Mitochondrial Physiology Network 8.2: 1-17

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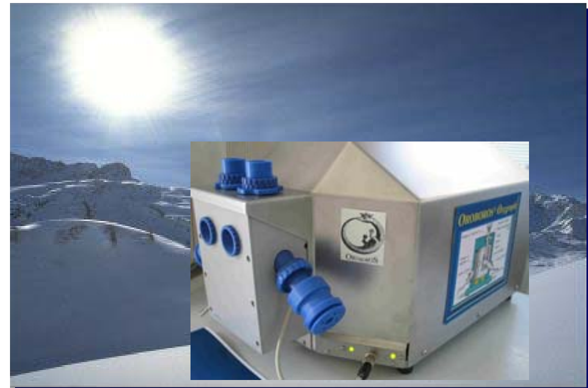
Mitochondrial Physiology (MiP) Workshop on High-Resolution Respirometry



27-31 March, 2003 Schröcken, Vorarlberg, Austria

OROBOROS INSTRUMENTS, Schöpfstr. 18, A-6020 Innsbruck, Austria.

Programme

Thursday, 27. March

Registration and informal evening at Hotel Mohnenfluh.

Friday, 28. March

08:30 - 11:45 **Erich Gnaiger (Innsbruck)** www.oroboros.at - **the *Oxygraph-2k* and hot topics in MiP.** MiP-2 and MiP-9

12:00 Ski break (bus leaves at 12:11 from Hotel Tannberg)

16:15 -19:00 **Erich Gnaiger and Christoph Schinagl (Innsbruck) *Oxygraph-2k*: Instrument demonstration.**

19:00 Dinner

20:30 **Hot topics in Mitochondrial Physiology - MiP presentations.**

Full attention (left to right): *Jie, Walter, Maria del Mar, Jordi, Ilka, Christoph, Pavel, Karin and Eveline.*

Jordi Bermudez (Departament Ciències Fisiològiques II, University of Barcelona, Spain) Protective effects of fructose-1,6-P2 against hepatic injury induced by galactosamine. MiP-1

Rodrigue Rossignol (INSERM-EMI, Université Victor Segalen-Bordeaux 2, F) Mitochondrial remodelling occurs in cancer cells forced to derive energy from OXPHOS. MiP-10

Naïg Gueguen (INRA Unité Mixte de Recherche sur le Veau et le Porc, Saint Gilles, France) Functional properties of mitochondria from different contractile fiber types. MiP-4

Maria del Mar González-Barroso (CNRS-UPR, Faculté de Médecine Necker-Enfants Malades, Paris, F) The Uncoupling proteins in mice. MiP-3

Walter Rossmanith (Inst. Anatomy, University Vienna, A) Mitochondrial function in paraplegics with long-term denervated degenerated muscles and effects of the use of electrical stimulation to restore standing.

Ješina Pavel (Center for Integrated Genomics, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, CZ) Hydrogen peroxide production by mitochondrial glycerophosphate dehydrogenase (mGPDH). MiP-7

Saturday, 29. March

What's up? Left to right: *Mathias, Kristin, Klaus, Naïg, Mikael* and *Eduardo.*

08:30 - 12:00 **Hands-on experiments with the *Oxygraph-2k* - the basis of High-Resolution Respirometry: Data acquisition; static and dynamic calibration; instrumental background control.**

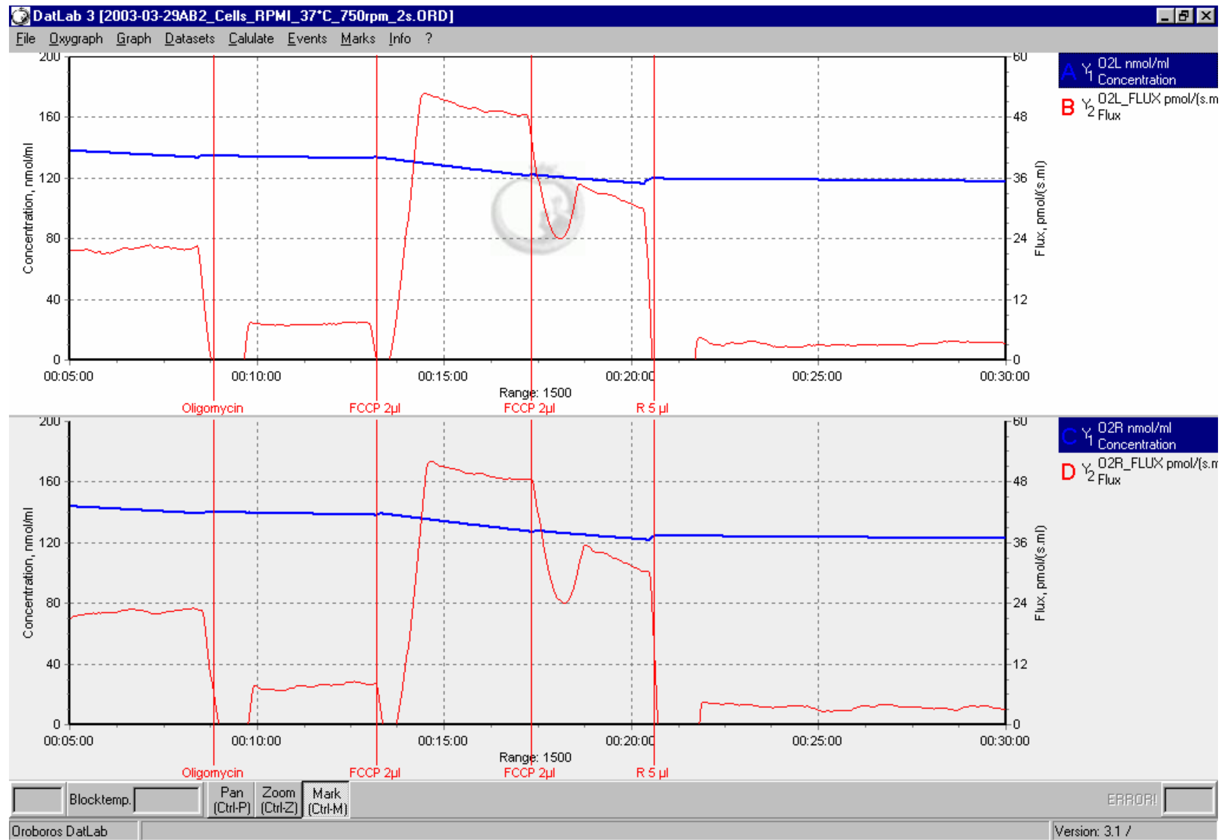
12:00 Lunch

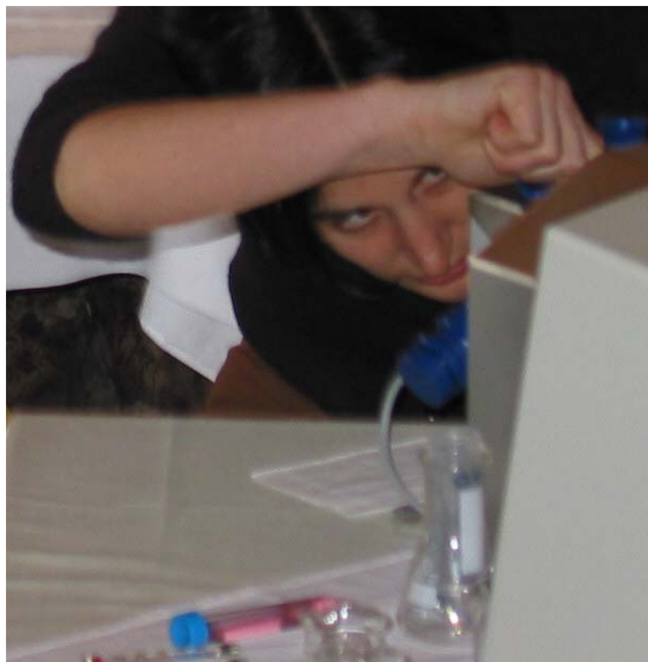
13:30 - 14:30 **Kathrin Renner (Innsbruck) Experimental demonstration with the *Oxygraph-2k*: High-Resolution Respirometry with leukemia cells - A. The experiment.**

Kathrin and the Oroboros *Oxygraph-2k* in action. The results are shown on the following page. For further details see *Mitochondrial Physiology News* **8.9** (2003) A high-resolution respirometry workshop-experiment with leukemia cells: Routine respiration, oligomycin-state 4, uncoupling, and rotenone inhibited states.

Literature: Renner K, Kofler R, Gnaiger E (2002) Mitochondrial function in glucocorticoid triggered T-ALL cells with transgenic Bcl-2 expression. *Molec. Biol. Rep.* 29: 97-101.

Renner K, Amberger A, Konwalinka G, Kofler R, Gnaiger E (2003) Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. *Biochim. Biophys. Acta* 1642: 115-123.





Hands-on experiment during the *Oxygraph-2k* workshop. *Kathrin* closes the chamber and takes a close look through the window – are there any gas bubbles in the medium?

Top: Screen shot from the demo-experiment with lymphoblastoma cells suspended in culture medium (RPMI). On-line display of results with *DatLab 3.1* for both chambers operated as replicate experiments in parallel (top and bottom graph for left and right chamber, respectively, over a 30 min period). Thick lines (blue): oxygen concentration [µM]; thin lines (red): oxygen flux [pmol·s^{-1}·ml^{-1}]. Vertical lines are events for additions of *(1)* oligomycin (2 µl), reducing endogenous respiration to resting State 4o (inhibition of ATP synthase); *(2)* FCCP, stimulating respiration to the uncoupled State 3u; *(3)* a second FCCP titration illustrating inhibition by excess uncoupler concentrations; and *(4)* inhibition by rotenone (inhibitor of complex I). Note some salient features of high-resolution respirometry: Low respiratory activities (small changes of oxygen concentration over 20 min) yield highly reproducible information on oxygen flux in the two chambers. Even inhibited flux (rotenone) is measured with high accuracy and stability.

14:30 - 15:30 Valentin R. Zhelyaskov and Jie Sun (World Precision Instruments, Sarasota, USA) Detection of free radicals and reactive oxygen species (ROS). MiP-11

Valentin inserting the WPI NO sensor into the chamber of the *Oxygraph-2k* (left), *Jie* expands the topic of electrochemical sensors to WPI free radical and ROS determination kits (right).

16:00 - 17:00 Hot topics in Mitochondrial Physiology - MiP presentations.

Kristin Heerlein (Department of Internal Medicine, Medical University Clinic Heidelberg, DE) Hypoxia reduces cellular oxygen consumption and Na/K-ATPase activity of alveolar epithelial cells. MiP-5

Kathrin Renner (Tyrolean Cancer Research Institute, Innsbruck, A) Apoptosis and mitochondrial function in glucocorticoid triggered leukemia cells with transgenic Bcl-2 expression. MiP-8

Eveline Hütter (Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, A) Respiration, coupling, ROS and aging in primary fibroblasts. MiP-6

17:00 - 23:00 Snowshoe walk and evening in the *Alpmuseum uf m Tannberg*
Special fee: 15.- Euro.
From Schröcken via Alp Felle to Alp Batzen (2 h walk).
19:00 Welcome at the *Alpmuseum.*
20:30 Dinner at Hotel Körbersee - www.koerbersee.at.
Hiking boots are recommended.

Josef Staggl (Schröcken) takes the lead.

Batzen **www.alpmuseum.at**

Sunday, 30 March

09:15 - 11:45 **Experimental demonstration with the *Oxygraph-2k*: High-Resolution Respirometry with leukemia cells - B. Evaluation.**
Demonstration: *DatLab* for data analysis
12:00 Ski break (bus leaves at 12:11 from Hotel Tannberg)
16:15 – 19:00 **Hands-on practice in groups: *Oxygraph-2k* handling and sensor service. / *DatLab* analysis.**
19:00 Dinner

20:30 **Workshop conclusions**
Integration of scientific presentations by several delegates was generally considered a valuable opportunity for scientific exchange on topics of MiP. In particular, PhD students and young invetigators appreciated this opportunity for a first presentation of their work in an international but informal setting. The travel to the quite remote location of Schröcken did not present a major obstacle and was rewarded by the Alpine scenery, and opportunities for discussions in an undisturbed and personal atmosphere, for skiing, and a snowshoe walk to the Alpmuseum. The training with the *Oxygraph-2k* and *DatLab* software were extended with some participants until departure on Monday noon. This extended specific explanations to a small group of participants, who asked for more opportunities for hands-on experience.

A summary discussion around the Oroboros *Oxygraph-2k*: *Jie, Christoph, Lukas, Valentin and Philipp.*

Monday, 31 March

09:15 - 11:45 **Training: Instrumental tests and *DatLab* analysis.**
12:00 **Departure**

CONTENTS: OVERVIEW ON HIGH-RESOLUTION RESPIROMETRY
Erich Gnaiger, Innsbruck

Introduction: Mitochondrial and cellular respiratory physiology – new challenges for high instrumental performance.
High-resolution respirometry – what makes the difference? Presentation of the new OROBOROS *Oxygraph-2k*
- Low oxygen and measurement of cellular oxygen consumption – pushing the limits of detection.
- Optimum system design - the OROBOROS *Oxygraph*.
- On-line recording of oxygen concentration and flux; linear slope versus oxygen flux as a function of time.
- The concept of high-resolution calibrations – overview on instrument demonstration.

OROBOROS *Oxygraph-2k:* On-line instrumental performance
- Instrumental background: measurement and correction as a function of p_{O_2}.
- High resolution of repsiratory flux at various steady-states.
- Conceptual and methodological advantages of measurement at physiological low levels of oxygen.

- High time resolution for kinetic analyses: Determination of the time constant, dynamic corrections.
-

Polarographic oxygen sensor and *Oxygraph* service

- Cleaning of anode and cathode.
- Electrolyte and membrane application.
- *Oxygraph* - instrumental maintenance.

***DatLab* – the specialized software for High-Resolution Respirometry: Data acquisition and analysis.**

Meet and Discuss with:

Philipp Gradl, WGT Electronics - where the OROBOROS *Oxygraph-2k* is produced. Custom-designed modifications of chambers, stoppers, accessories can be discussed and elaborated.

Lukas Gradl, software security networks – where *DatLab 3.1* is coded. Your specific ideas, demands and suggestions can be discussed in a group of experts.

Travel

Arrivals: Thursday, 27. March 2003

Munich-Schröcken: Transfer Innsbruck-Schröcken (departures in Innsbruck airport, 17:00; Restaurant Rififi, Schöprstr. 14, 19:30; depending on specified arrival times in Innsbruck; c. 2.5 h by car).

Zurich-Schröcken: Train from Zurich to Bregenz; transfer Bregenz-Schröcken (departure Bregenz train station, 15:20 (c. 1 h and 15 min by car; in case of late changes call Hotel Mohnenfluh: Tel. +43 5519-203; in Austria: Tel. 05519-203).

Departure: Monday, 31. March 2003

Transfer Schröcken-Innsbruck, 12:00, according to departure times in Innsbruck.

Transfer Schröcken-Bregenz, 09:00 and 18:00, according to departure times in Bregenz.

Weather

Sub-freezing temperatures are normal. Sunshine may be very strong – bring sunglasses and sunscreen, even if you do not plan to go skiing. Protect yourself against wind and potential snowfalls (gloves, warm jacket, etc.).

Registration

Registration fee: EURO 350.-

Presentations: A beamer, slide projector and flip chart will be available for presentations. Coffee breaks will be included according to the progress of the workshop programme.

Accomodation and Location

Schröcken: www.snowworld.net/

Hotel Registration: Organized through registration. The number of rooms at the special rate in Hotel Mohnenfluh is limited. If necessary, further accomodation may be arranged in the nearby Hotel Tannberg or in appartment houses. All meals are arranged jointly in Hotel Mohnenfluh.

Hotel Mohnenfluh www.mohnenfluh.at; Tel.: +43 5519 203; hotel@mohnenfluh.at

❷ Two-room appartment shared by two participants, breakfast, lunch and dinner: EURO 65,- per night per participant.

❶ Single room, breakfast, lunch and dinner: EURO 75,- per night.

❹ Two-room appartment shared by four participants, breakfast, lunch and dinner: EURO 60,- per night per participant.

Breakfast will be served between 07:30 and 08:30 (Friday and Saturday; and between 08:00 and 09:15 (Sunday).

Skiing www.intermaps.com/skimaps/snowworld.

Bus trips are free from Schröcken to the skiing area of Salober, leaving at 12:11 at Hotel Tannberg (near Hotel Mohnenfluh). For the afternoon, the skiing pass is EURO 21.- for the skiing lifts of Salober and Warth. There is also excellent crosscountry skiing around lake Kalbelesee and Körbersee, as well as easy walking in magnificent winter scenery. Ski rental is available in Schröcken and at Salober (top ski is Euro 16.- (1 day), 30.- (2 days), 42.- (3 days) or 52.- (4 days). You can return to Schröcken by skiing or by the free bus (leaving 15:30 at Salober).

Contact

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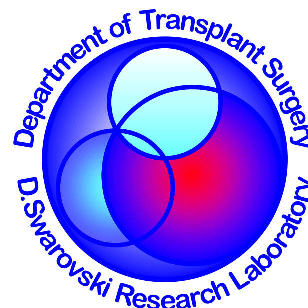

OROBOROS INSTRUMENTS
high-resolution respirometry

Oxygraph-2k

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Homepage: http://www.oroboros.at
Cooperation and Feedback in Science

Hot topics in Mitochondrial Physiology – MiP Abstracts

MiP-1. Protective effects of fructose 1,6-P2 against galactosamine-induced hepatic injury in rats

Cuesta Eduardo, Boada J, Perales JC, Roig T, ***Bermudez Jordi***
Unitat de Biofísica. Departament de Ciències Fisiològiques II. Universitat de Barcelona. L'Hospitalet de Llobregat 08907. Spain.

Fructose 1,6-biphosphate (F1,6BP) is a glycolytic intermediate that, when administered exogenously, protects organs and tissues against injuries induced by hypoxia/ischemia or toxic agents. In addition, the presence of F1,6BP in perfusion and preservation media increases the viability of grafts. Despite these beneficial effects, the mechanism is controversial. To provide new insights we studied the protective mechanisms of F1,6BP against galactosamine (GalN)-induced hepatitis in rats. The liver specificity of GalN is attributed to the high levels of galactokinase and UDP-glucose:galactose-1-P-uridyltransferase in hepatocytes. GalN metabolism impairs glycolysis and mitochondrial function, increases intracellular Ca^{2+} and ROS and depletes the ATP and uridine pools. Following the reduction of uridine pools, transcription activity and protein synthesis become arrested in hepatocytes, which increases the sensitivity of these cells to TNF-α. Inflammation then causes massive apoptosis of parenchyma cells and organ failure *in vivo*, which closely resembles viral hepatitis. F1,6BP pre-treatment rendered rats resistant to GalN injury. Studies *in vivo* and *in vitro* indicated that F1,6BP prevented GalN-induced oxidative stress and impairment of hepatocyte metabolism, thus maintaining ATP levels and reducing apoptosis. However, F1,6BP pre-treatment did not prevent the GalN-induced depletion of uridine pools in hepatocytes, so the liver cells remained highly sensitive to TNF-α, because of their inhibited transcription activity. We hypothesised that, in addition to the beneficial effects on the metabolism of hepatocytes, the protective effect of F1,6BP *in vivo* involves other cells types implicated in the inflammatory response of the liver. Our results suggest that the site of action of F1,6BP is the cell membrane and we provide evidence that F1,6BP inhibits the GalN-induced degranulation of mastocytes and activation of macrophages, reducing endotoxemia and inflammation in rats. The ability of F1,6BP to increase glycolysis and mitochondrial efficiency, preventing oxidative stress and apoptosis, confirms its potential use as a component of graft preservation media. In addition, the prevention of GalN-induced sepsis and inflammation in rats pre-treated with F1,6BP increases its interest as an anti-inflammatory drug.

MiP-2. NO effect on mitochondrial oxygen kinetics at low oxygen. *Oxygraph-2k* Workshop Report, University of Alabama at Birmingham 2002

Erich Gnaiger[1,2], *Jeannette E. Doller*[3], *Dave Kraus*[3], *Scruti Shiva*[4], *Paul S. Brookes*[4], *Victor M. Darley-Usmar*[4]
[1]*Department of Transplant Surgery, D. Swarovski Research Laboratory, University Hospital Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria; E-mail: erich.gnaiger@uibk.ac.at;* [2]*OROBOROS, Bioenergetics and Biomedical Instruments, Schöpfstr. 18, A-6020 Innsbruck, Austria;* [3]*Department of Biology, University of Alabama at Birmingham, Birmingham AL 35294-1170, USA;* [4]*Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

Most kinetic studies on the effect of NO on respiratory flux in isolated mitochondria or cells were restricted to high oxygen levels (>1-3 kPa; >10-30 µM O_2).[1] High-resolution respirometry resolves oxygen kinetics into the sub-micromolar range of oxygen concentration, which should contribute to resolving open problems on the kinetics and mechanism of competitive inhibition of cytochrome *c* oxidase by nitric oxide, NO. Specifically, a sigmoidal response of respiration to oxygen at 50 and 200 nM NO has

been reported in a study of isolated mitochondria in the range of air saturation to 30 µM O_2.[2] A single pilot experiment was carried out during an *Oxygraph-2k* workshop on high-resolution respirometry. Respiration of isolated rat liver mitochondria was inhibited by addition of NO, which increased the sensitivity to oxygen >25-fold when compared to the half-saturation oxygen pressure, p_{50}, in the absence of NO. Oxygen kinetics followed a monophasic hyperbolic function up to 2.2 kPa with NO (p_{50}=0.93 kPa), compared to the standard oxygen range to 1.1 kPa without NO (p_{50}=0.035 kPa; see also ref. 3). Adding an NO sensor to the *Oxygraph-2k* will further increase the potential of high-resolution respirometry. The *Oxygraph-2k* and *DatLab* software are designed to accommodate additional channels as an extension to the OROBOROS *BioenergeticsAnalyzer*.

1. Gnaiger E, Kuznetsov AV (2002) Mitochondrial respiration at low levels of oxygen and cytochrome *c*. *Biochem. Soc. Trans.* 30: 242-248.
2. Koivisto A, Matthias A, Bronnikov G, Nedergaard J (1997) Kinetics of the inhibition of mitochondrial respiration by NO. *FEBS Lett.* 417: 75-80.
3. Gnaiger E, Lassnig B, Kuznetsov AV, Margreiter R (1998) Mitochondrial respiration in the low oxygen environment of the cell: Effect of ADP on oxygen kinetics. *Biochim. Biophys. Acta* 1365: 249-254.

MiP-3. The uncoupling proteins in mice

***González-Barroso Maria del Mar**, Couplan E, Bouillaud F*
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Uncoupling protein-1 (UCP1) is responsible for heat production in brown adipose tissue. Studies made with transgenic mice have demonstrated that expression of UCP1 in muscle could lead to resistance to the obesity induced by a hyperlipidic diet.[1] Using a model of transgenic mice produced in the laboratory,[2] we observed a specific reduction of muscle mass. Muscles are differently affected according to their workload, and for example, the heart could support high level of UCP1 expression without showing obvious phenotype. This indicates that expression of UCP1 is not *per se* deleterious to ATP formation, but could affect muscle differentiation, when muscles are poorly recruited for contraction. *Ucp2* and *Ucp3* are two genes coding for proteins closely related to UCP1. Many experiments using recombinant expression or reconstitution support the hypothesis that UCP2 and UCP3, like UCP1, are able to transport protons and therefore to uncouple partially mitochondria. However, our studies with isolated mitochondria from *Ucp2* knockout mice hardly support this hypothesis.[3] This contradiction will be discussed.

1. Li et al. (2000) *Nat. Med.* 6: 1115-1120.
2. Couplan et al (2002) *J. Biol. Chem.* 277: 43079-43088.
3. Couplan et al (2002) *J. Biol. Chem.* 277: 26268-26275.

MiP-4. Functional properties of mitochondria from different contractile fiber types

***Gueguen Naïg**, Lefaucheur L, Fillaut M, Herpin P*
INRA Unité Mixte de Recherche sur le Veau et le Porc, Saint Gilles, France

In order to better understand the relationships between mitochondria and the contractile apparatus, and notably the mechanisms that permit the matching of ATP production to its myofibrillar consumption, we studied mitochondrial functional properties in saponin-permeabilised fibers, in relation to the contractile fiber type, based on myosin heavy chain (MyHC) content. Four muscles from twenty 11 week old rabbits were used. Mitochondrial oxygen consumption of fiber bundles was measured polarographycally[1] and the proportion of the MyHC isoforms within these fibers was analyzed by electrophoresis.[2] Contractile fiber classes were determined using principal component analysis. Type I and IIa+IIx fibers presented a higher maximal oxidative capacity, both for the total oxidative capacity (Vmax/mg dry-weight) and per mitochondrion (Vmax/CS), than glycolytic ones (IIx and predominantly IIb). This was

associated with a significant improvement in the coupling between oxidation and phosphorylation (ACR). Mitochondria of type I fibers also showed an apparent km for ADP nearly 20-fold higher (100 ± 20 µM) than that of IIx and predominantly IIb fibers (6.1 ± 3 and 5.9 ± 1.5 respectively), the IIa+IIx fibers being intermediate with an apparent km of 15.5 ± 5 µM ($p<0.01$). In presence of 20 mM creatine, the km for ADP was strongly decreased in type I (-88%) and IIa+IIx fibers (–70%), whereas no modification was observed for the two other fiber types, demonstrating an increasing functional coupling between mi-CK and oxidative phosphorylation in fibers with high oxidative capacities.[3] Thus, unlike the glycolytic fibers, mitochondrial respiration of the I/oxidative fibers does not seem to be controlled by free sarcoplasmic ADP but rather by the PCr/Cr ratio. The IIa/oxido-glycolytic fibers present an intermediate situation. This results show that the differences between fiber types do not correspond only to quantitative differences (mitochondrial density) but also qualitative properties : the more a fiber presents a slow contraction speed and oxidative metabolism, the more the respiratory coupling is optimized. This notably occurs through changes in the mechanisms involved in the mitochondrial respiratory control.

1. Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS (1998) Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol. Cell. Biochem.* 184: 81-100.
2. Staron RS, Pette D (1993) The continuum of pure and hybrid myosin heavy chain-based fibre types in rat skeletal muscle. *Histochem.* 100: 149-153.
3. Zoll J, Koulmann N, Lahoucine B, Ventura-Clapier R, Bigard A-X (2002) Quantitative and qualitative adaptation of skeletal muscle mitochondria to increased physical activity. *J. Cell. Physiol.* 194: 186-193.

MiP-5. Hypoxia reduces cellular and mitochondrial oxygen consumption of alveolar epithelial cells

Heerlein Kristin[1], *Schulze Andreas*[2], *Bärtsch Peter*[1], *Mairbäurl Heimo*[1]

[1]Department Internal Medicine VII, Sports Medicine and [2]Department of Pediatrics I, Division of Metabolism and Endocrinology, University of Heidelberg, Germany

Hypoxia has been shown to inhibit alveolar Na-reabsorption by decreasing activity and copy number of transporters. The present study was designed to examine the significance of inhibition of ion transporters such as the Na/K-ATPase or protein synthesis for the saving of energy during oxygen deprivation and if exposure to hypoxia also affects mitochondrial function. Alveolar epithelial cells (A549 cells) were cultured in normoxia and hypoxia (24 h, 1.5% O_2). Cellular oxygen consumption (JO_2 [pmol/s*mg protein]) was measured in normoxia, hypoxia and after reoxygenation (15 min) using high resolution respirometry in intact as well as in digitonin-permeabilized cells. Already after 5 min of hypoxia JO_2 was decreased by about 20%, it was decreased further after 24 h of hypoxia. Reoxygenation of hypoxia exposed cells increased cellular JO_2. In normoxia, the Na/K-ATPase activity accounted for about 15% of JO_2 but Na/K-ATPase-related JO_2 did not change during hypoxia. JO_2 related to protein synthesis was reduced from 23% of total O_2-consumption in normoxia to 14% after 24 h hypoxia. Both, acute and chronic hypoxia decreased the activity of complexes I, II and III of the mitochondrial electron transfer chain. Reoxygenation from acute hypoxia caused partial recovery of complex I only but not of complexes II and III. No recovery of activity was seen after chronic hypoxia. Our findings demonstrate that oxygen consumption is reduced during 5 min and 24 h hypoxia by a decrease in ATP utilization and, possibly, also by the decrease in the capacity of mitochondria to produce ATP. Lack of full recovery of JO_2 upon reoxygenation after prolonged exposure to hypoxia might indicate adjustments on the level of gene expression.

MiP-6. Respiration, coupling, ROS and aging in primary human fibroblasts

Hütter Eveline[1], *Renner Kathrin*[2], *Gnaiger Erich*[3], *Pfister Gerald*[1], *Jansen Dürr Pidder*[1]

[1]*Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck;* [2]*Tyrolean Cancer Research Institute, Innsbruck;* [3]*D. Swarovski Research Laboratory, Department of Transplantation Surgery, University Hospital Innsbruck*

Human cells in primary culture have a finite lifespan, a phenomenon termed „replicative senescence". After about 50 population doublings, cells stop proliferation and arrest irreversibly in the G1 phase of the cell cycle. Cellular energy metabolism is an important aspect of aging, as shown by life span extension through caloric restriction. Analysis of the glycolytic pathway in young and old cells revealed age-associated changes in the activity of several enzymes. Staining cells with the oxidant-sensitive dye dihydrorhodamine showed that senescent fibroblasts exhibit oxidative stress, a possible consequence of metabolic imbalance. Based on these results, we wanted to know whether mitochondrial function is impaired in senescent cells. Mitochondrial respiratory function was analyzed by high resolution respirometry with the OROBOROS® *Oxygraph*. The experimental regime started with routine respiration, followed by inhibition of ATP synthase with oligomycin, and uncoupling by stepwise titration of FCCP. Finally, respiration was inhibited by sequential addition of rotenone and antimycin A.[1] Respiration per cell was highly increased in old fibroblasts, owing to increased mitochondrial content (citrate synthase activity) in line with an increase in cell size. Normalization of respiratory parameters by citrate synthase activity diminished several differences obtained when expressing results per cell number. The capacity of the respiratory chain, reflected by uncoupled respiration per citrate synthase, is unchanged in old and young fibroblasts. Oligomycin-inhibited respiration, however, was significantly increased in senescent cells. Further, senescent cells exhibit a slightly decreased uncoupling control ratio, and a decreased ratio between uncoupled respiration and oligomycin-inhibited respiration. This indicates a lower coupling state of mitochondria in senescent fibroblasts. Additionally, we performed series of control experiments using young fibroblasts arrested in G1 by contact inhibition. Comparing these cells with senescent cells, the difference in the coupling state is much more striking than between proliferating and senescent cellls. This results indicate that there is no loss of mitochondrial respiratory capacity in senescent fibroblasts. The coupling state is lower in old cells compared to young ones, which might be a consequence of oxidative stress. Interestingly, G1 arrested young fibroblasts exhibit a very high coupling state, a phenomenon which warrants further study.

1. Hütter E, Renner K, Jansen-Dürr P, Gnaiger E (2002) Biphasic oxygen kinetics of cellular respiration and linear oxygen dependence of antimycin A inhibited oxygen consumption. *Molec. Biol. Rep.* 29: 83-87.

MiP-7. Hydrogen peroxide production by mitochondrial glycerophosphate dehydrogenase (mGPDH)

Ješina Pavel[1], *Červinková Zusanna*[2], *Drahota Zdenek*[1], *Houštěk Josef*[1]

[1]*Center for Integrated Genomics, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague;* [2]*Department of Physiology, Medical Faculty, Charles University, Hradec Králové, Czech Republic*

Glycerophosphate (GP)-dependent, ferricyanide-induced hydrogen peroxide production was studied in mGPDH rich brown adipose tissue mitochondria. Relations between the rate of hydrogen peroxide production and total amount of hydrogen peroxide produced at different GP and ferricyanide concentrations were determined. It was found that the rate of hydrogen peroxide production increases with increasing GP concentration and decreases with increasing ferricyanide concentration. Total amount of hydrogen peroxide produced increases with increasing ferricyanide concentration, however, not proportionally, and the efficiency of this process (oxygen/ferricyanide ratio) strongly declines. In case of liver mitochondria, where mGPDH is very low, we found that

triiodothyronine activated mGPDH represents almost the same capacity for the saturation of the respiratory chain as Complex II. The increase of mGPDH activity induced by triiodothyronine correlated with an increase of capacity for glycerophosphate-dependent hydrogen peroxide production. As a result of hormonal treatment, a 3-fold increase in glycerosphosphate-dependent hydrogen peroxide production by liver mitochondria was detected by polarographic and luminometric measurements.

This work was supported by grants: COST 918.50, GAČR 303/03/0799, VZ11110003, MSM111500003.

MiP-8. Apoptosis and mitochondrial function in glucocorticoid triggered leukemia cells with transgenic Bcl-2 expression

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Mitochondrial damage with release of apoptogenic factors including cytochrome *c* has been implicated in cell death signalling pathways. To examine mitochondrial function in apoptotic cells, we applied high-resolution respirometry to human leukemia cells.[1] Mitochondrial changes induced by apoptogenic drugs were compared after cell cycle arrest in the G_1- and S-phase, induced by the glucocorticoid dexamethasone and nucleotide analogue gemcitabine. These chemotherapeutics exerted opposite effects on cellular respiratory capacity (71 % and 131 % of controls, respectively). Respiratory changes correlated with corresponding alterations in cell size (volume, protein content and lactate dehydrogenase activity) and mitochondrial content (citrate synthase and cytochrome *c* oxidase activity per cell). A functional mitochondrial membrane potential was maintained in all treatments, as deduced from high and constant respiratory uncoupling control ratios. Thus, at least the majority of mitochondria remained intact despite 30 % apoptosis. Bcl-2 over-expression protected dexamethasone-treated cells from apoptosis, without fully preventing the decline of respiration and cell size. Independent of Bcl-2 expression, however, gemcitabine-treatment resulted in identical rates of apoptosis, with increased cellular respiration and cell size. These results, therefore, provide conclusive evidence that cell cycle arrest is the main mechanism explaining alterations in respiratory capacity and enzyme activities per cell in the early phase of apoptosis.

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MiP-9. Oxygen dependence of rotenone/antimycin A-inhibited respiration and biphasic oxygen kinetics in cultured fibroblasts

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Biphasic oxygen kinetics in fibroblasts was quantitatively explained by a near-linear increase of rotenone- and antimycin A-inhibited oxygen consumption in the high-oxygen range, which was superimposed on the mitochondrial hyperbolic component of oxygen kinetics observed in the low-oxygen range.[1] These results suggest an increased production of reactive oxygen species and oxidative stress at elevated, air-level oxygen concentrations, which is reduced under more physiological intracellular low-oxygen

levels. The high oxygen affinity of mitochondrial respiration provides the basis for the maintenance of a high aerobic scope at physiological low-oxygen levels, whereas further pronounced depression of oxygen pressure inhibits mitochondrial oxidative phosphorylation under hypoxia. The apparent metabolic depression in terms of oxygen consumption of these cells at physiological oxygen levels, therefore, indicates the reduction of a predominantly non-mitochondrial component of oxygen uptake, which is not coupled to oxidative phosphorylation.[2] In conclusion, a narrow optimum window of intracellular oxygen concentration exists between conditions of oxidative stress at high oxygen and energetics stress under severe hypoxia.

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MiP-10. Mitochondrial composition, structure and function is modified in cancer cells forced to switch from glycolysis to oxidative phosphorylation

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Cells must respond to changes in environment, including availability of substrates for energy metabolism, if they are to survive. Here, we have examined the adaptations of a transformed cell line, HeLas, and a primary cell line, fibroblasts, when forced to change from generating ATP predominantly by glycolysis to exclusively by oxidative phosphorylation, as can occur in solid tumors. Our result show that Hela cells producing ATP by oxidative phosphorylation grew more slowly but presented increased respiratory rates; the pH of the matrix was lower by 0.4 pH units, and the reduction potential in the matrix space was lower i.e. 74% *vs*. 93% total reduced. These changes were measured *in vivo* by a variety of techniques, including the use of two novel ratiometric GFP biosensors. Along with the above functional changes, these cells had increased synthesis of most of the oxidative phosphorylation components, a twofold increase in the amount of cristal membrane but no overall increase in total number/amount of mitochondria and mitochondrial DNA. Both confocal and electron microscopy also revealed significant remodeling of the mitochondrial network i.e, the tubules were thinner in width, more interconnected, widely distributed and extensively looped while in cells living by glycolysis the mitochondrial mass was mostly perinuclear. Our data show that tumor metabolism can be determined by substrate availability and they establish that cancer cells adapt their mitochondrial system structurally and functionally to derive energy under glucose limitation. They also show how the pleomorphic, highly dynamic structure of the mitochondrion is related to oxidative phosphorylation. We compared our finding on HeLa cells with those for non transformed fibroblasts to help distinguish the regulatory pathways. These findings have significant implications for cancer treatment.

MiP-11. Detection of free radicals and reactive oxygen species (ROS)

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Amperometric Methods: Measurement of the concentration of free radicals and reactive oxygen species in vitro and in vivo has tremendous implications in the biological sciences. World Precision Instruments has developed a wide variety of electrochemical sensors for the detection of nitric oxide, oxygen, hydrogen peroxide, nitrosothiols etc. Among the various methods for detection of nitric oxide (NO), amperometric sensing includes numerous advantages such as: high selectivity, in situ real time recording, ease of use and calibration, fast response, a wide dynamic measurement range: 0.1 nM-100 μM, and a wide range of electrode sizes from nm to mm. During the last 10 years WPI NO sensors have been successfully used for measurements of NO in animals, plants, in varied tissues and organs: cell cultures, kidney, brain, blood, serum and blood vessels. Nitrite, nitrate, and nitrosothiols samples can be indirectly measured if the samples are processed appropriately. Free radical analyzer, model "Apollo 4000" recently introduced by WPI, presents a new concept in amperometric measurements. It constitutes a four-channel amperometric and data acquisition system and computer. Each channel can be configured to measure any of the several analytes WPI is capable of measuring - nitric oxide, oxygen, and hydrogen peroxide. ***Spectrophotometric and Luminescence Sensing Methods***: WPI has recently developed several reagent kits with which to measure nitric oxide production and superoxide/hydrogen peroxide concentrations. Nitric oxide is produced by nitric oxide synthases *in vivo*, and is rapidly metabolized into nitrate and/or nitrite. WPI's kit uses copperized cadmium to reduce nitrate to nitrite, which in turn reacts with Griess reagents to produce a stable azo compound with a characteristic chromophore. The total nitrate and nitrite concentration represents integrated amount of nitric oxide production over the experimental time period. Establishment of the existence of Superoxide Dismutase (SOD), and thus superoxide *in vivo*, is the first milestone in free radical physiology and pathophysiology. The most important source of superoixde is probably from the leakage in the electron transport chains within mitochondria, more specifically in Complex I and in Complex III. Superoxide is converted catalytically into hydrogen peroxide by Mn-SOD within mitochondria. Diffused hydrogen peroxide had been determined by the horseradish peroxidase-amplified luminescence method. WPI's hydrogen peroxide determination kit utilizes horseradish peroxidase amplified luminol chemiluminescence to measure hydrogen peroxide concentration as low as 75 nM. Our special formulation yields extremely slow decay of the luminescence intensity and thus permits convenient and accurate measure using simple luminometer. Using an increased amount of horseradish peroxidase, and thus a very short lifetime of the luminescence, hydrogen peroxide concentration can be monitored continuously in real time *in situ*. In conclusion, our amperometric instruments and reagent kits could be of great value in innovative research on nitric oxide regulation of the production of hydrogen peroxide in mitochondria.

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