Course on High-Resolution Respirometry

IOC-32. *Mitochondrial Physiology Network* 11.2: 1-12 (2006)

Last update: 2006-10-10

International Course on High-Resolution Respirometry: Oxygraph-2k, TIP-2k and DatLab 4

21-25 April 2006

Schröcken, Vorarlberg, Austria

The 32nd Course on High-Resolution Respirometry starts with a demo experiment using cultured cells, providing a practical overview of the **Oxygraph-2k**, with integrated on-line analysis by **DatLab 4**, and application of the **TIP-2k** in a FCCP titration. Emphasis will be placed on hands-on sessions to introduce the operational details of high-resolution respirometry.

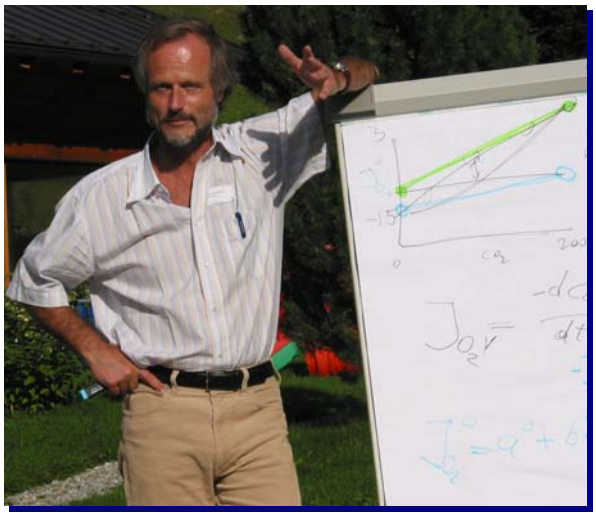
Experienced tutors guide small working groups step-by-step through the approach of high-resolution respirometry. Five Oxygraph-2k, four TIP-2k and several PCs are available for a do-it-yourself application of both hardware and software.

During lunch breaks, sufficient time is available for skiing, relaxing (snowshoe) walks and talks, to enjoy the refreshing scenery of the alpine environment, or use the spare time for specific tutorials.

Snowfall may interfere with snowshoe walks or skiing, but performance of the OROBOROS Oxygraph-2k is weather-independent. With DatLab 4 we accomplish data analysis on-line during the experiment, providing final results and their graphical presentation by the end of an experimental run. Thus we gain sufficient time to see the new Titration-Injection microPump TIP-2k in action and practice its simple and automatic operation.

Lecturers and Tutors

Susana Cadenas, Madrid, ES (*left*)
and Pavla Krivakova, Prague, CZ (*right*)



Erich Gnaiger, Innsbruck, AT

Programme

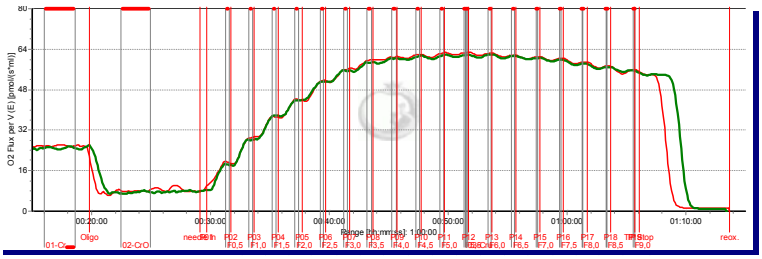
Friday, 21. April

Afternoon/Evening Check in at Hotel Mohnenfluh, 19:30 dinner.
Before/after dinner: Setting up the instruments with a glass of wine.

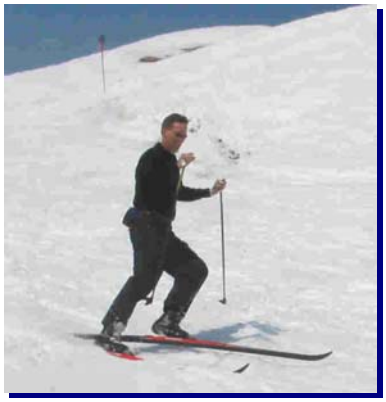
Saturday, 22. April

The final time schedule will depend on weather conditions and corresponding timing for breaks.

08:45 – 11:45 From switching on the Oxygraph-2k to the experimental result.
See **OROBOROS Protocols 2.1.A.** An experiment with high-resolution respirometry: Phosphorylation control in cell respiration. *MiPNet.* 10.4.



• Oxygraph-2k demo experiment with DatLab 4;
• Oxygen calibration;
• Addition of cells, closing the chambers;
• *Phosphorylation control titration:* Routine respiration (C_r), oligomycin-induced state (C_{rO}), uncoupled state (C_{ru}), inhibition by rotenone and antimycin A (C_{ruRA}); • FCCP titration with the TIP-2k; • Re-oxygenation.



12:00 - 16:00 Ski break (bus leaves at 12:22 from Hotel Mohnenfluh); alpine walks and talks; tutorials.
16:15 - 19:00 Working group session 1: Hands-on with the Oxygraph-2k (four instruments - eight parallel chambers): Cell respiration experiment.
19:30 Dinner
21:00 Hot topics in Mitochondrial Physiology (Abstracts pp 6-8).

***Left*: Terje Larsen with Norwegen crosscountry skies on the slope of Salober.**

Sunday, 23. April

08:45 - 11:45 **Working group session 2:** Hands-on experiments with the Oxygraph-2k - O2k-background.

12:00 – 16:00 Ski break (bus leaves at 12:22 and 13:04 from Hotel Mohnenfluh); alpine walks and talks; tutorials. Optional lunch at Hotel Körbersee - www.koerbersee.at.

***Left*: Susana Cadenas on skies - for the first time.**

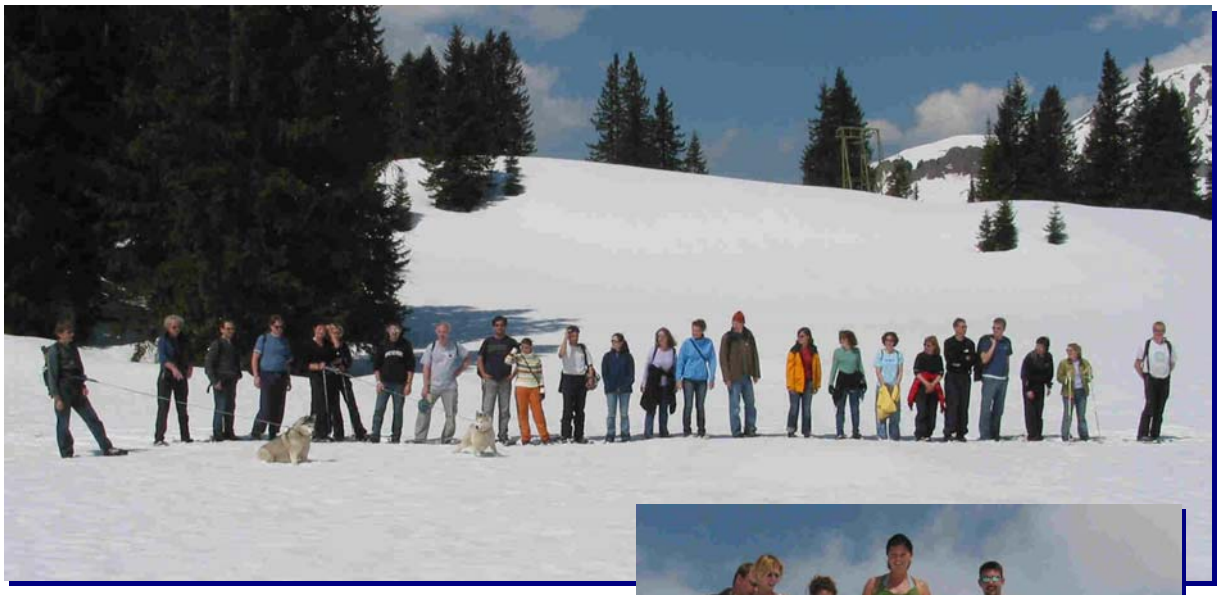
16:15 - 19:00 **Working group session 3:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4.

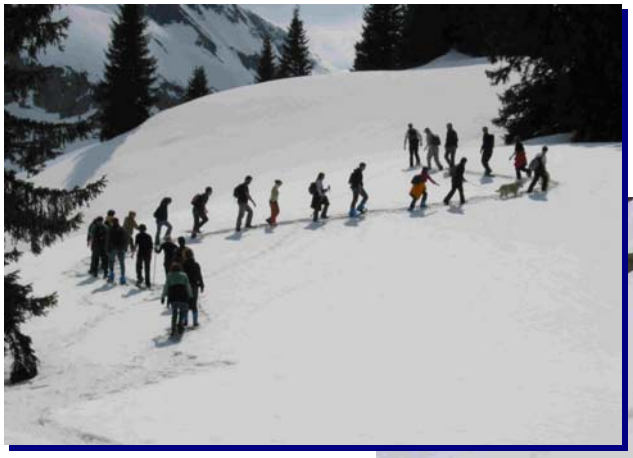
19:30 Dinner

21:00 Hot topics in Mitochondrial Physiology – see Abstracts.

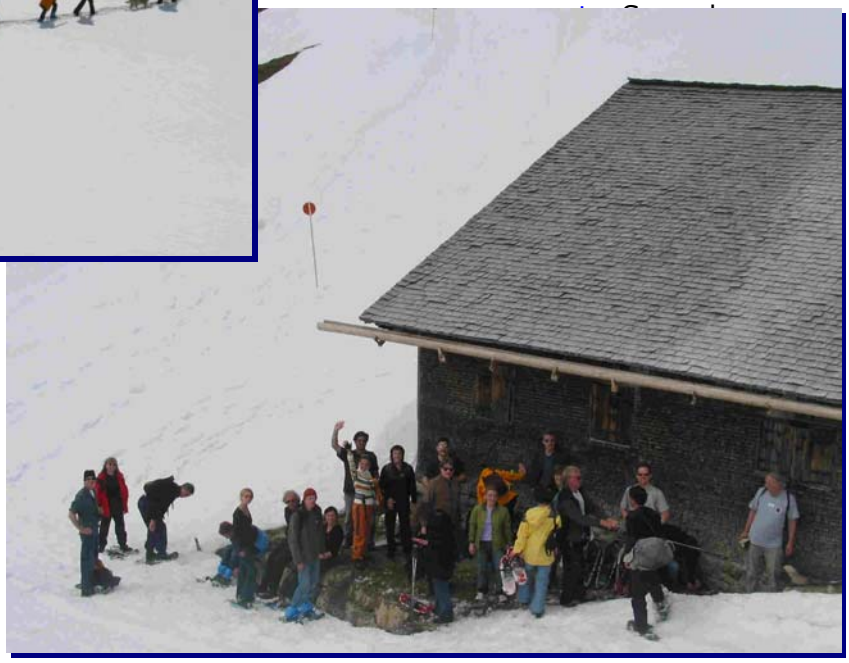
Monday, 24. April

08:45 - 12:30 **Working group session 4:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4.





13:00 – 17:00 **Snowshoe walk** from Schröcken to a welcome at the ***Alpmuseum uf m Tannberg*** - www.alpmuseum.a



17:30 - 19:30 **Working group session 5:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4.

20:00 Dinner at Hotel Mohnenfluh
Discussion - Summary - Conclusions

Tuesday, 25. April

Departure

CONTENTS: OVERVIEW ON HIGH-RESOLUTION RESPIROMETRY

Introduction: Mitochondrial and cellular respiratory physiology – new challenges for high instrumental performance.

High-resolution respirometry – what makes the difference? Presentation of the OROBOROS Oxygraph-2k

- Low oxygen and measurement of cellular oxygen consumption – pushing the limits of detection.
- Optimum system design - the OROBOROS Oxygraph-2k.
- DatLab 4: on-line recording of oxygen concentration and flux; linear slope versus oxygen flux as a function of time.
- DatLab 4: the specialized software for high-resolution respirometry; high-resolution calibrations.

OROBOROS Oxygraph-2k and TIP-2k: On-line instrumental performance

- Instrumental background: measurement and correction as a function of pO_2.
- High resolution of respiratory flux at various steady-states.
- The Titration-Injection microPump TIP-2k: automatic titrations.

- 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 (O2S) and O2k service

- Cleaning of anode and cathode.
- Electrolyte and membrane application.
- Oxygraph-2k and TIP-2k: instrumental maintenance.

Accomodation and Location

Hotel Mohnenfluh www.mohnenfluh.at; Tel.: +43 5519 203; hotel@mohnenfluh.at. The course takes place at Hotel Mohnenfluh (Sylvia Schramm-Strolz, *right*). Accomodation for all participants is arranged at Hotel Mohnenfluh and Hotel Tannberg. Breakfast and all meals will be served jointly at Hotel Mohnenfluh (cook Annelies; you will be served by Stephi and Romy).

Skiing

Warth-Schröcken - www.intermaps.com/skimaps/snowworld
Skiing lifts operate until Sunday, 23. April. Bus trips are free from Schröcken to the skiing area of Salober, leaving at 12:20/12:22 at Hotel Tannberg / Hotel Mohnenfluh (or 11:04/11:06). For the afternoon after 12:30, the skiing pass is € 22.50 for the skiing lifts of Salober and Warth. There is also excellent crosscountry skiing around lakes Kalbelesee and Körbersee, as well as easy walking in magnificent winter scenery. Ski rental is available in Schröcken and at the skiing lift Salober. Top ski (+boots) is € 16.- (+7.-; 1 day), 30.- (+12.-; 2 days), 42.- (+17.-; 3 days) or 52.- (+22.-; 4 days). You can return to Schröcken on skies (depending on snow conditions) or by the free bus (leaving 15:30 at Salober).

Weather Sunny days in spring may be warm, but sub-freezing temperatures are possible in April. Sunshine is very strong – bring sunglasses and sunscreen, even if you do not plan to go skiing. Protect yourself against wind and potential snowfall or rain (gloves, jacket, etc.).

Further information Introductory course material is available on our homepage www.oroboros.at.

Contact

Erich Gnaiger, PhD
Department of Transplant Surgery
D. Swarovski Research Laboratory
Innsbruck Medical University
Innrain 66/6
A-6020 Innsbruck, Austria
Tel: +43 512 504 24623 (24626)
Fax: +43 512 504 24625
E-mail: erich.gnaiger@uibk.ac.at
www.mitophyiology.org

OROBOROS INSTRUMENTS
high-resolution respirometry
Oxygraph-2k

Schöpfstrasse 18
A-6020 INNSBRUCK, Austria
Tel/Fax: +43 512 566796
E-mail: instruments@oroboros.at
Homepage: http://www.oroboros.at
Cooperation and Feedback in Science

Participants

Brasseur Gael, Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France; brasseur@ibsm.cnrs-mrs.fr

Braun Valerie, OROBOROS INSTRUMENTS, Schöpfstr. 18, A-6020 Innsbruck, Austria; instruments@oroboros.at

Cadenas Susana, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, CNIC, Melchor Fernández Almagro, 3, 28029 Madrid, Spain; scadenas@cnic.es – Abstract MiPNet-1

Cleary Joanne, University of Michigan, 930N University Ave, Ann Arbor MI 48104, USA; jdonohoe@umich.edu

Galkin Alexander, Universitatsklinikum Frankfurt, Institut fur Biochemie I, ZBC, Theodor-Stern-Kai 7, Haus 26, 4 Stock, Molekulare Bioenergetik, 60590 Frankfurt am Main, Germany; galkin@zbc.kgu.de

Gnaiger Erich, Innsbruck Medical University, Dept. Transplant Surgery, D. Swarovski Research Laboratory, Innrain 66/6, 6020 Innsbruck, Austria; and OROBOROS INSTRUMENTS, Schöpfstr. 18, A-6020 Innsbruck, Austria; erich.gnaiger@uibk.ac.at – Abstract MiPNet-3

Grégori Gérald, Chargé de Recherche, Laboratoire de Microbiologie, de Géochimie et d 'Ecologie Marines, CNRS UMR 6117, 163 Avenue de Luminy - Case 901- Bât TPR1 - Entrée G, 13288 Marseille cedex 9; gerald.gregori@com.univ-mrs.fr

Hansen Lillian Lund, Department of Medical Biochemistry and Genetics, University of Copenhagen, 3 Blegdamsvej, 2200 Copenhagen, Denmark; lhlh@imbg.ku.dk

How Ole-Jakob, Department of Medical Physiology, Institute of Medical Biology, Faculty of Medicine, University of Tromsø, 9037 Tromsø, Norway; ojh000@asp.uit.no

Hunger Miriam, OROBOROS INSTRUMENTS, Schöpfstr. 18, A-6020 Innsbruck, Austria; csad7037@uibk.ac.at

Keeney Paula, Lab Manager, Center for the Study of Neurodegenerative Diseases, Department of Neurology, University of Virginia, P.O. Box 800394, Charlottesville, VA 22908-0394, USA; pmk@cms.mail.virginia.edu

Krivakova Pavla, Faculty of Medicine, Dept. of Physiology, Charles University, Simkova 870, 500 38 Hradec Kralove, Czech Republic; krivakovap@lfhk.cuni.cz – Abstract MiPNet-2

Larsen Terje, Department of Medical Physiology, Institute of Medical Biology, Faculty of Medicine, University of Tromsø, 9037 Tromsø, Norway; Terje.Larsen@fagmed.uit.no

Lenaers Ellen, Department of Movement Sciences, Postbus 616, Universiteitssingel 50, 6200 MD Maastricht, The Netherlands; ellen.lenaers@bw.unimaas.nl

Lopez Anna, Dept. de Fisiologia, Instituto de Biotecnologia, Universidad de Granada, Avenida de Madrid, 11, E-18012 Granada, Spain; anillalopezramirez@hotmail.com

Palacino James, Novartis Institutes for BioMedical Research, Inc., Models of Disease Center, Neuroscience Group, 100 Technology Square, Cambridge, MA 02139, USA; james.palacino@novartis.com

Petrov Svetlana, Molecular Pharmacology, Byggnad HB4, AstraZeneca R&D Mölndal, 431 83 Mölndal, Sweden; svetlana.petrov@astrazeneca.com

Phielix Esther, Department of Human Biology, Maastricht University, Universiteitssingel 50, P.O. Box 616, NL-6229 ER Maastricht, The Netherlands; Esther.Phielix@HB.unimaas.nl – Abstract MiPNet-4

Sauerwein Hans, AMC, Dept of Endocrinology & Metabolism, F5-170, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; h.p.sauerwein@amc.uva.nl

Scheibye-Knudsen Morten, Department of medical biochemistry and genetics, Section A, University of Copenhagen, 3 Blegdamsvej, 2200 Copenhagen, Denmark; morten@forsoegsperson.dk
Schoonderwoerd Kees, Department of Clinical Genetics, Erasmus MC, Postbus 1738, 300O DR Rotterdam, The Netherlands; g.schoonderwoerd@erasmusmc.nl
Serlie Mireille, AMC, Deptartment of Endocrinology & Metabolism, F5-170, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; M.J.Serlie@amc.uva.nl
Sluiter Wim, Department of Biochemistry, Erasmus MC, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; w.sluiter@erasmusmc.nl
Subarsky Patrick, Candidate, Dept. of Medical Biophysics, University of Toronto,Office: Rm. 10-112, Ontario Cancer Institute/Princess Margaret Hospital, 610 University Ave., Toronto, ON, M5G 2M9, Canada; subarsky@uhnres.utoronto.ca - Abstract MiPNet-5
Swenson Lara, University of Michigan, 930N University Ave, Ann Arbor MI 48104 USA; lmclaugh@umich.edu
Therkelsen Ib, Department of Medical Biochemistry and Genetics, University of Copenhagen, 3 Blegdamsvej, 2200 Copenhagen, Denmark; ib@imbg.ku.dk

Hot topics in Mitochondrial Physiology – MiPNet Abstracts

MiPNet 1. Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: instrumental comparison including high-resolution respirometry.

Enara Aguirre[1], Felix Rodriguez-Juarez[1], Erich Gnaiger[2], Susana Cadenas[1]
[1]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Biology of Nitric Oxide Laboratory, Melchor Fernandez Almagro 3, 28029 Madrid, Spain; [2]Innsbruck Medical University, Dept. General and Transplant Surgery, D. Swarovski Research Laboratory, Innrain 66/6, A-6020 Innsbruck, Austria; and OROBOROS INSTRUMENTS

At low oxygen levels, mitochondrial respiration is controlled by the nitric oxide (NO)-cytochrome c oxidase (COX) signaling pathway, since NO is a membrane-permeant second messenger and competitive inhibitor of COX [1]. It is now well established that oxygraphs, with Teflon-coated stirrer bars and other plastic materials of high oxygen solubility, yield high rates of oxygen back-diffusion into the chamber when oxygen levels decline, causing artefacts of respiratory measurements. High-resolution respirometry with the OROBOROS Oxygraph-2k (O2k) reduces such back-diffusion by at least an order of magnitude, and incorporates automatic instrumental background corrections, treating the 'closed' chamber essentially as an open system with oxygen transport between the aqueous phase and the system boundary [2]. For measurement of NO in experimental chambers, however, the same instrumental problem of gas exchange between hydrophobic plastic materials and the aqueous medium has not been addressed, despite the high partition coefficient of NO between aqueous and organic phases [3].

To address these problems, we incorporated an NO sensor (ISO-NOP, WPI) into a Hansatech oxygraph chamber and a high-resolution respirometer (O2k), for simultaneous recording of respiration and NO. The NO sensor was calibrated by addition of known concentrations of KNO_2 under reducing conditions (KI/H2SO4) at 37 ºC and the response of the NO sensor in terms of accuracy, stability and reproducibility of the signal was compared between the two chambers. Measurements were taken in 1 ml (Hansatech) or 2 ml (O2k) closed chambers at 37 ºC, using their standard Teflon- or PEEK-coated stirrer bars, respectively. The titanium stopper of the O2k chamber was replaced by a polyvinylidenfluorid (PVDF) stopper, including a second inlet (2 mm diameter) for the NO sensor in addition to the capillary used for extrusion of gas bubbles and titration of chemicals.

The PVDF stopper showed identical characteristics to titanium in terms of minimum back-diffusion of oxygen in aerobic-anaerobic transitions, can be cleaned with 70 % and pure ethanol, and offers increased flexibility for accommodation of various additional

electrodes for multi-sensor applications. We compared the response of the NO sensor in the determination of the release of NO from a chemical source (DETA-NO) and the endogenous release from controlled intracellular NO production. We determined the inhibition of respiration caused by NO under physiological oxygen concentrations using conventional and high-resolution respirometry [2].

1. Mason M.G. et al. (2006) *Proc. Natl. Acad. Sci. USA* 103: 708-713.
2. Gnaiger E. (2001) *Respir. Physiol.* 128: 277-297.
3. Liu X. et al. (1998) *Proc. Natl. Acad. Sci. USA* 95: 2175-2179.

MiPNet 2. Oxidative damage of isolated rat hepatocytes by tert-butylhydroperoxide.

Pavla Křiváková[1], Tomáš Roušar[1], Otto Kučera[1], Halka Lotková[1], Anna Lábajová[2], Zuzana Červinková[1], Zdeněk Drahota[3]

[1]Department of Physiology, Faculty of Medicine, Charles University, Hradec Králové, Czech Republic, [2]Institute of Pathological Physiology Faculty of Medicine, Charles University, Prague, Czech Republic, [3]Institute of Physiology and Center of Integrated Genomics, Academy of Sciences of the Czech Republic. Prague, Czech Republic

Liver is the main target organ for toxic effects of various compounds. Oxidative stress is one of the most important mechanisms through which hepatotoxins induce cell death. Effective protection of cellular damage induced by oxidants requires more information about reactions involved in this process. Tert-butylhydroperoxide (tBHP) has been widely used as a model compound to mimic the effect of oxidative stress in various cell types. This organic hydroperoxide is metabolized in cells into free radicals and causes cellular injury. The aim of our work was to characterize toxic injury of isolated rat hepatocytes induced by tBHP.

Hepatocytes were isolated from male Wistar rats by two-step collagenase perfusion. A portion of cells was used for measurement of O_2 consumption (OROBOROS Oxygraph-2k) and for evaluation of mitochondrial membrane potential, MMP (tetraphenylphosphonium selective electrode). Remaining hepatocytes were cultivated in collagen coated Petri dishes. To estimate the rate of toxic injury, we measured TBARS, LDH, MMP (Rho 123, JC-1), and GSH/GSSH (HPLC).

Tert-butylhydroperoxide increased lipoperoxidation which preceded LDH leakage and decreased the activity of respiratory Complexes I and II, MMP and GSH/GSSG. Respiratory Complex I activity was much more sensitive to the peroxidative action of tBHP than the activity of Complex II. We found that the mechanism of the tBHP effect on mitochondrial membrane potential was dependent on the respiratory substrates. We can suppose two different mechanisms: One of them is inhibition of respiratory complex I and the second one is mitochondrial permeability transition pore opening.

MiPNet 3. Control of mitochondrial respiratory capacity. Q-junction at the oxidative phosphorylation highway.

Ashley Naimi[1,2], Cindy Writght[2], Helene Lemieux[3], Assegid Garedew[4], Jakob Troppmair[1], Pierre U Blier[3], Jean-Claude Tardif[5], Hans Sondergaard[6], Carsten Lundby[6], Jose A Calbet[7], B Saltin[6], Jorn Helge[6], Flemming Dela[6], Robert Boushel[2], Erich Gnaiger[1,4]

[1]Innsbruck Medical Univ., Dept. Transplant Surgery, D. Swarovski Research Lab., Innrain 66, 6020 Innsbruck, Austria. [2]Dept. Exercise Science, Concordia Univ., Montreal Qc, H4B 1R6 Canada; [3]Lab. Biol. Évolutive, Univ. Québec, Rimouski (Québec), Canada; [4]OROBOROS INSTRUMENTS, A-6020 Innsbruck, Austria; [5]Montreal Heart Institute, Montréal (Québec), Canada; [6]Copenhagen Muscle Research Centre (CMRC), Rigshospitalet, DK-2200 Copenhagen N, Denmark; [7]Dept. Physical Education, Univ. Las Palmas, Gran Canaria, Spain. - erich.gnaiger@uibk.ac.at

Electron flow in the mitochondrial respiratory chain drives proton translocation through the inner mitochondrial membrane, building a membrane potential and proton motive force which in turn fosters the power for oxidative phosphorylation. Metabolic maps in bioenergetics carefully point out that, in contrast to a linear arrangement of respiratory complexes, input into the electron transport chain proceeds in parallel through Complexes I and II (CI+II, and other flavoproteins) into the Q-cycle. The implications of this Q-junction on mitochondrial respiratory control are not sufficiently recognized in bioenergetics and metabolic flux control analysis. The Q-junction emerges

now as a novel paradigm of respiratory control in mitochondrial physiology, based on high-resolution respirometry (OROBOROS Oxygraph-2k [1]) in permeabilized cells and tissue preparations. Our recent studies of mouse myocardial fibers (0.7 mg [2]), human skeletal muscle fibers (1-5 mg [3]), permeabilized NIH3T3 fibroblasts ($0.5 \cdot 10^6$ cells [4]) and other cell types show that ADP-activated respiration with malate+glutamate or pyruvate (classical State 3) increases up to 2-fold after addition of succinate. Parallel electron input converging at the Q-junction shares flux control with the phosphorylation system, and corresponds to mitochondrial substrate supply *in vivo*. By establishing the reference state of maximum coupled respiration, parallel electron input into the Q-junction provides the proper basis for (i) quantifying excess capacities, metabolic thresholds, and interpreting flux control by various enzymes (e.g. COX) and functional units (phosphorylation system [5]), and (ii) evaluation of specific enzymatic defects in mitochondrial respiratory physiology and pathology. The design is discussed of a general protocol for multi-substrate/inhibitor titrations, which takes into account the concept of the Q-junction.

1. Gnaiger E (2003) Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. *Adv. Exp. Med. Biol.* 543: 39-56.
2. Lemieux H, Garedew A, Blier PU, Tardif J-C, Gnaiger E (2006) Temperature effects on the control and capacity of mitochondrial respiration in permeabilized fibers of the mouse heart (EBEC-2006 abstract).
3. 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. http://www.mitophysiology.org/index.php?naimia.
4. Gnaiger E, Wright-Paradis C, Sondergaard H, Lundby C, Calbet JA, Saltin B, Helge J, Boushel R (2005) High-resolution respirometry in small biopsies of human muscle: correlations with body mass index and age. *Mitochondr. Physiol. Network* 10.9: 14-15. http://www.mitophysiology.org/index.php?gnaigere.
5. Rasmussen UF, Rasmussen HN, Krustrup P, Quistorff B, Saltin B, Bangsbo J (2001) Aerobic metabolism of human quadriceps muscle: *in vivo* data parallel measurements on isolated mitochondria. *Am. J. Physiol. Endocrinol. Metab.* 280: E301–E307.

MiPNet 4. Lipid-induced mitochondrial dysfunction in type 2 diabetes.

Esther Phielix

Department of Human Biology, Maastricht University, Universiteitssingel 50, P.O. Box 616, NL-6229 ER Maastricht, The Nederlands - Esther.Phielix@HB.unimaas.nl

Skeletal muscle is responsible for a major part of postprandial glucose uptake, and resistance of the muscle towards the action of insulin is a common characteristic of type 2 diabetes mellitus. It has been suggested that a reduced fat oxidative capacity lead to insulin resistance of skeletal muscle. Indeed, a reduced fat oxidative capacity has been reported in type 2 diabetic patients and in their first-degree relatives. In addition, plasma free fatty acids (FFA) levels are increased in obese subjects and diabetic patients. Together with a reduced fat oxidative capacity this will result in accumulation of fatty acids and triacylglycerol (TG) in non-adipose tissues, such as liver, heart and skeletal muscle. The accumulation of triglycerides in muscle, termed intramyocellular lipid (IMCL), has been shown to correlate very strongly with insulin resistance. Several investigators have suggested that fatty acid intermediates, such as diacylglycerol and fatty acyl CoA, interfere with insulin signaling, leading to insulin resistance.

The cause of the decreased fat oxidative capacity still remains unknown. However, it is hypothesized that reactive oxygen species (ROS) produced during mitochondrial respiration lead to lipid peroxidation of the IMCL. In turn, these lipid peroxides can damage the mitochondria thereby further reducing oxidative capacity. Alternatively, it has been shown that a set of oxidative genes under control of the transcription factor PPAR-γ co-activator -1 (PGC-1) is downregulated in diabetic patients and this may underlie the reduced oxidative capacity in type 2 diabetic patients.

The aim of this project is to investigate mitochondrial functioning, lipid peroxidation and gene expression in diabetic patients, first-degree relatives of diabetic patients and healthy controls. Mitochondrial function will be determined, *in vitro* with use of the Oxygraph-2k and *in vivo* with the ^{31}P- magnetic resolution spectroscopy (MRS).

Tumor cell intravasation is dependent on available oxygen and does not correlate with hypoxia-induced expression of VEGF.

Patrick Subarsky, Richard P. Hill
Medical Biophysics, University of Toronto and Ontario; Cancer Institute, Princess Margaret Hospital, Toronto, ON, Canada, M5G 2M9 - subarsky@uhnres.utoronto.ca

Rationale: Metastatic dissemination from a primary tumor is a significant cause of treatment failure in cancer patients. Tumor hypoxia has been shown to predict for disease-free survival in head and neck, cervical carcinomas, and soft tissue sarcomas. Furthermore, some studies have identified a relationship between tumor hypoxia and distant metastatic disease. We were interested in characterizing the potential link between hypoxic exposure and tumor cell migration and intravasation.

Methods: Invasion across the basement membrane matrix, Matrigel, was analyzed using a transwell invasion assay under hypoxic conditions to model the process of intravasation from a primary tumor across a tissue basement membrane. The human tumor cell lines, HT1080 (fibrosarcoma) and MDA MB231 (breast carcinoma), used in the study were tested for their hypoxic response following exposure to various O2 levels (0.2, 1, 2, 3, 4, and 5%) for a period of 24 hours. VEGF protein levels were quantified by ELISA assay as a measure of a known hypoxia response.

Results: Both cell lines had constitutive expression of VEGF under normoxic conditions and demonstrated up-regulation upon exposure to hypoxia, with maximal levels of VEGF protein observed in cells exposed to 2% O2. HT1080 cells had a greater ability than MDA MB231 cells to invade through the Matrigel layer under all conditions, however exposure to low O2 (0.2 to 2.0%) conditions significantly decreased the ability of HT1080 cells to invade compared to oxic controls of 5 or 21% O2. MDA MB231 cells did not demonstrate significant changes in invasion, compared to the oxic controls, under low oxygen conditions although, exposure to 0.2% O2 showed a trend towards increased invasion. There was a significant increase in invasion at 4% O2, suggesting that even small decreases in oxygenation can alter the invasive potential of tumor cells. To investigate cellular differences in invasion despite a similar hypoxic response as measured by VEGF production, the activity of matrix metalloproteases, MMP-2 and MMP-9, were examined by gelatin zymography. HT1080 expressed abundant levels of both MMP-2 and MMP-9 in their active forms, however no differences were observed when cells were exposed to the hypoxic range of O2 conditions. MDA MB231 cells expressed active MMP-9, but active MMP-2 was undetectable. There were no differences in MMP-2 or MMP-9 levels at the various O2 concentrations suggesting that they are not responsible for the observed differences in invasion of the two cell lines during hypoxic exposure. Recent literature suggests that MT1-MMP may be hypoxia-regulated and we are currently investigating the expression of this protease in these two cell lines. Our current observations of increased invasion at intermediate levels of oxygen raise the question whether changes in gene expression at very low oxygen levels are relevant to cellular processes involved in metastatic intravasation.

Correspondence

"We are delighted with our O2k! It is obviously a very sensitive piece of equipment and the DatLab software makes using it very easy. The respiratory state of our cell lines will be important information for our group. I must be honest though, with no background in respirometry or with the O2k, I would have been lost without all the very helpful instruction I received in Schroecken. Thank you for making our use of this fine machine possible!" *Paula Keeney*

"First of all, thank you, everybody, for a most pleasant meeting in Schröcken!

Concerning some details of the Oxygraph time derivative, I have performed a small experiment called "Stirrer.dld", from which two Excel-files, "Stirrer-A" and "Stirrer-B" evolves. The Excel-files are more or less identical, simply Chamber A and B on the same instrument. The following references to figures concerns the file "Stirrer-A". Oxygraph users have noticed, that upon rapid changes in Oxygen concentration (as seen by switching the stirrer on and off) the Oxygraph time derivative seems to lag behind, and, as pointed out by Alexander G., even stretches to a much longer time span than the duration of the oxygen changes themselves would suggest. On discussing this topic with Erich G., it appears that the Oxygraph employs a calculation procedure which, at any time point, uses the preceeding 40 data points to determine the time derivative. The procedure also seems to introduce a good deal of smoothing, and for most pracical purposes, in which equilibration evolves over a slow time span of minutes, the built-in time derivative is robust and easy to read. However, if need be, there are other ways of calculating the time derivative. For comparison with the Oxygraph, I have used a procedure called "Deriv" which is an inherent part of software "IDL" (Interactive Data Language). Code for Deriv is attached to this mail as well for the benefit of anyone curious. It is a 3-point Lagrange numerical derivative, which closely follows the Oxygen changes in time, but the outcome is far more noisy than the Oxygraph procedure." - *Ib Therkelsen*

Discussion *(Erich Gnaiger)*: High time-resolution is necessary in various kinetic studies. Most common applications of high-resolution respirometry, however, require only information on respiratory flux over a period of time when metabolic activity is constant (O2k-Manual O2k.E). Standard settings in DatLab 4 are optimized for such applications. DatLab 4 offers a simple on-line solution for improving the time-resolution, by reducing the data recording interval (standard is 2 seconds) to the minimum of 0.2 s (setting in the Oxygraph Control window; see O2k-Manual O2k.A, page 11). As the data recording interval is reduced, the flux appears more noisy, but represents transitions more accurately and reduces the apparent time-delay. Most routine applications aim at obtaining average flux over short periods of time (some minutes), for which application the high smoothing effect of the standard DatLab 4 settings are suitable.

Off-line, DatLab 2 offers numerous options for optimizing analyses between the opposite demands on high time-resolution versus low noise of flux. While the on-line filter in DatLab 4 is fixed (Savitzky-Golay smoothing filter applied on the 40 preceeding data points), off-line options are many.

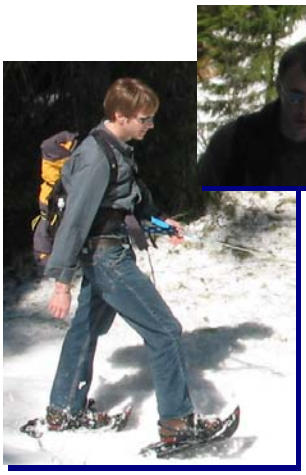
(1) A deconvolution is possible on the raw signal, applying a calibrated first-order exponential time constant (simply applying the 'stirrer test'; see O2k-Manual O2k.G).

(2) Subsequent to time correction (signal deconvolution), 'mild' polynomial smoothing or 'strong' arithmetic smoothing may be applied on the oxygen signal, with variable number of data points (the more data points, the stronger the smoothing effect).

(3) The slope can then be calculated over a variable number of data points, not merely selecting the only on-line option for preceding data points, but including leading data points (symmetrical fit) which avoids the apparent delay in the trace of flux.

(4) For the specific application of oxygen kinetics, an elaborate script (macro) applies an optimized strategy for high time-resolution combined with sufficient filtering of noise (O2k-Manual O2k.H).

"Thanks a lot for the lovely time we had at the Oroboros respiration course. I came back to Sweden and today is my second working day. We started to talk about the first experiments with the OROBOROS instrument and decided that the best way to start will to repeat the PCT-protocol with the cell suspension of 32D cells according to the protocol we got from the course." *Svetlana Petrov*



"I would like to thank you for the excellent respirometry course you have organized." *Alexander Galkin*

"I wanted to thank you for a very informative and fun O2k Course last week." *Lara Swenson*

"Thanks again for your organization of the High-Resolution Respirometry workshop. As you already know, I thoroughly enjoyed myself, learning a great deal of science and enjoying the mountain air." *Patrick Subarsky*

"This week in Austria has been fabulous. It's been the first time that I´ve left Spain for working and it wasn´t really an effort, just the opposite, I´ve enjoyed a lot and I´m glad we´ve come. Thank you very much for being so friendly and kindly with us. Now this week is part of the good old days of my life. Thanks for everything." *Ana López Ramírez*

"I am back to the lab and want to thank you one more time for this wonderfull workshop. It was great and the people were so nice." *Gérald Grégori*