IOC-36. *Mitochondrial Physiology Network* 11.6: 1-14 (2006)

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International Course on High-Resolution Respirometry *and* *MiPNet Workshop*

13-17 Dec 2006

Schröcken, Vorarlberg, Austria

The 36th Course on High-Resolution Respirometry started with a demo experiment with isolated mitochondria, 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 was placed on hands-on sessions to introduce O2k-high-resolution respirometry.

Experienced tutors guide small working groups step-by-step through the approach of high-resolution respirometry. Five Oxygraph-2k, three 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 (snow??), relaxing walks and talks, to enjoy the refreshing scenery of the alpine environment, or use the spare time for specific tutorials.

Snowfall may contribute to or 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 Titration-Injection microPump TIP-2k with new feedback-control in action and practice its simple and automatic operation.

Tutors and Invited Guest Lecturers

Susanne Arnold, Aachen, DE
Robert Boushel, Montreal, CA
Jeannette E. Doeller, Birmingham, Alabama, US
Erich Gnaiger, Innsbruck, AT
Steven C. Hand, Baton Rouge, Louisiana, US
David Kraus, Birmingham, Alabama, US
Hélène Lemieux, Rimouski, CA

MiPNet Hot Topics Speakers

Tiia Anmann, Tallinn, EE
Enrico Calzia, Ulm, DE
Michael Gröger, Ulm, DE

Programme

Wednesday, 13. December

Participants arriving in Innsbruck (and not immediately taken from the airport to Schröcken): Welcome reception at the OROBOROS INSTRUMENTS office, Schöpfstr. 18. Not later than **16:00** Departure: 2 hour drive from Innsbruck to Schröcken, across Arlberg Pass (St. Anton, St. Christoph), Flexenpass (Zürs, Lech), from Warth across the Tannberg Pass (Salober, lake Kalbelesee), to Schröcken. Usually th road from Lech to Warth is closed in winter, but now open due to exceptionally low snowfall.

Participants arriving in Bregenz: Meeting point at Bregenz train station, 1 hour drive to Schröcken.

Afternoon/Evening Check in at Hotel Mohnenfluh, 19:30 dinner.
After dinner: Welcome; setting up the O2k-instruments with a glass of wine.

Thursday, 14. December

08:45 – 13:00 From switching on the Oxygraph-2k to the experimental result.

See Protocol for the O2k Demo Experiment (below).

See also: **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 mitochondria, closing the chambers; • *Respiratory and phosphorylation control titration:* State 2-3-4; • Re-oxygenation;.• Uncoupler titration with the TIP-2k; • Inhibition by antimycin A.

13:15 – 15:00 Walk 1 Schröcken: Hotel Mohnenfluh, Tannberg bridge, Oberboden, Schmitte, return.

15:15 Invited Lecture - **MiPNet 1**: **Steven C. Hand** (*Baton Rouge, Louisiana, US* and *Innsbruck, AT*)
Depression of mitochondrial respiration during diapause: Potential limitations by substrate availability and the phosphorylation system.

16:00 - 19:00 **Working group session 1:** Hands-on with the Oxygraph-2k (four instruments - eight parallel chambers): Mitochondrial respiration experiment.

19:30 Dinner

21:30 Hot topics in Mitochondrial Physiology

MiPNet 2: **Anmann Tiia** (*Tallinn, EE*) Different kinetics of the regulation of respiration in permeabilized cardiomyocytes and in HL-1 cardiac cells.

MiPNet 3: **Michael Gröger** (*Ulm, DE*) Oxidative induced DNA damage.

MiPNet 4: **Enrico Calzia** (*Ulm, DE*) Organ protection in ischemia-reperfusion models.

Friday, 15. December

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

12:00 – 16:00 Walk 2. Bus leaves at 12:22 from Hotel Tannberg; walk from Salober/Kalbelesee to Hotel Körbersee - www.koerbersee.at (lunch recommendation: Käsfladen)

15:15 Hotel Körbersee - Invited Guest Lecture - **MiPNet 5: Susanne Arnold** (*Aachen, DE*) Oxidative energy production in neural cells - metabolic communication between astrocytes and neurons.

Welcome at the *Alpmuseum uf m Tannberg*..

Alpmuseum uf m Tannberg, Batzen www.alpmuseum.at

7:30 - 19:00 **Working group session 3:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4.

19:30 Dinner

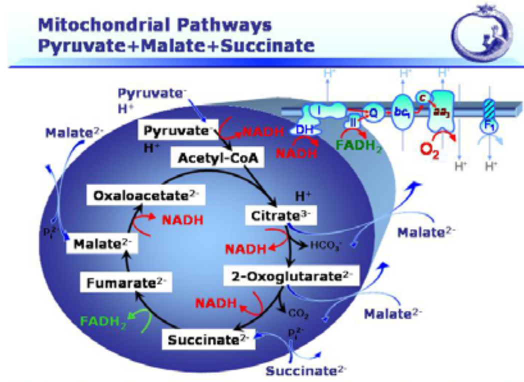
21:30 Hot topics in Mitochondrial Physiology

MiPNet 6: **Robert Boushel** (*Montreal, CA* and *Copenhagen, DK*) Patients with Type 2 Diabetes have normal mitochondrial function in skeletal muscle.

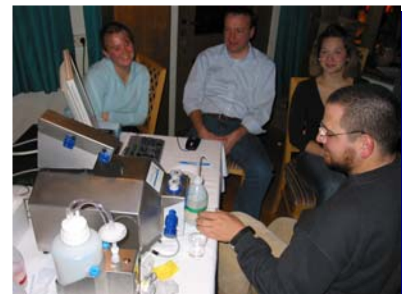
MiPNet 7: **Hélène Lemieux** (*Rimouski, CA* and *Innsbruck, AT*) High excess capacity of cytochrome *c* oxidase in permeabilized fibers of the mouse heart.

Saturday, 16. December

08:45 – 09:30 **MiPNet 8:** **Gnaiger Erich** (*Innsbruck, AT*) Combining respiratory substrates for Complex I+II. A transition from bioenergetics to mitochondrial physiology.



09:45 – 11:45 **Working group session 4:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4; special tasks.



12:00 - 16:00 Walk 3 (bus leaves at 12:22 from Hotel Mohnenfluh); skiing.

16:30 - 18:45 **Working group session 5:** Hands-on experiments with the Oxygraph-2k, oxygen sensor service, O2k-assembly; DatLab 4.

19:30 Dinner

21:30 Hot topics in Mitochondrial Physiology

MiPNet 9: **Jeannette E. Doeller** (*Birmingham, Alabama, US*) Hydrogen sulfide: From toxin to protectant.

MiPNet 10: **David Kraus** (*Birmingham, Alabama, US*) Hydrogen sulfide as a vasoactive signal: Clues from multisensor respirometry.

Discussion - Summary – Conclusions: Postponed

Sunday, 17. December

- Departure to Innsbruck and Bregenz

- Or: O2k MultiSensor working group
- Visit to Lech; with Michael Manhart, Skilifte Lech

Monday, 18. December

- Departure to Innsbruck
- 17:30 Office OROBOROS INSTRUMENTS - farewell party
- 18:00 X-mas market a the Golden Roof
- 20:00 Office OROBOROS INSTRUMENTS dinner buffett.

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.

The Protocol for the O2k Demo Experiment

Mitochondria	Isolated mitochondria (*Artemia fransciscana* embryos in the post-diapause stage) are incubated at 25 °C in the Oxygraph-2k, with 2 ml of mitochondrial medium. The medium is adjusted to the high physiological salt concentration of these animals living at extremely high salinity (Salt Lake, Utah). To investigate the effect of inhibiting complex I by rotenone on respiration with succinate, rotenone is titration into one chamber, and pure ethanol into the other chamber. Then identical titration regimes are applied for both chambers.
State 2	10 mM succinate is added to induce State 2 (no adenylates).
State 3	After titration of 0.25 mM ADP, flux increases steeply to a maximum (State 3) and returns to State 4 as all ADP is phosphorylated to ATP. After a re-aeration of the medium, another transition is induced to State 3 with 0.25 mM ADP. The second peak of flux may be higher than the first State 3 level, owing to activation of succinate dehydrogenase. Immediately after maximum flux is reached, just at the time when it starts to decline, a high concentration of 2.5 mM ADP is added to test for a possible incomplete saturation of State 3 flux by ADP, and to prolong the observation of a time-dependence of state 3 flux.
State 3*c*	Addition of 10 µM cytochrome *c* provides a test for the intactness of the outer mitochondrial membrane, as indicated by the lack of stimulation or respiration by external cytochrome *c*.
State u*c*	Subsequently, FCCP is titrated in steps of 0.5 µM (manually or using the TIP-2k), to test for a possible increase of flux in State u (uncoupled) compared to State 3 (coupled), which is expected in cases when the phosphorylation system is limiting (ANT, ATP synthase, phosphate transporter).
Residual	Finally, addition of antmycin A to uncoupled mitochondria inhibitis Complex III and reduces respiration to a minimum.

Results **http://www.oroboros.at/index.php?ioc36-demo**

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.

Skiing

Warth-Schröcken - http://www.snowworld.at/.
The Ski Opening is scheduled in the skiing area for the 15th of December. Still we are waiting for the new snow. 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 Snowfall and sub-freezing temperatures are expected in December. Sunshine may be 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.

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Oxygraph-2k

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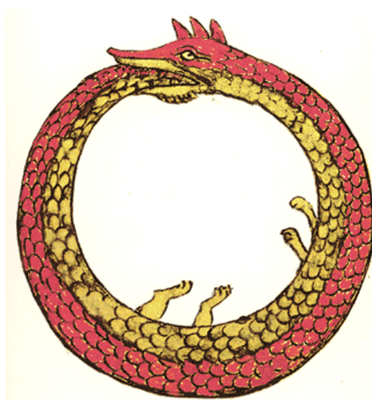
Cooperation and Feedback in Science



IOC36-participants and high-jump on the way from Salober/lake Kalbelesee to lake Körbersee, the peak of Widderstein in the background.

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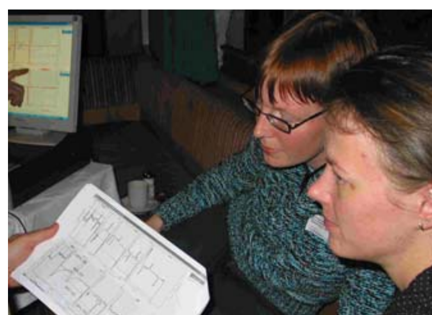

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Hot topics in Mitochondrial Physiology – MiPNet Abstracts

MiPNet 1. Depression of mitochondrial respiration during diapause: Potential limitations by substrate availability and the phosphorylation system.

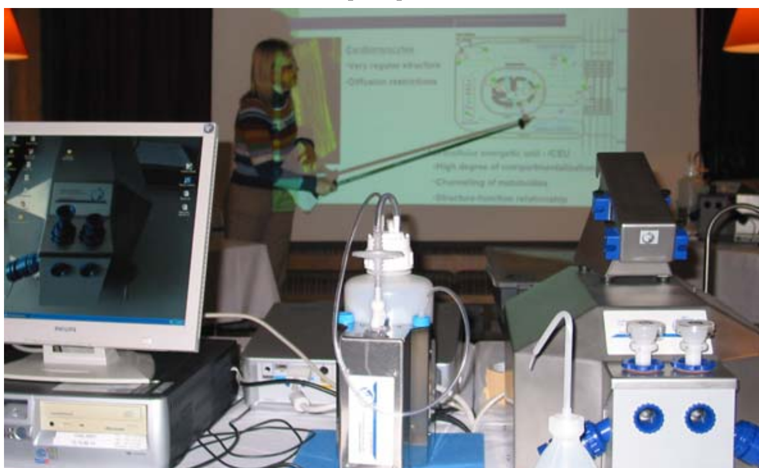
Steven Hand

Louisiana State University, Department of Biological Sciences, Baton Rouge LA, USA; Presently at sabbatical leave: Medical University of Innsbruck, Dept. General and Transplant Surgery, D. Swarovski Research Laboratory, Innsbruck, Austria.- shand@lsu.edu

Abstract

1. Hand SC, Gnaiger E (1988) Anaerobic dormancy quantified in Artemia embryos: A calorimetric test of the control mechanism. *Science* 239: 1425-1427.
2. Gnaiger E, Méndez G, Hand SC (2000) High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. *Proc. Natl. Acad. Sci. USA* 97: 11080-11085.

MiPNet 2. Different kinetics of the regulation of respiration in permeabilized cardiomyocytes and in HL-1 cardiac cells.



Tiia Anmann[1,2,3], Tuuli Kaambre[1], Peeter Sikk[1], Rita Guzun[3], Nathalie Beraud[3], Sophie Pelloux[4], Andrey V. Kuznetsov[5], Kalju Paju[6], Nadja Peet[6], Enn Seppet[6], Yves Tourneur[4] and Valdur Saks[1,3].

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The aim of this work is to compare the cellular regulation of mitochondrial respiration in permeabilized cardiomyocytes, where intermyofibrillar mitochondria are arranged in a highly ordered crystal-like pattern and the HL-1 cells from mouse heart. Two types of HL-1 cells were used 1) HL-1 beating cells (HL-1 B cells) which contain quite organized sarcomeres and represent somewhat of a hybrid between an embryonic and an adult myocyte and 2) HL-1 non-beating (HL-1 NB cells) without sarcomeres. Striking differences in the kinetics of respiration regulation by exogenous ADP between these cells was observed: the apparent Km for exogenous ADP was by order of magnitude lower in the permeabilized HL-1 NB cells without sarcomeres (25 ± 4 µM) than in permeabilized primary cardiomyocytes (360 ± 51 µM), and intermediate in normally cultured HL-1 cells (46 ± 15 µM). Very high sensitivity of the mitochondrial respiration for exogenous ADP was observed in permeabilized HL-1 NB cells, which is similar to that in isolated heart mitochondria (apparent Km for ADP is about 20 µM). Practically complete disorganization of regular mitochondrial arrangement was achieved in the adult permeabilized cardiomyocytes by short treatment with trypsin, which decreased very significantly the apparent K_m for exogenous ADP. The high value of the

apparent Km for exogenous ADP in regulation of mitochondrial respiration in permeabilized cardiomyocytes has been quantitatively explained by local restrictions of diffusion of ADP the level of the mitochondrial outer membrane and within the organized intracellular energetic units, ICEUs. High affinity for exogenous ADP in HL-1 NB cells demonstrates the absence of significant diffusion restrictions in these cells, in contrast to adult cardiomyocytes. Mitochondrial respiration was strongly activated by creatine in the permeabilized cardiomyocytes even in the presence of powerful competing pyruvate kinase - phosphoenolpyruvate system, while in the HL-1 NB cells the stimulatory effect of creatine not significant. The results of this study show that in normal adult cardiomyocytes intracellular local restrictions of diffusion of adenine nucleotides and metabolic feedback regulation of respiration via phosphotransfer networks are related to the complex structural organization of these cells.

MiPNet 3. **Oxidative induced DNA damage.**

Michael Gröger
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Oxidative stress may directly result from the exposure to hyperoxia [1,2], which typically occurs in organisms when breathing pure oxygen, especially under supra-atmospheric conditions (=hyperbaric oxygenation, HBO), or from a decreased antioxidant capacity. In principle, all biomolecules are susceptible to increased levels of reactive oxygen species (ROS). In the particular case of DNA, the enhanced oxidant burden leads to a damage resulting from the oxidation of single bases [3] as well as from DNA strand breaks [4].

Single cell gel electrophoresis (also commonly known as comet assay) is a simple and fast technique for quantifying DNA strand breaks at the level of single cells [5]. This technique is highly sensitive over a wide range of DNA-damage, thus detecting even low levels of injuries. By means of the comet assay it is possible to analyze the dose-dependent relationship between HBO and DNA damage as well as the quality of adaptation- and DNA-repair-mechanisms [6] *in vivo* and *in vitro*.

For example, in a recent in vivo study in a rat model we investigated the effects of an NO-donor (SIN-10) on the formation of DNA strand breaks during HBO; we could demonstrate that combining SIN-10 and HBO fairly doubled the DNA damage when compared to control conditions (HBO or SIN-10-administration alone) [7]. In a further study, the application of superoxide dismutase (SOD) in an ischemia/reperfusion model induced by thoracic aortic cross-clamping was shown to protect against the formation of DNA strand breaks in lymphocytes both *in vivo* and *in vitro* after HBO-exposure.

1. McCord JM (2000) The evolution of free radicals and oxidative stress. *Am. J. Med.* 108: 652-659.
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3. Cadet J, Berger M, Douki T, Ravanat JL (1997) Oxidative damage to DNA: formation, measurement, and biological significance. *Rev. Physiol. Biochem. Pharmacol.* 131: 1-87.
4. Aruoma OI, Halliwell B (1998) Molecular biology of free radicals in human diseases. OICA International, Santa Lucia, London.
5. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184-191.
6. Speit G, Hartmann A (1999) The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol. Biol.* 113: 203-212.
7. Gröger M, Speit G, Radermacher P, Muth CM (2005) Interaction of hyperbaric oxygen, nitric oxide, and heme oxygenase on DNA strand breaks *in vivo*. *Mutat. Res.* 572: 167-172.

MiPNet 4. Organ protection in ischemia-reperfusion models.

Enrico Calzia[1], Jochen Kick[2], Balazs Hauser[1], Hendrik Bracht[1], Florian Simon[2], Peter Radermacher[1], Hubert Schelzig[2]

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Thoracic aortic cross-clamping is a typical example of reactive oxygen species (ROS)-induced ischemia/reperfusion (I/R) injury [1], the most vulnerable organs being the spinal cord and the kidney [2]. The primary targets of ROS are enzymes, membrane lipids and the DNA [3], which results in cellular damage and apoptosis [4]. I/R-induced DNA damage presents as DNA base modification, single- and double-strand breaks, loss of purines, and impaired DNA repair [5].

Over the last years an experimental model of thoracic aortic cross-clamping has been developed in our institutions [6–8], which allows studying the degree of I/R injury induced to different organs and the efficacy of different pharmacological protective interventions. The most intriguing problem imposed by the experimental setup is to prevent left ventricular failure by tight control of blood pressure, which is achieved by continuous infusions of several anti-hypertonic drugs during the clamping manoeuvre.

By means of this model the role of arachidonic acid pathway activation in I/R injury and the effects of the cyclooxygenase-2 inhibitor Parecoxib have been investigated in a first series of 16 pigs [6]. These experiments revealed a beneficial influence of intrarenal Parecoxib infusion on kidney function, which was unrelated to macrocirculatory flow. In a further experiment the hypothesis that pre-treatment with a superoxide-dismutase (SOD) containing oral diet would reduce surgery and I/R-related DNA damage after thoracic aortic cross-clamping was tested [8]. In summary, this nutritional formula was indeed shown to reduce (1) DNA-damage related to surgery and I/R-injury, (2) apoptosis in the spinal cord white matter, and (3) regional venous acidosis, without, however, ameliorating organ function.

In conclusion, our model mimicks clinically relevant mechanisms of I/R-injury leading to organs dysfunction and hence source of serious complications in surgical patients. Consequently it offers a standardized tool for testing and developing well promising therapeutic strategies and options in a realistic context.

1. Gelman S (1995) *Anesthesiology* 82: 1026-1060.; 2. Gloviczki P (2002) *Cardiovasc. Surg.* 10: 434-441;
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5. Beckmann KB et al (1997) *J. Biol. Chem.* 272: 19633-19636; 6. Hauser B et al (2005) *Shock* 24: 476-481;
7. Hauser B et al (2006) *Shock* 25: 633–640; 8. Kick J et al (2006) *Intensive Care Med.* in press.

MiPNet 5. Oxidative energy production in neural cells - metabolic communication between astrocytes and neurons.

Susanne Arnold

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The brain is the organ with the highest energy demand in mammalian organisms. Two different brain cell types, neurons and astrocytes, are structurally, functionally, and metabolically tightly coupled with astrocytes playing a

central role in the regulation of cerebral energy metabolism in dependence on neuronal activity. Astrocytes express receptors and transporters for virtually every neurotransmitter thereby being able to sense neuronal activity. Application of neurotransmitters, for instance ATP, adrenaline, noradrenaline, to primary cortical astrocytes led to a cytosolic calcium increase followed by a depolarisation of the mitochondrial membrane potential and transient changes of NAD(P)H levels reflecting mitochondrial calcium uptake, stimulation of energy consumption and production. Based on different astrocytic and neuronal function and energetic requirements these two cell types show differences in the regulation of mitochondrial energy production which can be correlated with cell survival under pathological conditions.

Cytochrome c oxidase (COX) is the terminal enzyme of the mitochondrial respiratory chain and thereby involved in oxidative energy production. COX catalyses the electron transfer from ferrocytochrome c to oxygen, a process coupled to the translocation of protons across the inner mitochondrial membrane and subsequently to production of ATP, an indirect product of COX, through the ATP synthase. Mammalian COX is composed of three catalytic, mitochondrially encoded and ten regulatory, nuclear encoded subunits. The regulatory COX subunit IV plays an important role in adjusting energy production to cellular energetic requirements by binding of ATP to the N-terminus of subunit IV thereby causing an allosteric inhibition of COX activity at high energy level, i.e. high ATP/ADP ratio [1]. COX subunit IV exists in two different isoforms (IV-1 and IV-2 [2]). While isoform IV-1 is ubiquitously transcribed in all adult mammalian tissues, isoform IV-2 showed low, but cell type specific transcription levels in the brain with highest levels of this isoform in neurons and only marginal amounts in astrocytes. Expression of COX IV-1 is paralleled by the allosteric inhibition of COX by ATP at high energy levels, whereas COX IV-2 expression caused an abolition of this allosteric COX inhibition as determined by polarographic measurements of solubilized mitochondria. Consequently, COX IV-2 expression suppressed the sensitivity of COX to its allosteric regulator ATP and overruled the regulation of COX by the cellular energy level. We suggest that expression of COX IV-2 makes neurons more vulnerable to energetic substrate deficits than astrocytes expressing COX IV-1 and enables astrocytes to sense energetic demands and adapt energy production to energetic requirements in the brain.

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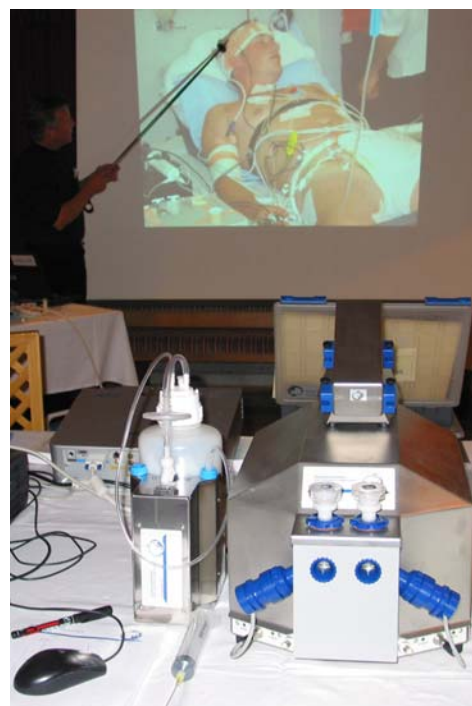
MiPNet 6. Patients with Type 2 Diabetes have normal mitochondrial function in skeletal muscle.



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Insulin resistance and type 2 diabetes are ostensibly associated with mitochondrial dysfunction. Yet there is a paucity of data on direct measures of mitochondrial O_2 flux in human cells. High resolution respirometry (OROBOROS Oxygraph-2k, Austria) was used to measure O_2 flux capacity of permeabilized muscle fibers from biopsies of the quadriceps in healthy humans (n=8; age 58 ± 2 yrs [mean ± SE]; body mass index 28 ± 1 kg/m^2; fasting plasma glucose 5.4 ± 0.2 mM) and patients with type 2 diabetes (n=11; age 62 ± 2 yrs; body mass index 32 ± 2 kg/m^2; fasting plasma glucose 9.0 ± 0.8 mmol/l). O_2 flux expressed per mg muscle fresh weight during ADP-stimulated state-3 respiration was lower (P<0.05) in patients with type 2 diabetes with complex I substrate (glutamate+malate; 31 ± 2 vs. 43 ± 3 pmol O2·s^{-1}·mg^{-1}),

and also with parallel electron input (glutamate+malate+succinate) from complex I+II (63 ± 3 vs. 85 ± 6 $pmol \cdot s^{-1} \cdot mg^{-1}$). Further increases in flux capacity were observed with uncoupling by FCCP, but were again lower (P<0.05) in type 2 diabetics (86 ± 4 vs. 109 ± 8 $pmol \cdot s^{-1} \cdot mg^{-1}$). However, when O_2 flux was normalized for mitochondrial DNA content or citrate synthase activity there were no differences in oxidative phopsphorylation or electron transport capacity between patients with type 2 diabetes and healthy controls. There is a normal mitochondrial function in type 2 diabetes. Blunting of coupled and uncoupled respiration can be attributed to lower mitochondrial content in type 2 diabetes.

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MiPNet 7. High excess capacity of cytochrome c oxidase in permeabilized fibers of the mouse heart.

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Metabolic flux control analysis and the concept of excess capacity of enzymes over pathway flux are related by the functional threshold, at which damage or inhibition of an enzyme reduces excess capacity to a minimum and starts to limit overall flux through the pathway. Excess capacity of cytochrome *c* oxidase (COX) varies between tissues, but little is known about differences between species. In particular, information is lacking on mitochondrial respiratory function in the mouse heart, despite the fact that transgenic mice provide increasingly important animal models. Permeabilized muscle fibers were prepared from the left ventricle of a single mouse heart, and measured in OROBOROS Oxygraph-2k instruments in parallel at 4, 25, 30, 37 and 40 °C (N≥4). Threshold plots were constructed from azide titrations of flux through the electron transport chain (parallel e-input into complexes I+II with pyruvate+glutamate+malate+succinate and uncoupling by FCCP), versus COX (0.5 mM TMPD+2 mM ascorbate after uncoupling and inhibition by rotenone+malonate+antimycin A). Azide was used, since inhibition of COX by cyanide is reversed by pyruvate particularly at low oxygen levels. The inhibition constant, K_i, of COX for azide was 0.1 mM at 37 °C, increasing from 4 to 40 °C over two orders of magnitude. COX velocity measured with TMPD+ascorbate was 1.3-fold of maximum electron transport capacity of the respiratory chain at 25 to 40 °C, and 3.3-fold at 4 °C. In contrast, linear extrapolations of threshold plots revealed a COX excess capacity of 1.6-fold over pathway flux in the range of 30 to 40 °C, increasing to 1.8- and 7.6-fold at 25 °C and 4 °C, respectively. Application of complex I substrates only, would yield an apparent COX excess capacity of >3-fold over pathway flux (at 30 and 37 °C), since parallel e-input through complex I+II doubled flux compared to complex I substrates. Taken together, COX excess capacity in myocardial fibers of the mouse was significantly higher than in fibers of rat heart or human skeletal muscle. Results obtained under hypothermic incubation conditions of permeabilzed fibers may be extrapolated to physiological

temperature of 37 °C with caution only. The very high COX excess capacity under hypothermia (4 °C) may compensate for hypothermic hypoxia by decreasing the p_{50} of mitochondrial respiration in parallel to the decreased p_{50} of hemoglobin and myoglobin. The present study yields an important baseline for further investigations of mitochondrial function in the mouse heart, including genetic models of acquired and inherited mitochondrial defects.

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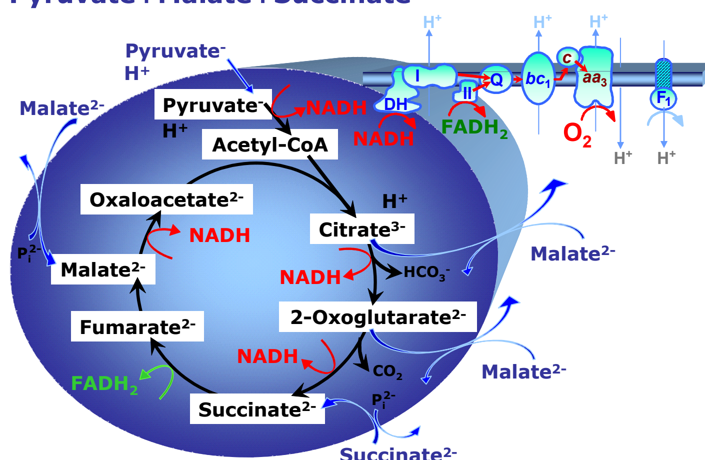
MiPNet 8. Combining respiratory substrates for Complex I+II. A transition from bioenergetics to mitochondrial physiology.



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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.

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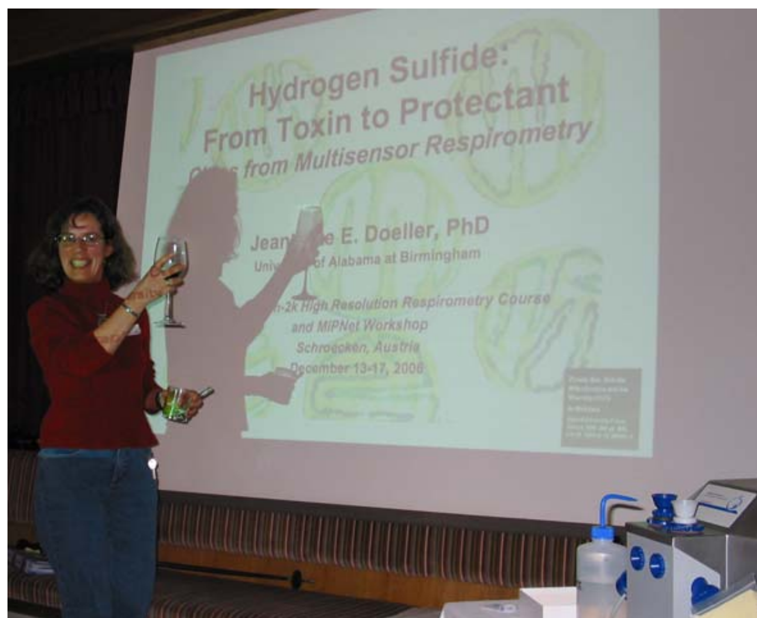
MiPNet 9. Hydrogen sulfide: From toxin to protectant.



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The path of hydrogen sulfide (H_2S) research has taken several surprising turns. For many years, H_2S was thought of as a poison, with toxicity resulting from heme group binding, thus inhibiting both O_2 delivery by hemoglobin and myoglobin, and electron transport to O_2 by cytochrome *c* oxidase. The human olfactory system protects against H_2S toxicity by detecting ppb H_2S. H_2S has also been known for years as an energy source primarily for prokaryote thiobacillus metabolism. Then in the late 1970s, the discovery of deep ocean hydrothermal vents boosted the nascent field of H_2S physiology as large communities of gutless animals were found to rely on H_2S as a primary energy source fueling their prokaryotic chemosynthetic endosymbionts. The study of H_2S physiology continued with symbiont-containing and non-symbiont-containing animals from other H_2S-rich environments. Then surprisingly from the 1990s on, reports began to appear stating that many animals including humans contain H_2S levels important for physiological health, and that in fact high or low H_2S levels result in human pathology. H_2S is now considered a cell signal on par with nitric oxide (NO) [1]. It is produced in all mammalian tissues tested, is capable of post-translational protein modifications via heme ligation and thiol interactions, and has effects on the nervous, cardiovascular, hepatic, pulmonary, and gastrointestinal systems. More recently, H_2S was shown to put laboratory mice into a reversible hibernation-like state [2]. This startling 2005 finding with important medical implications was recently corroborated in 2006, and both reports made headline news. But historically, humans have appreciated the beneficial effects of H_2S for centuries as visitors to sulfide mineral springs and spas where H_2S is absorbed dermally. In addition, the beneficial effects of dietary garlic, also known for centuries, are most likely mediated by H_2S as we have recently shown that garlic compounds are rapidly metabolized to H_2S by many cell types [3]. Using multisensor O2k-respirometry with the novel polarographic hydrogen sulfide sensor (PHSS) developed in our laboratory [4], we are focused on the accurate physiological and real-time measurement of H_2S and its interactions with O_2 in order to understand the beneficial effects of H_2S on the vascular system (see abstract by David Kraus) as well as the regulation of cellular and systemic H_2S homeostasis. Data pertaining to the mechanisms of H_2S production, consumption and capacitance in mammalian systems will be presented. It has also been recently proposed that H_2S may be responsible for the greatest extinction event on earth [5], and recently shown that H_2S is responsible for the preservation of organic molecules over geologic time [6], thus substantiating the claim that H_2S is both toxin and protectant.

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MiPNet 10. Hydrogen sulfide as a vasoactive signal: Clues from multisensor respirometry.

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Hydrogen sulfide (H_2S) has recently been shown to have a signaling role in vascular cells [1]. Like nitric oxide (NO), H_2S is enzymatically produced by amino acid metabolism and can cause post-translational modification of proteins, particularly at thiol residues. Molecular targets for H_2S include K_{ATP} channels, and H_2S may interact with heme proteins such as cyclooxygenase. It is well known that the reactions of NO in the vasculature are O_2-dependent but this has not been addressed in most studies designed to elucidate the role of H_2S in vascular function. This is important since H_2S reactions can be dramatically altered by the high concentrations of O_2 used in cell culture and organ bath experiments. In order to test the hypothesis that the effects of H_2S on the vasculature are O_2-dependent, we have measured real time levels of H_2S and O_2 in multisensor O2k-respirometry and vessel tension experiments as well as the associated vascular responses. A novel polarographic hydrogen sulfide sensor (PHSS) developed in our laboratory was used to measure H_2S levels [2]. We determined that in rat aorta, H_2S concentrations that mediate rapid contraction at high O_2 levels cause rapid relaxation at lower physiological O_2 levels. At high O_2, the vasoconstrictive effect of H_2S suggests that it may not be H_2S per se but a putative vasoactive oxidation product that mediates constriction [3].

H_2S may also mediate vasoactivity via interaction with S-nitrosothiols, again in an O_2-dependent manner. To test this hypothesis, we used multisensor O2k-respirometry to measure real time levels of H_2S, NO and O_2 in experiments in which H_2S was added to the model compound S-nitroso-glutathione (GSNO) at different O_2 levels. We found that H_2S addition caused rapid stoichiometric NO release from GSNO in an O_2-dependent manner. NO release and GSNO disappearance were independently measured spectrophotometrically. Three-dimensional analysis of the disappearance rates of H_2S and NO at different O_2 levels showed a dramatic interaction between H_2S and NO at high

O_2 that leads to much faster rates of disappearance of each component compared to rates of each component with O_2 alone [4].

The pathology of endotoxemia results in rapid hemodynamic alterations that are exacerbated by dysregulation of vascular tone to vascular signals [5]. The increased level of tissue oxidation products resulting from increased iNOS activity alters the response of vascular smooth muscle to H_2S signaling. We found that vessels from endotoxemic rats exhibited a higher H_2S production rate compared to healthy vessels, and instead of exhibiting H_2S-mediated vasorelaxation, they exhibited rapid constriction even under low O_2 conditions, similar to the response of healthy vessels at high O_2. This complex chemistry should contribute to the vasoactive effects of H_2S, NO and O_2 *in vivo*, and is best studied in real time with multisensor O2k-respirometry.

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