant over prolonged periods of time, on the other hand, have the problem of oxygen gradients at severe oxygen limitation. To address these methodological challenges, we applied continuous injections of air saturated aqueous medium (TIP-2k) into the chamber of an OROBOROS Oxygraph-2k (Austria). Redox states of mitochondrial cytochromes (cytochrome *b*, *c*, aa_3) were determined by measurement of difference spectra at wavelengths of 405-630 nm through a flexible glass-fibre light guide of the O2C spectrophotometer (LEA Medizintechnik, Germany). The apparent K_m for oxygen was 0.3-0.4 µM O_2 obtained from aerobic-anoxic transitions, and fully agreed with the steady state kinetics observed over 120-600 s of continuous oxygen injection. Oxygen concentrations were maintained at constant levels of 400 down to 50 nM O_2. A progressive reduction was monitored of mitochondrial cytochromes, corresponding to steady state respiration of 10-50 % of maximal rates. This combination of methods provides a valid reference for interpreting cytochrome spectra obtained in the intact organ during hypoxia and pathological states.

4-03L Kinetic model of nitric oxide inhibition of cellular respiration in intact cells.

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Physiological concentrations of nitric oxide (NO) inhibit mitochondrial Complex IV (cytochrome *c* oxidase, CcO) in a reversible manner that is competitive with oxygen [1]. Thus, mitochondrial respiration is controlled by the NO-CcO signaling pathway. Using HEK 293 cells expressing the inducible isoform of the nitric oxide synthase (iNOS) as a model in which NO is produced inside the cells in a finely controlled manner [2], we performed a detailed respirometric study of inhibition of respiration by NO at physiological oxygen levels (30 µM) and oxygen kinetics in the low oxygen range at concentrations of NO up to 1.8 µM, corresponding to pathological conditions. An electrochemical NO electrode (ISO-NOP, World Precision Instruments, Stevenage, Herts., U.K.) was introduced into the chamber of a high-resolution respirometer (OROBOROS Oxygraph-2k, Innsbruck, Austria). The higher sensitivity and reproducibility of oxygen concentration and respiration measurements at low oxygen concentrations (hypoxia) and during transitions to anoxia provided by high-resolution respirometry is in part achieved by on-line correction for instrumental background oxygen dynamics and for the time response of the oxygen sensor [3]. The combined technology allows simultaneous recording of respiration as a function of oxygen concentration and NO production evaluated by extracellular measurement. Oxygen flux at 30 µM oxygen in control cells was 16 ± 0.9 pmol.s^{-1}.10^{-6} cells (n = 55), decreasing to 4.6 ± 0.9 pmol.s^{-1}.10^{-6} cells (n = 7) in the presence of 1.8 µM NO. Hyperbolic oxygen kinetics of respiration was characterized by the p_{50} of 0.071 ± 0.004 kPa and J_{max} of 15.5 ± 1.6 pmol.s^{-1}.10^{-6} cells (n = 5) in the absence of NO. Induction of the cells to produce 1.8 µM NO, decreases the affinity of COX for oxygen and significantly alters energy metabolism which may have important pathophysiological consequences.

A kinetic model of NO inhibition of cellular respiration was developed. Both competitive reversible binding of NO to reduced cytochrome *c* oxidase (CcO) and uncompetitive binding to oxidized CcO were taken into account. Data analysis, by means of standard least squares non linear minimization routines (Matlab, the MathWorks inc., South Natick, MA, USA), showed that the best fit to the experimental data requires the affinity of CcO for O_2 to be modulated by NO bound to the enzyme, such that the species with NO bound to the uncompetitive site has higher K_m than uninhibited CcO, consistent with the inhibitory activity of NO. Both oxidized states of CcO are catalitically competent, as required by the observation that residual activity is present under high NO and low O_2 concentrations. The model has predictive value and integrates the complex chemistry of the enzyme and physiological adaptations of the cell. Interestingly, addition of NO scavengers reveals that NO has an activation effect in cells, which partially compensates for inhibition.

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4-04L Ischemia-reperfusion injury in heart mitochondria.

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4-05L Mitochondrial dysfunction in pathologies of the human heart and comparison with mitochondrial respiratory control in the mouse heart.

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Modern advances in the diagnosis of mitochondrial dysfunction make it possible for clinical laboratories to perform routine analyses of OXPHOS defects in cardiac tissues of patients undergoing heart surgery. Tissue samples are removed routinely in standard surgery (atrium cannulation, heart transplantation), providing an underutilized resource for research on mitochondrial pathologies. Extending such studies helps to reduce the research-related demand for invasive tissue biopsies. We evaluated cardiac mitochondrial respiration in small tissue samples from patients without coronary heart disease to advanced stage heart failure (HF, dilated cardiomyopathy). Coupled *OXPHOS* capacity and uncoupler-induced maximum respiration (capacity of the electron transport system; *ETS*) were measured by high-resolution respirometry in permeabilized fibers (atrial appendage, left and right ventricle). ADP-stimulated flux through Complex I was only 0.4 of *ETS* capacity, indicating a strong limitation by the phosphorylation system, in striking contrast to the flux control pattern observed in murine animal models. Maximum *ETS* capacity was observed with a combination of NADH-related substrates plus succinate, supporting convergent electron input through Complexes I and II simultaneously into the Q-cycle [1]. HF mitochondria were tightly coupled, as evaluated by respiratory control in the absence of ADP. Fibers from atrial appendage showed lower respiratory fluxes with all substrate combinations compared to left and right ventricle. Respiratory control patterns (flux ratios for specific substrates relative to ETS capacity with convergent electron flow) were similar in the three tissues of individual explanted hearts. Large-scale screening of mitochondrial defects in cardiomyopathies requires the cooperation of multiple clinical centers. Quantitative comparability of results requires standardization of tissue preparation, incubation conditions, protocols and application of general criteria for instrumental quality control. Our study may be considered as an

important step towards establishing a basic protocol for *OXPHOS* control analysis as a diagnostic tool for evaluation of the role of mitochondria in heart failure.

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1. Gnaiger E, ed (2007) *Mitochondrial Pathways and Respiratory Control.* OROBOROS MiPNet Publications, Innsbruck: 96 pp.

4-05P Functional consequences of genotypic differences studied by high-resolution respirometry in small samples of permeabilized skeletal muscle fibers.

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Loss of the HIF prolyl hydroxylase Phd1 is known to lower mitochondrial respiratory capacity with Complex I-related substrates in mouse skeletal muscle, with the consequence of increased hypoxia tolerance through reduced generation of oxidative stress [1]. Here we report the general methodological basis for studying phenotype/genotype relationships with limited amounts of tissue. A multiple substrate-uncoupler-inhibitor titration protocol was optimized as a single assay for high-resolution respirometry on 1.5 mg wet weight of permeabilized soleus fibers (37 °C). Based on oxygen kinetics analysis, an experimental oxygen range between 500 to 200 µM was chosen to avoid oxygen limitation of respiration by development of a hypoxic core within the fiber bundle [2]. Lack of cytochrome *c* stimulation indicated integrity of the outer mitochondrial membrane. Maximum ADP-stimulated flux of 82±24 (SD) pmol.s^{-1}.mg^{-1} was obtained with the substrate combination pyruvate+glutamate+malate+succinate, supporting respiration through Complexes I+II (CI+II) [3]. *OXPHOS* capacity was not limited by the phosphorylation system as shown by lack of further stimulation by uncoupling. *OXPHOS* capacity supported by Complex I or Complex II substrates alone was only 0.73 and 0.81 of maximal CI+II flux. These flux ratios were highly preserved throughout the variation of respiration among assays, providing evidence for variation of mitochondrial density in different fiber bundles. Identity of mitochondrial content, however, is suggested by equivalent Complex II respiration and citrate synthase activities in muscles from knockout and wild-type mice. NADH-linked respiration compensating for leak was 0.24 of CI+II OXPHOS capacity. In the knockout, OXPHOS capacity was reduced by 14 % and the relative leak was preserved at 0.23, providing evidence against any mild uncoupling as a possible mechanism of reducing ROS production. These results demonstrate the general potential of high-resolution respirometry and *OXPHOS* control analysis for functional diagnosis of mitochondrial defects.

This abstract was presented at: Mitochondrial Medicine 2008; Indianapolis, USA; June 2008.

1. *Aragonés et al. (2008) Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat. Genet. 40: 170-180.*
2. *Gnaiger E (2003) Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. Adv. Exp. Med. Biol. 543: 39-55.*
3. *Gnaiger E, ed (2007) Mitochondrial Pathways and Respiratory Control. OROBOROS MiPNet Publications, Innsbruck: 96 pp.*

4-06P High fat fed heart failure animals have enhanced mitochondrial function and acyl-CoA dehydrogenase activities.

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We have previously shown that administration of high fat in heart failure (HF) increased mitochondrial respiration and did not alter left ventricular (LV) function [1]. PPARα is a nuclear transcription factor that activates expression of genes involved in fatty acid uptake and utilization. We hypothesized that elevated mitochondrial respiration in high fat fed HF rats is due to increased expression of β-oxidation enzymes (short- (SCAD), medium- (MCAD), and long- (LCAD) chain acyl-CoA dehydrogenase). Rats

underwent ligation or sham surgery and were fed normal (10 % energy from fat; SHAM, HF) or high fat diet (60 % energy from fat; SHAM+FAT, HF+FAT) for 8 weeks. Subsarcolemmal and interfibrillar mitochondria were isolated from the LV and oxygen consumption was assessed using a Clark-type oxygen sensor at 30 °C. State 3 respiration using lipid substrates octanoylcarnitine (0.2 mM) plus malate (5.0 mM) and palmitoylcarnitine (0.04 mM) plus malate (5.0 mM) was increased in HF+FAT compared to SHAM+FAT and HF respectively (242±21, 246±21 vs 183±8, 181±6 and 193±17, 185±16 nAO·min^{-1}·mg^{-1}). State 4 respiration using palmitoylcarnitine was not different between groups, but was elevated in HF+FAT vs SHAM+FAT using octanoylcarnitine (53.8±3.8 vs 67.5±5.9 nAO·min^{-1}·mg^{-1}). Despite decreased *mcad* mRNA in HF and HF+FAT, MCAD protein was not different between groups, and MCAD activity increased in HF+FAT (65.1±2.7 vs 81.5±5.4 nmol·min^{-1}·mg^{-1}). Activities of SCAD and LCAD were also elevated in HF+FAT though *scad* and *lcad* mRNA expression were not different. SCAD, MCAD, and LCAD activities correlated to increased state 3 respiration using both lipid substrates. In conclusion, enhanced mitochondrial respiration associated with high fat may result from increased activation of acyl-CoA dehydrogenases, but is not due to increased mRNA or protein expression. High fat in normal animals did not adversely affect mitochondrial respiration or the expression and activity of enzymes involved in β-oxidation.

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4-07P Uncoupling effect of lauryl sulfate on mitochondria.

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The mechanism of uncoupling by lauryl sulfate (LS) has been studied in rat liver and heart muscle mitochondria. We found that electron transport can be uncoupled by low (70 μM) LS concentration in a fashion completely arrested by the ATP/ADP antiporter inhibitor carboxyatractylate (CAtr). On the other hand, uncoupling by two-fold higher LS concentration was not sensitive to CAtr. Addition of oleate desensitized mitochondria to low LS so that addition of bovine serum albumin (BSA) became necessary to recouple mitochondria. These results can be explained by assuming that low LS releases endogenous fatty acids from some mitochondrial depots and these fatty acids are responsible for uncoupling. As to high LS, it causes a non-specific (CAtr-insensitive) damage to the mitochondrial membrane.

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4-08P Measurement of mitochondrial respiratory capacity in permeabilized muscle fibers from cold adapted mice.

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Background: Mitochondrial respiration is a principal feature determining tissue metabolic health. Adaptation to cold is associated with brown fat thermogenesis and with an increase in UCP3 expression in muscle mitochondria [1,2]. However, adaptive changes of muscle mitochondrial respiration in response to prolonged cold stress are not fully elucidated. This prompted us to investigate the mitochondrial respiration in mice adapted to a cold environment.

Methods: Mitochondrial respiration in soleus muscle of male mice adapted to cold (4 °C) for 30 days (n=4) was compared to that of control mice housed at an ambient

temperature of 25 °C (n=4). Muscle fibers (6.09±0.11 mg) were permeabilized by saponin treatment (50 µl/ml, 15 min, 4 °C). Oxygen measurements were performed in MiR05 respiration medium at 37 °C using an oxygen sensor (Oxytherm, Hansatech, UK) and the following substrate-inhibitor protocol [3]: G_N (5 mM) +D (5 mM) +S_D (5 mM) +Omy (2 µg/ml) +c (19 µM) +Mna (2 mM) +KCN (10 mM). Addition of cytochrome *c* did not increase respiration.

Results: Cold adaptation was not associated with a modulation of *LEAK* respiration (G_N). However, respiration rate with glutamate increased after addition of ADP 5.2-fold (G_D/G_N) in cold acclimatized mice and 8.8-fold in control mice. The maximal *OXPHOS* capacity in the coupled ADP-activated state was reached by the substrate combination of glutamate and succinate (GS_D). Respiration with glutamate (complex I substrate, G_D) represented 77.9 % and 71.8 % of the maximal respiratory rate in cold adapted and control mice, respectively. Succinate (complex II substrate), however, contributed only 22.1 % and 28.2 % to the rate in cold adapted and control mice. Interestingly, oligomycin inhibited GS_D respiration rate only slightly - by 8 % (cold acclimatized) and 12 % (control). Administration of malonate, inhibiting the utilization of succinate, led to 83.1 % and 76.7 % decrease of the maximal (GS_D) respiration rate in cold adapted and control mice, respectively. The residual oxygen consumption after addition of KCN was 5.6% (control mice) and 7.4% (cold adapted mice) of the maximal respiration rate (GS_D).

Conclusions: These results indicate a tendency toward lower respiratory capacity of soleus muscle mitochondria in response to prolonged cold stress. Similar experiment was done by Mollica et al, 2005. They measured state 3 and state 4 respiration in the presence of succinate as a substrate in skeletal muscle mitochondria isolated from cold exposed (15 days) and control rats. They did not notice any significant changes between groups, but state 3 and state 4 respiration rate had a tendency toward lower respiration rate in cold acclimatized mice, similar as we had.

Abbreviations: G_N glutamate without ADP, D addition of ADP, S_D succinate in the presence of ADP, Omy oligomycin, *c* – cytochrome *c*, Mna – malonate

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4-09L Depressed endogenous mitochondrial respiration stimulates elevated cardiac superoxide production in spontaneously hypertensive rats.

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Much attention has been focused on mitochondrial energy production (ATP), and its export (to form creatine phosphate) and consumption in failing heart muscle. However, less interest has been directed towards these functions in the compensated hypertensive heart, where functional complications are less obvious yet may provide insight to disease ontogeny. Hearts from twelve-month old Spontaneously Hypertensive Rats (SHR) with compensated cardiac hypertrophy were compared with those from age- and sex-matched normotensive Wistar-Kyoto (WKY) rats. *Ex vivo* working hearts from SHR showed reduced compliance and impaired responses to increasing preload (Frank-Starling response). High resolution respirometry of permeabilized LV fibres from hypertrophied

SHR hearts revealed elevated State 3 (with excess ADP) respirational flux with glutamate and malate, and increased uncoupled flux rates with glutamate, malate and succinate and elevated cytochrome c oxidase flux rates. These data conform to the concept that cardiac mitochondria from SHR are functionally better than those from WKY rats. However, SHR fibre affinities (apparent K_M) for ADP were 54% greater, while endogenous respirational flux (ATP fuelled) and actomyosin ATPase activities were 15% and 50% lower respectively than those observed in WKY fibres. Superoxide ($O_2{\cdot}^-$) production when fibres respired endogenously was 15% greater in SHR fibres than WKY fibres, as was concomitant tissue lipid peroxidation. These data indicate that impaired ATP turnover in hard-working SHR hearts may starve mitochondria of ADP. Slowed ADP supply may consequently impair mitochondrial respiratory complexes and elevate $O_2{\cdot}^-$ production, which then progressively damages the SHR heart.

Day 5: Mitochondrial molecular physiology

5-01L Mitochondria and apoptosis: Energy-limited states and signaling for cell death.

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Correlations that implicated mitochondrial involvement in apoptosis have accumulated for some time, but it was in 1996–1997 when it became clear mitochondria were not just bioenergetic organelles but also controlled life and death decisions in the cell. Cytochrome *c* (cyt-*c*) release by mitochondria in the progression of mammalian apoptosis sparked the realization that mitochondria play a critical gatekeeper role. We will briefly discuss the apoptotic machinery for the three most well-studied systems, those of *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian cells. At least five factors that reside in the mammalian mitochondrion are involved in caspase-dependent and -independent Programmed Cell Death (PCD). Depending on the nature of the death signal, these factors are released through permeabilization of the outer, or inner and outer, mitochondrial membrane. The NADH-oxidase AIF and the endonuclease EndoG translocate to the nucleus where they are involved in chromatin condensation and DNA degradation. Other effectors impact the PCD machinery in a caspase-specific fashion. SMAC/DIABLO releases caspases from inhibition by IAPs. IAPs are intrinsic regulators of the caspase cascade and are the only known endogenous proteins that regulate the activity of both initiator (caspase 9) and effector (caspase 3, caspase 7) caspases. The serine protease Omi contributes to PCD in two ways. Omi neutralizes inhibition of caspases by IAPs, and also contributes to caspase-independent apoptosis through its protease activity. Within the intermembrane space, cyt-*c* is essential for oxidative phosphorylation, but after release into the cytoplasm, it initiates the assembly of the apoptosome, i.e. the molecular machinery that activates caspase 9.

Mitochondrial outer membrane permeabilization (MOMP) in mammals involves a complex interplay between the pro- and anti-apoptotic proteins belonging to the Bcl-2 family. Bcl-2 family proteins are divided into three subfamilies: multi-domain anti-apoptotic (e.g. Bcl-2, Bcl-xL), multi-domain pro-apoptotic (e.g. Bax, Bak) and pro-apoptotic BH3-only proteins (e.g. Bid, Bad). MOMP is mediated by the pore-forming proteins Bak and Bax, whose activation is promoted by BH3-only proteins. In non-apoptotic cells Bak is tail-anchored to the outer mitochondrial membrane, whereas Bax is mostly cytosolic. During apoptosis Bax translocates to the mitochondrion where it changes conformation and inserts into the outer membrane. Bax and Bak undergo conformational changes, oligomerize and form pores in the outer membrane. Secondly, when mammalian mitochondria are exposed to high Ca^{2+} in the presence of the co-activator P_i, especially when accompanied by adenine nucleotide depletion and a reduced $\Delta\Psi$, the opening of the mitochondrial permeability transition pore (MPTP) can occur.

Oxidative stress as a result of the generation of ROS is another modulator. The increase in permeability of the inner membrane to solutes causes swelling of the matrix, rupture of the outer membrane, and release of pro-apoptotic factors from the intermembrane space.

Cellular conditions experienced during energy-limited states – elevated calcium, shifts in cellular adenylate status, compromised $\Delta\Psi$ – are precisely those that trigger, at least in mammals, opening of the MPTP. How is activation of mitochondria-based pathways for the signaling of apoptotic and necrotic cell death avoided under conditions of hypoxia, anoxia, diapause, estivation and anhydrobiosis? Embryos of the brine shrimp, *Artemia franciscana*, survive extended periods of anoxia and diapause, and evidence indicates that opening of the MPTP and release of cyt-*c* do not occur. Further, caspase activation in this crustacean is not dependent on cyt-*c*. Its caspases display regulation by nucleotides that is consistent with 'applying the brakes' to cell death during energy limitation. Unraveling the mechanisms by which organisms in extreme environments avoid cell death may suggest possible interventions during disease states and biostabilization of mammalian cells.

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Readings:

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5-02L Integrated mitochondrial function and the clinical utility of polarographic analysis.

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5-03L Protein targeting and import into mitochondria: The process, its conserved nature, and potential for mitochondrial stabilization.

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Only approximately 1% of all mitochondrial proteins are encoded by the mitochondrial genome. Proteome analyses suggest about 800 (yeast) up to 1500 (human) different proteins are associated with mitochondria. Thus the vast majority of mitochondrial proteins are encoded by the nucleus and must be imported and targeted to one of four locations: outer membrane, intermembrane space, inner membrane or matrix. Precursor proteins can be separated into two main classes: (a) preproteins destined for the matrix, and a smaller number heading to the inner membrane and intermembrane space, carry a N-terminal, positively-charged, cleavable presequence; (b) Precursor proteins destined for the outer membrane, and many intermembrane space and inner membrane proteins, carry various internal targeting signals. These precursors do not have cleavable extensions, and have the same primary sequence as the mature proteins.

The translocase of the outer mitochondrial membrane (TOM) is a central entry gate for practically all nuclear-encoded mitochondrial proteins. After recruitment to and passage through TOM, precursor proteins follow one of three major pathways. (a) Preproteins with a presequence (those of this type destined for the matrix) are transferred to the translocase of the inner membrane (TIM23 complex; channel forming). TIM23 cooperates with mt Hsp70, which represents the core of the presequence translocase-associated motor (PAM). (b) Precursors of hydrophobic proteins of the inner membrane (e.g., the ANT) utilize chaperone-like proteins (Tim9, Tim10, Tim12) and the insertion machinery of the inner membrane (TIM22 complex; the 'carrier protein' translocase). c) Precursor proteins with complicated topologies (e.g., porin, Tom40) are first imported via the TOM complex to intermembrane space, then via small Tims (9, 10) are passed on to the SAM Complex (sorting and assembly machinery) for insertion into the outer

membrane. Other smaller proteins with cysteine motifs are aided in their targeting to the intermembrane space by the mitochondrial import and assembly machinery (MIA; main component Mia40), which promotes the formation of intramolecular disulfides and initiates the mature conformation of these proteins.

The Tom40 pore is conserved among mitochondria of animals, fungi, and plants. Recently, we have discovered a Late Embryogenesis Abundant (LEA) protein in embryos of the brine shrimp *A. franciscana* that is targeted to the mitochondrion via a N-terminal leader sequence (29 AA). LEA proteins are known for their ability to stabilize biological structures against stress incurred during drying. Expressing a fusion protein composed of the leader sequence from this LEA protein and GFP (green fluorescent protein) shows that this chimeric protein is imported into mitochondrial network of human hepatoma cells (HepG2/C3A). The results indicate the highly conserved nature of the protein import machinery for mitochondria of mammalian and invertebrate cells [perhaps including TIM23, PAM and the mitochondrial processing peptidase (MPP)] and indirectly, the targeting sequence as well. A potential application for the sugar trehalose and this mitochondrial-targeted LEA protein (AfrLEA3) for stabilization of mitochondria during freezing and drying will be discussed.

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5-04P Differential inhibition of energy-producing pathways of hepg-2 cells by 3-bromopyruvate.

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The effects of the alkylating agent 3-bromopyruvate (3-BrPA) on ATP producing pathways were studied using HepG2 cells. 3-BrPA decreases HepG2 viability (10 % with 10–100 μM and 25 % with 300–1000 μM when the cells were incubated for 60 min. At longer times (180 min), the range of decrease in viability were about 60 % with 10–100 μM and 75 % with 300–1000 μM. The LD_{50} for 3-BrPA was 2.1±0.12 mM for 60 min and 0.076±0.04 for 180 min. The hexokinase-II activity, measured in the mitochondrial fraction following the [^{32}P]glucose-6-P formation in the presence of 150 μM 3-BrPA, was not affected. However, incubation of HepG2 cells with 150 μM for 30 min decreased in 60 % lactate production. Cells were incubated with 3-BrPA as above in glucose-free (DMEM) medium containing 2 mM glutamine as oxidizable substrate (GFM-cells) or glucose-free (DMEM) medium supplemented with 5 mM glucose (GM-cells) and the basal respiration was 35.0±2.1 (GM-cells) or 51.0±3.2 (GFM-cells) pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells (SE; N=5). A decrease of 22 % or 50 % of basal respiration by 3-BrPA was detected in GM or GFM cells, respectively. Respiration measured in the presence of 1 μg/mL oligomycin was increased only in GM-cells (from 11.0±1.5 to 17.0±1.7 pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells; N=5). The oligomycin-inhibited respiration was also increased by 3-BrPA treatment in permeabilized HepG2 cells in GM. Maximum uncoupled respiration induced by the proton ionophore FCCP (0.5 or 1 μM) was decreased by 3 BrPA treatment only in GFM intact or digitonin-permeabilized cells (from 57.7±3.7 to 31.7±4.3 pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells for intact cells; N=4). 3-BrPA decreased respiratory control ratios either in GM or GFM cells. In digitonin-permeabilized cells, Complex I supported respiration was decreased by 50 % in GFM-cells and Complex II supported respiration was inhibited by 50 % in both media. Our results suggest that glycolysis and specific sites of mitochondria play a role in 3-BrPA induced HepG2 death. The toxic effects of 3BrPA depend on the oxidizable substrates supplied to cells.

5-05P **PKA stimulation protects transformed cells from apoptosis induced by glucose depletion, by re-activating mitochondrial activity.**

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The link between cancer genetics and abnormal use of glucose by tumors is opening a new scenario in which bioenergetics would contribute to, and sustain, malignant transformation [1].

We showed that *K-ras* transformed mouse fibroblasts are exquisitely sensitive to glucose deprivation, showed reduced oxidative phosphorylation ability and an enhanced apoptosis under glucose depletion as compared to their normal counterparts [2]. Ras-dependance of the observed phenotypes was confirmed by analysis of transformed cells that expressed a Ras-inhibitory dominant negative GEF [3]. Moreover our results suggested that the metabolic rerouting observed in transformed cells is due to both transcriptional and post-translational mechanisms [2,4]. More recently we have found that mitochondria of transformed cells are partially deranged. Such derangement, as revealed by a specific set of experiments, seems to be a consequence of a reduced activity of respiratory chain Complex I (unpublished data). Therefore, to evaluate the relevance of the transcriptional and the post-translational events in such respiratory chain alterations, time-dependent changes in transcriptome and specific activation of PKA pathway - able to control mitochondrial activity - of normal and transformed cells growing in media supplemented with either high (25 mM) or low (1 mM) initial glucose concentration has been performed. Such analysis allow us to relate mitochondria dysfunction with an extensive change in expression of genes encoding oxidative respiration chain proteins (studied by Affymetrix analysis) and with a lack of a post-translational modification able to positively regulate the mitochondrial activity. Indeed, as we show in this report, stimulation of PKA activity by Forskolin treatment, of both normal and transformed cells grown in 25 and 1 mM glucose, protects transformed cells from apoptosis induced by glucose deprivation, by enhancing the mitochondrial potential, the intracellular ATP levels and decreasing the intracellular ROS accumulation.

We therefore hypothesized that enhancement of PKA activity in transformed cells induces their survival under glucose depletion by both transcriptional and post-translational mechanisms.

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5-06P **Seleno compounds prevents the brain energy impairment induced by methylmercury poisoning in adult mice.**

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Methylmercury (MeHg), an organic form of mercury, has been shown to cause severe and irreversible neurobehavioral and neuropsychological disorders in both humans and animals. The objective of the present work was to investigate the potential protective

effect of chronic subcutaneous administration of sodium selenite, Na_2SeO_3, and diphenyl diselenide, $(PhSe)_2$, on the respiratory chain function in adult Swiss albino male mice exposed to MeHg poisoning. Adult male mice were treated with MeHg dose of 40 $mg \cdot L^{-1}$ diluted in drinking water during 21 days and the seleno compounds were daily subcutaneously injected (5 $\mu mol \cdot kg^{-1}$). Control animals received drinking water *ad libitum*. The activities of Complexes I, II, II-III and IV (CI-IV) were assessed in mitochondrial preparations from mice cerebral cortex 24h after the last subcutaneous administration. Enzyme activities were measured spectrophotometrically. It was initially demonstrated that MeHg administration provoked a significant inhibition of the electron transport chain in mitochondrial preparations by diminishing the activities of CI-IV. Next, we observed that $(PhSe)_2$ chronic co-administration significantly prevented the reducing MeHg effect on the activities of CI-IV of the respiratory chain in cortical mitochondrial preparations. On the other hand, the inorganic seleno compound showed a differential effect; while it prevented the inhibition of the activity of CII-III and CIV, it potentiated or not modified the inhibitory effect of MeHg on CI and CII, respectively [CI: $F_{(5,26)}=40.93$; $P<0.0001$; CII: $F_{(5,25)}=13.16$; $P<0.0001$; CII-III: $F_{(5,21)}=5.55$; $P<0.01$; CIV: $F_{(5,21)}=5.55$; $P<0.01$]. Altogether, these data provide evidence for the first time, that the inhibitory effect of MeHg poisoning on the electron transfer chain in mice cerebral cortex might be prevented by using seleno compounds. This strongly suggests that impairment of brain energy metabolism might be involved in the mechanisms by which MeHg causes neurotoxicity and that, particularly, $(PhSe)_2$ could be considered as a neuroprotective agent in MeHg-induced brain poisoning, probably because of the highly nucleophilic activity of its reduced form, phenylselenol, which could directly react with MeHg.

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5-07P Periodontitis: Role of oxidative phosphorylation.

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Periodontitis is an inflammatory disease affecting the tissues that surround the teeth, as a result of complex interactions between pathogenic bacteria and the host's immune response. There is increasing evidence for the involvement of reactive oxygen species in the pathogenesis of inflammatory disorders, including periodontitis where mtDNA deletions have been identified [1]. Therefore, we aimed to look at the mitochondrial dysfunction; with respect to clinical, radiological, biochemical, cell biology and genetics aspects of periodontitis. We have examined a total of 92 patients, of which 30 chronic periodontitis patients were selected for the above analysis. The institutional ethics committee approved this study and informed written consent was obtained from each participant. All clinical parameter scores of the tissue sampled areas were significantly higher ($P<0.001$) than in the control group. Complete mtDNA sequencing revealed a total of 16 novel mutations in 25 patients, of which A4234T, A7796G and G8115R were missense mutations. Mitochondrial inner membrane depolarization is one of the major alterations observed in the patients [2]. Mitochondrial inner membrane potential was analyzed by FACS using fluorochrome 3.3`dihexyloxacarbocyanine iodide (DiOC6). We observed a decrease in the mitochondrial inner membrane potential of up to 40 % in patients compared to controls. Heat Shock Protein (HSP) 60 is a mitochondrial matrix protein, involved in the folding and correct assembly of polypeptides into multimeric enzymes [3]. Analysis of HSP 60 by immunoblotting revealed complete absence of HSP 60 in 3 out of 17 patients. However, the cytosolic HSP 70 levels were normal compared with controls. Cytochrome *c* oxidase (Complex IV) protein shows differential expression in the patients compared with the controls.

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5-08S **Mitochondria and the future of death.**

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Death in the past was largely digital, that is all-or-nothing, because people mainly died young from infectious diseases, starvation or violence. But death is now becoming an analogue process, because people are dying old and slowly from degenerative disease driven by aging. Mitochondria contribute to this process by producing reactive oxygen species, by regulating cell death and by becoming dysfunctional with age.

Cell death has in the past also been thought to be digital. But we are now starting to recognise a continuum between different forms of cell death, and even reversible forms of 'death'. Apoptosis has been considered as a form of cell death, to which a cell is irreversibly committed once cytochrome c is released from the mitochondria. However, apoptosis can also be regarded as a means of signalling to phagocytes, which may in principle be reversible up to the point of phagocytosis. We find that mitochondrial cytochrome c release does not inevitable commit a cell to death because if the cell reduces the cytosolic cytochrome c then caspase activation is blocked. Similarly caspase activation does not inevitably commit a cell to death, because the caspases can be inactivated by endogenous oxidants. Phosphatidylserine flip to the outer leaflet of the plasma membrane is also a reversible process, as long as phagocytyes are not present to eat the cell. We find that many aspects of apoptosis are fully reversible in neurons. This leads to the conclusion that apoptosis can (in some circumstances) be reversible and is not always a form of cell death.

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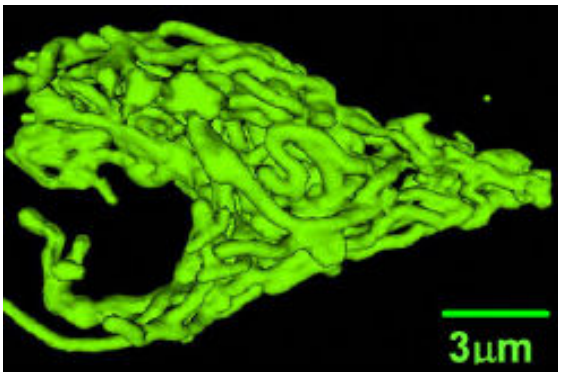
MiP*Art* – Mitochondrial Physiology and Art

Mythochondrion (Patricia Karg)



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