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Mitochondrial Physiology

The Many Faces and Functions of an Organelle

Edited by
Erich Gnaiger

MiP2005
4th Conference on
Mitochondrial Physiology
Schröcken, Vorarlberg, Austria

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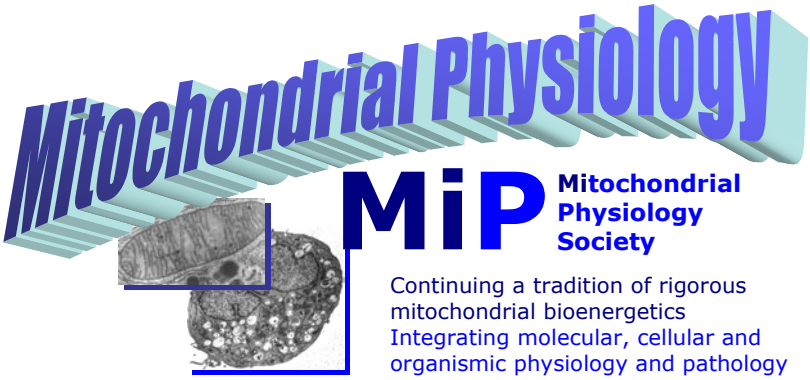
Focus

Continuing rigorous mitochondrial bioenergetics; integrating molecular, cellular and organismic physiology and pathology - mitochondrial function in health and disease. Presentations by established scientists and young investigators. In the face of a tremendously increased interest in MiP2005, the max. number of participants was increased from 100 to 160. Irrespectively, the number of oral presentations remains limited to provide time for discussions.

The 3rd MiP Conference was held in 2003 (Schröcken, Austria), at which occasion the MiP Society was formed. With your on-line registration, you may choose to become a member of the MiP Society:

www.mitophysiology.org



Mitochondrial Physiology

www.mitophysiology.org

The Mitochondrial Physiology Society has been founded in 2003, to organize MiP Conferences and MiP Workshops. These "MiP Events" focus on common and cell-specific functions of mitochondria at molecular, organelle, cellular, tissue and organ levels. Thus we meet the increasing demands for integrating current developments in the methodologically and conceptually complex field of mitochondrial physiology. Mitochondrial research has recently undergone a dramatic renaissance, due to a whole series of discoveries revealing its central roles in cell death, disease pathology, aging, thermogenesis, oxidative stress, cell signalling, cellular regulation and cell bioenergetics. Functional genomics and proteomics is putting an urgent emphasis on integrative understanding of the link between genes/molecules and cellular/organisms functions. Because these topics require continuous technical advances there will also be an emphasis on new methodological developments, quantitative methods of analysis in mitochondrial research including mathematical modeling, proteomics and the use of tools for measuring multiple parameters in an experiment. This multidisciplinary approach requires a new forum for high-level training courses, bringing together scientists working in divergent disciplines, to talk together about mitochondrial function and dysfunction, to resolve differences, to develop research agendas, to spread new knowledge and techniques, and to develop collaborations.

Until recently, mitochondrial respiratory function was mainly studied in research laboratories specializing on the bioenergetics of isolated mitochondria and bacteria, energy transformation in transmembrane gradients and characterization of the various complexes of the respiratory chain. Today these methods and concepts are expanded to applications on intact or permeablized cells and tissue preparations, and are widely used by groups with different research and clinical orientation, particularly in molecular cell signalling. The series of MiP Events will help to provide the expertise necessary for successful application of the techniques and concepts on mitochondrial bioenergetics in the wider context of molecular and cell biology, exercise physiology, diagnosis of mitochondrial diseases, and evaluation of mitochondrial function in oxidative stress, ischemia-reperfusion, aging and numerous areas (such as apoptosis) of key-involvement of mitochondria.

Erich Gnaiger, Ph.D.
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MiP2005

Chairpersons receive a MiP2005 present if their session is successfully started and completed within the **time scheduled** in the programme, and if they guide discussions such that contributions are made to clarify **what MiP is**.

10+5 min Presentations: Speakers receive a MiP2005 present if their presentation proves that they selected their 10 (max. 12) **best slides**, if the PowerPoint slides are readable from the far back, and if transmission of the key message for MiP is completed within **600 s** (use of SI units is encouraged). +5 min will be reserved for discussions. This should particularly provide poster presenters an additional opportunity to point out connections to their related messages.

Posters receive a MiP2005 present if the poster reveals focus on the **essential** detail and **visible** perspective on MiP.

YIP - Young Investigator Presentations: Students are invited to participate in the competition for YIP awards. (1) Selection of a YIP for a 10+5 min oral presentation is a MiP award, based on submitted abstracts. (2) Six YIP posters are selected during the meeting for 5-min presentations in Session 12 (5+5 min), and these young investigators receive MiP awards.

Instrument Exhibition: Specific instrument exhibitions are organized in the lunch breaks and evenings, in connection with poster presentations.

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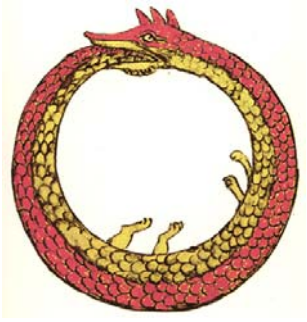

Walks and talks at MiP2003: Erich Gnaiger (top), Eiji Takahashi (left), Guy Brown and Cecilia Giulivi (middle), Evi Hütter and Steve Hand (right).

UNITED MITOCHONDRIAL DISEASE FOUNDATION

September 9, 2005

Dear Colleagues:

On behalf of the Board of Directors and Staff of the United Mitochondrial Disease Foundation (UMDF), we are pleased to offer these words of congratulations and support.

At UMDF, our mission is our driving force:

> To promote research and education for the diagnosis, treatment, and cure of mitochondrial disorders and to provide support for affected individuals and families.

It is for this reason that we are proud to provide support for this important scientific meeting which has been organized by the Mitochondrial Physiology Society under the Chairmanship of Prof. Erich Gnaiger. We understand that good science knows no borders and we are committed to a world united in the struggle to unravel the medical mystery of mitochondrial disorders.

Thank you for your talent, energy, and fervent desire to help find a cure. We truly believe that we are on the verge of medical breakthroughs that will change our understanding of disease in the 21st century. This is a task of no small proportion; and we must all pull together if we are to make a difference.

Again, please accept our profound thanks for your good work. Enjoy the conference, this wonderful setting, and please remember the thousands upon thousands of children and adults who are counting on us for a better future or, sadly, any future at all.

Yours toward a cure,

Charles A. Mohan, Jr.
Chair, Board of Directors

With all best wishes,

J. Thomas Viall
CEO/Executive Director

The United Mitochondrial Disease Foundation • 8085 Saltsburg Road, Suite #201 • Pittsburgh, PA 15239
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Mission Statement

To promote research and education for the diagnosis, treatment, and cure of Mitochondrial Disorders and to provide support to affected individuals and families

Welcome to MiP2003 - The music group of Schröcken provided a warm welcome to MiP2003 in the evening after arrival. The greetings of the conductor Heinz Feuerstein are translated by Erich Gnaiger (left).

MiP2003 Session in the lecture hall - Opening lecture by Graham Kemp, chaired by Guy Brown (top right). Kathrin Renner assists in the presentations (foreground).

Left table 1: Zdenek Drahota (edge); left side: Josef Houstek and Miriam Palacios; right side: Veronica Hollis, Marisol Quintero, Jacques Leibowitch, Andrea Dlasková, Petr Jezek.

Left table 2: front: Pavel Jesina, Petr Pecina, Alena Vojtisková, Olga Matejková, Ondrej Kuda, Marek Böhm; back side: Sebastian Vogt, Susanne Arnold, Dieter Brdiczka (behind), Maik Hüttemann, Bernhard Kadenbach, Cecilia Giulivi (standing), Katrin Staniek, Hans Nohl.

Left table 3: Adrian Lambert, Peter Schönfeld, Stefanie Jarolim (behind), Julian Pakay, Franz Hartner, Julius Liobikas (behind), Marina Jendrach, Pavel Golik, Oliver Speer (behind), Fréderic Bouillaud (standing).

Right table 1: front: Guy Brown; left: Shey-Shing Sheu; back: Steve Hand, Michael Verkhovsky, Jordi Bermudez (behind), Hans van Beek, Robert Boushel (edge).

Right table 2: left: Barbara Santoro; back: Daniela Curti, Zemfira Gizatullina, Irina Shabalina, Vilma Borutaite, Jerzy Duszynski, Marzia Arese (behind).

Right table 3: left: Domenico Boffoli; back: Brigitte Haffner, Ove Eriksson (behind), Eveline Hütter, Uwe Schlattner (behind), Fernanda Ferreira, Marko Vendelin (behind), Nazzareno Capitanio, Claudia Piccoli (front), Eiji Takahashi (behind).

Mitochondrial Physiology: The Many Faces and Functions of an Organelle

0-01. The many faces of mitochondiral physiology - Welcome to MiP2005.

Erich Gnaiger[1,2]
[1]*Swarovski Research Lab., Dept. Transplant Surgery, Innsbruck Medical Univ.;* [2]*OROBOROS INSTRUMENTS, Innsbruck, Austria. – erich.gnaiger@uibk.ac.at*

The idea for a first MiP workshop originated during an European training course in Grenoble, France in summer 1999, and was held in Schröcken, Vorarlberg, Austria in Sept. 1999, with 14 presentations and about 30 participants. Another small MiP workshop was organized as a satellite meeting to the European Bioenergetics Conference (EBEC) and BioThermoKinetics Meeting (BTK) in Arcachon, France, in Sept. 2002.

The Mitochondrial Phyisology Society was founded in 2003 as a spontaneous result of the successful MiP2003 Conference held on 12-16 September 2003 in.
As a continuation, MiP2007 will

While the little village of Schröcken (1269 m above sea level), with 233 inhabitants and 800 touris beds is well known for winter sports in the Arlberg region, it is quite remote from international airports, may be inaccessible under extreme weather conditions due to avelanches, land slides and flooded roads (such as in August 2005), and might be difficult to reach during occasional snowfalls even in September.

Like MiP2003, organization of the 4th Conference on Mitochondrial Physiology (MiP2005) started with the difficult perspective of a "zero-budget meeting". Fortunately, this changed as a results of fruitful discussions during a a meeting with the board of the United Mitochondrial Disease Foundation (UMDF) during the Mitochondrial Medicine Conference in St. Louis (US) in June 2005. In the name of the Mitochondrial Physiology Society, I want to thank the UMDF for their interest in future collaborations between UMDF and MiP, and for their generous financial support of MiP2005. In cooperation with all MiP members, contributors and particularly those who received financial support for their contribution to MiP, we will make all possible efforts to turn this great support into a growing seed spreading our contributions world-wide. "*Think Mitochondria*" (UMDF) and "*Integrating molecular, cellular and organismic physiology and pathology*" (MiP) are common denominators of our common interests. Whereas MiP has its main roots in fundamental mitochondrial bioenergetics and was established to integrate the rich scientific tradition of bioenergetics with the new challenges of mitochondrial physiology, the UMDF takes the integrative concept of "*Think Mitochondria*" several steps further to the clinitian, to the familiy, to the individual patient. UMDF provides exquisitly the "nuts and bolts" for research in mitochondrial medicine (C.A. Mohan Jr., *Think Mitochondria*, Vol. 1) - MiP can contribute by its world-wide network of scientific excellence. Without any doubt, therefore, the expanding fields of mitochondrial physiology and mitochondrial medicine – MiP and UMDF – can benefit mutually from an explicit co-operation. A specific mission of MiP in this respect is the extension of quantitative diagnostic approaches to evaluate mitochondrial function in health and disease.

0-02. The future of mitochondrial physiology.

Guy C Brown

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It is not entirely clear what mitochondrial physiology is, but it may be the study of what mitochondria do.

A Brief History of Mitochondrial Physiology:

1774: Joseph Priestly & Antoine Lavoisier, discover oxygen and respiration.
1857: Rudolph Kölliker, pioneer of light microscope, finds mitochondria in muscle.
1890: Richard Altmann, develops mitochondrial stain, postulates genetic autonomy.
1898: Carl Benda develops crystal violet as mitochondria-specific stain. Coins name.
1912: Otto Warburg, identifies respiratory enzyme, crude mitochondrial isolation.
1923-1933: David Keilin, identifies cytochromes and redox chain.
1940-1943: Albert Claude, isolates intact liver mitochondria and microsomes.
1948-1951: Albert Lehninger, locates β-oxidation, TCA and oxphos in mitochondria.
1952: George Palade, uses EM to define inner, outer membranes and cristae.
1950's: Britton Chance, control of mitochondrial respiration.
1961: Peter Mitchell, chemiosmotic theory.
1972: Denham Harman, mitochondrial free radical theory of aging.
1974: David Nicholls, mitochondrial proton leak regulates BAT heat production.
1981: Lynn Margulis, endosymbiotic theory of mitochondrial origins.
1981: Fred Sanger, mtDNA sequence
1986: Kay Tanaka et al, first mtDNA diseases reported.
1996: Xiaodong Wang, reports cytochrome *c* release central to apoptosis.

I have no idea what the future of mitochondrial physiology is, but I am sure it will be as surprising and unpredictable as it's past. I will propose some wacky ideas about mitochondria, not because I necessarily think they are true, but because I don't know that they are false.

Mitochondrial viruses: can viruses hang out in mitochondria?
Mitochondrial autonomy: can mitochondria exit and enter cells?
Mitochondrial identity crisis: can mitochondria fuse with other cell membranes?
Mitochondrial differentiation: cells differentiate and change state, do mitochondria?
Mitochondrial vibrations: is energy moved around by vibrational energy transfer?
Mitochondrial lightning: is the electric field large enough to cause ionization?

0-03. Role of intramitochondrial ROS in long-distance transmission of the apoptotic signal.

Vladimir P Skulachev

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A simple method to study interaction of two cell cultures has been developed when two coverslips were placed side by side in a Petri dish, one coverslip with apoptogen-treated cells (the inducer coverslip), and another with non-treated cell (the recipient coverslip). TNF, staurosporine and H_2O_2 treatment of cells on the inducer coverslip is shown to initiate apoptosis on the recipient coverslip. Such an effect is increased by a catalase inhibitor aminotriazole and is arrested by addition of catalase or by pre-treatment of the cells with nanomolar concentrations of mitochondria-targeted cationic

antioxidant MitoQ, which specifically arrests the H_2O_2-induced apoptosis. The MitoQ action is abolished by an uncoupler preventing accumulation of MitoQ in mitochondria. It is concluded that ROS produced by mitochondria in the apoptotic cells initiate the H_2O_2 release from these cells. The H_2O_2 released is employed as a long-distance cell suicide signal messenger. In processing of such a signal by the recipient cells, mitochondrial ROS production is also involved. It is suggested that the described phenomenon may be involved in expansion of the apoptotic region around the damaged part of the tissue at heart attack and stroke as well as in organoptosis, i.e. disappearance of organs during ontogenesis.

1. Pletjushkina OY, Fetisova EK, Lyamzaev KG, Ivanova OY, Domnina LV, Vyssokikh MY, Pustovidko AV, Vasiliev JM, Murphy MP, Chernyak BV, Skulachev VP (2005) *Cell Death Differ.* (in press).
2. Pletjushkina OY et al. (2005) *Biochemistry (Moscow)* (submitted).

Session 1: Integrated Cardiovascular and Mitochondrial Physiology. I. Skeletal Muscle, Exercise, and Endurance

1-01. Repeated static contractions to fatigue increases mitochondrial vulnerability towards oxidative stress.

Kent Sahlin[1,2,3], M Mogensen[2], JS Nielsen[2], M Tonkonogi[1,3,4]

[1]Stockholm Univ. College Physical Education and Sports, Stockholm, Sweden; [2]Inst. Sport Sciences Clinical Biomec., Univ. Southern Denmark, Odense; [3] Dept. Physiology Pharmacology, Karolinska Intitutet, Stockholm, Sweden; [4]Dept. Health Social Sciences, Dalarna Univ., Falun, Sweden. - kent.sahlin@ihs.se

The effect of repeated static exercise (RSC) on mitochondrial function and SR Ca^{2+} kinetics was investigated in human muscle. Ten male subjects performed 5 sustained static contractions at 66 % of maximal voluntary contraction force (MVC) to fatigue with 10 min rest in between. Muscle contractility was measured pre- and post-exercise with MVC and transcutaneous electrical stimulation. Mitochondria isolated from muscle biopsies (pre-, 0 and 24 h post-exercise) were analyzed for respiratory function [with and without prior exposure to H_2O_2 (ROS)], mitochondrial resistance to Ca^{2+} induced pore opening (MCaR) and in vitro sarcoplasmatic reticulum (SR) Ca^{2+} uptake and release.

RSC had no effect on mitochondrial function. However, mitochondria isolated from muscle samples taken after RSC were more vulnerable to ROS as demonstrated by reduced respiratory control index (RCI=state 3/state 4), reduced P/O ratio and reduced maximal rate of oxidative phosphorylation (oxphos) (P<0.05). After 24 h recovery P/O ratio and oxphos were restored, whereas RCI remained depressed and uncoupled respiration was elevated. MCaR was related to % type II fibres (myosine heavy chain II) but was not affected by RSC. RSC resulted in altered muscle contractility (reduced MVC, twitch force, 20/50 Hz force ratio and faster force relaxation) which remained 3 h post-exercise. SR Ca^{2+} uptake rate was lower 0 h post-exercise (P<0.01 vs. 24 h post-exercise) and could not explain the faster force relaxation.

It is concluded that RSC does not affect mitochondrial function but increases the vulnerability of mitochondria towards ROS. It is suggested that this is a consequence of augmented ROS formation and associated depression of scavenger substances during RSC, which is an ischemia-reperfusion type of exercise. The depressed 20/50 Hz force ratio and the slow recovery of muscle contractile function suggest that fatigue is related to non-metabolic factors e.g. failure of excitation-contraction coupling.

1-02. Mitochondrial regular arrangement in muscle cells: a "crystal-like" pattern.

Nathalie Béraud[1], M Vendelin[1,2], K Guerrero[1], VA Saks[1,3]

[1]Group Quantitative Structural Bioenergetics, Lab. Fundamental Applied Bioenergetics, INSERM E0221, Joseph Fourier Univ., Grenoble, France; [2]Inst. Cybernetics, Tallinn Technical Univ., Akadeemia 21, 12618 Tallinn, Estonia; [3]Lab. Bioenergetics, National Inst. Chemical Physics Biophysics, Akadeemia 21, Tallinn, Estonia. - nathalie.beraud@ujf-grenoble.fr

The aim of this work is to characterize quantitatively the arrangement of mitochondria in heart and skeletal muscles. For this, we studied confocal images of mitochondria in non-fixed cardiomyocytes and fibers from soleus and white gastrocnemius muscles of

adult rats. The arrangement of intermyofibrillar mitochondria was analyzed by estimating the densities of distribution of mitochondrial centers relative to each other (the probability density function).

In cardiomyocytes (1820 mitochondrial centers marked), neighboring mitochondria are aligned along rectangle, with distance between the centers equal to 1.97 ± 0.43 μm and 1.43 ± 0.43 μm in longitudinal and transversal directions, respectively.

In soleus (1659 mitochondrial centers marked) and in white gastrocnemius (621 pairs of mitochondria marked), mitochondria are mainly organized in pairs at the level of I-band. Due to such organization, there are two distances characterizing mitochondrial distribution in longitudinal direction in these muscles. The distance between mitochondrial centers in longitudinal direction within the same I-band is 0.91 ± 0.11 μm and 0.61 ± 0.07 μm in soleus and white gastrocnemius, respectively. The distance between mitochondrial centers in different I-bands is est ≈ 3.7 μm and ≈ 3.3 μm in soleus and in gastrocnemius, respectively. In the transversal direction, the mitochondria are packed considerably closer to each other in soleus than in white gastrocnemius | the distance is equal 0.75 ± 0.22 μm (soleus) and 1.09 ± 0.41 μm (gastrocnemius).

Our results show that intermyofibrillar mitochondria are arranged in highly ordered crystal-like pattern in a muscle-specific manner with relatively small deviation in the distances between neighboring mitochondria. This is consistent with the concept of the unitary nature of the organization of the muscle energy metabolism [b].

1. Vendelin M, Béraud N, Guerrero K, Andrienko T, Kuznetsov A, Olivares J, Kay L, Saks V (2005) Mitochondrial regular arrangement in muscle cells : a "cristal-like" pattern. *Am. J. Physiol. Cell. Physiol.* 288: C735-C767.
2. Saks V, Kaambre T, Sikk P, Eimre M, Orlova E, Paju K, Piirsoo A, Appaix F, Kay L, Regitz-Zagrosek V, Fleck E, Seppet E (2001) Intracellular energetic units in red muscle cells. *Mol. Cell. Biochem*. 356: 643-657.

1-03. High-resolution respirometry in small biopsies of human muscle: correlations with body mass index and age.

Erich Gnaiger[1], C Wright-Paradis[2], H Sondergaard[3], C Lundby[3], JA Calbet[4], B Saltin[3], J Helge[3], R Boushel[2]

[1]D. Swarovski Research Lab., Dept. Transplant Surgery, Innsbruck Medical Univ., Austria; [2]Dept. Exercise Science, Concordia Univ., Montreal, Canada; [3]Copenhagen Muscle Research Centre, Denmark; [4]Dept. Physical Education, Univ. Las Palmas, Gran Canaria, Spain. - erich.gnaiger@uibk.ac.at

Aerobic exercise and several aspects of life style influence mitochondrial respiratory function in human muscle, in addition to effects of age, gender and genetic background. In the present study, a significant part of the variability in respiration of human mitochondria [1] was explained by analysis of readily accessible background information on 25 healthy human subjects (19 males and 6 females; 22 to 46 years). Based on a novel multi-substrate/inhibitor protocol, this approach advances the functional analysis in mitochondrial physiology and pathology.

A protocol for high-resolution respirometry (with two or three OROBOROS Oxygraph-2k operated in parallel) was designed for quantification of mitochondrial respiratory capacities in permeabilized muscle fibers obtained from small needle biopsies (2 to 6 mg per run; 2 or 4 runs per subject). Cell membranes were selectively permeabilized [2], and lack of respiratory stimulation by cytochrome *c* indicated an intact outer mitochondrial membrane (Fig. 1). Measurements were performed at 30 °C in the range of 20 to 50 kPa oxygen pressure (210 to 530 μM), to avoid oxygen limitation [3]. In this range, autoxidation of ascorbate and TMPD was a linear function of oxygen, which was applied for correction of chemical background oxygen flux.

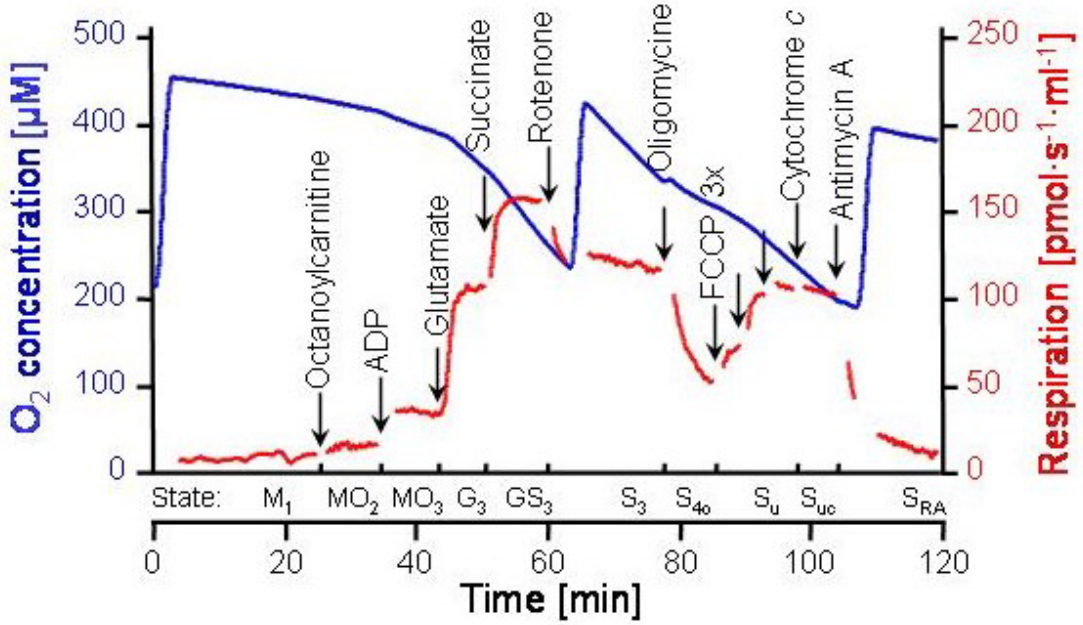

Fig. 1. Oxygen concentration and oxygen flux per volume of permeabilized fibers (vastus lateralis, 5.0 mg wet weight) in medium MiR05 containing malate. The titration steps are shown by arrows (continued with titrations of ascorbate and TMPD; not shown), and the corresponding states are defined on the time axis. Oxygenations were performed with pure oxygen purged into the gas phase of the intermittently opened chamber.

ADP-stimulated respiration with malate+octanoylcarnitine (state OM_3) was 46 % compared to further addition of glutamate (state GM_3). An additive effect was exerted by parallel complex I+II electron input (the GS_3/GM_3 ratio was 1.6), since respiration with succinate/rotenone (S_3) was only 1.1 times the state GM_3 (Fig. 1). In a variation of this protocol, FCCP was titrated upon state GS_3, yielding a further 44 % increase (and a corresponding GS_u/GM_3 ratio of 2.4). State GS_3, therefore, reflects the capacity of the phosphorylation system, in agreement with results on isolated mitochondria [4]. The coupled state GS_3 represents the physiologically relevant upper limit of respiration, providing parallel complex I and II input in accordance with an operational TCA cycle. The physiological excess capactiy of COX, expressed as the COX/GM_3 ratio was 2.7, whereas the COX/GS_3 ratio was 1.4. Respiratory adenylate control ratios were identical with octanoylcarnitie (OM_3/OM_2) and succinate (S_3/S_o).

State GS_3 declined significantly as a function of body mass index (BMI; body weight/hight^2) in the 19 males, which explained ~60 % of total variability. BMI was independent of age, as was the GS_3 respiratory capacity. Fatty acid oxidation capacity (state OM_3), however, declined significantly with age (males and females combined), thus extending a study on isolated mitochondria [1] to a surprisingly narrow range of ages. Consideration of BMI and age, therefore, improves the diagnostic resolution of functional mitochondrial respiratory analyses.

Supported by the Copenhagen Muscle Research Centre and Fonds de la Recherche en Sante Quebec (FRSQ), Concordia Univ., The Natural Science and Engineering Research Council of Canada (NSERC).

1. Rasmussen UF, Krustrup P, Kjaer M, Rasmussen HN (2003) Human skeletal muscle mitochondrial metabolism in youth and senescence: no signs of functional changes in ATP formation and mitochondrial oxidative capacity. *Pflugers Arch. – Eur. J. Physiol.* 446: 270-278.
2. Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Mark W, Steurer W, Saks V, Usson Y, Margreiter R, Gnaiger E (2004) Mitochondrial defects and heterogeneous cytochrome *c* release after cardiac cold ischemia and reperfusion. *Am. J. Physiol. Heart Circ. Physiol.* 286: H1633–H1641.
3. Gnaiger E (2003) Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. *Adv. Exp. Med. Biol.* 543: 39-55.
4. 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.

1-04. Local adaptations in mitochondrial substrate-specific O_2 flux capacity with prolonged, low intensity, whole-body endurance training.

Robert Boushel[1], C Wright-Paradis[1], H Sondergaard[2], JA Calbet[3], B Saltin[2], J Helge[2], E Gnaiger[4]

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Endurance training elevates peak muscle oxygen uptake by means of hemodynamic increases in oxygen delivery and local increases in muscle capillary and mitochondrial density. In addition, training enhances the rate of muscle fat oxidation for energy supply during submaximal exercise [1]. The purpose of this study was to investigate the temporal adaptations in whole body and local arm and leg muscle oxidative capacity and substrate utilization at several stages of low intensity endurance training. Seven healthy Danes skied for 6 hours daily at 65 % of maximum heart rate over 42 days in the polar region of northern Greenland. High-resolution respirometry (OROBOROS Oxygraph-2k) allowed quantification of mitochondrial respiratory capacities from saponin-permeabilized skeletal fibers (2-6 mg) of the deltoid and vastus muscles [2].

At baseline, state 3 O_2 flux (in the presence of ADP) with parallel electron input into complexes I+II (glutamate, malate and succinate) was higher in the vastus compared to the deltoid (54 ±7 vs. 37±2 pmol·s·$^{-1}$mg^{-1}, respectively; P<.05). At training day 7, state 3 phosphorylation capacity was unchanged in the deltoid but reduced to 31±5 pmol·s^{-1}·mg^{-1} in the vastus (P<.05). After 42 days of skiing, flux capacity was the same in both muscles (38±3 pmol·s^{-1}·mg^{-1}). State 3 O_2 flux with octanoylcarnitine+malate was also higher in the vastus compared to deltoid at baseline (13±0.5 vs 10±0.5 pmol·s^{-1}·mg^{-1}, respectively; P<.05), but after 42 days of skiing both muscles had equal flux capacity with fat substrate (12.3±0.8 vs. 12.7±1 pmol·s^{-1}·mg^{-1}). Despite a daily energy expenditure of ~25,000 kJ (~6,000 kcal) over the 42 day ski sojourn, there were no changes in muscle mass, whole body V_{O2max} or substrate utilization measured by whole body respiratory quotient, nor whole-leg and arm V_{O2max}.

These data reflect that (1) prolonged low intensity endurance exercise induces local adaptations to equalize substrate-specific muscle phosphorylation capacity preferentially towards energy sustainability rather than for peak respiratory power, and (2) high-resolution respirometry provides insight into local, muscle-specific adaptations not detectable with whole body or limb measures of oxidative capacity or substrate utilization. Considering previous findings indicating that mitochondrial function is not a limiting factor for V_{O2max}, these local muscle adaptations support the novel concept of exercise-specific muscle 'metabolic fitness'. In addition to the importance of cardiovascular fitness, these findings may have important implications for health.

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1-05. Mitochondrial studies *in situ* reveal a novel mechanism of dysfunction: uncoupling in aged muscle.

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Studies of mitochondria in biochemically purified organelles or permeabilized fibers *in vitro* are not always made under conditions mimicking their normal *in situ* environment of intact muscle. We developed NMR and optical spectroscopic methods to quantify mitochondrial function directly in intact muscle of humans and mice to establish a "gold standard" by which to identify and quantify abnormal function. We focused on aging because muscles in the elderly have reduced aerobic ATP synthetic capacity. We showed a substantial decline in aerobic phosphorylation capacity due to both reduced mitochondrial volume and reduced ATP synthesis capacity per mitochondrial unit. The latter is an example of dysfunction, not merely reduced function. Most work on mitochondria in aged muscle focuses on respiration. However, our recent *in vivo* experiments revealed significant uncoupling of ATP synthesis and respiration with age in both mouse and human muscle as we now describe.

In order to evaluate the effect of mitochondrial dysfunction on energetics in intact muscle, the following characteristics need to be quantified: ATP energy store generated, oxygenation, capacity for ATP synthesis by mitochondria, and coupling of phosphorylation to respiration (P/O ratio). In aged human and mouse muscle there is little change in total creatine and a tendency for ATP to be reduced with the consequence that the energy stores are maintained with minimal decrement in chemical potential. In young and aged muscle myoglobin is normally not fully saturated indicating that mitochondria function *in vivo* at very low p_{O2} (<8 torr, 1.1 kPa). The rate of PCr resynthesis following a metabolic perturbation is reduced in aged muscle and phosphorylation is decreased in elderly muscle per unit mitochondrial volume.

In addition our results also revealed the novel finding that phosphorylation is uncoupled to respiration in aged muscle. Mouse young adult leg muscle has a P/O = 2.2 whereas it is reduced to 1.1 in 30 month old mice. In elderly human muscle our recent data reveal the tibialis anterior muscle is normally well-coupled (P/O ~ 2.5) whereas the first dorsal interosseous of the hand can be substantially uncoupled in the same individual with P/O~1.9 for subjects >65 years. There is also a reduction in resting energy demand because oxygen consumption is not increased with uncoupling. The differences found in human individuals can be exploited to characterize the underlying mechanisms and their functional consequences.

To summarize, our work identified uncoupling of oxidative phosphorylation in elderly muscle (a mitochondrial dysfunction) as a new mechanism accounting for the reduced capacity of ATP synthesis characteristic of muscle in elderly subjects. This mechanism is in addition to the known loss of mitochondrial mass (sarcopenia, reduced mitochondrial volume) and respiratory chain capacity. Our ability to measure coupling *in vivo* in different human muscles allows us to design new experiments to identify variation in the effects of aging on mitochondrial function. We are actively exploring the mechanisms involved in this novel observation of uncoupling in both mouse and human muscle and their functional consequences.

1-06. Role of gender and caloric restriction in mitochondrial respiration-phosphorilation capacities and biogenesis in rat skeletal muscle.

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Previous work has shown the existence of a sexual dimorphism in the mechanisms in charge of the adaptation to food deprivation periods in rodents. Accordingly, females may have evolved adaptations to better withstand caloric restriction, a frequent condition in nature, probably because they are subjected to more severe selection pressures during time of food supply than males [1]. One of these mechanisms is the great ability of females to conserve energy in periods of food restriction, with their consequent higher energy efficiency [2,3]. Changes in this efficiency can be linked to changes in the necessary mechanisms to obtain sufficient energy, which are the mitochondrial oxidative-phosphorylation (OXPHOS) complexes.

In this work, we studied the role of gender and caloric restriction in the modification of mitochondrial respiration-phosphorilation capacities and mitochondrial biogenesis in rat skeletal muscle. For this purpose, Wistar rats of both genders were subjected to a three months of 40 % caloric restriction diet and compared to control rats fed ad libitum. Skeletal muscle gastrocnemius were removed, and we measured the nuclear and mitochondrial DNA (mtDNA) contents, the enzymatic activities of mitochondrial respiratory chain complexes I, III, IV, and ATPase (OXPHOS system), as well as the expression and protein content of several genes involved in mtDNA replication, transcription, and mitochondrial function, such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factors 1 and 2 (NRF-1, NRF-2), mitochondrial transcription factor A (TFAM), mitochondrial single DNA binding protein (mtSSB) and cytochrome *c* oxidase subunits I and IV (COX I, COX IV). The results indicated that mitochondrial muscle biogenesis was not altered either by gender or by three months of caloric restriction diet. Besides, skeletal muscle of female rats had a higher mitochondrial respiratory chain machinery and phosphorylation capacity than males, which is not due to differences in mitochondrial mass, but due to a higher cellularity index.

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1-07. Cycling efficiency is positively correlated to type I fibre content and negatively correlated to UCP3 protein but not to mitochondrial efficiency determined *in vitro*.

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The biochemical efficiency of the mitochondrion (P/O ratio) is an important determinant for the overall efficiency of the cell. Numerous factors have an influence on the mitochondrial efficiency such as the choice of substrate and the structural and biochemical composition of the inner mitochondrial membrane. There are some data from animal and cell culture studies, which indicate that the P/O ratio is influenced by muscle fibre type composition and content of UCP3 proteins [1,2]. Previous studies have shown

a negative correlation between the efficiency during cycling and UCP3 expression and a positive correlation between cycling efficiency and % type I fibres [3,4]. These studies indicate, that individual differences in cycling efficiency could be related to the mitochondrial efficiency. Training is known to decrease the UCP3 protein content and may also to some extent alter the fibre type distribution (increased oxidative fibres). These training induced changes should in theory increase the cycling efficiency of trained subjects. However, previous studies have shown contradicting results. The purpose of this study was to investigate the hypothesis that individual variations in cycling efficiency is related to mitochondrial efficiency. Furthermore we wanted to test the hypothesis that trained subjects have a higher cycling efficiency compared with untrained subjects.

On minimum two occasions trained (n = 9) and untrained (n = 9) subjects completed a submaximal cycle test at 40, 80 and 120 W. Based on the oxygen consumption the energy expenditure (EE) was calculated for assessment of individual cycling efficiency (gross-efficiency, GE; work-efficiency, WE; and delta-efficiency, DE). GE and WE were determined at all intensities. DE was calculated as the slope of the linear relationship between the EE and the work loads accomplished by the subject. GE, WE and DE were determined as the percentage conservation of energy in external work. Biopsies were taken on a separate day and used for determination of the mitochondrial respiratory efficiency, UCP3 protein content and fibre type distribution. Mitochondrial efficiency was determined during state 3 and submaximal respiration (constant rate of ADP infusion) with pyruvate+malate (Pyr) or palmitoyl-L-carnitine+malate (PC) as substrates. The relationship between the submaximal mitochondrial respiration and the resulting P/O ratio was fitted to a logarithmic function. Significance was considered at $P<0.05$.

Trained subjects had a higher maximal mitochondrial respiration when the respiration was expressed per kg wet weight. The relationship between the mitochondrial P/O ratio and the absolute respiration showed that the P/O ratio increased with increasing respiration. There was no significant difference between trained and untrained subjects in their mitochondrial efficiency. Untrained subjects had a significantly higher amount of UCP3 protein compared to trained individuals, but there was no difference in the fibre type distribution. UCP3 protein was negatively correlated with DE (r = -0.48), WE at 80 W (r = -0.49) and WE at 120 W (r = -0.56). Furthermore, WE was positively correlated to the % type I fibres at 80 W (r = 0.57) and at 120 W (r = 0.53). However, GE, WE and DE were not correlated to the mitochondrial efficiency determined during submaximal or maximal respiratory rates. Furthermore, there were no differences in GE, WE or DE between trained and untrained subjects.

It is concluded that cycling efficiency is correlated to the mitochondrial UCP3 protein and fibre type distribution. However, these correlations could be caused by fibre type differences in the content of UCP3 protein [5]. It is also concluded that mitochondrial efficiency is lower at low rates of respiration. This may be explained by an increased membrane potential, which triggers an increased proton leak. Individual cycling efficiency was not correlated to mitochondrial efficiency determined *in vitro* and there was no difference between trained and untrained subjects in their cycling efficiency. The results indicate that cycling efficiency *in vivo* is not influenced by the mitochondrial efficiency as determined *in vitro*.

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1-08. Polymorphisms in the mtDNA hypervariable region related to individual difference in endurance performance.

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It is well known that trainability of exercise widely differs from one person to another. The individual difference is evidently determined not only by environmental factors such as life-style and habitual meals, but also by genetic factors [1-2]. It is hypothesized that there are genes affecting endurance capacity and their responses to regular exercise. Searching the factors causing the individual difference is considered meaningful in terms of a more accurate prescription for how to forecast and evaluate the exercise capacity. The purpose of this study was to investigate whether the polymorphisms in the hypervariable region I of mitochondrial DNA (mtDNA HVR-I) related to individual difference in the aerobic capacity. 94 elite endurance athletes and 92 healthy controls participated in this study. The single nucleotide polymorphisms（SNPs）of mtDNA HVR-I and the VO_2max were determined. The relation between V_{O2max} and SNPs in the endurance athletes and their controls was analyzed. The polymorphism in the mtDNA HVR-I was decided based on the 'Cambridge sequence'. The total numbers of SNPs of mtDNA HVR-I were 19 variable sites. The subjects were classified into two groups at each variable site, the Cambridge sequence group and the non-Cambridge sequence group. The results indicated that V_{O2max}/kg were significant difference between Cambridge and non-Cambridge sequence groups at nucleotide positions 16362, 16085, and 16297 (P<0.05). The male athletes with non-Cam sequence at nucleotide position 16297 has a significant lower V_{O2max}/kg, while the female athletes with non-Cam sequence at nucleotide positions 16362 and 16085 have significant lower V_{O2max}/kg. In contrast of endurance athletes, V_{O2max} of the healthy controls were difference between Cam and non-Cam sequence groups at nucleotide position 16298 (P<0.05), but after body weight revising, the difference of V_{O2max}/kg was not significant at this position. The controls with non-Cam sequence at nucleotide position 16219 has a significant lower V_{O2max}/kg (P<0.05). In conclusion, we suggest that several polymorphisms in mtDNA HVR may relate to individual differences in endurance capacity and trainability, as SNPs markers, nucleotide positions of 16298, 16129, 16362, 16085 and 16297 related to individual difference of human aerobic endurance and their trainability [3]. As a rare unique heteroplasmic SNPs site in the endurance athletes, nucleotide position 16085 obviously is an important gene marker. Those mtDNA markers are significant for forecasting and assessing the athletic capability.

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1-09. A feedback molecular regulation of uncoupling on ROS generation in muscle mitochondria during the prolonged exercise.

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The mitochondrial electron leak is an important source of endogenous ROS in exercise. The recent researches show that effect of 'mild uncoupling' of respiratory chain is involved in mitochondrial anti-oxidation event [1]. The purpose of present research is to investigate whether mitochondrial mild uncoupling is induced and/or activated during strenuous exercise and to explore its possible molecular regulation.

SD rats were divided into 5 groups to rest or run for 45, 90, 120 and 150 min respectively on the treadmill according to the incremental protocol and sacrificed at rest or immediately after every exercise time course. The following parameters were determined: 1) Separated mitochondrial State 4 respiration rate in the presence of malate and glutamate by using Clark Oxygen Electrode; 2) ROS generation of separated mitochondria by fluorometric probe; 3) Expression of UCP-3 mRNA in muscle homogenate and its protein in mitochondria using RT-PCR and Western-Blotting respectively.

(1) Mitochondrial ROS generation were significant higher at 45, 90, 120 min than at rest ($P<0.05$, $P<0.001$, and $P<0.01$, respectively) with the peak at the point of 120 min, then obviously declined at 150 min subsequently ($P<0.001$). (2) In a parallel change, state 4 rate increased significantly when exercising for 90 and 120 min ($P<0.01$ and $P<0.001$ respectively) and lowered in exercising to 150 min ($P<0.001$). (3) There were remarkably higher levels of UCP-3 mRNA at 90, 120 and 150 min ($P<0.001$, $P<0.01$ and $P<0.01$ respectively), and following UCP-3 protein contents increased at 120 and 150 min (both $P<0.001$).

According to the observed changes, we hypothesize that ROS may contribute to activated and/or induced expression of UCPs. A functioning of the UCPs may cause mild uncoupling in response to matrix superoxide and other oxidants during exercise, leading to increased proton leak and feedback decreased superoxide production. This complicated and precise feedback loop would constitute a self-limiting cycle to protect against excessive superoxide production [2], leading to early protection against oxidation and regulation of cellular and mitochondrial redox.

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1-10. Effect of coenzyme Q_{10} supplementation on heart and liver mitochondrial function during exercise.

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Several studies have showed the effect of supplement of coenzyme Q (CoQ) on ability of exercise, but the effect of supplement of coenzyme on mitochondria function in exercise is unclear [1,2]. This investigation will research the effect of Coenzyme Q_{10} on mitochondrial function during exercise from the point of view of biochemistry and bioenergetics.

SD rats were divided into four groups: normal control group (NC); supplement Q control group (QC); normal exercise group (NE); supplement Q and exercise group (QE). Exercise group rats were forced to run on the treadmill and were sacrificed at 120 min after exercise. The parameters were following: (1) Mitochondria CoQ concentrations in heart and liver were analyzed by HPLC [3]. (2) The activities of syntheses were measured with luciferase-luciferin. (3) The initial rates of proton ejection and electron transferring were measured with potassium ferricyanide as an electron acceptor. (4) ROS generation rate were determined by fluorometric probe. (5) MDA concentrations were measured in myocardium, livers and serum.

(1) In coenzyme Q_{10}-treated rats, the coenzyme Q_{10} level of mitochondria increased significantly in myocardium and liver; the coenzyme Q_9 level of mitochondria increased significantly in myocardium also. (2) Comparing with normal control group, QC and QE group $H^+/2e$ and H^+-ATPase synthesis activity increased significantly in myocardium and liver. Comparing with NE group, QE group $H^+/2e$ increased significantly in myocardium. The results indicated that supplement of exogenous coenzyme Q improves the coupling of oxidative phosphorylation and increases the rate of mitochondria ATP synthesis activity. (3) Supplementing CoQ increased significantly ROS generation in rats in stage of exercise. The level of MDA of mitochondria increased significantly after treat with coenzyme Q_{10} in myocardium and livers. There are no differences in the level of MDA in serum in each group.

Supplement of exogenous coenzyme Q_{10} can increase the level of CoQ in mitochondria and the efficiency of energy transduction and the rate of ATP synthesis in mitochondria. At the same time, it increases production of free radicals and level of LPO.

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Session 2: Integrated Cardiovascular and Mitochondrial Physiology. II. Oxygen Delivery, Hypoxia, and Altitude

2-01. Measuring and modelling the effects of altered O_2 delivery on muscle metabolism.

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I consider four main issues, based as far as possible on quantitative analysis of published data in terms of models of mitochondrial function and regulation *in vivo*.

(A) Methodological issues in measurement of O_2 delivery and O_2 usage in vivo: There are several ways to study muscle 'metabolism (by which I mean ATP turnover in the service of force generation) *in vivo*. First, for oxidative metabolism, there are whole body V_{O2} measurements, and (less convenient) invasive arteriovenous difference (AVD) measurements of muscle O_2 consumption [5]. Second, ^{31}P magnetic resonance spectroscopy (MRS) can measure ATP production by oxidative and nonoxidative means (the latter including glycolysis to lactate and adjustments of transient ATP demand/supply imbalance by changes in phosphocreatine (PCr) concentration) [1,10,11]. There is a tradition of assessing 'mitochondrial capacity' (inferred maximal rate of oxidative ATP synthesis *in vivo*) by analysing relationships between oxidative ATP synthesis rate and the concentrations of metabolites of presumed regulatory relevance (e.g. ADP) or their correlates (e.g. PCr) [11,13,16]. Third, near-infrared spectroscopy (NIRS) and ^{1}H MRS of deoxymyoglobin can be used to report tissue PO_2. NIRS estimates of muscle O_2 content can be used to measure rates of O_2 usage [22], and to make semi-quantitative inferences about vascular O_2 supply abnormalities [11,12]. Recently NIRS has been suggested as a measure of O_2 AVD, and in combination with V_{O2} has been used to estimate the kinetics of blood flow [4]. (I argue that this is not a useful calculation, for algebraic reasons, and because in the data analysed [4] blood flow is always tightly coupled to O_2 use). Interpretation of NIRS data [12] is still hampered by disagreement about whether the signal is mainly from capillary deoxyhaemoglobin or myocyte deoxymyoglobin; if the latter [21], then like ^{1}H MRS of deoxymyoglobin [19], NIRS usefully reports p_{O2} in the vicinity of the mitochondrion (see (D) below). Fourth, ^{13}C MRS labelling methods can be used to measure TCA cycle flux *in vivo* [9], although current estimates of basal oxidative ATP synthesis by this method are substantially too high, for technical reasons. The same is true of a fifth technique, ^{31}P MR saturation transfer, which can be used with ^{13}C MRS to assess mitochondrial coupling *in vivo* [17].

(B) Understanding oxidative ATP synthesis rates in relation to metabolite concentrations: None of these methods tell us all we want to know, but some key measurements are available. I want to ask to what extent we understand these, a question with several aspects. First, can we fit our measurements of rates and metabolite concentrations into a model consistent with known biochemistry and physiology, both classical, and the newer insights of Metabolic Control Analysis (MCA)? Probably yes, to some extent. Secondly, how much explanatory power do such models have? Does e.g. measured [ADP] predict the rate of mitochondrial O_2 consumption? In some circumstances, yes e.g. within a single experiment. However, there is substantial

variation between published human studies in inferred maximal rates and relationships between ATP synthesis rates and e.g. [ADP]. Some of this is due to disagreement about resting [PCr] measured by ^{31}P MRS (necessary for calculation of [ADP]), and some is no doubt methodological in other ways, but what remains might be interesting physiology. In addition to longstanding disagreements about candidate force-flow relationships, it is argued (in conformity with MCA [3]) that 'parallel-activation' or 'feed-forward' influences are important in control of mitochondrial ATP synthesis *in vivo* [14], detectable in ^{31}P MRS experiments as a lack of correlation between fluxes and concentrations, and in particular as flux changes 'too large' for the changes in e.g. [ADP] [14]. This conclusion can be avoided if one posits a higher-order force-flow relation [7], although whether this corresponds to mitochondrial behaviour *in vitro* remains controversial. Recently attention has been focussed [20] on the direct effects of PCr and free Cr on O_2 consumption *in vitro*, independent of [ADP] [23]. Implications for mitochondrial regulation *in vivo* are linked to mitochondrial creatine kinase [20], although no detailed predictions have been made. While this is in theory a way in which a hyperbolic flux-ADP relationship *in vitro* could appear *in vivo* as the sigmoid relationship [7] needed to explain the dynamic–range problem without parallel activation, the required effect is much larger than has been shown *in vitro*. However, our understanding of these mechanisms remains incomplete.

(C) Understanding oxidative ATP synthesis rates in relation to glycolysis: A third question is, given some task-defined ATP demand, can we predict, nontrivially, how ATP generation will be split between oxidative and glycolytic means? One approach is to use the measurements we have (e.g. pH, PCr, ADP) to predict the oxidative ATP synthesis rate, and then infer glycolytic ATP synthesis essentially by difference. As well as the problems already mentioned, this is hampered by disagreement about how much the pH change which accompanies lactate accumulation itself reduces mitochondrial capacity [6,8]. Assessing this definitively requires agreement on the relevant flux-force relationships, which, as we have seen, is lacking. A related approach, concentrating on possible activators of glycolysis, is hampered by similar disagreement about whether open- or closed-loop influences dominate [2,15], and if the latter, which metabolites measurable *in vivo* are potential predictors of flux *in vivo*.

(D) Modelling the effect of impaired O_2 delivery on oxidative metabolism: This is physiologically and pathologically important. There have been semi-quantitative approaches. In vascular disease [11] and experimental hypoxia [18] the mitochondrial capacity inferred from ^{31}P MRS measurements is reduced at low cell PO_2 inferred from NIRS or ^{1}H MRS (see (A) above), apparently consistent with the known dependence of mitochondrial metabolism on the concentration of its substrate O_2 [10]. At present, though, a rate of O_2 usage cannot be 'read off' from cell PO_2 and (say) [ADP], any more than it can be from [ADP] and pH in exercise where [see (B) and (C) above], although pH falls, p_{O2} is not 'limiting'.

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2-02. Spatial profiles of mitochondrial oxygen consumption in myocardium *in situ* during ischemia.

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In the normal heart myocardial blood flow and oxygen consumption are both heterogeneous, but highly matched to each other. Myocardial ischemia and infarction as a result of coronary stenosis are major causes of death in humans. Myocardial infarction may show a patchy pattern. We investigated how oxygen delivery, as reflected by coronary blood flow, matches to oxygen consumption at the local level during increasing partial coronary stenosis of the left anterior descending (LAD) artery in porcine left ventricle.

In *in vitro* and *in vivo* myocardium we have demonstrated that TCA cycle and other metabolic fluxes can be quantitated from a single high-resolution carbon-13 NMR spectrum in a small tissue sample, making it possible to assess spatial patterns of oxygen consumption [1,2]. Heart muscle samples are taken exactly 5.5 min after the start of infusion of 13-enriched acetate. A high-resolution NMR spectrum is obtained from tissue sample extracts, showing single carbon NMR peaks, often split into multiplets due to J-coupling between adjacent isotopes. The NMR free induction decays are analyzed in the time domain and yield absolute line intensities. The multiplet intensities are analyzed by a model for isotope traffic in the TCA cycle and communicating amino acid pools. The 160 differential equation model is integrated for each point on a trajectory in parameter

space to find an optimal fit. Flux quantitation therefore depends on a pre-steady-state of carbon-13 isotope enrichment in glutamate.

In small (<1 ml) tissue samples, blood flow was measured with radioactive microspheres, and oxygen consumption in the same sample with the carbon-13 method. In one group of animals LAD pressure was 65-70 mmHg downstream of the stenosis (group I, n = 7), and in another group LAD pressure was 30-35 mmHg (group II, n = 7). During normal perfusion, blood flow (~5 ml·min^{-1}·g^{-1} dry mass) and oxygen consumption (~19 μmol·min^{-1}·g^{-1} dry mass) are well correlated (r=0.85). In group I, blood flow decreased by 31.9 % on average during stenosis, in group II by 40.9 %. The correlation between blood flow and oxygen consumption on a local level decreased markedly with decreasing blood flow and with decreasing LAD pressure downstream of the stenosis.

We conclude that during progressing partial coronary occlusion, in addition to the global decrease of the oxygen supply-to-consumption ratio, oxygen delivery to oxygen consumption matching is increasingly heterogeneous. This implies that local vasodilation reserve is not uniformly matched to regional demand and that during stenosis some areas are more affected by ischemia than others.

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2-03. Oxygen- and flux-dependence of ROS-formation of lung alveolar epithelial cells.

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ROS are produced in the cytoplasm and by mitochondria [3]. However, results are divergent on changes in ROS production upon varying cellular oxygen supply [1,2]. In the present study we measured ROS-formation during 15 min (37 °C) exposure of lung alveolar A549 cells to hypoxia (2% O_2), normoxia (21 % O_2) and hyperoxia (35 % O_2) by chemiluminescence (CL; 30 μM lucigenin) and ESR-spectroscopy (CMH, CPH, ACP, PPH; 500 μM). Both, CL and ESR indicate the lowest accumulation of ROS during exposure to hypoxia and an increase in ROS-formation with increasing oxygenation. It has to be noted, however, that decreasing the concentration of CMH also decreases the rate of ROS-formation in normoxic cells. In contrast to results obtained by CL and ESR, DCF-fluorescence was increased in hypoxia. In all cases, return to 21 % O_2 increased ROS-formation in hypoxic but decreased ROS in previously hyperoxic cells. N-acetyl-cysteine decreased CL in all states of oxygenation but oxygen-dependency of CL was still apparent. Decreased ROS-formation in hypoxia was associated with slightly increased NADH, measured by fluorescence microscopy, and a decrease in cellular oxygen consumption, measured by high-resolution respirometry (OROBOROS Oxygraph). In intact cells, addition of rotenone and antimycin A increased ROS-formation, whereas stimulation of complex I and II respiration by increasing ADP decreased ROS measured by CL in digitonin-permeabilized cells. Both, in the non-stimulated and ADP-stimulated state, ROS-formation was lower in hypoxia than normoxia. Our results confirm apparent discrepancies on changes in ROS formation upon changing oxygenation that can only be explained on the basis of different species of ROS that are seen by different detection methods. It might also be, however, that different ROS-indicators affect the rate of cellular ROS-formation in different ways, which needs further exploration.

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2-04. The effect of hypoxia upon the expression pattern of isoforms and the kinetics of cytochrome *c* oxidase in astrocytes and neurons.

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The brain is the organ with the highest energy demand in mammalian organisms. Neurons and astrocytes, two different brain cell types, are structurally, functionally, and metabolically tightly coupled with astrocytes playing a central role in regulation of cerebral energy metabolism in dependence on neuronal activity. Oxygen is, besides glucose, the most important substrate to fulfil neuronal energetic requirements and also the substrate of cytochrome *c* oxidase (COX), the enzyme that is engaged in mitochondrial oxidative energy metabolism. 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 energetic requirements by binding of ATP to the N-terminus of subunit IV and thereby causing an allosteric inhibition of COX activity at high energy level, i.e. high ATP/ADP ratio [1]. It was found that this COX subunit is expressed in isoforms (IV-1 and IV-2). While isoform IV-1 is ubiquitously transcribed in all adult mammalian tissues including brain, isoform IV-2 showed so far high transcription levels only in the lung [2].

Besides the expression of COX IV-1 isoform in astrocytes and neurons from mouse and rat brains, we detected also mRNA transcripts for the COX IV-2 isoform in neurons pointing at a cell type specific expression of COX subunit IV isoforms in the brain. Under conditions of oxygen deprivation mRNA transcription of COX IV-2 is induced in astrocytes and upregulated in neurons. So far, yeast has been the only organism known to express two isoforms (Va and Vb), homologous to the mammalian subunit IV, in dependence on oxygen concentration [3]. The functional consequences of an increased expression of COX IV-2 isoform, that structurally differs from COX IV-1 isoform in its N-terminus, are reflected in the abolition of allosteric inhibition of COX by ATP at high ATP/ADP levels. We conclude that the expression of COX IV-2 isoform under hypoxia suppresses the sensitivity of COX to its allosteric regulator ATP and overrules the regulation of COX by the cellular energy level.

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2-05. How Epo-overexpressing mice adapt to chronic excessive erythrocytosis.

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Excessive erythrocytosis is usually associated with hypertension and thromboembolism resulting in severe cardiovascular complications. The key player of regulating red blood cell number is erythropoietin (Epo) that controls red blood cell production in a HIF-1-dependent manner. To test the impact of hypoxia-independent expression of Epo, we generated a transgenic (tg) mouse line termed tg6 that, due to constitutive expression of human Epo, reached hematocrit values of 0.8 to 0.9 without alteration of blood pressure, heart rate or cardiac output. In tg mice plasma volume was not elevated whereas blood volume was 25 % of the body weight compared to 8 % in wildtype (wt) siblings. While plasma viscosity did not differ between tg and wt, tg whole-blood viscosity increased to a lower degree (4-fold) than expected. Apart from the nitric oxide-mediated vasodilatation, adaptation to high hematocrit in tg mice involves regulated elevation of blood viscosity by increasing erythrocyte flexibility.

Knowing that Epo exerts also non-erythropoietic but tissue-protective effects, we tested the impact of several insults on our tg6 mice. We observed that elevated Epo levels protected from light-induced (but not from inherited) retinal degeneration as well as from experimentally induced myocardial infarction. Finally, we provide evidence that upon exposure of tg6 mice to 6 % oxygen, cerebral Epo regulated the hypoxic ventilatory response.

2-06. Mitochondrial respiratory function in human skeletal muscle fibers studied at high altitude.

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This study examined the effects of acclimization to hypoxia (4559 m) on mitochondrial substrate utilization and respiratory function. The Bergstrom technique was used to obtain muscle biopsies of the vastus lateralis from 10 healthy Danish male subjects (25± 2 yrs) at sea level and again after 6 to 9 days at high altitude. High-resolution respirometry (OROBOROS Oxygraph-2k [1]) allowed quantification of mitochondrial respiratory capacities from saponin-permeabilized skeletal muscle fibers (2-6 mg).

At sea level, state 3 respiration (in the presence of ADP) with parallel electron input into respiratory complexes I+II (glutamate, malate and succinate) was 58±4 pmol·s^{-1}·mg^{-1}), 1.6-fold higher than with glutamate+malate or succinate+rotenone. Flux with complex I+II substrates indicates the capacity of the phosphorylation system as shown by the 1.5-fold higher respiration after uncoupling by FCCP. These findings obtained with permeabilized muscle fibers agree with results on isolated mitochondria [2]. Respiratory capacity with octanoylcarnitine+malate was 48 % of that with glutamate+malate. Respiratory coupling was quantified through the stimulation by ADP or inhibition of ATP synthase by oligomycin and subsequent uncoupling by FCCP. Respiratory control ratios with succinate or octanoylcarnitine were 2.8. Compared to these results at sea level, 6 to 9 days of high altitude exposure did not induce a detectable change in any of the respiratory capacities nor in coupling. These findings indicate that mitochondrial function is not a limiting factor for V_{O2max} during early acclimatization to high altitude,

strengthening the concept of a dominant role for systemic oxygen delivery during intense dynamic exercise [3].

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2-07. Different regimen of intermittent hypoxia training (IHT) as modulators of heart mitochondrial membrane permeability transition.

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IHT is believed to induce myocardial protection. We hypothesized that inhibiting of mitochondrial permeability transition pore (mPTP) opening by means of antioxidant system promotion is one of the key elements of such protection, and mitochondrial swelling rate depends on degree of hypoxic exposure. Four groups of male adult Wistar rats participated in the study. Animals of Gr. 1 underwent daily sham IHT (control group). Gr. 2–4 were exposed for 2 weeks to IHT in two regimen: R_1- breathing in normobaric chamber with 11 % O_2 gas mixture, 15-min sessions with 15 min rest intervals, 5 times daily (Gr. 2); R_2 – breathing with 8 % O_2, 5-min sessions with 15 min rest intervals, 5 times daily (Gr. 3-4). Gr. 1-3 were examined next day after IHT, Gr. 4 – in 45 days after IHT stopping. Phenylarsineoxide (PAO)-induced mitochondrial swelling rate was investigated spectrophotometrically (λ = 520 nm) in isolated heart mitochondria by a decrease in their optical density after 20 min of incubation with PAO. The intensity of lipid peroxidation and antioxidant defense mechanisms in rat organism were estimated before and after IHT by measuring of malon dialdehyde (MDA) content and the activity of superoxide dismutase (SOD) and catalase (CAT) in blood and liver. It was shown that the training with moderate hypoxia (R_1) did not essentially influence mPTP opening nor free radical production: The magnitude of mitochondrial swelling in Gr. 2 under incubation with PAO in concentrations 10^{-5} and 10^{-4} mol/l was almost the same as in Gr. 1; MDA content decreased by 10 and 20 % in blood and liver, respectively; a slight increase in superoxide dismutase activity by 29 и 23%, a decrease in catalase activity by 9 % (ns) and 21 %, respectively, was observed. The training with more severe hypoxia (R_2) provoked a two-fold decrease in PAO-induced mitochondrial swelling (Gr. 3 compared with Gr. 1). And even in 45 days (Gr. 4) the protective effects of R_2 on mitochondria were well-preserved. These effects were completely abolished in the presence of cyclosporin A (10^{-5} mol/l), indicating that mitochondrial swelling was due to mitochondrial permeability transition pore opening. Simultaneously, R_2 training caused pronounced increases in MDA content both in blood and liver by 67 and 32 %, respectively, and considerable augmentation in activities of SOD (49 and 32 %) and CAT (18 and 43 %). Moreover, in 45 days the activity of SOD exceeded initial levels three-fold both in blood and liver. Taken together, moderate intermittent hypoxia which provokes some inhibition of lipid peroxidation with slight increase in antioxidant activity, does not influence PAO-induced mitochondrial swelling. A more severe regime of IHT, stimulating both the increase of lipid peroxidation and strongly pronounced augmentation of antioxidant system, causes a stable increase in resistance of mitochondrial membrane to PAO. Probably, intensified free radical production during more severe hypoxia could serve as a trigger in signal transduction cascades and lead to increase in antioxidant defence and

thus to mitochondria protection. IHT can be used as protective procedure preventing mitochondria from damage.

2-08. Structure and function of muscle mitochondria at endurance training connected with intermittent hypoxia.

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Endurance training (ET) induces muscle mitochondrial biogenesis and adaptations of mitochondrial function [1]. ET in hypoxia is thought to modulate these effects [2]. Intermittent hypoxic training (IHT) has been recently shown to improve oxygen transport to and within muscle cells [3]. But, the effects of this modality of hypoxia exposure on muscle mitochondria structure and respiration at ET are poorly understood. The aim of this study was to compare muscle mitochondrial adaptations induced by severe ET combined with IHT to those occurring with ET only at the same relative workload.

Male adult Wistar rats were subjected to swimming training for 4 weeks (30 min daily, the workload corresponded to 70-75 % V_{O2max}). The IHT course was added in the last 2 weeks of ET; the rats underwent the IHT sessions: breathing with hypoxic mixture containing 12 % O_2 for 15 min with 15-min rest intervals, 5 times daily. We found that ET+IHT induced a greater increase in the numerical density, the volume density, and size of mitochondria in the red gastrocnemius compared to the similar effects of ET. Whereas ET without IHT stimulated preferential adaptation of the subsarcolemmal mitochondria, ET+IHT affect both the subsarcolemmal and intermyofibrillar mitochondria. In case of combined action of ET+IHT, the internal structure of mitochondria in terms of compartmental spaces and membranes was well-preserved. ET+IHT led to a highly expressed increase in the values of mitochondrial respiratory control (J_3/J_4) and ADP/O ratio under α-ketoglutarate oxidation compared to values under succinate oxidation. The combination of ET with IHT, therefore, was found to be the most productive model for stimulating mitochondrial biogenesis and increasing of the NADH-dependent oxidation pathway role in muscle energy production.

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2-09. Effects on gene regulation by reactive oxygen species during intermittent hypoxic training.

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Genetic mechanism relevant to reactive oxygen species (ROS) during intermittent hypoxic training were studied, so that the theory on exercise and hypoxic acclimatization is provided.

One hundred and twenty male Sprague-Dawley rats were randomly divided into three groups: normoxia group, acute hypoxia group and intermittent hypoxic group. Normoxia group included normoxia control and normoxia training sub-groups; acute hypoxia group included acute hypoxia control, acute hypoxia exercise and acute hypoxia training sub-groups; intermittent hypoxic group included intermittent hypoxic control and intermittent hypoxic training sub-groups. Therefore there are totally 12 sub-groups, each one with 10 rats. During the 4 weeks experimental period, we employed the 14.5 % and 12.6 % concentrations of oxygen (equal to altitude 3000 m and 4000 m respectively) in the

hypoxic chamber. The rats of acute hypoxia training were introduced to treadmill running on an incline of 0 at 25 m/min for 1 h every day for 4 weeks. The rats of intermittent hypoxic were exposed to hypoxia for 12 h every day for 4 weeks. In addition, intermittent hypoxic training rats were kept running out of chamber with 1 h training bouts at the speed of 25 m/min every day. Mitochondria ROS were assessed by methods of DCFH-DA. The mRNA level of HIF-1α, VEGF, NF-κB (p65), c-fos, c-jun, MnSOD, CuZnSOD, GSH-Px in heart tissue and EPO in kidney tissue were investigated by RT-PCR.

The results show that ROS generation is required for the induction of HIF-1 mRNA. During hypoxia, these findings reveal that mitochondria-derived ROS are both required and sufficient to initiate HIF-1α stabilization during hypoxia. And hypoxia activated transcription of EPO, VEGF-MnSOD, NF-κB, c-fos, c-Jun. We therefore conclude that ROS participate in the signalling pathways involved in the activation of multiple transcription factors. It is also indicated that there is a close relation between hypoxic acclimatization and gene expression of HIF-1, EPO, VEGF, MnSOD, NF-κB, c-fos, c-Jun and hypoxic acclimatization.

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2-10. Effect of muscle mitochondria heterogeneity on O_2 transport in muscle fiber at rest and work.

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Muscle O_2 supply is very heterogeneously distributed with high values near capillary and low ones at the lethal corner. Using the ideas concerning O_2 supply in some biological objects we tried to examine the possible ways of p_{O2} equalization in muscle fibers with mitochondrial heterogeneity.

Contemporary literature is lacking a unified opinion as to physiological role of the two mitochondrial populations - subsarcolemmal (SS) and intermyofibrillar (IMF) - in skeletal and cardiac muscle which differ in their morphological and biochemical properties [1]. The aim of the study is to investigate the impact of heterogeneity of the mitochondria on muscle O_2 supply by means of mathematical modeling [2]. We have calculated the p_{O2} distribution in myocytes under different values of the apparent Michaelis constant for SS and IMF clusters, both varying within the range of 0.5 to 10 mmHg and mitochondria oxygen capacities $q_{O2,ss}$ and $q_{O2,IMF}$ varying within the range of 1 to 30 ml·min^{-1}·100 g^{-1} at rest and work.

Our investigation at rest revealed that a 2-fold increase in oxygen consumption rate in the SS mitochondria only with unchanged oxygen utilization rate in IMF mitochondria may markedly displace the estimated p_{O2} histogram to the left. So we suggest that an increased O_2 consumption in the SS mitochondrial cluster (for example, by partial uncoupling of respiratory chain in mitochondria of SS cluster) allows to burn 'extra' oxygen, converting the energy to heat, which is readily eliminated by capillary blood flow and by heat dissipation from the fiber surface near blood capillaries.

Investigation at work reveals that one of the approaches for equalizing p_{O2} distribution and increasing p_{O2} at the lethal corner is to change the values of $q_{O2,ss}$ and $q_{O2,IMF}$. According to computations, a significant increase in $q_{O2,ss}$ gives only a slight increase in p_{O2} at the lethal corner. In this case, a worthy strategy of fight for oxygen might be

linked with the hyperplasia of the mitochondria that are located near capillaries and densely packed under the sarcolemma. Another approach is to change the values of K_m for O_2 in mitochondrial SS and IMF. The calculations have demonstrated that a rise of $K_{m,SS}$ by 6 to 8 times changes the O_2 gradients in the fiber, increases the p_{O2} value at the lethal corner, reduces the percentage of tissue zones with low values p_{O2} and shifts the p_{O2} histogram to the right. Since SS-mitochondria take only small volume of muscle fiber, they can, without noticeable damage to general energy production, increase oxygen flux inside the fiber to IMF mitochondria thereby performing an important function of equalizing the cell p_{O2}. It contributes to the reduction of oxygen debt production and weakens the local hypoxia in a working skeletal muscle.

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Session 3: Signalling To and From the Mitochondria. I. Nitric Oxide and Calcium

3-01. Interactions of nitric oxide with mitochondrial cytochrome oxidase : a complete kinetic model requires binding to both haem and copper.

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The nitric oxide signalling pathway is classically mediated by activation of soluble guanylate cyclase. However, in 1994 it was additionally shown that mitochondrial oxygen consumption by cytochrome oxidase is reversibly inhibited by NO in a manner apparently competitive with the oxygen tension [1]. Inhibition of mitochondrial respiration by NO at cytochrome oxidase has since been implicated in a wide range of physiological processes, [2-3] including redox signalling, oxygen sparing and brain blood flow-metabolism coupling. It was initially suggested that NO inhibited by binding to the ferrous haem a_3 oxygen binding site. However, in 1997 we demonstrated that NO could also interact with an oxidised copper centre in the enzyme (Cu_B) that does not bind oxygen [4]. A recent paper has attempted to explain all the extant literature data within a simple one-site competitive model [5]. Here we report experimental and modelling studies that demonstrate that such a model is inconsistent with NO interactions with the enzyme. Although NO inhibition is always oxygen sensitive, the sensitivity is not consistent with a pure competitive interaction. Non-competitive interactions at a second (Cu_B) site are required and these effects predominate at low oxygen consumption rates and high pO_2. We describe a complete kinetic model of NO inhibition of cytochrome oxidase which, given the cellular NO, O_2 and oxygen consumption rates, describes not only the expected degree of inhibition of the enzyme, but also the nature of the inhibited state. Surprisingly at low NO concentrations (<50 nM) the copper interaction is preferred over the haem even at relatively low oxygen tensions (20 μM). Nitric oxide:copper reactions are therefore crucial in maintaining NO control of mitochondria throughout the *in vivo* range of mitochondrial oxygen consumption rates and oxygen tensions.

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3-02. Differential sensitivity of cytochrome *c* oxidase and guanylate cyclase to endogenous NO at physiological oxygen concentrations.

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Nitric oxide (NO) stimulates cGMP production by binding to the haem iron of soluble guanylate cyclase. NO also binds to the binuclear center of cytochrome *c* oxidase reversibly inhibiting mitochondrial oxygen consumption in competition with oxygen. Recent work suggests that guanylate cyclase is significantly more sensitive to being activated by NO than cytochrome *c* oxidase is to being inhibited at physiological p_{O2} [1]. The aim of this study was to determine the sensitivity of both enzymes to endogenously produced NO at physiological oxygen concentrations (30 μM O_2). We used HEK 293 cells transfected with the inducible isoform of the NO synthase gene under the control of a tetracycline-inducible promoter. In this system, NO is generated inside the cells at different levels for extended periods of time. The amount of NO produced was within the physiopathological range (up to 1.3 μM). NO-stimulated cGMP production at 30 μM O_2 was measured inside hypoxic chambers while mitochondrial oxygen consumption was determined by high-resolution respirometry (OROBOROS Oxygraph-2k). Under physiological conditions of p_{O2}, the NO concentration giving half-maximal activation (EC_{50}) of guanylate cyclase was around 3 nM, whereas that required to achieve 50 % inhibition of respiration (IC_{50}) was determined to be 160 nM from a control value of 14 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells. These data show that, in our system, the IC_{50} of cytochrome *c* oxidase is more than 50-fold higher than the EC_{50} of soluble guanylate cyclase to endogenous NO, thus confirming previous studies using NO donors which suggest that the latter enzyme is more sensitive than mitochondrial respiration to NO at physiological oxygen tension.

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3-03. Crosstalk between mitochondria and ER: nitric oxide and calcium.

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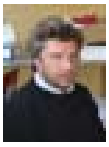
Studies with isolated mitochondria are performed at artificially high pO_2 (220 to 250 μM oxygen), although this condition is hyperoxic for these organelles. It was the aim of this study to evaluate the effect of hypoxia (20-30 μM) on the calcium-dependent activation of 2-oxoglutarate dehydrogenase (or 2-ketoglutarate dehydrogenase; OGDH) and mitochondrial nitric-oxide synthase (mtNOS). Mitochondria had a P/O value 15% higher in hypoxia than that in normoxia, indicating that oxidative phosphorylation and electron transfer were more efficiently coupled, whereas the intramitochondrial free calcium concentrations were higher (2-3-fold) at lower pO_2. These increases were abrogated by ruthenium red indicating that the higher uptake via the calcium uniporter was involved in this process. Based on the difference on $K_{0.5}$ for calcium for mtNOS and Krebs cycle (for oxoglutarate dehydrogenase 0.16 μM and mtNOS ~1 μM), mitochondria can produce nitric oxide at relatively "high calcium" microdomains. Nitric oxide, by binding to cytochrome oxidase in competition with oxygen, decreases the rate of oxygen consumption. This condition is highly beneficial for the following reasons: i, these mitochondria are still able to produce ATP and support calcium clearance; ii, it prevents the accumulation of ROS by slowing the rate of oxygen

consumption (hence ROS production); iii, the onset of anoxia is delayed, allowing oxygen to diffuse back to these sites, thereby ameliorating the oxygen gradient between regions of high and low calcium concentration. In this way, oxygen depletion at the latter sites is prevented. This, in turn, assures continued aerobic metabolism by activating the calcium-dependent dehydrogenases.



3-04. Agonist induced mitochondrial ATP production critically depends on the trans mitochondrial Ca^{2+} flow rate.

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Cell stimulation with an IP_3 generating agonist rapidly evokes increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyto}$) which are partially transferred into the lumen of mitochondria in many different cell types. Recent studies have suggested that such an elevation of the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) is crucial to stimulate mitochondrial oxidative phosphorylation. Supposable targets regulated by $[Ca^{2+}]_{mito}$ are intra-mitochondrial dehydrogenases and metabolite carriers in the mitochondrial inner membrane. Eventually, Ca^{2+} triggered enhancement of these activities leads to increased levels of adenosinetriphospate (ATP) [1]. Ca^{2+} induced mitochondrial ATP production requires first of all sufficient Ca^{2+} uptake into mitochondria, which is ensured by the negative membrane potential across the mitochondrial inner membrane of energized mitochondria and a positioning of mitochondrial Ca^{2+} uptake channel(s) close to sites of Ca^{2+} release and Ca^{2+} entry. Recent studies have suggested that mitochondrial uptake of entering Ca^{2+} is essential to maintain Ca^{2+} inhibited capacitaive Ca^{2+} entry (CCE) [2]. Thus, most of the Ca^{2+} that enters the cell is immediately taken up by subplasmalemmal energized mitochondria. However, the contribution of CCE to the activation of mitochondrial ATP production is not known. Surprisingly, it has been found that upon cell stimulation with an IP_3 generating agonist, $[Ca^{2+}]_{mito}$ increases transiently despite a sustained elevation of $[Ca^{2+}]_{cyto}$. Recently we showed that in endothelial cells the transient nature of $[Ca^{2+}]_{mito}$ elevation crucially depends on the activity of the mitochondrial Na^+/Ca^{2+}-exchanger (NCX_{mito}), extracellular Na^+ and the activity of sarco-endoplasmic reticulum Ca^{2+} ATP-ase (SERCA) [3]. Utilizing mitochondrial targeted firefly luciferase (Luc-mt) as a reliable sensor of changes in ATP levels, the described mitochondrial Ca^{2+} cycling could be compared to that of mitochondrial ATP production upon cell stimulation with an IP_3 generating agonist. Agonist induced mitochondrial oxidative phosphorylation crucially depended on the presence of extracellular Ca^{2+} , while just IP_3 mediated transfer of Ca^{2+} from the ER to mitochondria was insufficient to stimulate mitochondrial ATP biosynthesis. Surprisingly, if the mitochondrial Ca^{2+} signal was prolonged by either removal of extra-cellular Na^+ or by an inhibition of NCX_{mito} with CGP 37157, mitochondrial ATP production in response to the agonist was diminished. In line with these findings, SERCA inhibition during cell stimulation, which also impairs mitochondrial Ca^{2+} extrusion, abolished the agonist induced increase of mitochondrial ATP, too. These findings demonstrate that the agonist induced mitochondrial ATP biosynthesis is impaired if mitochondria are forced to elevate $[Ca^{2+}]_{mito}$ during stimulation with an IP_3 generating agonist, indicating that mitochondrial Ca^{2+} clearance contributes to agonist induced mitochondrial ATP production. Therefore it is tempting to speculate, that mitochondrial ATP production rather benefits from the mitochondrial Ca^{2+} turnover than from sustained mitochondrial Ca^{2+} accumulation upon cell stimulation.

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3-05. Molecular structure of the contact sites formed between mitochondria and ER. Evidence for the PTP role in mitochondrial Ca^{2+} uptake.

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The contacts between mitochondria and endoplasmic reticulum (ER) play an important function in cell metabolism – they secure a direct calcium transmission from ER to the mitochondria. Upon opening of the inositol 1,4,5-triphosphate (IP3)-gated channels of the ER, the mitochondrial surface becomes exposed to a higher concentration of Ca^{2+} than that in the bulk cytosol. This enables the uptake of calcium by mitochondria *via* low affinity calcium uniporter [1]. In this study we tried to find answers to the following three questions: How do the contacts between mitochondria and the ER look like? Is the mitochondrial permeability transition pore (PTP) located in the proximity of these contacts? Is PTP the main target for calcium originating from the ER during stimulation of the cell.

For this purpose we studied protein components of mitochondria-associated membranes (MAM fraction) which can be a "junction bridge" between ER and mitochondria. Then, we investigated the roles of the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane and of the adenine nucleotide translocase (ANT), components of the PTP, in modulating mitochondrial calcium response. We overexpressed VDAC and three isoforms of ANT (ANT-1, ANT-2, ANT-3) in HeLa cells and analyzed calcium homeostasis, mitochondrial membrane potential and structure of mitochondria and the endoplasmic reticulum (ER). In our a paper [2] we have proposed that VDAC is a key determinant of Ca^{2+} permeability at ER-mitochondria contacts and is thus responsible for exposing calcium uniporter of the inner mitochondrial membrane to the large $[Ca^{2+}]$ gradients needed for rapidly accumulating Ca^{2+} in mitochondria upon cell stimulation. Based on this and on our recent results, we propose that VDAC increases calcium permeability of the ER-mitochondria contact sites. On the other hand, we observe large, cyclosporin A sensitive, reduction of mitochondrial calcium uptake in ANT-1 and ANT-3 transfected cells. The cytosolic calcium response after agonist stimulation is as in control cells. Moreover, overexpression of ANT-1 and ANT-3 (but not ANT-2) induces mitochondrial fragmentation. This and other our results could clarify the role of a larger complex including VDAC, ANT, cyclophilin D, the peripheral benzodiazepine receptor and members of the Bcl-2 family in the interaction between mitochondria and ER, and the possibility that some of these proteins are a part of the molecular machinery docking mitochondria to Ca^{2+} stores.

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3-06. Calcium activation of the malate-aspartate shuttle in tissues expressing distinct isoforms of the mitochondrial aspartate-glutamate carrier.

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Aralar and citrin are the human isoforms of the aspartate-glutamate carrier (AGC) involved in the NADH Malate-Aspartate shuttle (MAS) for the transfer of reducing equivalents from citosol to mitochondria [1-4]. Aralar and citrin have several EF-hands motifs in a long amino terminal extension that faces the intermembrane space. We have tested a possible role for extra mitochondrial calcium in activation of MAS, as the step catalyzed by the AGC is the only one irreversible of that pathway. To this end, we have measured the calcium sensitivity of the shuttle reconstituted in isolated mitochondria, in the presence of ruthenium red to inhibit the calcium uniporter. Aralar and citrin have different expression patterns [5], and we have studied the kinetics of calcium activation in tissues with only one isoform. Mitochondria of tissues which express only aralar (namely brain and muscle) exhibit a calcium activation of MAS of 3 fold, with an $S_{0.5}$ of about 340-230 nM, whereas in liver, where only citrin is present, the increase of activity due to calcium was lower (1.5-fold) with a $S_{0.5}$ of 100-140 nM. In the heart, where both isoforms are coexpressed, calcium activation is also observed ($S_{0.5}$ 230 nM, 3.5-fold activation), and appears to be due to aralar since it disappears completely in Aralar-deficient mice.

So far, calcium signalling in mitochondria is believed to take place mainly through calcium uptake in mitochondria across the calcium uniporter (CU), followed by activation of three dehydrogenases (pyruvate, isocitrate and α-ketoglutarate) of the mitochondrial matrix that results in an increased production of mitochondrial NADH. Calcium uptake through CU requires relatively high calcium concentrations [6], higher than those activating MAS. In conclusion, MAS is activated by calcium concentrations smaller than those activating the CU-mitochondrial dehydrogenase pathway, specially through aralar as AGC. Therefore, it is an adequate mechanism to transduce small citosolic calcium signals to the mitochondria. On the other hand, the very low $S_{0.5}$ for calcium in liver mitochondria in which citrin is the only AGC suggests that MAS would be constitutively activated at the normal resting $[Ca^{2+}]_i$.

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3-07. Subplasmalemmal mitochondria modulate the activity of plasma membrane Ca^{2+} extrusion machinery.

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Mitochondria actively participate to the cellular calcium homeostasis and modulate the pattern of agonist-induced calcium signals by their ability to sequester and release calcium. In particular during agonist stimulation, the Ca^{2+} released from the ER is taken up by mitochondria and subsequently returned back to the ER, thus preventing its depletion [1]. As well, the Ca^{2+} that enters the cell transit through mitochondria toward the ER, short-circuiting the cytosol [2]. The calcium fluxes between mitochondria and the plasma membrane or the ER are difficult to study because, in most mammalian cells, mitochondria assemble into a dynamic network constantly remodeled by fusion and fission reactions. To study this dynamic Ca^{2+} connexion, we overexpressed a protein of the mitochondrial fission machinery, hFis1, and measured Ca^{2+} within organelles with GFP-based Ca^{2+} sensors. hFis1 overexpression induces a rapid fragmentation of mitochondria which then cluster around the nucleus, leaving subplasmalemmal ER regions devoid of mitochondria. We previously showed that this remodeling did not impair the ability of mitochondria to accumulate the calcium released from the ER in HeLa cells [3]. The cytosolic Ca^{2+} signals elicited by histamine were unaltered as long as Ca^{2+} was present in the extracellular medium, but were significantly blunted when Ca^{2+} was removed from the medium. Upon Ca^{2+} withdrawal, the free ER Ca^{2+} concentration, $[Ca^{2+}]_{ER}$, decreased rapidly and cells with fragmented mitochondria were unable to respond to repetitive stimulations. The loss of stored Ca^{2+} was due to an increased activity of the extrusion machinery (Ca^{2+}-ATPase and/or Na^{+}/Ca^{2+} exchanger) and was associated with an increased influx of Ca^{2+} and Mn^{2+} across Ca^{2+} entry channels. The increased Ca^{2+} influx compensated the loss of stored Ca^{2+}, and brief Ca^{2+} additions between successive agonist stimulations fully corrected subsequent histamine responses. We proposed that the lack of subplasmalemmal mitochondria disrupts the transfer of Ca^{2+} from plasma membrane channels to the ER, and that the resulting increase in subplasmalemmal $[Ca^{2+}]$ enhances Ca^{2+} extrusion mechanisms.

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3-08. Mitochondrial ATP regulation at fertilization.

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In mammalian oocytes, attachment of the sperm to the oocyte induces drastic changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) consisting of a single relatively long lasting (~5 min) rise in $[Ca^{2+}]_i$ followed by short repetitive transients of $[Ca^{2+}]_i$ lasting several hours. These changes in $[Ca^{2+}]_i$ at fertilization are called Ca^{2+} oscillations and appear to be a prerequisite for the normal development of embryos. Delayed fertilization (postovulatory aging) of oocytes significantly affects both embryonic development and Ca^{2+} oscillations [1,2]. Because Ca^{2+} oscillations depend on Ca^{2+} release and reuptake in the endoplasmic reticulum (ER) and the latter relies upon ATP availability in the ER, we

undertook the present study to address the role of intracellular ATP regulation at fertilization in the delayed fertilized mouse oocyte. Measurement of $[Ca^{2+}]_i$ was conducted using fluorescent dye fura-PE3 while intracellular ATP concentration ($[ATP]_i$) was continuously assessed in single oocytes from changes in intracellular free Mg^{2+} concentration measured by Mg^{2+} sensitive dye magnesium green (MgG). At fertilization, MgG fluorescence was transiently increased concomitant with the first transient elevation of $[Ca^{2+}]_i$ indicating a relative decrease in $[ATP]_i$. In the fresh oocyte (oocytes recovered from the oviduct 12.5 hrs after hCG injection), it was quickly followed by a significant decrease below the baseline indicating a relative increase in $[ATP]_i$. In contrast, in the aged oocytes (oocytes recovered from the oviduct 18.5 hrs after hCG injection), such a decrease in MgG fluorescence was not observed. In the separate experiment, ATP content in the fresh and aged oocytes was determined *in vitro* by the luciferin-luciferase assay. Intracellular ATP contents measured *in vitro* were comparable in the unfertilized fresh and aged oocytes. Intracellular ATP content at 5 hrs after fertilization was increased in the both oocytes, where the fresh oocyte showed a significantly higher value than the aged oocyte. From these results, we conclude that fertilization shifts the set point of intracellular ATP regulation in the fresh oocyte so that abrupt increases in ATP utilizations at fertilization are effectively buffered. In contrast, the aged mouse oocytes failed to readjust the level of intracellular ATP at fertilization. Relative deficiencies of ATP at fertilization may lead to the altered Ca^{2+} oscillations pattern and poor developmental potency commonly noted in the aged oocyte [3].

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3-09. Mitochondrial nitrogen radical synthesis by a NOS-independent mechanism.

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Mitochondria are suspected to produce nitric oxide (NO) and reactive nitrogen species (RNS), which regulate the action of the respiratory chain. However, the existence of a distinct mitochondrial NO synthase enzyme (mtNOS) is debated and the mechanism by which mitochondria produce RNS is unclear [1]. We hypothesized that not mtNOS, but the respiratory chain enzymes are responsible for RNS production. Diaminofluorescein (DAF) was applied for the assessment of RNS production in isolated mouse brain, heart, and liver mitochondria and also in a cultured neuroblastoma cell line. Fluorescence was detected by confocal microscopy and flow cytometry. Respiring mitochondria produced reactive nitrogen species, which were inhibited by catalysts of peroxynitrite decomposition. Mitochondria from different regions had varying morphology, but their DAF fluorescence was similar. Withdrawal of arginine and calcium or the application of nitric oxide synthase inhibitors failed to decrease DAF fluorescence. In contrast, disrupting the integrity of the organelles or withdrawing respiratory substrates markedly reduced RNS production. Inhibition of Complex I abolished the DAF signal, which was restored in the presence of Complex II substrates. Inhibition of the respiratory complexes downstream from the ubiquinone cycle or even dissipating the proton gradient had no effect on DAF fluorescence indicating that the redox state of ubiquinone significantly

affects the reaction. We conclude that mitochondria from brain, heart and liver are capable of significant RNS production via the respiratory chain rather than through an arginine-dependent mitochondrial nitric oxide synthase.

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3-10. Minocycline does not inhibit calcium-induced mitochondrial permeability transition.

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Minocycline, a semisynthetic tetracycline, has been shown to be neuroprotective in certain models of ischemic and neurodegenerative disease and has potential for clinical use. It has recently been suggested that minocycline acts through direct inhibition of calcium-induced mitochondrial permeability transition (mPT) [1,2]**.** One striking feature of the key studies performed on isolated brain (and liver) mitochondria is the extraordinarily high concentration/dose of minocycline needed to prevent the mitochondrial release of proteins following various triggering events (e.g. calcium, tBID and oxidants) as compared with experiments utilizing cell culture systems. These findings have likely triggered the tendency to use higher and higher doses of minocycline in animal models, e.g. 180 mg/kg i.p. in a recent study on spinal cord injury [3].

In the present study we revisited the conclusion that minocycline directly prevents the induction of mPT and release of pro-apoptotic proteins in brain-derived rodent mitochondria. The objectives were to investigate the effect of a wide concentration/dose range of minocycline on calcium-induced mPT under both energized and de-energized conditions, calcium and tBID-induced release of cytochrome *c* as well as evaluate the effect of minocycline on normal mitochondrial respiratory function and calcium transport.

We demonstrate that minocycline at high concentrations interfere with mitochondrial respiration and calcium transport. Minocycline at relevant concentrations does not inhibit permeability transition or cytochrome *c* release following a calcium insult when mitochondria are respiring on malate and glutamate. These results were confirmed in a de-energized model where induction of mPT is independent of mitochondrial respiratory capacity, thereby allowing a very wide range of minocycline concentrations to be tested (up to 250 µM, 10 µmol/mg mitochondrial protein).

We conclude that minocycline does not inhibit mitochondrial permeability transition. Minocycline may prove to be a beneficial neuroprotective agent but its mechanisms of action are still open for discussion and care must be taken to choose relevant doses in animal experiments that can be translated to safe human clinical use.

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3-11. The ER Ca^{2+}-pumps and the mitochondrial Na^+/Ca^{2+}-exchange function in a close relationship of interdependency.

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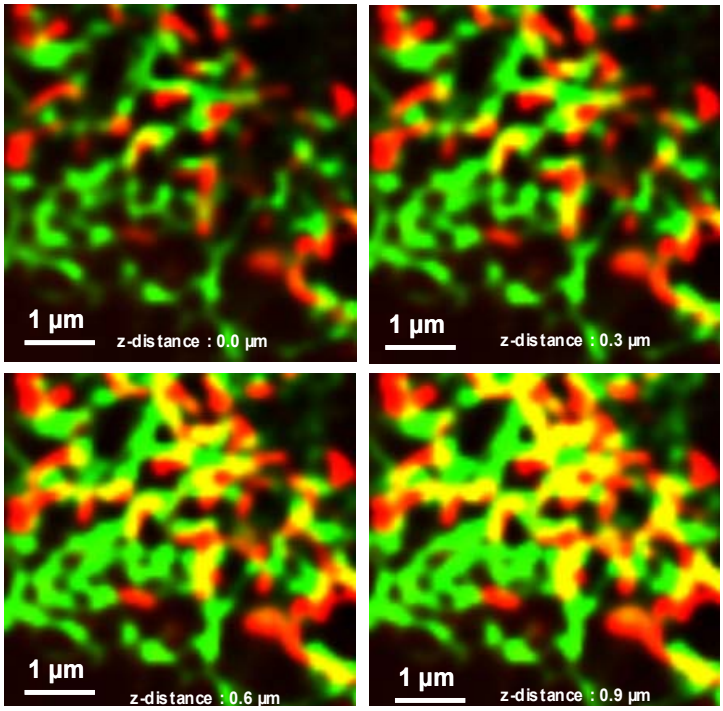
Intracellular Ca^{2+} controls a remarkable number of signalling pathways within cells. The question is how signalling specificity is maintained with the use of such a promiscuous messenger. In many cells spatial and temporal Ca^{2+} gradients are dynamically generated and thought to play crucial roles in achieving signalling specificity [1]. Growing evidence suggests that local Ca^{2+} communication between the endoplasmic reticulum (ER) and mitochondria is of utmost importance for the formation, maintenance and control of specific local Ca^{2+} microdomains within a cell. However, the molecular mechanisms of Ca^{2+} signal transmission between these organelles are only fractionally decoded and thus still subject of acute research [2]. Recently we showed that mitochondria deliver extracellular Ca^{2+} towards the ER Ca^{2+} pumps (SERCAs) during cell stimulation with an IP_3-generating agonist [3]. Thereby mitochondria seem to imbibe Ca^{2+} close to Ca^{2+} entry channels at the plasma membrane and subsequently release the absorbed Ca^{2+} via the mitochondrial Na^+/Ca^{2+}-exchanger (NCX_{mito}). Inhibition of NCX_{mito} using the benzothiazepin derivate CGP 37157 prevented complete Ca^{2+} refilling of the ER during cell stimulation. In addition, SERCA inhibition during cell stimulation with an IP_3-generating agonist increased mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) at once. This effect was clearly attenuated by NCX_{mito} inhibition indicating that the mitochondrial Ca^{2+} elevation upon SERCA inhibition is accomplished by the NCX_{mito} working in reversed mode. In addition this result implicates that the ER Ca^{2+} pumps function in a close relationship of interdependency to the NCX_{mito}. Thus it is tempting to speculate, that both Ca^{2+} transporters located at different organelles are also physically linked (see figure) at least during cell stimulation. Such a coupling of these proteins might be accomplished by yet unknown scaffolding-, anchoring- or adaptor-proteins and may explain the observed functional interdependency.



Images were taken using an array confocal laser scanning microscope (ACLSM). Endothelial cells (Ea.hy926) expressing YC4-ER and mtDsRed (Mitochondria in red, ER in green) were analysed. The orange colour indicates possible contact sites between both organelles.

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3-12. Mitochondrial abnormalities in a PC12 cell model of Alzheimer's disease.

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Mitochondrial dysfunction plays an important role in the pathogenesis of Alzheimer's Disease (AD) and other neurodegenerative disorders. The histopathological hallmarks of AD are extracellular senile plaques, consisting mainly of amyloid beta (Aß) peptide, and intracellular neurofibrillary tangles (NFT), consisting of hyperphosphorylated tau protein. Other characteristics are oxidative stress, diminished brain metabolism, reduced synaptic density, chronic inflammation and neuronal loss in selected brain regions. Today it is still unknown, whether intracellular or extracellular Aß is responsible for the oxidative stress-induced neurotoxic effects on mitochondria [1]. It also might be possible that an altered distribution of APP in subcellular compartments plays a crucial role for enhanced oxidative stress in the AD brain.

We investigated the chronic effects of the Swedish double mutation in the beta amyloid precursor protein (APP) (APPsw) and human wildtype APP (APPwt) on mitochondrial function and acute effects of supplementary stress ($Aß_{1-42}$, ER and oxidative stress) on mitochondrial calcium ($[Ca^{2+}]_m$) and mitochondrial membrane potential ($\Delta\psi_m$) in PC12 cells. We determined the APP expression, Aß production and secretion into the extracellular compartment under basal conditions. APPwt and APPsw PC12 cells express the same amount of APP, but Aß production and secretion is 3-5 fold increased in APPsw PC12 cells due to the mutation [2]. Then, we investigated the chronic effects of APP and Aß on mitochondria and found no differences on basal $[Ca^{2+}]_m$. But the APPsw cells exhibit a significant reduced $\Delta\psi_m$ compared to wt and vector PC12 cells. We also found reduced ATP levels and reduced cytochrome *c* oxidase activity. Acute stimulation with secondary insults showed only small effects on $[Ca^{2+}]_m$, but distinct changes in $\Delta\psi_m$ especially on APPsw cells. Only stimulation with Thapsigargin led to a significant enhanced calcium uptake into the mitochondria in APPsw cells. Our findings support the hypothesis that APP or Aß might affect the mitochondrial calcium homeostasis and energy metabolism in a way, which enhanced the vulnerability of the cells for oxidative stress and other secondary insults during AD.

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3-13. Selenium modifies calcium homeostasis in cells undergoing metabolic and genetic mitochondrial stress.

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Calcium signalling plays an important role in various physiological and pathological processes. Mitochondria could transiently accumulate an appreciable amount of calcium and thereby affect cellular calcium homeostasis [1,2]. Many mitochondrial diseases can modify cellular Ca^{2+} fluxes.

We have studied intracellular calcium signals in following osteosarcoma cell lines: 1. wild type, 2. depleted of mtDNA (Rho0), 3. 98 % heteroplasmy NARP mutant, 4. wild type starved. We measured basal and activated cytosolic calcium levels, an organization of mitochondria within the cell, the mitochondrial membrane potential, and ATP content of the cell.

We have found that in Rho0 cells mitochondrial stress causes disruption of mitochondrial filaments, decrease of mitochondrial membrane potential and increase in basal cytosolic calcium concentration. In starved cells we have observed reduced responses to thapsigargin, SARCA inhibitor. It seems that in these cells ROS production was also altered.

Oxidative stress and imbalance between free radical generation and detoxification may play a pivotal role in the pathogenesis of many mitochondrial diseases. Mitochondrial produced superoxide is a major cause of cellular oxidative damage. Therefore, we have investigated how selenium, a potent antioxidant, influences the osteosarcoma cells under genetic and metabolic stress conditions. Moreover, selenium is an essential component of several enzymes and has been linked to regulatory functions in cell growth, cytotoxicity, and transformation possibly involving redox regulation. We have investigated effects of selenium on calcium signaling in thapsigargin and CCCP treated cells. We have found that selenium reduces calcium release from intracellular stores but causes higher basal cytosolic Ca^{2+}. These effects were highly dependent on selenium concentration.

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3-14A. Effects of Amyloid beta peptides on mitochondrial function in glial cells in culture: mitochondria as targets of calcium and oxidative stress.

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Amyloid beta (Aβ) peptides accumulate in the CNS in Alzheimer's Disease. We have used digital fluorescence imaging techniques to examine the effects of Aβ peptides in mixed cultures of neurons and astrocytes from rat or mouse hippocampus. The Aβ caused sporadic increases in intracellular calcium concentration in astrocytes while neurons remained quiescent [1]. Aβ also increased the rate of generation of free radical species in astrocytes but not neurons [2]. The increase in ROS generation was attributable to the calcium dependent activation of an NADPH oxidase expressed in the astrocytes. Western blots and immunofluorescence studies showed that the astrocytes express gp91 ^{phox}, p67 ^{phox} and p40^{phox}, the membrane and soluble subunits of the phagocytic NADPH oxidase. We also found that mitochondria undergo complex depolarisations in the astrocytes in response to the ROS generation by the oxidase. This raises the more general question concerning the consequences of ROS generation for the mitochondria of cells that generate large amounts of ROS 'professionally'. In the astrocytes, Aβ peptides caused a slowly progressive dissipation of mitochondrial potential. This was dependent on ROS generation by the oxidase - it was blocked by antioxidants, by inhibitors of the oxidase (apocynin, DPI, AEBSF) and was absent in cells cultured from gp91 ^{phox} knockout transgenic mice [3]. The loss of potential was also prevented or reversed by the addition of additional mitochondrial substrates to all complexes of the chain - by glutamate, methyl succinate or pyruvate, strongly suggesting that the depolarisation must be due to impaired mitochondrial substrate supply. Superimposed on the slow progressive loss of potential were abrupt transient depolarisations. These could be very large, lasting 10s of seconds or even a few minutes. Most such events were fully reversible, although some led to cell death and lysis. Simultaneous measurements of mitochondrial potential and intracellular calcium showed that these events invariably accompanied a calcium transient and they were abolished in the absence of calcium. They were also abolished by antioxidants, by inhibitors of the NADPH oxidase, in cells from the gp91^{phox} knockout mice, and by cyclosporin A, none of which altered the calcium signal. Therefore, the oxidant stress generated by activation of

the oxidase sensitises mitochondria to calcium signals causing openings of the mitochondrial permeability transition pore which may be transient and reversible.

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Session 4: Signalling To and From the Mitochondria – II.

4-01. Reconstitution of kinase signaling in mitochondria - how mitoKATP opening inhibits permeability transition opening.

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Pharmacological preconditioning can be triggered by an intracellular signaling pathway in which G_i-coupled surface receptors activate a cascade including PI3K, eNOS, guanylyl cyclase and protein kinase G (PKG) [1]. Activated PKG opens the mitochondrial K_{ATP} channel (mitoK_{ATP}) and mitoK_{ATP} opening causes increased production of reactive oxygen species (ROS) [2,3], which then go on to activate other kinases. The steps between PKG and mitoK_{ATP} opening are unknown, as are the steps downstream of mitoK_{ATP}.

We found that exogenous PKG + cGMP induces mitoK_{ATP} opening in isolated heart mitochondria to the same extent as K_{ATP} channel openers such as diazoxide or cromakalim. This effect was blocked by mitoK_{ATP} blockers — 5-HD, glibenclamide, and TPP^+, by the PKG-selective inhibitor KT5823, and by protein kinase C (PKC) inhibitors chelerythrine, Ro318220, and the highly selective PKC-peptide antagonist, V_{1-2}. We also found that mitoK_{ATP} is opened by the PKC activators 12-phorbol 13-myristate acetate and H_2O_2. We conclude that PKG is the terminal cytosolic component of the signaling pathway and that it transmits the cardioprotective signal from cytosol to inner mitochondrial membrane by a pathway that includes PKC-.

K_{ATP} channel openers or activators of PKG or PKC inhibited MPT opening, and this effect was also mediated by a PKC-. Inhibition of MPT opening was prevented by MPG, indicating that the signal was transmitted by a mitoK_{ATP}-dependent increase in H_2O_2.

The effect of PKG + cGMP requires an intact outer membrane, whereas the effects of the two PKC-s do not. Indeed both effects of PKC- activation — mitoK_{ATP} opening and MPT inhibition — were observed in mitoplasts, implying that these PKCs are tightly bound to the inner membrane. This partial resolution of the mitochondrial portion of the cardioprotective signaling pathway should enable us to identify the kinase that phosphorylates mitoK_{ATP}.

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4-02. Modulation of energy transfer between mitochondria and myofibrils by changes of the cardiac work.

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In the heart, the energy supplied by the mitochondria to the myofibrils is continuously adjusted to the contraction requirement for a wide range of cardiac loads. This process, finely tuned over a broad range of mechanical requirement, is mediated by

a complex specialized enzyme system. The contribution of creatine kinase (CK) shuttle in transferring the energy between mitochondria and myofibrils may vary with the changes in cardiac work. For example, we have observed that the export of ATP from mitochondria to myofibrils is bypassing the CK shuttle if ATP synthesis is partially inhibited [1,2].

The aim of this study was to determine the dependence of the energy transfer pathways between mitochondria and myofibrils on cardiac workload. We studied the energy transfer pathways by analyzing the 31P-NMR magnetization transfer data using Bloch-McConnell equations [3]. The data was acquired on isovolumetric Langendorff perfused hearts. The work was varied by changing the external calcium concentration [Ca] (from 0.5 mM to 4.0 mM) and/or by beta adrenergic stimulation. In our analysis, we computed the share of energy transfer through different pathways, consistent with the observed magnetization changes. For this, mathematical models were composed taking into account the forward and reverse CK reactions, ATPase activity as well ATP synthesis in mitochondria. Several possible energy transfer pathways were considered with different levels of intracellular compartmentation. The simplest model considered in our study was the non-compartmentalized model exchanging magnetization between PCr, ATP, and Pi (three-site model). The most complex one consisted of three compartments for ATP (mitochondrial matrix, cytoplasm, and myofibrillar compartment), three isoforms of CK, ATPase and ATP synthase connecting compartmentalized ATP with PCr and Pi.

At low work, ([Ca]=0.5 mM), all considered energy transfer schemes were able to reproduce the measured magnetization transfer within the experimental errors. This includes the simplest considered scheme (the three-site model) as well. However, when the beta adrenergic stimulation was used with high [Ca]=4.0 mM, the simple non-compartmentalized model was not able to fit the data: ATP compartmentalization must be taken into account. When compartmentalized models were used, it was possible to separate the fluxes through two isoforms of CK - the mitochondrial and the cytoplasmic one. According to the experimental data, the total forward rate of CK reaction is almost constant if the cardiac work is changed by increasing [Ca] from 0.5 mM to 4.0 mM. However, the forward and the backward rates of subcellular CK isoenzymes are changing with cardiac work. Namely, a rise in cardiac work increased both mitochondrial PCr production in mitochondria and myofibrillar PCr utilization at constant global CK flux.

In addition, the compartmentalized models allow one to find the fraction of ATP which is transported as PCr by the CK shuttle and directly as ATP from mitochondrial matrix to myofibrils. Our analysis of the data suggests that both pathways (direct export of ATP from the matrix to myofibrils and the CK shuttle) are probably used, but depending on the work their proportion might vary. In extreme conditions of a work demand exceeding mitochondrial ATP synthesis capacity (beta stimulation and high calcium): global CK flux decrease and ATP is mainly exported directly.

These findings suggest that the CK shuttle is able to support the energy transfer, except in extreme conditions. This may have implications in understanding the process of cardiac pathology.

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4-03. Comparison of the effects of nonesterified fatty acids and of their ethanolamine amides (*N*-acylethanolamines) on isolated mitochondria and on mitochondria within intact cells, including triggering of apoptosis.

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Nonesterified long-chain fatty acids are excellent energy-providing respiratory substrates for numerous tissues but they are also well-known protonophores and uncouplers of oxidative phosphorylation for isolated mitochondria and mitochondria within intact cells [1]. Elevated concentrations of fatty acids can elicit cell death, both necrotic and apoptotic [2]. *N*-Acylethanolamines (NAEs) form a class of important fatty acid derivatives that have recently attracted attention because of their formation and accumulation in injured heart and brain [3]. The main representative of these compounds, *N*-arachidonoylethanolamine (also called anandamide) may have signalling functions and also acts as ligand for cannabinoid receptors. The present work compares some effects of nonesterified fatty acids with those of NAEs.

In isolated rat heart mitochondria NAEs were much weaker protonophores than corresponding nonesterified fatty acids [4]. In contrast, in the presence of micromolar concentrations of Ca^{2+}, long-chain unsaturated NAEs, *N*-arachidonoylethanolamine and *N*-oleoylethanolamine, were potent openers of the mitochondrial permeability transition pore. They also acted as weak inhibitors of mitochondrial respiration, in particular of complex I of the respiratory chain. As weak uncouplers, NAEs decreased the rate of the formation of reactive oxygen species (ROS) by respiring mitochondria. However, *N*-arachidonoylethanolamine partly prevented the drastic decrease of ROS formation produced by chemical uncouplers. In this respect, it exerted a similar, though much weaker, effect as that by known inhibitors of complexes I and III, rotenone and antimycin A.

Acting on cells in culture, arachidonic acid and, to a lesser extent, oleic acid elicited apoptotic cell death in the following cell types: rat hepatoma AS-30D, mouse Ehrlich ascites carcinoma, human lymphoblastoid Jurkat cells and human leukemia HL-60 cells. However, mouse neuroblastoma N2a cells appeared resistant to arachidonic and oleic acids. Interestingly, this cell line was also more resistant to ultraviolet irradiation than the former cell lines. Apoptosis induced in susceptible cell lines by arachidonic and oleic acids proceeded along the mitochondrial pathway characterized by release of cytochrome *c* from mitochondria to the cytosol and activation of caspase-3.

Conflicting information exists in the literature on proapoptotic and antiproliferating activity of *N*-arachidonoylethanolamine. In the present study we were unable to induce apoptosis by NAEs in rat myoblasts H9c2, primary rat neonatal cardiomyocytes and mouse neuroblastoma N2a cells. Further studies in this line are, however, required.

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4-04. Mitochondrial GTP metabolism - a main function of NDP-kinase D?

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Isoenzymes of hexameric nucleoside diphosphate kinase (NDPK/nm23) function in NTP-biosynthesis (NDP + ATP <-> NTP + ADP) and have additional, multi-facetted roles in cell signaling, proliferation and differentiation [1]. Detailed subfractionation of rat liver cells and surface plasmon resonance spectroscopy [2] with recombinant protein revealed that the NDPK-D isoenzyme [3] has a unique intramitochondrial localization at the inner mitochondrial membrane, where it firmly binds to acidic phospholipids, mainly cardiolipin. The latter high affinity interaction (K_D about 30 nM) is due to the NDPK-D-specific arginine 90 in a basic RRK motif at the surface of the NDPK hexamer, since a R90D mutation abolishes cardiolipin interaction. Latency assays with liver, HEK and HeLa mitochondria suggest that most NDPK-D is oriented towards the intermembrane and cristae space, while a variable fraction may be oriented towards the matrix space. The physiological role of NDPK-D was analyzed with HeLa cell lines that can express NDPK-D under the control of an inducible tetracycline (tet) promoter. Mitochondrial respiration from control cells was only weakly stimulated with NDPK substrate TDP, while it was strongly stimulated in NDPK-D overexpressing tet-treated cells, together with a decrease in K_m (ADP). Thus, NDPK-D uses NDP nucleotides to locally regenerate ADP in the mitochondrial intermembrane space, which in turn stimulates oxidative phosphorylation. From the NTP generated, mainly GTP could be important for GTP-dependent processes in the intermembrane compartment, like e.g. GTP-binding proteins involved in mitochondrial dynamics.

We propose a model for NDPK-D participating in GTP-export from the matrix space and GTP-regeneration in the intermembrane space by association of the NDP kinase hexamer with adenylate translocator of the inner membrane via cardiolipin patches, similar to proteolipid complexes formed by octameric mitochondrial creatine kinase [4].

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4-05. Inter-genomic cross talk between the mitochondria and nucleus in aging and cancer.

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As an important part of 'free-radical theory of aging' mitochondria play a fundamental role both as producers and targets of ROS. Mitochondrial DNA (mtDNA) is extremely susceptible to ROS damage compared to genomic DNA because of ROS production by the mitochondria, the absence of protective histones and limited DNA repair. Thus individuals accumulate mtDNA mutations as they age which leads to compromised mitochondrial function.

To understand the biological consequences of mtDNA mutation accumulated during aging we have created mitochondrial gene knock out cell lines (ρ°). Our study suggests

that ρ° cells demonstrate characteristic features of cellular aging. Mitochondrial gene knock out ρ° cells showed typical morphology associated with aging such as increased size and elongated appearance. They have increased senescence-associated β-Gal activity, lipofuscin pigment and plasminogen activator inhibitor-1 expression. Consistent with their decreased proliferation, the expression of mitotic cyclins was decreased and that of cdk inhibitors was increased. Retinoblastoma (Rb) hypophosphorylation and decreased telomerase activity were also noted. Using this cellular model and cybrid cell technology, we provide evidence that (1) inactivation of mitochondrial genes leads to chromosomal instability (CIN) that are present in a variety of human tumors and (2) mitochondrial gene knockout cells show transformed phenotype. Our study also demonstrates that mitochondrial genetic status plays a key role in regulation of a multifunctional protein APE1 (also known as Ref1 or HAP1) involved in transcription and DNA repair in the nucleus and the mitochondria. Our study revealed that altered expression of APE1 in ρ° cells and tumorigenic phenotype can be reversed by exogenous transfer of wild type mitochondria in ρ° cells. Furthermore, we demonstrate that APE1 expression is altered in variety of primary tumors. Taken together, these studies suggest that inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis and that APE1 mediates this process. Our study support the mitochondrial theory of aging and suggest that ρ° cells can serve as an *in vitro* model for cellular aging.

4-06. A toxic halogenated cysteine *S*-conjugate has deleterious effects on mitochondrial physiology.

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Many toxicants impair mitochondrial physiology leading to a decrease in energy production. Such a malfunction could lead to progressive organ failure. Humans are continuously exposed to reactive electrophiles derived from the environment, medications and food, and as natural metabolites within the body. Many are detoxified through acetylation of the corresponding cysteine *S*-conjugate to the mercapturate, which is excreted. However, if the cysteine *S*-conjugate contains a good leaving group at the sulfur, a *β*-elimination reaction may compete with the acetylation reaction. Enzymes that catalyze this elimination reaction are known as cysteine *S*-conjugate *β*-lyases. These enzymes convert susceptible cysteine *S*-conjugates to pyruvate, ammonium and a sulfur-containing fragment (RSH). If the eliminated fragment is not particularly reactive, the parent cysteine *S*-conjugate is not generally toxic. On the other hand, if RSH is reactive, the parent cysteine *S*-conjugate is potentially toxic. Halogenated alkenes, such as tetrafluoroethylene, are examples of environmental and workplace contaminants that are toxified (bioactivated) at least in part by the action of cysteine *S*-conjugate *β*-lyase(s) on the corresponding cysteine *S*-conjugate. The cysteine *S*-conjugate derived from tetrafluoroethylene [(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC)] is converted by cysteine *S*-conjugate *β*-lyases to an RSH fragment that is a thioacylating agent, particularly of protein lysine residues. TFEC (and other halogenated cysteine *S*-conjugates) are mitochondrial toxicants, presumably as a result of the presence of cysteine *S*-conjugate *β*-lyase activity in these organelles [1,2]. A major cysteine *S*-conjugate *β*-lyase in mitochondria is mitochondrial aspartate aminotransferase (mitAspAT) [3]. In the presence of TFEC the enzyme is inactivated on average after several thousand turnover events [3]. Exposure of rat kidney *in vivo* and PC12 cells in culture to TFEC results in selective inhibition of mitochondrial enzymes of energy metabolism, including mitAspAT, aconitase, and the E2 and E3 components of the α-ketoglutarate dehydrogenase complex (KGDHC) [reviewed in 4].

In the present work, isolated rat liver mitochondria energized with succinate were incubated in the presence or absence of TFEC. Four physiologically important mitochondrial parameters (O_2 uptake, Ca^{2+} flux, mitochondrial membrane potential ($\Delta\psi_m$), and swelling) were simultaneously measured using a uniquely designed multiparameter chamber. A concentration- and time-dependent disruption of these parameters by TFEC was observed: (1) inhibition of mitochondrial respiration, (2) Ca^{2+} release from mitochondrial matrix, and (3) dissipation of $\Delta\psi_m$. Both the lag-period and the degree of maximal swelling of mitochondria induced by TFEC were decreased with increasing concentration and time of pre-incubation. KGDHC and mitAspAT were both inhibited by ~20 % in liver mitochondria exposed to TFEC. No change was found in the activities of glutamate- or malate dehydrogenases.

Possible exposure to endogenous and exogenous electrophiles (that can be bioactivated via the cysteine *S*-conjugate *β*-lyase pathway) may lead to selective loss of enzymes involved in energy metabolism. This loss may contribute to (or exacerbate) the mitochondrial dysfunction associated with aging and many neurodegenerative diseases.

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4-07. *v*-Raf antagonizes impairment of mitochondrial respiratory function following growth factor removal.

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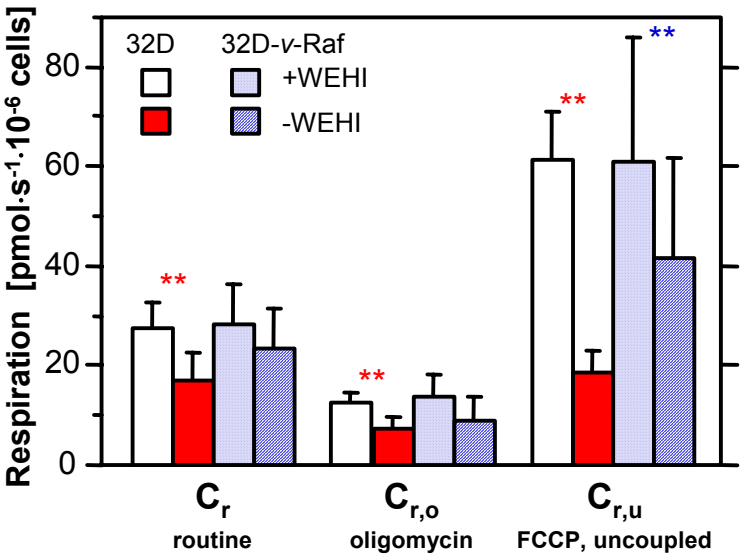
The cell's ability to respond to extrinsic stimuli depends on the provision of adequate energy. Increasing evidence suggests that cellular energy production itself is subject to regulation by extrinsic signals through signaling pathways, which control cell proliferation and survival. In tumors, where signaling components are frequently affected by mutations, an increased dependence on glycolytic energy has been recognized long ago and more recent experiments suggested direct targeting of components of the glycolytic machinery as one of the underlying mechanisms [1,2]. Indirect evidence from studies on the control of cell survival by C-Raf suggests a role for this kinase in maintaining mitochondrial integrity during apoptosis induction [3]. These experiments also suggested cooperation in this process with two other major guardians of cell survival, Bcl-2 and PKB. To test for direct effects of C-Raf on mitochondrial energy production we performed high resolution respirometry on the mouse pro-myeloid 32D cell line. These cells strictly depend on IL-3 for growth and survival. IL-3 removal results in growth arrest and subsequent apoptosis, which can be prevented through over-expression of the oncogenic form of C-Raf, *v*-Raf. In the comparison of the effects of growth factor withdrawal on mitochondrial respiratory function in 32D cells *versus* 32D cells protected by *v*-Raf, our experimental design focused on early time points before cells bocome irreversibly committed to cell death.

Cells were incubated in RPMI 1640 + 10% FCS supplemented with penicillin-streptomycin and 2 mM L-glutamine without IL-3 for a period of 8 h at a cell density of $0.5 \cdot 10^6$ cells·ml^{-1}. Controls were cultured in the same medium supplemented with IL-3 (15 % WEHI). During the 8 h time interval, no significant difference in viability (<4 % trypan blue or annexin V staining) was observed between control and growth factor deprived cells. The respiratory activities of intact cells were measured using the OROBOROS Oxygraph-2k for high resolution respirometry. After recording cellular routine

respiration (C_r) in the respective incubation media, ATP-synthase was inhibited by oligomycin ($C_{r,o}$), followed by a stepwise FCCP titration to achieve maximum uncoupled respiration in intact cells ($C_{r,u}$). Respiration was then inhibited by rotenone and antimycin A. Data (means ± SD) were analysed by a paired t-test.

Activities of citrate synthase (mitochondrial matrix marker enzyme) and lactate dehydrogenase (glycolytic marker enzyme) per million cells remained unchanged, irrespective of IL-3 withdrawal, indicating that mitochondrial content and glycolytic capacity were maintained. Analysis of ERK and AKT phosphorylation, two main signaling effectors of the IL-3 receptor, revealed no measurable decline in their activities at the end of the WEHI starvation period. However, a significant decrease in cell volume (measured by CASY®) was observed in 32D (0.93 ± 0.10 pL versus 0.76 ± 0.05 pL) but not in cells protected by v-Raf. Regardless of the significant decrease in cell size, the protein content of 32D cells remained unaffected upon IL-3 withdrawal.

Mitochondrial respiratory function of IL-3-deprived 32D cells dropped significantly in all respiratory states (Fig. 1). Routine and oligomycin-inhibited respiration of 32D-*v*-Raf cells deprived of IL-3 were not significantly lower compared to their controls with IL-3 (Fig. 1).



The uncoupling control ratio (UCR = $C_{r,u}/C_r$) is a sensitive indicator for the integrity of mitochondrial function in intact cells. In 32D controls, the UCR declined from 2.3 ± 0.23 to 1.23 ± 0.61 after growth factor removal. In contrast, the UCR of 32D-*v*-Raf cells remained unaffected by IL-3 withdrawal. The inverse of the respiratory control ratio ($C_{r,o}/C_{r,u}$), an index of the extent of oligomycin inhibited leak rate of respiration relative to the maximum capacity of the respiratory chain, was significantly different between controls (0.20 ± 0.01) and growth factor deprived 32D cells (0.43 ± 0.18), indicating primarily the loss of respiratory capacity, and providing indirect evidence for simultaneous partial uncoupling [4].

Even though IL-3 withdrawal showed a significant effect on the respiratory rates of the different respiratory states of 32D cells, the oxygen kinetics in coupled intact cells was not significantly affected, with p_{50} values of 0.044 ± 0.001 kPa for controls and 0.039 ± 0.006 kPa for IL-3 deprived cells.

The decline of mitochondrial respiratory capacity comprised an early event in the pathway to apoptosis after growth factor withdrawal, before the onset of inactivation of the main signaling effectors of the IL-3 receptor. This time course suggests a primary role of mitochondrial respiratory function in these cells. Our results clearly demonstrate that IL-3 withdrawal severely compromises mitochondrial respiratory function in a fashion that is almost completely suppressible by v-Raf. This for the first time suggests a direct link between the key mitogenic and survival kinase C-Raf and mitochondrial energy homeostasis.

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4-08. Modulation of mitochondrial respiratory parameters by the human papillomavirus type 16 E7 oncoprotein.

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Changes in cellular carbohydrate metabolism are a hallmark of malignant transformation and represent one of the earliest discernible events in tumorigenesis. We previously showed that the E7 oncoprotein targets the glycolytic key regulator pyruvate kinase subtype M2 (M2PK) and this leads to downregulation of M2PK activity and changes in the glycolytic flux [1]. There is evidence that different oncogenes, such as ras and HPV 16 E7, cooperate in a complex way to establish the metabolic phenotype of tumor cells which supports the proliferative state [2,3].

We analyzed, whether mitochondrial oxidative phosphorylation, representing another important energy producing system, is influenced by the HPV 16 E7 oncogene. We used a cell line derived from normal rat kidney (NRK) cells, called 14/2 cells, which contain a hormone inducible expression vector for HPV-16 E7 [4]. All experiments were performed after 4 h of dexamethasone-induced E7 expression [2]. Mitochondrial respiratory function was analyzed by high-resolution respirometry with the OROBOROS® Oxygraph-2k. 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 [5].

Cellular routine respiration was decreased after 4 h induction of the E7 protein, whereas oligomycin-inhibited and uncoupled respiration remained unchanged. This resulted in an increased uncoupling control ratio, and a decreased phosphorylation respiratory control ratio (RCRp [5]). The activity of the mitochondrial matrix marker enzyme citrate synthase was comparable between induced an uninduced cells, which confirmed the results obtained when relating respiratory parameters per cell number. Taken together, these data suggest that the observed phenomenon might be linked to modulations of cellular metabolism by the human papillomavirus. However, the exact mechanisms how an oncoprotein can influence the activity of the respiratory chain remain to be elucidated.

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4-09. Keratinocyte-specific knockout of the Tfam protein allows to elucidate the role of the mitochondrial respiratory chain for cell proliferation *in vivo*.

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The Tfam protein controls the amount of mitochondrial DNA (mtDNA) in the cell [1-3]. Without mtDNA, the mitochondrial respiratory chain is not functional, because mtDNA encodes 13 of its ca. 80 polypeptide subunits. A HeLa cell line without mtDNA (ρ0) shows a profound proliferation defect, an effect which is however not simply due to energetic or biosynthetic problems, since levels of ATP, UTP, heme containing proteins and FeS cluster enzymes were normal. These results point to a hitherto unknown role of the mitochondrial respiratory chain in cell proliferation. To elucidate this role *in vivo*, we have bred mice with a keratinocyte-specific deletion of the Tfam protein by crossing mice having exons 6 and 7 of the TFAM gene flanked by loxP-sites (2) with mice carrying the Cre-recombinase transgene under control of the keratinocyte-specific human K14 promoter. At the day of birth, Tfam protein is still detectable, but older knockout animals show a progressive loss confined to the epidermis. At day 6 after birth, the mtDNA-encoded subunit II of cytochrome oxidase is not detectable any more. The epidermis gradually gets thinner, its epidermal stem cell compartment, the basal layer, is disordered and hair follicles fail to develop normally. The knockout mice stop gaining weight at day 3 and die between day 4 and day 7. The tongue epithelium is also disordered and papillae show a progressive degradation from day 0 to day 6. The animals develop an ulceration at the back of the tongue, the resulting pain probably inhibiting food intake, leading to the observed weight loss and ultimately death. Considering the proposal by some authors that the epidermis is a physiologically anaerobic tissue [4], the drastic effect of ablating respiratory chain activity in keratinocytes is even more striking. These results show that an intact mitochondrial respiratory chain is essential for cellular proliferation *in vitro* and proliferation and differentiation *in vitro* and *in vivo*.

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4-10. Mitochondrial hyperpolarization: a checkpoint of T-cell life, death, and autoimmunity.

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Activation, proliferation, and cell death pathway selection of T lymphocytes depend on reactive oxygen intermediates (ROI) production and ATP synthesis which are tightly regulated via the mitochondrial transmembrane potential ($\Delta\psi_m$). Mitochondrial hyperpolarization (MHP) and ATP depletion represent early and reversible steps in T cell activation and apoptosis. By contrast, T lymphocytes of systemic lupus erythematosus (SLE) patients exhibit persistent MHP, cytoplasmic alkalinization, increased ROI production, and ATP depletion that mediate enhanced spontaneous and diminished activation-induced apoptosis and sensitize lupus T cells to necrosis. Necrotic, but not

apoptotic, cell lysates activate dendritic cells and may account for increased interferon-α production and inflammation in lupus patients. MHP is proposed as a key mechanism of pathogenesis and target for pharmacological intervention in SLE. Persistent MHP was associated with increased mitochondrial mass and increased mitochondrial and cytoplasmic Ca^{2+} content in T cells and enhanced NO production by monocytes of lupus patients. Activation of T cells through the T cell receptor initiates a biphasic elevation in cytosolic free Ca^{2+} concentration, a rapid initial peak observed within minutes and a plateau phase lasting up to 48 h. In response to CD3/CD28 costimulation, rapid Ca^{2+} fluxing was enhanced while the plateau phase was diminished in lupus T cells. NO-induced mitochondrial biogenesis in normal T cells enhanced the rapid phase and reduced the plateau of Ca^{2+} influx upon CD3/CD28 costimulation, thus mimicking the Ca^{2+} signaling profile of lupus T cells. Mitochondria constitute major Ca^{2+} stores and persistent MHP and NO-dependent mitochondrial biogenesis may account for altered Ca^{2+} handling by lupus T cells. Coordinated changes in expression of genes encoding members of the electron transport chain (ETC) and anti-oxidant defenses underlie mitochondrial dysfunction and dominate the altered gene expression profile of lupus lymphocytes. Members of the ETC may serve as novel target for pharmacological intervention in SLE.

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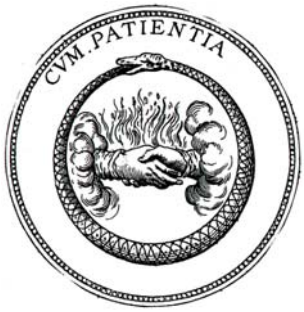

Session 5: Organization of the Mitochondrial Respiratory Chain and Control of Oxidative Phosphorylation

5-01. cAMP-dependent phosphorylation of catalytic subunit I of cytochrome *c* oxidase switches off enzyme activity.

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Signaling pathways targeting mitochondria are poorly understood. To examine whether the cAMP-dependent pathway affects cytochrome c oxidase (COX), the terminal enzyme of the electron transport chain, cow liver COX was purified in the presence of theophylline, a phospho-diesterase inhibitor, leading to high cAMP levels. Using anti-phospho antibodies, we showed that COX subunit I is tyrosine-phosphorylated in the presence of theophylline but not in its absence. The site of phosphorylation, identified by mass spectrometry, was Tyr-304 of COX subunit I. Subunit I phosphorylation leads to decrease of V_{max} and shifts the reaction kinetics from hyperbolic to sigmoidal such that COX is fully inhibited up to 10 μM cytochrome *c* substrate concentrations, even in the presence of allosteric activator ADP. To assess our findings with the isolated enzyme in a physiological context, we tested whether treatment with the physiological starvation signal glucagon leads to COX inactivation. Measurements using cow liver tissue or fed human HepG2 cells revealed dramatic inhibition of COX activity upon treatment with glucagon. Similar results were obtained with cow tissue. Interestingly, cells starved overnight already showed sigmoidal kinetics in the presence of ATP. Thus, the glucagon receptor/G-protein/cAMP pathway regulates COX activity. We tested the effect of asthma drug theophylline on cow lung tissue applying concentrations used in therapy and observed COX inhibition and decreased ATP levels. Our findings may provide a mechanism for theophylline action during asthma, where airway constriction, which requires energy, is weakened due to COX inhibition, leading to decreased ATP production through oxidative phosphorylation.

5-02. 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.

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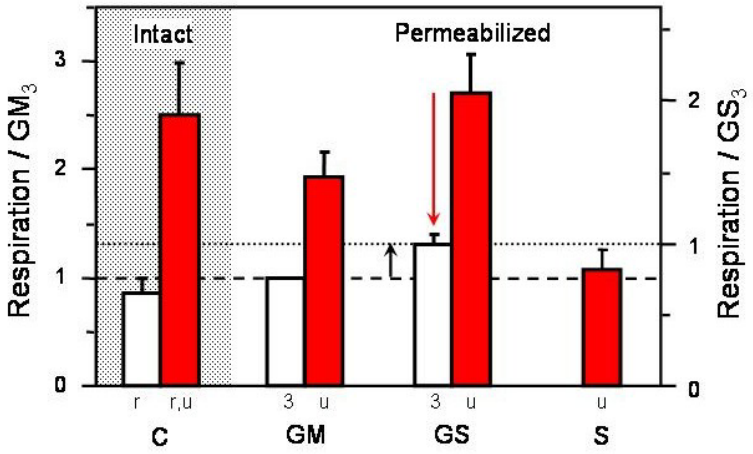
Oxygen consumption increases between 10-15 fold from resting to maximal in mammals, largely reflecting the rest-work transition in muscle tissues [1]. In a variety of cultured cells, in turn, routine respiration can be stimulated 3-fold by uncoupling oxidative phosphorylation, corresponding to an uncoupling control ratio (UCR) of 3 [2,3]. Uncoupled respiration of intact cells has been considered as the gold-standard for evaluation of maximum capacities *in vivo*, to avoid any artifacts from the loss of essential metabolites, disruption of the cytoskeletal structure, and channeling of respiratory

substrates to the organelles [4]. The definition of a physiological reference state has important implications when interpreting values for the excess capacity of specific enzymes in the electron transport chain, as well as phenotypic effects of various genetic and acquired mitochondrial disorders [4,5]. In the present study, we addressed the problems encountered when using uncoupled respiration as a physiological reference [2-4], and resolved fundamental discrepancies of respiratory capacity in intact and permeabilized cells [4-6].

Oxygen flux was measured in NIH3T3 fibroblasts at a density of $0.3 \cdot 10^6$ cells· ml^{-1}, using high-resolution respirometry (OROBOROS Oxygraph-2k) and optimized titration regimes with various substrates and inhibitors for evaluation of mitochondrial respiratory function.



Routine respiration of intact cells (C_r) measured in fibroblasts suspended in culture medium increased after uncoupling (state $C_{r,u}$) by a factor (UCR) of 2.9 ± 0.2 SD. This maximum flux in non-permeabilized cells was 2.5-fold higher than the coupled flux, measured in digitonin permeabilized cells with saturating ADP and complex I substrates (glutamate+malate, state GM_3 vs $C_{r,u}$; Fig. 1). This confirms previously reported differences [4]. Since the uncoupled cell as a model for maximum coupled respiration (state 3) is questionable, we investigated state 3 respiration and uncoupling with various substrate combinations in permeabilized cells. Uncoupling by FCCP, in the presence of glutamate+malate, stimulated state 3 respiration 2-fold. This flux through complex I was still below uncoupled respiration of intact cells. Complete agreement was reached only with uncoupling and parallel electron input through complexes I and II with glutamate+malate+succinate (GS_u versus $C_{r,u}$; Fig. 1). Coupled respiration (GS_3), however, was merely half of the uncoupled respiratory capacity (Fig. 1). Compared to state 3 with complex I substrates only (GM_3), parallel electron input increased respiratory capacity by a factor of 1.3. The additive effect was also shown by comparison with respiration in the presence of rotenone plus succinate (S_u). Taken together, (1) the uncoupled state in intact and permeabilized cells showed that combined glutamate and succinate (without rotenone) was essential for adequate substrate supply, (2) the pronounced increase in respiration after uncoupling compared to state 3 indicated the quantitative importance of the phosphorylation system in respiratory control [7], and (3) the excess capacities of complexes III and IV are at least 1.9 when related to the physiological reference state of coupled respiration, much higher than that derived from uncoupling control ratios [4]. Our results thus resolve controversies on the applicability of permeabilized cells as *in vivo* models in mitochondrial physiology.

A direct "$V_{O2,max}$" (state 3) is inaccessible in intact fibroblasts because maximum ADP stimulation cannot be achieved without permeabilization of the plasma membrane. However, agreement between uncoupled respiration in intact and permeabilized cells was obtained with parallel electron input through complexes I and II, which corresponds to the operation of the TCA cycle and the mitochondrial substrate supply *in vivo*. Not the uncoupled, but the coupled state 3 with parallel electron input thus yields a measure of maximum capacity of mitochondrial respiration. This approach provides a physiological reference state, analogous to the $V_{O2,max}$ obtained by ergometry during maximal aerobic exercise. According to previous interpretations, a UCR of 3 would indicate that routine respiration amounts to merely 33 % of total capacity. Based on our results, however, routine respiration operates much closer to physiological capacity, i.e. at 77 % of the physiological reference state GS_3. Maximum respiratory capacity is under tight control by the phosphorylation system, which is eliminated by uncoupling. In this light, conventional

applications of the UCR must be reconsidered with reference to accepted concepts of adenylate control in mitochondrial respiration. A comparable pattern of the effects of parallel electron input and uncoupling is observed in permeabilized human muscle biopsies ([7]; Session 1). Since routine coupled and uncoupled respiration cannot be measured in muscle biopsies, cultured cells provide a unique model for contrasting respiratory capacities observed *in vivo* and after permeabilization. NIH3T3 cells, therefore, provide a valid model for evaluation of the physiological reference state of mitochondrial respiratory capacity. Parallel complex I + II electron input in coupled and uncoupled states represents an important addition to the conventional titration protocols applied for the diagnosis of mitochondrial respiratory control in health and disease.

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5-03. Role of mitochondrial network organization in the regulation of energy production in living human cells: a multi-approach study.

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Recent advances in cell biology have helped to create a picture of mitochondrial organization whereby the organelle exists as a single dynamic network (mt-network), either forming a reticulum or a fragmented collection of vesicles (Figure 1). Several observations have revealed that its tridimensional organization is variable in human living cells, both under normal or pathological situations. However, little is known about the determinants and the bioenergetic consequences of these changes. Also, the fuctionning of mitochondria as a network, even heterogeneous, needs to be demonstrated. Such analysis is complex since mitochondria participate in a multiplicity of cellular functions such as energetics, ROS production, calcium signaling or apoptosis. Changes in mt-network organization can be observed in association with different physiological

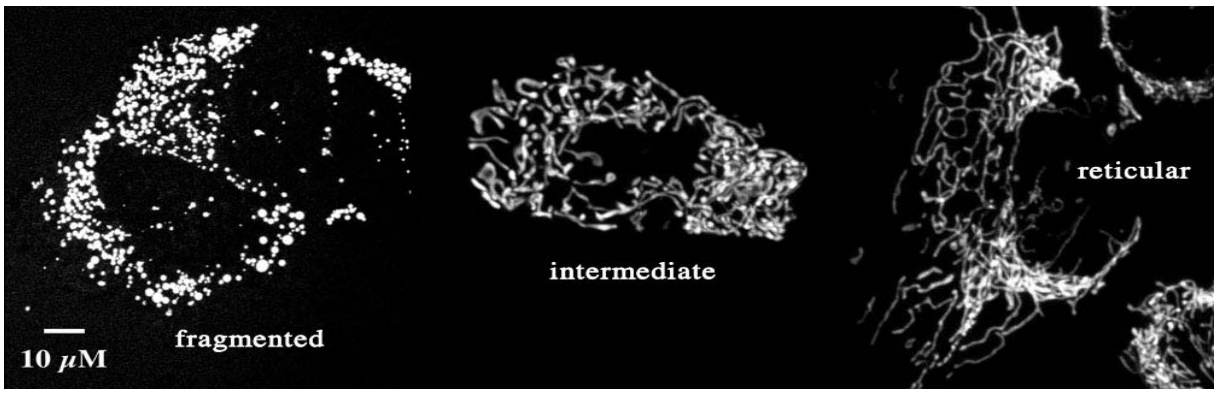

Figure 1: Three main configurations of the mitochondrial network in living HeLa cells stained with a matrix targeted GFP.

situations including cell cycle, cytoskeletal trafficking, energy status, pathology or apoptosis (see Table 1). These changes can be mediated by fission and fusion proteins, but their regulation is poorly understood. The sole contribution of physicochemical transitions in mitochondrial lipid bi-layers also remains to be investigated. Here we present our work on the relationships between the energy status of the cell and mitochondrial network organization. Using fluorescence microscopy and ratiometric GFP

Table 1: Changes in mt-network organization under various physiological, pathological or experimental situations.

	TRIGGER	METHODOLOGY	MT-NETWORK	CELLULAR IMPLICATIONS	REF.
ENERGY STATUS	Change in the type of cellular energy substrate: oxidative versus glycolytic	Ratiometric GFPs, pH and redox sensinsitive, TEM and confocal analysis	*In oxidative mode:* thin tubular, branched. *In glycolytic mode*: larger tubules, peri-nuclear	Adaptation of mt-network to the carbon source.	[1] [2]
CELL CYCLE	Synchronisation of human cells in culture (143B)	Mitotracker labelling	Fragmentation before S phase, reticular again in G1	Mt-network could be a check point of cell cycle	[3]
	Analysis in budding yeast	Destabilization of actin cables or the mitochore	Three classes of mitochondrial motility	Mitochondrial movement requires actin cables	[4]
	Induction of apoptosis by staurosporin, and silencing of fusion or fssion protein	RNAi OPA1 Confocal analysis	Fragmented, vesicular, cytochrome c release	Loss of ⍰Ψ, higher sensitivity to apoptosis	[5]
		- RNAi DRP1(fission) - RNAi Fis1 (fission)	Elongated	Resistance to apoptosis (staurosporin or actimomycin D)	[6]
PATHOLOGY	Oxphos diseases	Monoclonal antibodies Immunofluorescence	Abnormal: peripheric, ragged, fragmentation	Alteration of mt-network profile in Oxphos deficient cells	[7]
OXPHOS ALTER-ATIONS	Rotenone treatment of Helas and MRC5s	ratiometric GFP pH and redox sensitive	Vesicular with "donuts"	Increased redox potential, decrease in respiration.	Present study
	CCCP treatment of Helas and MRC5s	Immuno fluorescence	Fragmented	⍰Ψis necessary for fusion	[8]
	Treament with 10µM ß-amyloids on MRC5s	GFP ratiometric pH and redox sensitive	Fragmented	Alteration of mt-network profile in Oxphos deficient cells	Present study
	Misarrangment of F1F0-ATPsynthase (Yeast)	Cross linking of ATP synthase	Fragmented	Abnormal cell division and mt-DNAtransmission to buds	[9]
MONITORING OF mt - FUSION OR FISSION PROTEINS	Repression of fusion protein mfm2	Immuno fluorescence Anti-sense ARNm	Fragmented and clustering	reduced glucose oxidation, ⍰Ψand cell respiration	[10]
	Over expression of fusion protein mfm1	Immuno fluorescence Fluorescence microscopy	Super connectivity	Mixing of whole mitochondria content	[11]
	Over expression of fusion protein mfn2	Immuno fluorescence	Clustering around the nucleus	Mfn2 GTPase regulates or mediates mitochondrial fusion	[12]
	Silencing of fusion proteins mfm 1and 2	RNAi Mfn1 and 2 cell fusion and GFP	Unmixing of mitochondrial populations from two cells	Unmixing of whole mitochondria content	[8]
	Overexpression of the fission protein Drp 1	Overexpression	Fragmented	Alteration of calcium signaling	[13]

biosensors we observed, in living human cells, specific and opposite transformations of mitochondrial overall structure (as well as internal organization) in response to *in situ* activation or inhibition of mitochondrial energy production using different effectors such as glucose deprivation, or treatment with rotenone, KCN, antimycin A, and amyloid peptide. For comparison, we looked at the mt-network features in a variety of cells taken from patients with a mitochondrial disease. We present also novel results we obtained on a human cell line where DRP1, a protein involved in fission of the mt-network was stably knocked out using the tet-induced siRNA technology. In these cells, we observed important changes in mitochondrial membrane fluidity and mt-network organization, associated with the uncoupling of oxidative phosphorylation and the impairement of cell proliferation in absence of glucose.

Taken together, our observations point out toward a strong implication of mt-network organization in the regulation of OXPHOS, under normal or pathological situations.

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5-04. Depression of oxidative phosphorylation under normoxia during invertebrate diapause and strategies for mitochondrial stabilization.

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In response to cues signifying the approach of winter, adult brine shrimp (*Artemia franciscana*) produce encysted embryos that enter diapause. Diapause is a specific type of dormancy that is genetically programmed into the life cycle, is controlled by endogenous factors, and is characterized by developmental and metabolic arrest. Diapause affords profound tolerance to severe environmental stresses. We show that respiration rates of diapause embryos collected from the field (Great Salt Lake, Utah, USA) are reduced up to 92 % compared with non-diapause embryos when measured under conditions of normoxia and full hydration [1]. However, mitochondria isolated from diapause embryos exhibit rates of state 3 and state 4 respiration on pyruvate that are equivalent to those for non-diapause embryos with active metabolism; a reduction in state 3 and state 4 respiration (15 % – 27 %) was measured with succinate for two of three collection years. Respiratory control ratios for diapause mitochondria are comparable to or higher than those for non-diapause embryos. The P:O flux ratios are statistically identical. Our calculations suggest that respiration of intact, non-diapause embryos is operating close to the state 3 oxygen fluxes measured for isolated mitochondria. For one collection year, cytochrome *c* oxidase (COX) activity was 53 % lower in diapause mitochondria; the minimal impact of this COX reduction on mitochondrial respiration appears to be due to the 31 % excess COX capacity in *A.*

franciscana mitochondria. Transmission electron micrographs of embryos reveal mitochondria that are well differentiated and structurally similar in both states. As inferred from the similar amounts of mitochondrial protein extractable, tissue contents of mitochondria in diapause and postdiapause embryos are equivalent. Taken together, metabolic depression during diapause cannot be fully explained by lower mitochondrial densities in vivo or by the properties measured for isolated mitochondria. Rather, downregulation may be due to reversible inhibition of oxidative phosphorylation in vivo that is not maintained in isolated mitochondria (e.g., via phosphorylation of cytochrome *c* oxidase subunit 1 [2], a diffusible inhibitor, or substrate limitation).

In nature, diapause embryos and their mitochondria are commonly exposed to prolonged periods of anoxia and severe desiccation [cf., 3]. During these periods of restricted energy flow, structure and functional capacity of mitochondria must be retained and opening of the mitochondrial permeability transition pore (MPTP) avoided if embryos are to survive and recover [4]. Trehalose content in *A. franciscana* embryos is approximately 400 mM. This sugar is well known for its ability to stabilize macromolecules during drying and has been used extensively to improve the desiccation tolerance of mammalian cells. We show evidence that trehalose is present in the matrix of mitochondria from *A. franciscana* embryos. To test whether trehalose improves desiccation tolerance of mammalian mitochondria, we introduced trehalose into the matrix of isolated rat liver mitochondria by reversibly permeabilizing the inner membrane using the MPTP. Measurement of the trehalose concentration inside mitochondria using high performance liquid chromatography shows that the sugar permeated rapidly into the matrix upon opening the MPTP. The concentration of intra-matrix trehalose reaches 0.29 mmol/mg protein (~190 mM) in 5 min. Mitochondria, with and without trehalose loaded into the matrix, were desiccated in a buffer containing 0.25 M trehalose by diffusive drying. After rehydration, the inner membrane integrity was assessed by measurement of membrane potential with the fluorescent probe JC-1. Results show that following drying to similar water contents, the mitochondria loaded with trehalose have significantly higher inner membrane integrity than those without trehalose. These findings suggest the presence of trehalose in the matrix improves desiccation tolerance of isolated mitochondria.

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5-05. Functional significance of mtDNA evolution.

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Mitochondrial DNA is a powerful tool for the investigation of the population dynamics of animal species. Although evolutionary and population genetic studies often assume that mtDNA undergoes neutral or nearly neutral evolution, the important roles of all 13 mtDNA-encoded peptides in cellular ATP production suggest that mtDNA could have

significant metabolic and fitness consequences [1]. Different methods have been used to detect natural selection. Statistical analysis of mtDNA sequences is a powerful analytical tool but possesses little power in detecting functional significance of specific mtDNA substitutions. Physiological studies on cellular or animal models can highlight how divergent combinations of mtDNA and nuclear background interact together, tease apart the relative roles of environmental and genetic factors in cytonuclear interactions and assess the adaptive value of different mitochondrial haplotypes. For example, various studies have shown that the repopulation of mtDNA-less cells with mtDNA of different species leads to different levels of respiratory function restoration depending on the phylogenetic distance between the mtDNA donor species and the recipient [2]. In populations of invertebrates (copepods and drosophila) laboratory crosses or microinjections revealed either an unpredictable pattern of hybrid breakdown, or fitness advantage of specific mitochondrial mitotypes (for a review see ref. 1). The former suggests that genomes co-adaptation arises during the course of stochastic population differentiation while the latter could suggest specific adaptations to local environments. Wild introgressed populations offer a rare opportunity to study the potential adaptive value of mtDNA in a natural environment. A natural population of brook char *(Salvelinus fontinalis)* has been found that possesses the mitochondrial DNA of Arctic char *(Salvelinus alpinus*) with no sign of nuclear introgression. This introgression could be highly significant because Arctic char is a cold adapted species, whereas brook char is more temperate and the introgressed population is found in the northern distribution range of brook char. Complete sequencing of the mitochondrial genome of Arctic and brook char revealed 47 amino acid substitutions between the species with only one in the cytochrome oxidase complex and 45 in the NADH oxidase complex. We therefore estimated the temperature sensitivity of muscle mitochondrial respiration rate as well as the maximal activity of enzymes of the electron transport system and Krebs cycle in both species and their hybrids to assess the functional impact of mitochondrial introgression on energy metabolism.

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5-06. Importance of primary reactions in cytochrome *c* oxidase for mitochondrial physiology.

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Cytochrome *c* oxidase is a molecular energy transduction device. It catalyzes the reduction of dioxygen to water, and conserves the released energy into an electrochemical proton gradient ($\Delta\mu_{H+}$) that subsequently drives the synthesis of ATP. The enzyme requires four electrons and four protons to convert O_2 into water. These electrons and protons are transferred into the O_2 reduction site from opposite sides of the membrane, generating $\Delta\mu_{H+}$. In addition, for each O_2 molecule reduced, the enzyme translocates four protons across the membrane, a process that doubles the efficiency of $\Delta\mu_{H+}$ formation.

Cytochrome c oxidase evolved as an efficient energy conserving molecular machine, and as a result of this it cannot spend redox energy on oxygen binding. The dissociation constant of oxygen from the binuclear site of cytochrome c oxidase is on the order of 1 mM. At the same time apparent oxygen affinity measured as K_m for oxygen is below 1 μM [1]. The presentation shows how molecular design can solve such a discrepancy. The second point of the presentation is to show by analyses of primary electron transfer

events why the enzyme "professionally' reducing oxygen never generates oxygen reactive species [2].

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5-07. ph dependence of the various phases of the proton pump of cytochrome *c* oxidase.

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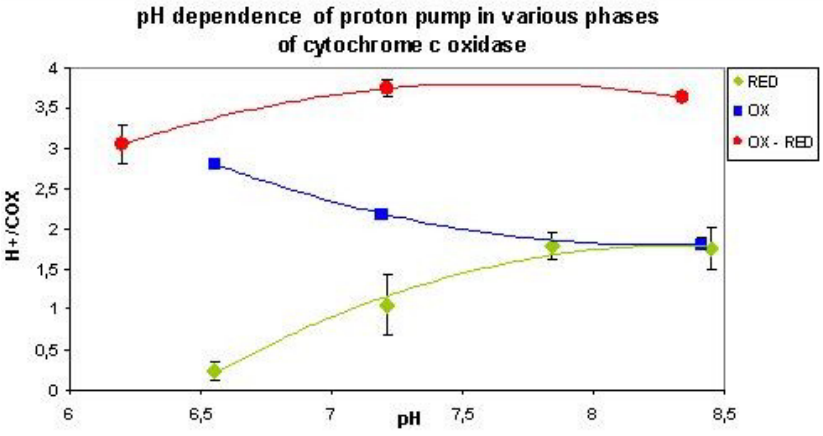
Cytochrome *c* oxidase (COX) catalyses the reduction of dioxygen to water by ferrocytochrome *c*. This reaction is coupled to the translocation of up to $1H^+/e^-$, $4H^+/O_2$ across the coupling membrane from the inner to the outer aqueous phase [1,2]. The catalytic cycle of COX can be divided into two phases: a reductive phase and an oxidative phase. A study is presented on the pH-dependence of proton transfer of COX reconstituted in vesicles, associated with the oxidation phase, the reduction phase and the oxidation-rereduction rapid transition.

The reconstitution of cytochrome *c* oxidase, purified from beef heart mitochondria [3], in phospholipid vesicles was performed by the cholate dialysis method [4] at different internal pH. Simultaneous recording of absorbance and pH changes were carried out with a diode-array spectrophotometer (settled in the multiwavelength mode) and a combined electrode respectively [5].

The experimental data show (1) an increase of the H^+/COX ratio from ≈0 at acidic external pH to ≈2 at alkaline external pH in the reductive phase; (2) a decrease of the H^+/COX ratio from ≈2.7 at acidic external pH to ≈1.7 at alkaline external pH in the aerobic oxidative phase. Experiments carried out when changing the internal or the external pH, indicate that the H^+/COX ratio depends essentially on the external pH. In the rapid oxidation-rereduction transition the results reveal a bell-shaped external pH-dependence of the H^+/COX ratio with a maximum of ≈4 at pH 7.25 (see also [1]).



The results are discussed in terms of presently debated models, in which roles of cooperative H^+/e^- coupling are envisaged at the metal centers and H_2O formation at the a_3-Cu_B binuclear center [2,6,7].

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5-08. Regulation of cytochrome *c* oxidase kinetics *in vivo* by phosphorylation of subunits via different signal cascades in bovine heart and isolated rat cardiomyocytes.

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The control of respiration by the availability of ADP is generally explained by Peter Mitchells chemiosmotic hypothesis via the mitochondrial membrane potential. A new control of respiration by ATP, independent of the membrane potential, was found by Arnold and Kadenbach [1], based on binding of ATP to subunit IV of cytochrome *c* oxidase (COX). High cellular ATP/ADP ratios induce an allosteric inhibition of COX activity, which, however, is only found with the phosphorylated enzyme. The allosteric ATP-inhibition was postulated to keep the mitochondrial membrane potential $\Delta\psi_m$ at low levels, thus preventing the formation of reactive oxygen species (ROS) [2]. Lee et al. [3] described recently a correlation between cAMP-dependent phosphorylation of Tyr304 of subunit I and the allosteric ATP-inhibition of COX in liver. We investigated the effect of wortmannin (inhibitor of phosphatidylinositol-3 kinase, PI3K), PP2 (inhibitor of non-receptor tyrosine kinases) and IBMX (inhibitor of phosphodiesterases) on ATP-inhibition of COX and phosphorylation of subunit II in bovine tissue. Ground bovine heart was incubated at 4 °C under shaking with cell culture medium and corresponding additions. From the incubated tissue mitochondria were isolated and suspended in 25 mM phosphate buffer containing 0.1 % Tween 20 and protease- and phosphatase-inhibitors. The kinetics of ascorbate respiration was measured polarographically at increasing concentrations of cytochrome *c* in the presence of 5 mM ADP as well as 5 mM ATP with an ATP-regenerating system (PEP and PK). Wortmannin and PP2 turned off the ATP-inhibition, whereas IBMX stimulated it. While PP2 completely prevented phosphorylation of subunit II, wortmannin and IBMX had little effect on its phosphorylation. This was shown by Western Blot with an antibody against phosphotyrosine after immunoadsorption of the holoenzyme from laurylmaltoside-solubilized mitochondria with a monoclonal antibody against subunit IV. From the results we conclude that the ATP-inhibition of COX could be turned on by phosphorylation via 3 different signal cascades: (1) via the G-receptor/cAMP pathway, (2) via the PI3K/Akt pathway, and (3) via a nonreceptor tyrosine kinase pathway. Corresponding experiments were performed with isolated adult rat cardiomyocytes, obtained by Langendorff-perfusion with collagenease. Tyrosine-phosphorylation of subunit IV was found to be turned off completely by incubation of cardiomyocytes with wortmannin, accompanied by abolition of ATP-inhibition of COX.

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5-09. The early stage of Complex I assembly is linked with Complex III and IV supercomplex.

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Mitochondrial respiratory chain complex I is the largest complex of the oxidative phosphorylation (OXPHOS) system with about 46 subunits. This complex has a L-shaped structure, with an arm buried in the mitochondrial inner membrane and the other arm

protruding in the mitochondrial matrix. According to a recently proposed model of a modular complex I assembly, these two parts are preassembled independently and then combined to form a full complex [1].

In order to study this model, we used a mouse cell line with a frameshift mutation in the ND6 mitochondrial complex I subunit gene and a consequent lack of ND6 synthesis. Results obtained by Western blot after PAGE and BN-PAGE showed a lack of full complex I assembly, confirmed by absence of (1) complex I enzyme activity and (2) O_2 consumption on pyruvate-malate. In addition, we found some assembly intermediates for the matrix arm but not for the membrane arm, which confirmed the assembly sequence proposed earlier [1].

Moreover, we found a high molecular weight complex I assembly intermediate including the 17kD subunit. Further investigations revealed a specific interaction between this subunit and supercomplex III and IV. This result was obtained only in our cell model but not in control cells, which may underline a putative early step for complex I assembly, involving complex III and IV. Indeed, the 17 kD subunit that is the first protein implied in the membrane arm assembly pathway [1] may need to be associated to these complexes in order to be associated with the ND6 subunit. This is stressed by the fact that in our ND6 deficient cell line, we found also an increase of complex III and complex IV activity and protein amount, reinforcing the hypothesis of a link of these two complexes in complex I assembly.

As already shown [2], OXPHOS supercomplexes are significant not only for a biochemical goal like substrate channeling but also for assembly and stabilization of protein complexes. Indeed, they suggested that a lack of complex III assembly had an effect on complex I assembly. Putting these results together, we propose that the association of the complex I 17 kD subunit and supercomplex III+IV may be the first step of complex I assembly.

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5-10. Functional role of excess capacity of cytochrome *c* oxidase in isolated mitochondria and tissue preparations: heart and liver.

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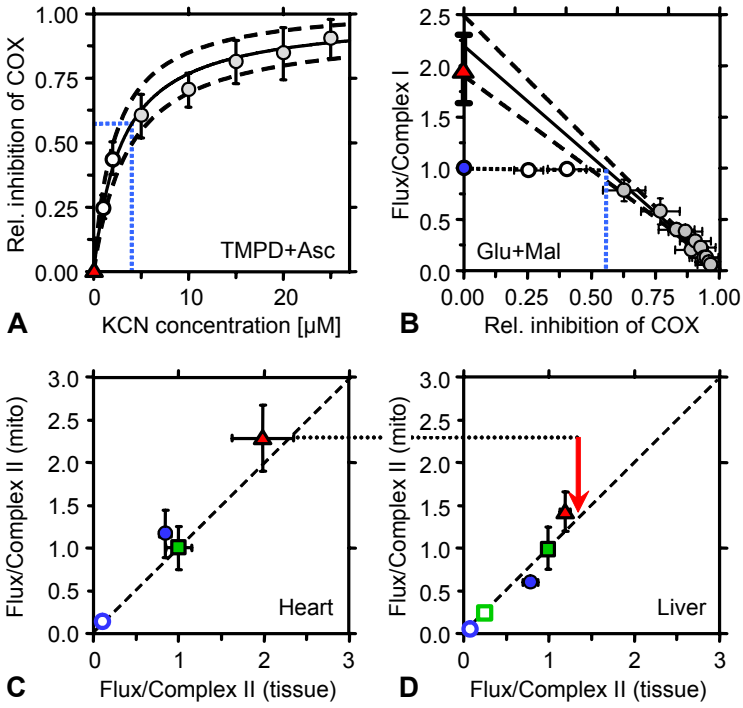
Flux control through the mitochondrial respiratory chain is shared by several enzymes, as related to the excess capacity of single steps over pathway flux. Excess capacity of cytochrome *c* oxidase (COX) remains controversial, apparently depending on the use of isolated mitochondria, permeabilized cells or muscle fibres, and living cells [1-3]. In the present study with high-resolution respirometry (OROBOROS Oxygraph-2k), these discrepancies were resolved by (1) application of optimized titration regimes for evaluation of mitochondrial respiratory function, and (2) direct comparison of isolated mitochondria and permeabilized tissue preparations.

COX reaction velocity was measured at state 3 (COX_3; antimycin A, 2 mM ascorbate, 0.5 mM TMPD, 1 mM ADP; Fig. 1A). The TMPD concentration was matched for the assessment of COX excess capacity, as shown by KCN threshold plots (Fig. 1A and B). When COX reaction velocity was related to flux with succinate (state S_3; complex II input, with rotenone), COX excess capacity was higher in rat heart than liver in both, isolated mitochondria [2] and permeabilized tissue preparations (Fig. 1C and D). The COX_3/S_3 flux ratio was 2.0-2.3 in heart and 1.2-1.4 in liver. Using state 3 respiratory flux

with pyruvate or glutamate and malate (state PM_3 or GM_3; complex I input) as reference, COX excess capacities were unchanged in heart and permeabilized pig liver tissue. In isolated rat liver mitochondria, however, the COX_3/PM_3 ratio was significantly increased to 2.4 (and the COX_3/GM_3 ratio was even higher). The low COX excess capacity in liver as related to succinate respiration represents more closely a functional COX excess capacity, due to the well-known fact that succinate is a "better" substrate in isolated liver mitochondria, and pyruvate+malate respiration is preserved better in permeabilized liver tissue [4].



The excess capacity of COX was functionally related to an increase in oxygen affinity of mitochondrial respiration [2], considered to be a relevant parameter in mitochondrial physiology [5].

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5-11. Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species: implications for the use of exogenous ubiquinones as therapies and experimental tools.

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Mitochondrial reactive oxygen species (ROS) production plays a central role in oxidative damage and redox signalling. Consequently exogenous antioxidants, such as ubiquinones, have been widely used in mitochondrial studies as both potential therapies and useful research tools. However, the effects of exogenous ubiquinones can be difficult to interpret because in addition to being antioxidants they can also be pro-oxidants or facilitate respiration by acting as electron carriers. Recently we developed a mitochondria-targeted ubiquinone ($MitoQ_{10}$) that accumulates within mitochondria due to the membrane potential, greatly increasing its effectiveness as an antioxidant. $MitoQ_{10}$ has been used to prevent mitochondrial oxidative damage and to infer the involvement of mitochondrial ROS in signalling pathways. However, uncertainties remain about the mitochondrial reduction of $MitoQ_{10}$ to its antioxidant ubiquinol form, the extent of its oxidation by the respiratory chain and its pro-oxidant potential.

To address these issues, we have compared MitoQ analogs of varying alkyl chain lengths (MitoQ$_n$, n = 3, 5, 10, 15) with untargeted exogenous ubiquinones. We found that MitoQ$_{10}$ could not restore respiration in ubiquinone-deficient mitochondria, while untargeted ubiquinones could. This occurred because oxidation of MitoQ analogs by complex III was minimal, probably due to their limited partition into the core of phospholipid bilayers. In contrast, MitoQ analogs were well reduced by complex II and glycerol-3-phosphate dehydrogenase, and this rate of reduction depended on chain length. Because of its rapid reduction and negligible oxidation, MitoQ$_{10}$ persisted *in situ* in its active ubiquinol form, making it an effective antioxidant against lipid peroxidation, peroxynitrite and superoxide in the lipid phase. Paradoxically, exogenous ubiquinols also autoxidize to generate superoxide but we show this requires their deprotonation to the ubiquinolate anion in the aqueous phase. Consequently, in the presence of phospholipid bilayers, ubiquinol hydrophobicity determines the balance between antioxidant and pro-oxidant reactions. Superoxide production by MitoQ$_{10}$ within intact mitochondria was insufficient to damage aconitase, but did lead to hydrogen peroxide production and nitric oxide consumption, both of which may affect cell signalling pathways. Finally MitoQ$_{10}$ was not an effective antioxidant against peroxides, even though it can prevent H_2O_2-induced cell death. Our results provide a comprehensive description of how exogenous ubiquinones interact with mitochondria and ROS and help clarify the interpretation of experiments using these compounds. These findings have broad implications for the rational design and use of exogenous ubiquinones as therapies and as research tools to probe mitochondrial function.

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5-12. In quest for the origin of thermal sensitivity of electron transport in heart mitochondria.

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Adaptative changes in the composition of cellular and mitochondrial lipids are a widespread feature of thermal adaptation in ectothermic animals (review by [2]) and hibernating mammals [1]. Compositional changes allow to preserve membrane fluidity even with solidifying impact of low temperature. Very few studies, however have addressed the effect of changes in mitochondrial membrane composition on the thermal sensitivity of enzymes located in the membranes as the electron transport chain. In the first part of our study, 4 groups of rats were fed with diets differing in the nature of fat and their antioxidant supplementation (Group 1: coconut oil; group 2: olive oil; group 3: fish oil and group 4: fish oil and probucol) in order to modify the composition of their cardiac mitochondrial membranes. After four months of feeding, complexes I (malate and pyruvate with or without cytochrome c added), II (succinate) and IV (ascorbate and TMPD) mitochondrial respiration where measured at three temperatures (5, 20 and 35 °C). The membrane composition has no impact on the thermal sensitivity of mitochondrial respiration for any of the complexes measured. However, complex II seems to limit the electron flux in the electron transport system at low temperature while the limitation is set by complex I at 35 °C. In contrast, complex IV demonstrated a high rate at any temperature and a very low thermal sensitivity compared to complex I and II.

In the second part of the project, we compared the thermal sensitivity of mitochondrial respiration and of different steps implied in this process (Vmax of complex I, II, III, IV, V, pyruvate dehydrogenase and citrate synthase) in the heart of rats and of a cold temperature ectotherm species, the Atlantic wolfish (*Anarhichas lupus*).

Mitochondrial respiration with malate and pyruvate as substrate was measure at 4 temperature in wolfish (5, 15, 25 and 35°C) and 6 temperatures in rats (5, 15, 25, 30, 35, 40 °C). Complex I mitochondrial respiration shows a high Q_{10} value between 5 and 15°C in rats (4.94 ± 0.34) compared with wolfish (2.52 ± 0.20). This high thermal sensitivity in rats could not be explained by the thermal sensitivity of any complex of mitochondrial respiration. The only measured process showing a similar thermal sensitivity than mitochondrial respiration in rat is the pyruvate dehydrogenase (Q_{10} of 4.66 ± 0.27). This enzyme allows the transition of pyruvate between the glycolysis and the Krebs cycle. These results suggest that mitochondrial respiration at low temperature could be limited by the processes implied in the supply of reducing equivalent ($NADH_2$ and $FADH_2$) to the respiratory chain.

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5-13. Assessment of mitochondrial function and respiratory complex function using mitochondrial containing blood cells following membrane permeabilization.

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Assessing mitochondrial function for the diagnosis of metabolic disorders and inborn errors of metabolism is generally performed using a muscle biopsy. In the pediatric population this generally means an invasive surgical procedure with substantial emotional stress and concomitant morbidity. What is needed is a method for assessing mitochondrial function that is less invasive and usable on functional (coupled) mitochondria.

From normal healthy adult volunteers, we obtained 50 ml of blood into EDTA containing vacutainer tubes. The red blood cells were separated using the Lymphoprep method from Granier®. Membrane permeabilization was accomplished in the oxygraph chamber using 40 µg/ml saponin. The mitochondrial containing cells were then assessed for oxidative phosphorylation by measuring oxygen consumption in the presence of specific inhibitors or substrates. Cells were aliquoted and frozen in the presence of 20 % DMSO, quickly frozen on dry ice slurry, and stored for a minimum of 24 hours, at –70 °C, and tested in the oxygraph.

Coupled respiration was 1.16 ± 0.35 $\mu M \cdot min^{-1} \cdot g^{-1}$ protein for the freshly isolated cells and 0.95 ± 0.14 $\mu M \cdot min^{-1} \cdot g^{-1}$ protein for the frozen cells. Rotenone (complex I inhibitor) produced a 29 % decrease in respiration and atractyloside (adenine nucleotide translocase inhibitor) produced a 52 % decrease in respiration. Frozen cells retained their ability to be inhibited by rotenone and atractyliside (43 and 38 % respectively).

We conclude from this study that the isolation of mitochondrial containing blood cells and saponin permeabilization of these cells results in coupled respiration of the mitochondria and provides an avenue for assessing the mitochondrial complex activity. Following freeze/thaw of these cells there is still coupled respiration and an opportunity for measuring coupled respiration and oxidative complexes.

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

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

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

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

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

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

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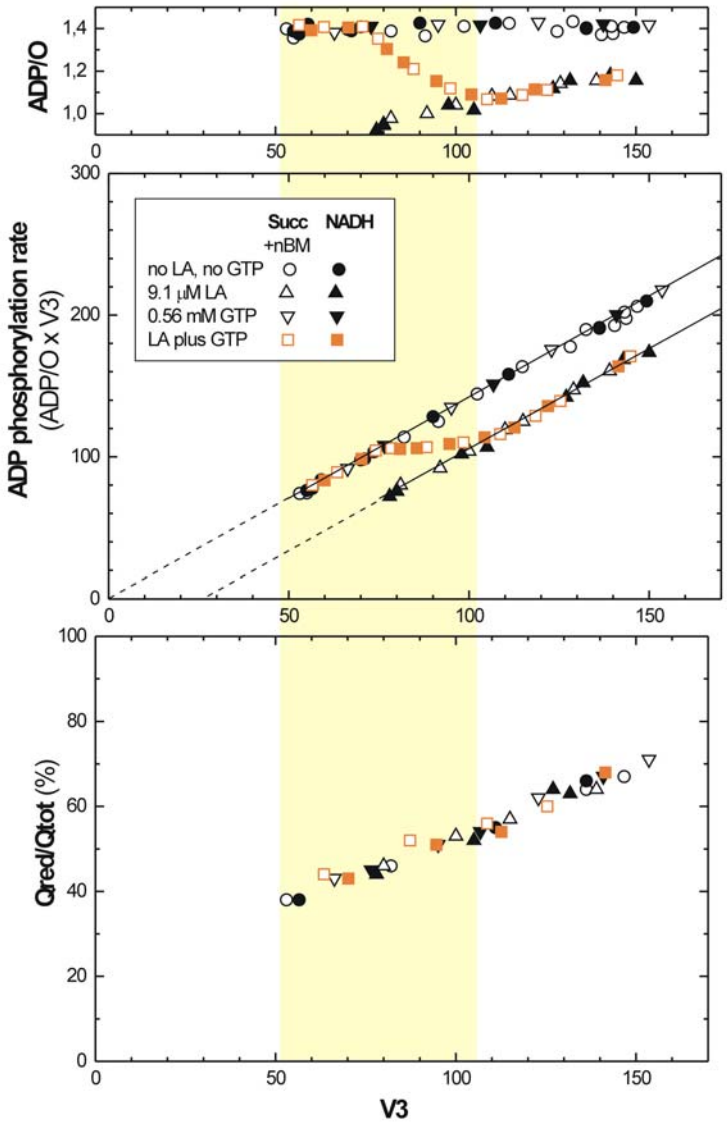
The mitochondrial respiratory chain of amoeba *Acanthamoeba castellanii*, a non-photosynthesizing amoeboid protozoon, like that of plant mitochondria, possesses energy-dissipating pathways like a cyanide-resistant alternative oxidase, non-phosphorylating rotenone-insensitive internal and external NADH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



inhibitors, i.e., when the rate of quinol (QH_2)-oxidizing pathway is decreased. On the contrary, the progressive decrease of state 3 respiration by declining respiratory substrate availability (by succinate uptake limitation or by decreasing external NADH concentration), i.e., when the rate of Q-reducing pathways is decreased, progressively leads to a full inhibitory effect of GTP (Fig. 1). Moreover, in *A. castellanii* mitochondria isolated from cold-treated cells, where a higher uncoupling protein activity is observed, the inhibition of the LA- induced proton leak by GTP is revealed for the same low value of the Q reduction level. These results are in agreement with those obtained for rat skeletal muscle [2] and potato tuber [3] mitochondria.

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

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

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

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

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

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

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

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

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

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6-06. Mitochondria are sensitive to carcinogenic chemicals.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6-10. Mitochondrial permeability transition in an invertebrate: Absence of a calcium-regulated pore in the face of profound Ca^{2+} storage.

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

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

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

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

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

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

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

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

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

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6-12. Hepatitis C virus core protein binds to mitochondria and induces Ca^{2+} uptake, ROS production, complex I inhibition, and permeability transition.

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

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

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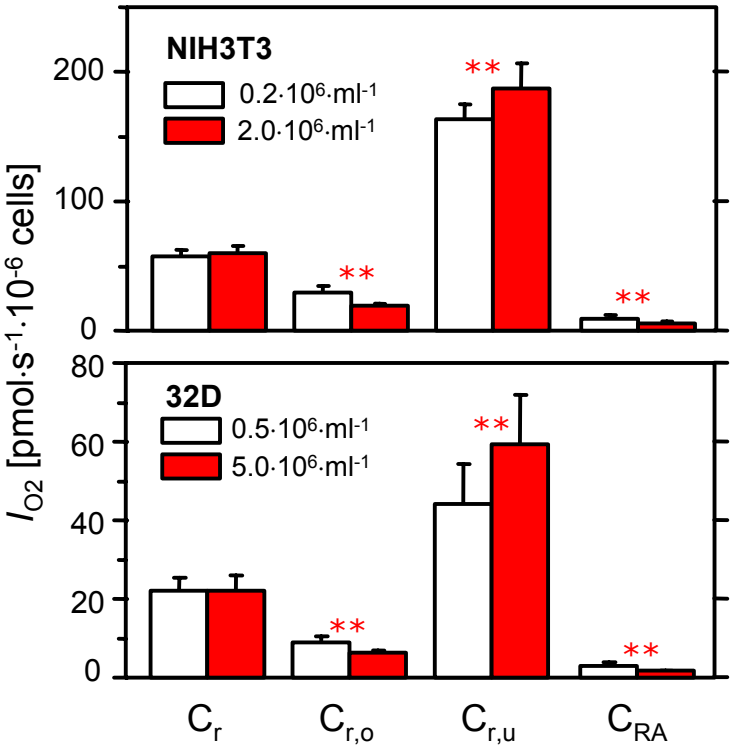
The uncoupling control ratio (UCR) is a sensitive index for the integrity of mitochondrial function in cells. The UCR is the respiratory ratio of uncoupled to physiologically controlled states, when intact cells are incubated in culture medium (routine respiration) or in the absence of exogenous energy substrates (endogenous respiration). Even in healthy control cells, large differences of UCR values are reported, with high UCR typically ranging from 2.5 to 3 at experimental cell densities of 0.2 to $1{\cdot}10^6$ ml^{-1} [1-3]. In contrast, UCR values of 1.1 to 1.9 are reported by other groups using experimental cell densities $>3{\cdot}10^6$ ml^{-1} [4,5]. Hypothetically, cell-cell signalling may exert a density-dependent effect on routine respiration in the physiologically controlled state, whereas removing respiratory control by uncoupling should eliminate such potential density-related effects.

We measured respiratory activities of intact NIH3T3 fibroblasts (adherent) and mouse pro-myeloid 32D (suspension) cells, each at two cell densities, using the OROBOROS Oxygraph-2k for high resolution respirometry. Cells were grown to a standardized density. Cells from each culture flask (N=12 for each cell type) were suspended at low and high experimental densities (0.2 and $2.0{\cdot}10^6{\cdot}$ml^{-1} for NIH3T3; 0.5 and $5.0{\cdot}10^6{\cdot}$ml^{-1} for 32D). After recording routine respiration of cells suspended in 2 ml culture medium, ATP-synthase was inhibited by oligomycin (2 µg·ml^{-1}). Increasing the oligomycin concentration had no effect on respiration. Subsequently, stepwise FCCP titration was performed up to the optimum FCCP concentration required for maximum stimulation of respiration. Optimum FCCP was 6.3 ± 1.4 and 7.8 ± 2.5 µM at low cell density, and decreased significantly at high density by a factor of 1.4 and 1.6 in NIH3T3 and 32D, respectively. Finally, respiration was inhibited by 0.5 µM rotenone and 2.5 µM antimycin A. Respiratory oxygen flux was corrected on-line for instrumental background (DatLab 4), which is a standard procedure in high-resolution respirometry [1] and is particularly important at low cell densities, to eliminate corresponding methodological artefacts. Data were analysed by a paired t-test and presented as means ± SD.

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



types (Fig. 1). This divergent effect of experimental cell density on respiration in the two consecutively induced metabolic states rules out experimental artefacts related to the oxygen measuring system and to potential errors in cell counting. The respiratory control ratio (uncoupled to oligomycin-inhibited respiration, $RCR_{u/o}$) decreased two-fold at low cell density, from 9.5 to 5.7 in NIH3T3 and from 10.0 to 5.0 in 32D cells. This significant change of the $RCR_{u/o}$ at constant routine respiration was in direct contrast to hypothetical expectations on the effects of cell density. These density-related effects were comparable in magnitude to changes in $RCR_{u/o}$ reported in the context of oxidative stress [2], senescence [3], or cell cycle arrest [3].

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

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

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

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Non-physiological increases in mitochondrial production of reactive oxygen species (ROS) are considered to be essential in the pathogenesis of several acute and chronic neurodegenerative diseases. Pathological calcium fluxes in neuronal cell death has been extensively investigated, but the role of calcium in mitochondrial ROS production is currently unresolved and both increases and decreases of the detection of ROS by calcium-loading in mitochondria have been reported [1,2].

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

dependent on availability of respiratory substrates, and improved accessibility of electron donors increased the O_2 utilization and ROS detection.

The mPT dependent increase of ROS can likely be attributed to both the loss of endogenous oxidant scavenging systems and escape of cytochrome *c*. However, challenging mitochondria with 1.4 and 8 µmol/mg calcium produced a similar extensive level of swelling but the latter dose led to a substantially greater ROS burst and to a significant inhibition of mitochondrial O_2 consumption following permeabilization. Thus, calcium overload combined with mPT is suggested to directly affect redox centers in the respiratory complexes. As the calcium-triggered ROS generation is dependent on inner membrane permeabilization it may be subject to pharmacological modulation of the mPT.

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

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

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

We have shown that the ability to inhibit ADP-stimulated respiration by DiazoFm (30 µM) and DiazoFp (30 µM) in concentrations 2.5- and 1.4-fold lower than those for diazoxide (30 µM) using succinate as a substrate. These effects were observed in the absence of K^+ in the medium The fluorine-containing K_{ATP} channel openers did not change the activity of succinate dehydrogenase significantly (by -7 %) compared to diazoxide (by -27 %). In other experiments it was established that DiazoFp activated ADP-stimulated respiration using 2- oxoglutarate as a substrate of oxidation (by -34 %). All K_{ATP} openers investigated showed an uncoupling effect irrespective of substrates used. This effect was more expressed when using succinate as a substrate and is abolished by the application of 5-hydroxydecanoate (200 µM), an inhibitor of the mitochondrial K_{ATP} channels. We have also shown that DiazoFm prevented the Ca^{2+}- dependent depression of oxidative phosphorylation.

Our investigations indicate that fluorine-containing K_{ATP} channel openers do not significantly affect the mitochondrial respiratory chain, especially the complex II. The uncoupling effect and decrease of depression of oxidative phosphorylation by Ca^{2+} can be considered a protective mechanism of action of the new fluorine-containing K_{ATP} channel openers.

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Session 7: Oxidative Stress and Mitochondrial Function

7-01. Pathophysiological role of mitochondrial glycerophosphate dehydrogenase.

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Mitochondrial glycerophosphate dehydrogenase (mGPDH) is an important regulatory device of cell intermediary metabolism. Its activity is very high in several mammalian tissues (brown fat [1], placenta [2], beta cells of pancreas [3]) however, in most tissues its expression is highly down-regulated. In spite of many speculations about its role in various physiological (thermogenesis [4]) and pathological (hyperthyroidism [5], cancer [6]) processes, the significance of remarkable variations of the mGPDH activity in various mammalian tissues is not clear.

We have reported that mGPDH represents a new site of ROS production in brown fat [6], liver [7] and placental mitochondria [8]. In this communication we present additional data indicating: (a) that ROS production measured by Amplex Red in the presence of Rotenone, antimycin A and myxothiazol is directly connected with mGPDH catalytic function, (b) that transfer of electrons from mGPDH to CoQ has different characteristics that that from succinate dehydrogenase, (c) that correlation of triiodothyronine concentration in serum, mRNA level for mGPDH in liver homogenate, mGPDH protein content and mGPDH activity in liver mitochondria after application of a single dose of triiodothyronine to euthyroid rats indicates that this enzyme is quickly eliminated from the mitochondrial membrane when the hormonal signal disappears.

On the basis of these data potential role of mGPDH in various pathological processes is discussed.

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7-02. ROS generation by mitochondrial respiratory chain - implication for neurodegenerative diseases.

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We have quantified the superoxide and H_2O_2 production rates of intact rat brain and skeletal muscle mitochondria under condition of oxygen saturation using *p*-hydroxyphenylacetate and Amplex red as fluorescent probes for H_2O_2 generation and hydroethidine as probe for superoxide formation. The localisation of superoxide producing sites was determined by evaluating the effects of SOD addition. In accordance with previous work [1], at comparable respiration rates and excellent functional quality of mitochondria we detected in brain mitochondria a high reversed electron flow-dependent H_2O_2 generation while the bc_1-complex-dependent H_2O_2 generation in the presence of succinate+antimycin was low. On the other hand, the reversed electron flow-dependent superoxide generation rate was small while the bc_1-complex-dependent superoxide production was considerable. In contrast, isolated skeletal muscle mitochondria showed at almost comparable reversed electron flow-dependent H_2O_2 generation more than ten-fold higher bc_1-complex-dependent superoxide and H_2O_2 generation. Our data are compatible with the following suppositions: (i) The major ROS generation site in complex I visible during reversed electron flow (very likely the FMN semiquinone moiety) is liberating superoxide predominantly to the mitochondrial matrix space. (ii) Similarly, the bc_1-complex-dependent superoxide generation site (the semiquinone at center 'o') liberates superoxide to both compartments with certain preference to the cytosolic space (in accordance with [2]). (iii) Muscle mitochondria, most likely due to their higher endogenous CoQ content, generate at comparable maximal rates of respiration considerable larger amounts of superoxide at center 'o' of complex III [3]. These findings imply a considerable role of mitochondrial complex I – dependent ROS generation for pathologies of the brain, like epilepsy and neurodegenerative diseases.

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7-03. Mitochondrial oxidative stress in genetically dyslipidemic mice.

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Atherosclerotic disease remains a leading cause of death in westernized societies, and reactive oxygen species (ROS) play a pivotal role in atherogenesis. Mitochondria are the main intracellular sites of ROS generation and are also targets for oxidative damage. Here, we show that mitochondria from atherosclerosis-prone, hypercholesterolemic LDL receptor knockout mice have oxidative phosphorylation efficiency similar to that from control mice, but have a higher net production of ROS and susceptibility to develop membrane permeability transition. Increased ROS production was observed in mitochondria isolated from several tissues, including liver, heart and brain and in intact mononuclear cells from spleen. In contrast to control mitochondria, knockout mouse mitochondria did not sustain a reduced state of matrix NADPH, the main source of antioxidant defense against ROS. Experiments in vivo showed faster liver secretion rates and de novo synthesis of triglycerides and cholesterol in knockout than in control mice, suggesting that increased lipogenesis depleted the reducing equivalents from NADPH and generated a state of oxidative stress in hypercholesterolemic knockout mice. These data

provide the first evidence of how oxidative stress is generated in LDL receptor defective cells, and could explain the increased LDL oxidation, cell death and atherogenesis seen in familiar hypercholesterolemia.

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7-04. Do mitochondrial RONS contribute to endotoxin mediated organ failure?

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An excessive production of reactive oxygen and nitrogen species (RONS) in mitochondria is considered to cause cell damage via necrosis or apoptosis. The aim of this study was to estimate the contribution of this mechanism in rats subjected to endotoxic shock. There are two common routes of endotoxin administration. Endotoxin introduced intraperitonially (i.p.) reaches first liver and then heart. In contrast, endotoxin introduced intravenously (i.v.) reaches first heart and then liver. Therefore, the first goal of this study was to clarify whether two routes of endotoxin administration influence rat liver and rat heart mitochondria (RLM and RHM) in the same manner or not. Sprague-Dawley rats weighing 280±21 g were subjected to LPS challenge 8 mg lipopolysaccharide (LPS)/kg (i.v.) and 20 mg LPS/kg (i.p.). These two doses resulted in an approx. similar mortality rate of 50 % and 30 % within 16 hours, respectively. The rats were sacrificed 16 h after LPS injection and mitochondria were prepared. Irrespectively of the route of LPS administration we observed similar changes in mitochondrial function: a significant increase in the rate of state 3 respiration in RLM, and a strong trend to a decrease in the rate of state 3 respiration in RHM. State 4 respiration rate was not influenced by LPS in both RLM and RHM. These changes were not substrate dependent (glutamate/malate vs. succinate), indicating that they do not originate from complex I or II. Thus, LPS did not have a deleterious effect on mitochondrial function. Using spin-trapping technique we detected a significant increase in RONS production in RLM, and no difference to controls in RHM. Therefore, RONS-mediated mechanisms seem to be plausible in the liver rather than in the heart. The second goal of this study was to find out whether increased RONS production in RLM has a coincidence with impaired cellular function in liver cells. We determined the release of a cytoplasmic enzyme (ALT) in blood as a marker of damaged cytoplasmic membrane of liver cells. In addition to mitochondrial function we determined enzymatic activity of cytochrome p450 in the microsomal fraction of the liver following dealkylation of 7-ethoxycoumarin. Increased mitochondrial RONS production in RLM was accompanied by increased levels of ALT in blood, suggesting the disintegration of the liver cell membranes, and by a decrease in p450 activity, suggesting a dysfunction of the endoplasmic reticulum. In contrast, mitochondrial function was not affected. We suggest that mitochondrial RONS can be involved in the oxidative damage of other subcellular organelles, but not mitochondria themselves. The latter is likely due to the strong antioxidant capacity of mitochondria.

7-05. Protection of oxidatively damaged mitochondria by novel chromanol-type antioxidants.

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Vitamin E is the most important lipophilic antioxidant protecting biomembranes from lipid peroxidation (LPO). Therefore, vitamin E and its derivatives are frequently used in the therapy or prevention of oxygen radical-induced diseases [1]. In the present study, novel chromanol-type antioxidants [2] such as the dimeric twin-chromanol, cis- and trans-oxachromanol as well as the well-known short-chain analogue of vitamin E, pentamethyl-chromanol, were tested for their antioxidative potency in rat heart mitochondria (RHM). As a prerequisite for a beneficial effect in mitochondria the tested substances should not disturb the highly sensitive function of the inner mitochondrial membrane. Bioenergetic parameters of isolated RHM, determined with the complex I substrates glutamate+malate, were not significantly changed in the presence of the highest concentration of chromanols under study (50 nmol/mg mitochondrial protein).

Exposure of RHM to an LPO-inducing system (50 µM cumene hydroperoxide plus 50 µM Fe^{2+}) significantly deteriorated their bioenergetic function. Alterations of bioenergetic parameters were partially abolished by preincubating RHM with antioxidants before adding the radical-generating system. In the lower concentration range twin-chromanol turned out to be more efficient than pentamethyl-chromanol, both being far more protective than cis- and trans-oxachromanol. Whether this protective effect was due to their antioxidative action was assessed from the measurements of protein-bound SH-groups, an indirect indicator of protein oxidation, and thiobarbituric acid-reactive substances, an indicator of LPO. For both parameters it was shown that oxidant-induced changes were partially prevented by a preincubation of RHM with chromanols.

Accumulation of increased levels of LPO products is usually accompanied or preceded by decreased α-tocopherol concentrations in lipid membranes [3]. HPLC measurements of α-tocopherol and its first stable oxidation product, tocopheryl quinone, in mitochondrial membranes have shown that α-tocopherol was rapidly consumed after the initiation of LPO. Pentamethyl-chromanol and twin-chromanol were similarly effective in preventing the decay of α-tocopherol and the increase of tocopheryl quinone levels in mitochondrial membranes. For both chromanols a concentration of 5 nmol/mg protein was required to protect endogenous α-tocopherol against oxidation, while a concentration of 1 nmol/mg protein was insufficient.

In conclusion, the new chromanol-type antioxidants, especially twin-chromanol, were able to improve bioenergetic and biochemical parameters of mitochondria exposed to oxidative stress.

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7-06. The preparation procedure does not influence significantly the function and morphology of liver mitochondria from control and endotoxic rats.

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Endotoxic shock is a condition in which the cardiovascular system fails to perfuse tissues adequately and cells fail to extract oxygen from blood. In each cell the mitochondria are the main oxygen sink and are thus responsible for building up an gradient which contributes to oxygen extraction from the blood. It is important, therefore, to determine adequately mitochondrial function during endotoxic shock and in other pathological states. A major problem is the isolation of mitochondria from tissues, which can be accompanied by a selective loss of a mitochondrial population with potential different size in control and experimental animals. The aim of this study was to check whether the preparation procedure exerts an influence to mitochondrial functions and morphology. Sprague Dawley rats were treated either with LPS 8 mg/kg (i.v. or i.p.). Untreated rats were used as a control. After 16 hours the livers were collected, homogenized, mitochondria were prepared and used in three experiments. The first experiment aimed at the comparison of mitochondrial respiratory parameters measured directly in liver homogenates and in isolated mitochondria using high-resolution respirometery (OROBOROS Oxygraph-2k). In these experiments we did not observe any significant difference between those parameters in homogenate and in mitochondria. This indicates that the isolated mitochondria represent the entire mitochondrial pool in liver homogenate. The second experiment aimed at the comparison of mitochondrial morphology in liver tissue and isolated mitochondria by means of electron microscopy. Staining was performed with uranyl acetate and lead citrate. The results showed that the swelling of mitochondria, typically observed in liver slices during endotoxic shock can clearly be seen in isolated mitochondria. In contrast, no swelling was observed in mitochondria isolated from control animals. This demonstrates that the morphological structure of mitochondria was not changed significantly during isolation procedure. The third experiment aimed at the comparison of the quality of mitochondrial preparations from control and LPS treated animals. We performed the analysis of typical respiratory chain markers (cyt b, cyt c1, cyt c and cyt a) in control mitochondria and mitochondria isolated from LPS treated rats by means of optical redox-difference spectroscopy using a dual-wavelength photometer. There was no significant difference between isolated mitochondria from control and LPS treated animals, indicating that there was no group-specific contamination with non-mitochondrial organelles. Together all these data suggest that the isolation of rat liver mitochondria from control and LPS treated rats does not significantly influence mitochondrial function and morphology and represent the whole mitochondrial pool in liver tissue.

7-07. Superoxide generating and cycling mechanism and its significance in energy partitioning of proton motive force in mitochondria.

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For the fist time, we showed that $O_2^{\cdot -}$ and H_2O_2 generation in mitochondria were increased nonlinearly with state 4 respiration, exhibiting a non-ohmic relationship with $\Delta\Psi$ [1]. We also found that $O_2^{\cdot -}$ generated directly by a single electron leak pathway in the respiratory chain was able to induce proton leak through HO_2 transfer across the membrane into the matrix, which is formed by interaction of $O_2^{\cdot -}$ and H^+ on the outer surface of the mitochondrial inner membrane [2]. Meanwhile, we proposed a hypothetical model of cooperation of 'reactive oxygen cycle' with the Q cycle and H^+ cycle to combine both processes of electron leak and proton leak in mitochondria [1-3]. Recently, a model was proposed [4], combining our 'reactive oxygen cycle' model and the Skulachev/Garlid/Jezek fatty acid shuttling model for UCP1, of uncoupling double-loops of H^+ and $O_2^{\cdot -}$ cycling across the mitochondrial inner membrane in order to elucidate the possible function of UCPs, activated by superoxide in the mitochondrial matrix [4]. Here, we provide novel experimental evidence that $O_2^{\cdot -}$ generated in rat liver mitochondria without UCP expression was able alone to serve as an endogenous protonophore to induce H^+ leak for diverting the energy of protonmotive force (Δp) for heat production. By investigating the link between mitochondrial state 4 respiration with succinate and the generation of $O_2^{\cdot -}$ (and H_2O_2) and heat production in both euthyroid and hyperthyroid rat liver mitochondria, as well as the effects of exogenous SOD on H^+ leak, H^+ pumping and heat production in these two types of mitochondria, we revealed that (1) hyperthyroid mitochondria show an obvious increase in state 4 respiration, 71 % higher ($P<0.01$) than euthroid controls, with no significant increase in state 3 respiration. The values of RCR and ADP/O decreased 23 % ($P<0.05$) and 29 % ($P<0.05$), respectively. These results show that the alteration in respiratory chain activity of hyperthyroid mitochondria mainly lies in state 4, known to be controlled by basal proton leak of mitochondria. (2) Proton leak rate and proton pumping activity were 51 % and 120 % higher, respectively, in hyperthyroid mitochondria than in euthyroid controls. Addition of SOD decreased the proton leak by 45 % for euthroid and 39 % for hyperthyroid mitochondria. However, SOD could give a further increase in proton pumping activity (by 45 % in euthyroid and 44 % in hyperthyroid mitochondria). The effects of SOD on both reactions exhibited a dose dependent and saturation mode, indicating that $O_2^{\cdot -}$ are involved in the process of H^+ translocation across the inner mitochondrial membrane. (3) Direct assay of $O_2^{\cdot -}$ during state 4 respiration in mitochondria revealed 40 % higher concentrations in mitochondria from hyperthyroid rat liver than in euthyroid controls. Both CCCP and nigericin depressed significantly the generation of $O_2^{\cdot -}$ and H_2O_2. In accordance with these observations the reduction of cyt c showed more increase in hyperthyroid than euthyroid mitochondria. (4) The heat production by succinate oxidation in hyperthyroid mitochondria measured by microcalorimetry was $13.0 \cdot 10^{-2}$ J/mg protein, which is 70 % higher than that of euthyroid mitochondria ($7.7 \cdot 10^{-2}$ J/mg protein). SOD reduced the 'extra' elevated heat production of hyperthyroid mitochondria to $7.6 \cdot 10^{-2}$ J/mg protein, which equals the value observed in euthyroid controls. The elevation of average heat production in hyperthyroid mitochondria is consistent with the increase in state 4 respiration (71 % higher), and with depressed average heat production (73 % lower), as well as depressed H^+ leak rate and increased proton pumping activity by SOD. These results confirm that the stimulation mechanism of proton leak and heat production in hyperthyroid mitochondria is due to $O_2^{\cdot -}$ induced H^+ leak in assciation with mitochondrial state 4 respiration, and $O_2^{\cdot -}$ formed in Q cycle could be served as an endogenous protonophore for the H^+ leak to dissipate the energy of Δp, as in the 'reactive oxygen cycle' model [1-3].

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7-08. Mitochondrial superoxide production is inversely proportional to complex I activity in human complex I deficiency.

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Respiratory chain dysfunction lies at the basis of severe clinical syndromes, presenting either at birth or in early childhood, especially affecting organs and tissues with a high-energy demand, including brain, heart and skeletal muscle. Among these disorders, isolated complex I deficiency (OMIM 252010) is the most frequently encountered enzyme defect. Structurally, complex I (NADH:ubiquinone oxidoreductase; E.C. 1.6.5.3) consists of 46 subunits, seven of which are encoded by the mitochondrial DNA and the remainder by the nuclear genome. In addition to defects in the mitochondrial DNA, mutations in nuclear genes (NDUFV1, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8) have been shown to be associated with isolated human complex I deficiency. In order to enhance our understanding of the pathophysiology of disorders of the human oxidative phosphorylation (OXPHOS) system, we study genetically characterized patient skin fibroblasts. A previous study [1] revealed an increased production of superoxide and enchanced lipid peroxidation in human skin fibroblasts chronically treated with the complex I inhibitor rotenone. Here we determined whether enhanced superoxide generation was associated with complex I deficiency in a collection of 24 pediatric patients. To this end we applied videomicroscopy to measure the superoxide-induced conversion of hydroethidine (HEt) into ethidium (Et) in living cells. Superoxide production was similar in five control fibroblast cell lines of different genetic origin and passage number, indicating that these factors are not major contributors to superoxide production in our assay. Analysis of patient fibroblasts displayed a significantly increased rate of superoxide production relative to control. Strikingly, this rate was inversely proportional to the residual enzymatic complex I but unrelated to complex III activity. This supports the idea that the superoxide detected, originates from complex I. Using Blue Native gel electrophoresis on CI deficient fibroblasts, we show that residual CI activity is linearly correlated to the amount of CI-39 kDa protein in fully assembled CI. Since superoxide production is inversely related to CI activity, which represents fully assembled and active CI protein, these findings support the idea that increased superoxide production is caused by a smaller amount of active (but less stable?) CI and not by normal amounts of 'leaky' CI.

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7-09. Relation between free iron and mitochondrial superoxide radicals during endotoxic shock.

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Oxidative stress plays an important role in the development of multiple organ failure in sepsis and endotoxic shock. Free iron is a metabolically active metal ion that increases the effects of free radical production by enhanced hydroxyl radical generation through the Fenton reaction with hydrogen peroxide. Mitochondrial superoxide radicals (SR) are considered as the main source of hydrogen peroxide. In this study we compared the levels of free iron and mitochondrial SR in endotoxic shock induced by intraperitoneal (i.p.) or intravenous (i.v.) LPS administration. Adult male Sprague-Dawley rats were injected with lipopolysaccharide (LPS) at a dose of 8 mg/kg (i.v. or i.p.) or 20 mg/kg LPS (i.p.). 16 hours after LPS application the levels of transferrin iron in blood, free iron in tissue, and mitochondrial SR were detected by means of electron paramagnetic resonance (EPR) spectroscopy. Transferrin iron levels in blood showed a significant decrease in all groups of LPS treated rats compared to control animals, suggesting the translocation of iron in tissues, mainly into tissue ferritin. However, part of this iron can appear as free iron. The injection of 8 mg LPS/kg (i.p.) did not result in an increase either in mitochondrial SR or in free iron levels. The injection of 20 mg LPS/kg (i.p.) resulted in a significant increase in both mitochondrial SR and free iron levels, accompanied by an increased mortality rate. The challenge with 8 mg LPS/kg (i.v.), also accompanied by an increased mortality rate, resulted in a significant increase in mitochondrial SR and in a trend to increase free iron levels. Since liver is the first target tissue for i.p. injection and lung for i.v., respectively, we determined free iron levels in lung after 8 mg/kg LPS i.v. administration, but did not find any change. Our data show that increased levels of free iron and mitochondrial SR in tissue may depend on the dose and the route of LPS administration and that under certain conditions free iron may amplify the oxidative potential of mitochondrial SR.

7-10. Influence of oxidative stress on osteoblasts cultured on either titanium or polystyrene.

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Titanium is a successful biomaterial used for many applications that possesses good biocompatibility and mechanical strength. It is covered by a surface layer of titanium dioxide. Mechanical disintegration of this stable inert oxide layer leads to fast reformation of titanium dioxide (corrosion). For that, the oxidation of titanium (anodic reaction) as well as the reduction of oxygen (cathodic reaction) is necessary. During the cathodic reaction at least intermediately, reactive oxygen species (ROS) can occur, which are able to affect interactions between titanium implant and surrounding tissue (monocytes/macrophages, osteoblasts, osteoclasts, endothelial cells). The surrounding cells can be activated (firstly transiently and secondly permanently) and production of reactive oxygen species by cells themselves can be induced.

In this study, we simulated oxidative stress by hydrogen peroxide and observed changes which occured in osteoblasts cultured on either titanium or polystyrene. We determined dose-dependent but material independent cytotoxicity, different reduced

glutathione levels and activation of different enzymes of oxidative defence and intracellular signalling.

7-11. MitoSOD: a novel mitochondria-targeted superoxide dismutase mimetic.

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Superoxide ($O_2^{\bullet -}$) is the proximal reactive oxygen species (ROS) generated by the mitochondrial respiratory chain, which is the major source of ROS in the cell. It is estimated that up to 1% of respiratory chain electrons generate superoxide (the one-electron reduction product of molecular oxygen) instead of contributing to the reduction of oxygen to water. Once generated, mitochondrial superoxide can react to form various other ROS, including hydrogen peroxide, peroxynitrite and the hydroxyl radical; together, these ROS can cause oxidative damage to all classes of macromolecules. For this reason, mitochondrial ROS are strongly implicated in mitochondrial pathology and in the ageing process. Detoxification of mitochondrial superoxide is therefore a promising therapeutic strategy; in addition, a mitochondria-specific superoxide scavenger would be an invaluable laboratory tool.

We have developed mitoSOD, a mitochondria-targeted version of the manganese superoxide dismutase mimetic M40403 [1]. MitoSOD is directed specifically to mitochondria by the inclusion of the triphenylphosphonium moiety, a lipophilic cation that drives the mitochondrial membrane potential-dependent accumulation of covalently linked compounds within the matrix (to 100-500x the cytosolic concentration) [2]. Previously, TPP-derived compounds have been shown to accumulate within mitochondria of diverse tissues upon oral delivery to mice [3], underscoring their potential utility as therapies for disorders of mitochondrial dysfunction. In this work the antioxidant efficacy of mitoSOD *in vitro* and within isolated mitochondria was characterised. MitoSOD was shown to compete with cytochrome c for reaction with superoxide, at concentrations comparable to M40403. In addition, mitoSOD competed with NO for reaction with mitochondrial membrane-derived superoxide.

The study of *in vivo* mitochondrial superoxide production and detoxification is compromised by the dearth of robust and specific intracellular superoxide probes. Oxidation of dihydroethidium to the red-fluorescent ethidium has been widely used as a measure of intracellular superoxide; however, it is highly susceptible to oxidation in air and is therefore not specific for superoxide. In addition, it does not localise specifically to mitochondria. However, it has recently been suggested that superoxide oxidises dihydroethidium to a fluorescent product that is distinct from ethidium, and is specific for superoxide [4]. We have investigated the feasibility of an HPLC-based assay for mitochondrial superoxide within cells, using mitoSOX, a commercially-available mitochondria-targeted derivative of dihydroethidium.

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7-12. Selective inhibitory effect of tBHP on respiratory chain enzymes in isolated rat hepatocytes and protective effect of SAMe.

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Oxidative stress is one of the most important mechanisms through which hepatotoxins induce cell death. Effective protection of cellular damage induced by oxidants requires better understanding of 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 in the cells metabolized into free radicals and induces an array of cellular dysfunctions including lipoperoxidation, oxidation of NAD(P)H and functionally important –SH groups, glutathion depletion and a number of other deleterious events leading to cell death [1,2]. The aim of this work was to characterize toxic injury of isolated rat hepatocytes induced by tBHP. We also tested the potential hepatoprotective effect of S-adenosylmethionine (SAMe). In the organisms, SAMe participates in various transmethylation and transsulphuration reactions.

Hepatocytes were isolated from Male Wistar rats by collagenase perfusion. Isolated cells were suspended in Krebs-Henseleit medium. For estimation of toxic injury we measured activity of respiratory Complex I and Complex II (OROBOROS Oxygraph-2k, AUT), mitochondrial membrane potential - MMP (Rho 123, JC-1), MDA (TBARS) and GSH/GSSG (HPLC).

Tert-butylhydroperoxide decreases the activity of respiratory Complex I and Complex II, MMP and GSH and increases lipoperoxidation. These changes are in proportional relation to the tBHP concentration and time of incubation. Respiratory Complex I activity is much more sensitive to the peroxidative action of tBHP than the activity of Complex II. We found that SAMe has protective effect against toxic injury of isolated rat hepatocytes induced by tBHP. It seems that this effect is ascribed more to transmethylation reactions than to transsulphurations.

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7-13. Evidence for increased superoxide production in atrial fibrillation detected by ESR and HPLC based assay.

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Atrial fibrillation is the most common cardiac arrhythmia. It is associated with a 5-6 fold increase in the incidence of a stroke, due to almost exclusively to thrombus formation in the left atrial appendage. We hypothesized that this decrease in $NO^{\bullet}$ may be due to increased superoxide ($O_2^{\bullet-}$) production and oxidative destruction of $NO^{\bullet}$. To address this hypothesis, we induced AF in pigs using rapid atrial pacing.

We studied the reaction of $O_2^{\bullet-}$ with a new spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), for analysis of $O_2^{\bullet-}$ production in suspension of cardiomyocytes and in isolated heart tissue. Parallel to ESR we investigated the superoxide formation using dihydroethidium and a newly developed HPLC method [1]. After 10 min of incubation, the intracellular concentration of CMH in cells reached 18.1 %. For loading the cells with DHE we incubated them for 20 min. Intracellular $O_2^{\bullet-}$

production was measured from PEG-SOD inhibited formation of 3-methoxy-carbonyl radical ($CM^{\bullet}$) or from selective DHE interaction product oxyethidium (OxyEt). A 1.8-fold increase in LAA $O_2^{\bullet-}$ from 80 to 140 a.u./mg tissue/10 min was confirmed using ESR and using formation of OxyEt. Treatment of cardiomyocytes with lactate leads to 3-fold increase in $O_2^{\bullet-}$ production in LAA.

We conclude that atrial fibrillation is associated with increased $O_2^{\bullet-}$ and decreased $NO^{\bullet}$ production in the left atrial appendage. Using new synthesized cyclic hydroxylamine CMH and DHE we detected intracellular $O_2^{\bullet-}$ production from mitochondria. High cell permeability and high reactivity with $O_2^{\bullet-}$ of CMH allow effective detection of low amounts of intra- and extracellular $O_2^{\bullet-}$. These findings we confirmed using dihydroethidium and new developed HPLC based assay.

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7-14A. Lysosomal ROS formation.

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Ubiquinone is inhomogenously distributed in subcellular biomembranes. Apart from mitochondria where ubiquinone was demonstrated to exert bioenergetic and pathophysiological functions unusually high levels of ubiquinone were also reported to exist in Golgi vesicles and lysosomes. In lysosomes the interior differs from other organelles by the low pH value which is important not only to arrest proteins but also to ensure optimal activity of hydrolytic enzymes. Since redox-cycling of ubiquinone is associated with the acceptance and release of protons we assumed that ubiquinone is a part of a redox chain contributing to unilateral proton distribution. A similar function of ubiquinone was earlier suggested by Crane to operate in Golgi vesicles. Support for the involvement of ubiquinone in a presumed couple of redox-carriers came from our observation that almost 70 % of total lysosomal ubiquinone was in the divalently reduced state. Further reduction was seen in the presence of external NADH. Analysis of the components involved in the transfer of reducing equivalents from cytosolic NADH to ubiquinone revealed the existence of a FAD-containing NADH-dehydrogenase. The latter was found to reduce ubiquinone by means of a b-type cytochrome. Proton translocation into the interior was linked to the activity of the novel lysosomal redox chain. Oxygen was found to be the terminal electron acceptor thereby also regulating acidification of the lysosomal matrix. In contrast to mitochondrial respiration oxygen was only trivalently reduced giving rise to the release of $HO^{\bullet}$-radicals. The role of this novel proton-pumping redox chain and the significance of the associated ROS formation has to be elucidated.

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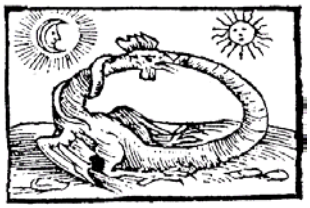

Session 8: Mitochondria in Ischemia-Reperfusion and Cardiomyopathy

8-01. The metabolic activators of mitochondrial ATP-dependent potassium channel and its role in cardioprotection.

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Cardiac disorders are a common cause of death in technically developed countries, so the search for principally new ways of their prevention and treatment is a problem of great concern. A development of new approaches based on the data of basic studies obtained lately is important in this perspective. It is well know that synthetic activators of the mitochondrial ATP-dependent K^+ channel (mitoK_{ATP}) have a clear cardioprotecting properties and prevented ATP disintegration in experimental myocardial ischemia [1]. In our earlier research we found that uridine-5′-diphosphate (UDP) is a physiological activator of the mitoK_{ATP} [2].

The goal of this study is to investigate a possible role of metabolic activators of mitoK_{ATP} in cardioprotection. We studied the cardioprotective properties of the precursors of UDP, uridine and uridine-5′-monophosphate (UMP). These precursors opposed to UDP possess the capacity to penetrate into the cell. On a model of acute myocardial infarction in rats the anti-ischemic activity of these preparations was revealed. It was shown that the myocardium infarct zone decreased in 1.9 and 3.5 times for uridine and UMP, respectively. The changes of the T-wave amplitude, electrophysiological characteristic of myocardium injuries during infarction, confirmed these data. The results obtained showed the same trend of changes of the T-wave amplitude under the treatment of the preparations. The inhibitors of mitoK_{ATP} glibenclamide and 5-hydroxydecanoic acid (5-HD) prevented the cardioprotective effect of uridine and UMP. This suggests that mitoK_{ATP} is involved in the realization of the anti-ischemic effect of uridine and its phosphonucleotide. It was also found that uridine and UMP affect the development of occlusion arrhythmias in rats, the effect of UMP being more pronounced. Since the antiarrhythmic action of 5-HD was less manifested, than the effect of glibenclamide we suppose that their antiarrhythmic action is associated mainly with the activation of the cell membrane ATP-dependent potassium channel (cellK_{ATP}). Thus both preparations can be considered as potential cardiotropic agents, but the mechanism of their action can be different.

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8-02. Effects of NO and estrogens on ischaemia-induced permeability transition in heart mitochondria.

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Apoptosis is known to contribute to ischaemia-reperfusion induced cell death in the heart. However, the mechanism of induction of apoptosis during ischaemia or reperfusion is still unclear. Recently we have shown that global ischaemia by itself (without reperfusion) can induce cytochrome c release from mitochondria and subsequent apoptosis through opening of mitochondrial permeability transition pore (MPT) [1]. In this study we aimed to investigate whether protective action of nitric oxide (NO) and estradiol, two well known cardioprotective agents, can be associated with their effect on MPT during heart ischemia.

NO depending on concentrations and other conditions can be cytotoxic or cytoprotective. For example, S-nitrosothiols can rapidly induce mitochondria-mediated apoptosis in the perfused heart [2]. In contrast, NO itself at relatively low concentrations can be cardioprotective. We found that short (3-5 min) pre-perfusion of hearts with micromolar concentrations of NO donor DETA/NO protected hearts from ischemia-induced cytochrome c release from mitochondria and subsequent respiratory inhibition and caspase activation. Similarly, perfusion of the hearts with 100 nM estradiol prevented the loss of cytochrome c from mitochondria and its accumulation in the cytosol as well as inhibition of mitochondrial respiration, caspase activation and nuclear apoptosis. In isolated mitochondria, estradiol prevented MPT related, high calcium induced loss of cytochrome. These data suggest that estrogens and NO can protect the myocardium against ischemia-induced apoptosis by inhibiting MPT.

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8-03. Role of matrix calcium accumulation in mitochondrial permeability transition in postconditioned versus preconditioned myocardium.

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Calcium is a major trigger of mitochondrial permeability transition. Inhibition of the mitochondrial permeability transition pore (mPTP) opening plays a key role in cardioprotection afforded by both postconditioning (PostC) and preconditioning (PreC). We investigated whether inhibition of mPTP in PostC and PreC might be related to a change in mitochondrial calcium after ischemia-reperfusion.

Anesthetized NZW rabbits (n=9/group) underwent either no intervention (sham) or 30 min of ischemia (I) followed by 1 h of reperfusion R. Animals underwent either no intervention (control, C), PreC by 5 min ischemia / 5 min reperfusion, or PostC with 4 episodes of 1 min ischemia / 1 min reperfusion performed after 1 min of reperfusion after the 30 min ischemia. At the end of the protocol, area at risk mitochondria were isolated by differential centrifugations. Total mitochondrial calcium content was assessed after mineralisation by HNO_3 and measured by Induced Coupled Plasma Atomic Emission Spectrometry (ICP AES). Using freezed/thawed detergent-treated mitochondria, ionized (free) mitochondrial calcium content was measured using a calibrated Ca^{2+}-selective microelectrode. We also assessed the Ca^{2+} resistance capacity CRC of mPTP defined as

the calcium overload required to induce mPTP opening. Moreover we checked mitochondrial integrity by electron microscopy.

In controls, CRC was significantly reduced with Ca^{2+} overload required for mPTP opening averaging 0.73 ± 0.16 µg calcium/mg of mitochondrial proteins versus 4.23 ± 0.17 in sham hearts (P<0.0001). PostC, as PreC attenuated CRC reduction with Ca^{2+} overload averaging 1.58 ± 0.14 and 1.91 ± 0.26 µg calcium/mg prot. respectively (P<0.005 versus C). When compared to shams (1.42 ± 0.09), total mitochondrial Ca^{2+} content was significantly increased in both controls (2.39 ± 0.43 µg calcium/mg prot.) and PostC (2.34 ± 0.37), but not in PreC group (1.29 ± 0.17). Similar patterns were obtained for ionized mitochondrial Ca^{2+} content with a significant difference (P<0.001) versus sham (0.16 ± 0.01 µg calcium/mg Prot.) in controls (0.61 ± 0.10) and PostC group (0.77 ± 0.15), but not in PreC (0.26 ± 0.05). Electron photomicrograph clearly showed a lot of swollen mitochondria with damaged cristae in control group myocardium, while many mitochondria in PreC and particularly in PostC group displayed intact membranes and dense matrix.

These data suggest that restoration of mitochondria integrity play a major role in cardioprotection by PreC and PostC treatment. Mitochondrial permeability transition may be regulated in different ways in postconditioning versus preconditioning since reduced susceptibility to calcium overload observed in PreC and PostC were not correlated with the same repartition of mitochondrial calcium in these 2 groups.

8-04. Mitochondrial function in cardiac ischemia-reperfusion injury and ischemic preconditioning.

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Mitochondrial dysfunction has long been recognized as a key event in cardiac ischemia-reperfusion (I-R) injury. In addition recent evidence has invoked a role for mitochondria in ischemic preconditioning (IPC). Several aspects of mitochondrial function have been investigated in both I-R and IPC, including: (i) the proton permeability (H^+) leak of the inner membrane, (ii) post-translational modifications to complex I, (iii) the role of nitric oxide ($NO^\bullet$) and its redox cohorts, and (iv) the regulation of ROS generation by O_2 tension and $NO^\bullet$. To accomplish this, several unique methodologies have been combined, including open-flow respirometry [1], proteomics, chemiluminescent $NO^\bullet$ detection and novel mitochondrially targeted drugs.

Results are summarized as follows: (i) H^+ leak is reversibly elevated in IPC via a UCP dependent mechanism, and is further elevated irreversibly in I-R by a mechanism involving AMP stimulation of the ANT, and transient PT pore opening. (ii) Thiols in the 75kDa subunit of complex I are damaged in I-R, and this leads to ROS generation without significant loss of enzymatic activity. (iii) Complex I can be S-nitrosated on specific subunits under various conditions. (iv) ROS generation by mitochondria does not increase under hypoxia, but actually decreases with O_2 tension. The ramifications of these findings for therapeutic intervention in I-R injury, in particular the use of mitochondrially-targeted antioxidants, will be discussed.

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8-05. Reversible redox dependent modulation of mitochondrial aconitase and proteolytic activity status during cardiac ischemia reperfusion.

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Aconitase, a citric acid cycle enzyme that converts citrate to isocitrate, belongs to the family of iron-sulfur containing dehydratases whose activities depend on the redox state of the cubane [4Fe-4S] cluster. Recent evidence indicates that mitochondrial aconitase can be reversibly inhibited or progress to irreversible inactivation and degradation in response to pro-oxidants [1]. Cardiac ischemia/reperfusion is associated with an increase in mitochondrial free radical production. In the current study, the effects of reperfusion-induced production of pro-oxidants on mitochondrial aconitase and proteolytic activity both in cytoplasm and mitochondria were determined to assess whether alterations represented a regulated response to changes in redox status or oxidative damage. Evidence is provided that ATP-dependent proteolytic activity in the mitochondria increases during early reperfusion followed by a time-dependent reduction in activity to control levels. These alterations in proteolytic activity parallel an increase and subsequent decrease in the level of oxidatively modified protein. However, proteasome activity is decreased upon the same times of reperfusion. Aconitase activity exhibited a marked decline in activity followed by reactivation during cardiac reperfusion. Loss and regain in activity involves reversible sulfhydryl modification. Aconitase was found to associate with the iron binding protein frataxin exclusively during reperfusion. *In vitro* frataxin has been shown to act as a chaperone protein that protects aconitase from $[4Fe\text{-}4S]^{2+}$ cluster disassembly, irreversible inactivation, and potentially degradation [2]. Thus, the response of mitochondrial aconitase and ATP-dependent proteolytic activity to reperfusion-induced pro-oxidant production appears to be a regulated event that would be expected to reduce irreparable damage to the mitochondria.

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8-06. Inhibition of electron transport during ischemia prevents mitochondrial ischemic damage and protects the heart.

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Mitochondrial dysfunction contributes to myocardial injury during ischemia (ISC) and reperfusion (REP). ISC damages the electron transport chain leading to a decrease in the rate of oxidative phosphorylation (OXPHOS). Reversible blockade of electron transport with amytal immediately before ISC attenuates ischemic damage to OXPHOS. We proposed that protection of OXPHOS during ISC will preserve OXPHOS and decrease myocardial damage during REP.

Langendorff perfused rat hearts were treated with amytal (2.5 mM bolus for 1 min immediately before ISC) or vehicle and underwent 25 min global ISC (37 °C) and 30 min REP without additional treatment. Subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria were isolated at end of REP to measure OXPHOS with glutamate as substrate. Left ventricular developed pressure (LVDP), diastolic pressure (DP) and lactate dehydrogenase (LDH) release were measured. Amytal pretreatment protected OXPHOS in SSM and IFM following ISC-REP with preserved state 3 and the decreased state 4 rates leading to improved respiratory control ratio (RCR). Amytal also prevented ischemic contracture (DP-ISC) and improved functional recovery during REP with increased left ventricular developed pressure (LVDP-REP) and decreased diastolic pressure (DP-REP). Amytal attenuated LDH release during REP and myocardial infarct size, indicating

decreased myocyte cell death. Thus, reversible blockade of electron transport during ISC preserves mitochondrial OXPHOS and mitigates myocardial damage during REP.

OXPHOS during REP [nAO·min^{-1}·mg^{-1}]	SSM State 3	SSM State 4	SSM RCR	IFM State 3	IFM State 4	IFM RCR
ISC-Rep (*n*=12)	122 ± 6	57 ± 4	2.3 ± 0.2	173 ± 10	74 ± 5	2.5 ± 0.2
Amytal+ISC-Rep (*n*=11)	172 ± 9*	35 ± 4*	5.4 ±0.5*	274 ±17*	43 ± 3*	6.6 ± 0.6*

Cardiac function	DP-ISC [mmHg]	LVDP-REP [mmHg]	DP-REP [mmHg]	LDH [mU·min^{-1}·g^{-1}]
ISC-REP (*n*=12)	54 ± 6	57 ± 4	37 ± 4	427 ± 60 (*n*=11)
Amytal+ISC-REP (*n*=11)	5 ± 1*	79 ± 5*	2 ± 1*	273 ± 28* (*n*=9)

Mean ± SEM. * *P*<0.05 vs. ISC+REP

8-07. Does endurance training limit rat heart mitochondrial dysfunction induced by *in vitro* anoxia-reoxygenation?

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Mitochondria are clearly involved in the physiopathology of many cardiac dysfunctions [3] and endurance training is known to improve the tolerance of the heart and specifically of heart mitochondria to in vivo oxidative-based insults [1,2]. However, the effect of endurance training on heart mitochondrial function submitted to in vitro anoxia-reoxygenation (A-R) is poorly understood. The present work intended to analyse the effect of moderate endurance treadmill training (14-wk) against rat heart mitochondrial dysfunction induced by in vitro A-R.

The respiratory parameters state 3, state 4, ADP/O and respiratory control ratio- RCR, as well as biochemical markers of protein oxidation (carbonyl groups) and lipid peroxidation (malondialdehyde) were determined in isolated mitochondria before and after 1 min anoxia followed by 4 min reoxygenation. Basal levels of heat shock protein 60 kDa (HSP60) and 70 kDa (HSP70) were measured in mitochondria and whole muscle homogenate, respectively.

A-R significantly impaired the rate of state 3 and state 4 respiration, as well as the RCR and ADP/O in the sedentary group. Nevertheless, mitochondrial state 3 respiration was significantly higher in trained than in the non-trained group both before and after A-R. The impairments in RCR, ADP/O ratio and state 4 induced by A-R in non-trained group were significantly attenuated in endurance-trained group. Oxidative modifications of mitochondrial proteins and phospholipids were found in sedentary group after A-R, although limited in trained group. Increased levels of mitochondrial HSP60 and tissue HSP70 accompanied the lower decrease in the respiratory function after A-R observed in trained group.

It is concluded that previous 14-wk endurance training limited the impairments on rat heart mitochondria caused by *in vitro* A-R.

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8-08. Mitochondrial mechanism of stress-induced cardiomyocyte injury.

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It is confirmed that stress induces cardiovascular dieases with cardiomyocyte injury, but its cellular and molecular mechanism remains unclear. In the present study the changes of cardiac mitochondria of stressed rats were characterized to explore the influence of stress on mitochondrial function and its role in cardiomyocyte injury. The results showed that stress induced the increase of the respiratory control rate (RCR) and oxidative phosphorylation efficiency (P/O) in the mitochondria of cardiomyocytes in the time and dose dependent fashion, and resulted in the disorder of intracellular Ca^{2+} balance. The stress induced alteration of mitochondrial membrane permeability transition (MPT) was also found, which in turn led to the release of cytochrome *c* from mitochondria to cytosol and activated caspase cascade in cardiomyocytes. Stress also increased the Fas expression and activated the Fas pathway through acting on the mitochondrial MPT. Mechanism. Bcl-2 overexpression in cardiomyocyte protected the mitochondria from stress injury and reduced the cardiomyocyte death, including apoptosis and necrosis, induced by stress. Hsp70 also depressed the Fas–mitochondria pathway to decrease the death rate of cardiomyocyte under stress loading. As a conclusion it appears that the mitochondria play an important role in the mechanism of stress induced cardiomyocyte injury. Both Bcl-2 and Hsp70 seem the key regulators in the mitochondrial mechanism of stress induced cardiomyocyte apoptosis. These findings imply the possibility that regulating the expression of Bcl-2 or Hsp70 to protect mitochondria acts as a new therapeutic principle in cardiovascular diseases.

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8-09A. Mitochondrial dysfunction and oxidative stress in chagasic cardiomyopathy.

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Chagasic cardiomyopathy (CCM), is a major public health threat in Latin America and Mexico, and is recognized as an emerging infectious disease in the U.S. Endomyocardial biopsies from patients in different clinical stages of the disease have suggested that myocardial inflammation and fibrosis play an important role in the pathogenesis of CCM. Because only a few, if any, parasites are detected during progressive CCM, other factors are believed to be involved in activation and/or sustenance of the inflammatory response. These factors are, however, not known.

Our recent studies have provided a new framework for understanding the initiation and progression of CCM. We have shown in experimental models that infection by *Trypanosoma cruzi* elicits mitochondrial dysfunction that is associated with oxidative modifications and altered activities of the respiratory chain complexes, generation of reactive oxygen species (ROS), and antioxidant/oxidant imbalance in the heart. Further, we have shown that scavenging of ROS diminishes the mitochondrial dysfunction-induced oxidative stress and, subsequently, is effective in limiting the inflammatory responses,

and the oxidative damage in the cardiomyocytes and heart tissue of infected mice. These studies suggest that ROS are critical in sustenance of the oxidative stress and elicitation of the pro-inflammatory cytokines in cardiomyocytes and may contribute to tissue fibrosis and cell death associated with chagasic disease development. Our other studies demonstrate the mitochondrial defects and antioxidant/oxidant imbalance are presented in the peripheral blood of the experimental animals and human patients infected by *T. cruzi*. These data show the effects of the cardiac cellular damage are reflected in the circulatory system. We would discuss the pathophysiological significance of mitochondrial oxidative stress in human chagasic disease development.

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Session 9: Mitochondria and Degenerative Diseases – I. Animal Models

9-01. Knockdown of COX5a in zebrafish phenocopies aspects of human COX deficiency.

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Zebrafish offer significant genetic, physiologic, and economic benefits as a model system in which to study human disease [1]. While mouse has been the traditional animal model of mitochondrial disease, we believe zebrafish will be a powerful new system in which to investigate outstanding questions in the field of mitochondrial pathophysiology [2].

In humans, defects in mitochondrial energy production, particularly by oxidative phosphorylation (OXPHOS), affect a wide variety of tissues including central and peripheral nervous system, ocular, cardiac, gastrointestinal, and skeletal muscle [2-3]. Deficiency in cytochrome c oxidase (COX), an integral part of the OXPHOS respiratory chain, is a component of many mitochondrial disorders [4]. We are using a morpholino antisense oligonucleotide to the COX5a subunit (COX5a-MO) to knockdown expression of COX5a and recapitulate human COX deficiency syndromes in zebrafish. Utilizing monoclonal antibodies we are able to follow tissue expression of COX5a in wildtype and MO-injected fish by immunohistochemistry and western blot, while monitoring the levels of COX using a monoclonal antibody against subunit 1 and total mitochondrial content in cells with an antibody to porin.

Preliminary results of COX5a knockdown in the developing zebrafish indicate that many of the tissues affected in humans with COX deficiency are also affected in zebrafish. Morpholino knockdown of COX5a results in stunted growth, motility impairment, pericardial edema, brain and eye abnormalities, failure to inflate the swim bladder, and no gastrointestinal tract development. The zebrafish model of COX deficiency will be useful for investigating the molecular and cellular basis of mitochondrial disease.

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9-02. APP transgenic mice exhibit mitochondrial dysfunction.

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Mitochondrial dysfunction underlies many common age-related diseases, including Alzheimer's disease (AD). AD is characterized by two major histopathological hallmarks, extracellular plaques of fibrillar ß-amyloid (Aß) peptides and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein [1,2]. In previous studies we could show that P301L tau transgenic mice exhibit mitochondrial

respiratory defects [3]. Furthermore, accumulation of Aß and oxidative stress seem to play central roles in the pathogenesis, by probably directly leading to mitochondrial dysfunction.

To investigate the contribution of Aß in AD-related neurodegenerative processes, we used isolated mitochondria of APP transgenic (tg) mice. These mice exhibits onset of Aß plaques at an age of 6 months, but intracellular Aß load is already increased at the age of 3 months. The non-physiological high levels of Aß found in transgenic mice lead to senile plaque formation like in their human counterparts, preceded by oxidative stress [4]. We detected decreased basal levels of mitochondrial membrane potential ($\Delta\psi_m$) in tgAPP mice compared to littermate non-tg control mice. Hydrogen peroxide and the nitric oxide donor sodium nitroprussid damaged the cells significantly by decreasing Psi_m in littermate non-tg control mice but not in tgAPP mice. Most probably, this is due to the preliminary insult caused by the chronic APP exposure. In addition, we observed decreased ATP levels behaving in a similar pattern after additional oxidative stress. Complementary, we observed a significant reduction of cytochrome *c* oxidase activity in 8 month old tg APP mice. In contrast, no differences in the NADH ubiquinone oxidoreductase activity between WT and tgAPP mice could be observed.

Our results further emphazise the important role of mitochondrial dysfunction in the pathogenesis of AD. Moreover, they indicate that Aß is already involved in these neurotoxic mechanisms before plaque formation occurs.

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9-03. Proteomic and biochemical analyses of diabetic human and obese rodent skeletal muscle mitochondria reveals altered concentration, oxidation, and activity of respiratory chain proteins.

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Altered mitochondria-related gene expression in skeletal muscle has been observed in Type 2 Diabetes (T2DM) patients compared to Insulin Sensitive (IS) individuals, implicating mitochondrial dysfunction in the development of insulin resistance (IR). Obesity-related and diabetes-related IR in skeletal muscle has been linked to intramyocellular lipid (IMCL) accumulation and impaired mitochondrial oxidative phosphorylation [1,2]. It is hypothesized that specific mitochondrial defects in skeletal muscle, including altered expression of individual respiratory chain complex proteins and increased reactive oxygen species (ROS) generation, are directly related to increased IMCL and insulin resistance.

Using proteomic and biochemical analyses, we have assessed whether there are altered expression, oxidative modification, and/or activity of respiratory chain protein complexes in 6 pairs of IS and T2DM patients matched for age, race, gender, and BMI and in an animal model of obesity using Zucker (fa/Br) obese rats. Mitochondrial preparations were subjected to 2-Dimension Blue-Native gel electrophoresis for optimal separation of membrane proteins [3,4]. Multiple proteins associated with respiratory

complexes I, III, IV, and V were found to be altered in T2DM compared to IS individuals and expression of complexes I and IV were decreased in the obese rat compared to lean littermates. Alterations in the ETC protein subunits could also increase ROS formation. Western blot analysis confirmed increased levels of oxidized mitochondrial thiols and increased protein carbonyl adducts in T2DM individuals compared to insulin sensitive controls and in the obese rodents compared to their controls which could further compromise function [5-7].

These abnormalities in respiratory chain protein expression and oxidation could affect function of respiratory complexes resulting in defects in substrate oxidation and accumulation of IMCL. The dramatic increases in respiratory protein oxidation in diabetes implicate increased ROS generation as a potential key mechanism for impaired insulin action and metabolism in insulin resistant humans.

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9-04. Hypertriglyceridemia in transgenic mice is associated with higher mitochondrial resting respiration and increased whole body CO_2 production and body temperature.

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High plasma levels of triglycerides lead to an increase in liver mitochondria resting respiration rate and predispose to mitochondrial permeability transition (MPT) [1]. In the present study, we demonstrate that spleen lymphocytes isolated from hypertriglyceridemic transgenic mice also present resting oxygen consumption rates 54 % higher when compared to spleen lymphocytes isolated from control mice. No significant alterations in the transmembrane mitochondrial potential or reactive oxygen species production could be detected through DHE or H_2DCF-DA fluorescence method. Fibrate treatment reduced triglyceride plasma levels by 50 % and normalized liver mitochondrial resting respiration. However, insulin treatment, which reduced triglyceride plasma levels by 30 %, did not correct the liver mitochondrial respiratory control in transgenic mice. When submitted to oxidative stress, liver mitochondria isolated from transgenic mice showed higher susceptibility to lipid peroxidation induced by Fe(II)/citrate than control liver mitochondria. In agreement with the results of mitochondria resting respiration (isolated and *in situ*), whole mice CO_2 production rate was higher in the hypertriglyceridemic transgenic than in control mice (14.8 ± 1.1 vs. 12.6 ± 1.5 $g \cdot kg^{-1} \cdot h^{-1}$, $P<0.05$, respectively). In addition, body temperature was also higher in transgenic than in control mice (37 ± 0.4 °C vs. 36.3 ± 0.2 °C, $P<0.05$, respectively). We propose that this faster catabolism may represent a regulated

adaptation to oxidize excess free fatty acids in these hypertriglyceridemic transgenic mice.

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9-05. A study of cytotoxicity of phytanic acid in mitochondria and astrocytes isolated from rat brain - possible mechanism in Refsum disease.

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In Refsum disease, a peroxisomal genetic disorder, the branched-chain fatty acid phytanic acid (3,7,11,15-tetramethylhexadecanoic acid; Phyt) accumulates at high levels (up to 1 mM) throughout the body. Clinical features suggest that Phyt exerts severe cytotoxicity, mostly in tissues with a high energy turnover. We studied the influence of non-esterified Phyt on various parameters of the energy metabolism in rat brain by using isolated mitochondria and brain cells.

Single-cell analysis applied to isolated hippocampal astrocytes reveals that Phyt (100 µM) drastically increases the inflow of extracellular Ca^{2+}, exerts strong depolarization of mitochondria *in situ*. Furthermore, cell viability of cultured astrocytes was significantly reduced after a 5h-exposure to Phyt. All these changes were not seen with the unbranched palmitic acid.

Isolated mitochondria become strongly deenergized by Phyt, applied at low concentrations (5 – 20 µM, i.e. 5 – 20 nmol/mg of mitochondrial protein). In energized mitochondria, deenergization is mainly due to protonophoric activity of Phyt. In addition, Phyt decreases state 3 respiration by inhibition of the electron flow within the respiratory chain and inhibition of the ADP/ATP exchange across the inner membrane.

As an important functional consequence of these findings, mitochondria preloaded with small amounts of Ca^{2+} (100 nmol/mg protein) become highly sensitized to rapid membrane permeability transition (MPT), even when only low concentrations of Phyt (below 5 µM) are applied. Depolarisation of the inner mitochondrial membrane and locking the ADP/ATP carrier in the matrix conformation most likely account for this sensitization to MPT.

Moreover, the interaction of Phyt with components of the respiratory chain raises strongly mitochondrial superoxide generation ($O_2^{\bullet-}$), an observation which is not seen with palmitic acid. Interaction of Phyt with complex I mostly contributes to Phyt-related $O_2^{\bullet-}$ generation. This conclusion is supported by (i) inhibition of NADH-ubiquinone oxidoreductase and (ii) decreased reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In addition, similar to antimycin A (complex III-inhibitor), Phyt increases complex III-related $O_2^{\bullet-}$ generation.

In conclusion, the observed harmful effects of Phyt on brain mitochondria and on astrocytes support the hypothesis that clinical features of Refsum disease are directly related to pathologically increased levels of Phyt.

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9-06. Mitochondrial biogenesis in rat embryo during placentation process.

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Mitochondrial biogenesis is a complex event that requires the coordinated regulation of both mitochondrial and nuclear genome by several transcriptional activators and coactivators [1]. Although important advances in this field have been achieved, the molecular pathways are not well known. In this sense, the mitochondria of rat embryo during placentation are a suitable model to further understand the mitochondrial proliferation-differentiation process, due to the important oxidative metabolism activation that takes place at this stage of development [2]. Thus, the gene expression of some proteins involved in mitochondrial replication, transcription and function, such as mitochondrial single strand DNA binding protein (mtSSB) [3], mitochondrial transcription factor A (TFAM) [4] and cytochrome *c* oxidase subunit I (COXI) respectively, have been investigated in rat embryo throughout gestational days 11, 12 and 13. We have shown that during the period studied there was a reduction in mtSSB mRNA levels accompanied by a great decrease in cellular mitochondrial DNA content (mtDNA). In addition to that, an important rise in the ratio between TFAM and mtDNA, and also in COXI relative gene expression was observed on gestational day 13. All these results together suggest that during the placentation period the rat embryo mitochondria reduce their proliferation and enter a differentiation stage by increasing their transcriptional activity probably through the physiological TFAM function as a mitochondrial transcription factor. To sum up, the present study supports the fact that embryo development is a physiological condition where mitochondrial biogenesis is well illustrated. Therefore, the current model could be of great interest for further understanding many unknown aspects of mitochondriogenesis, which should help understand the pathophysiology of mitochondrial diseases.

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9-07. The placental phenotype of a model of mitochondrial dysfunction in mice.

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Maternal nutrition is known to affect fetal growth and development and by restricting protein intake studies have demonstrated several metabolic abnormalities leading to insulin resistance. Pups maintained on a low protein diet during lactation exhibit a lower growth weight and a reduced insulin response to glucose challenge. Proteome analysis has revealed altered expression of 70 proteins by fetal malnutrition including proteins related to mitochondrial energy transfer and glucose metabolism. Placental size is increased and a higher placental/birth weight ratio is seen in gestational diabetes. In mothers with anaemia produce larger placentas and in smoking mothers there is a reduced placental weight but increased ratio of placental weight to birthweight.

A common variant in human mtDNA, at bp 16189, has been associated with type 2 diabetes, thinness at birth [1] and high placental weight in humans [2]. The T to C transition lies in the major non-coding region which contains control sequences for replication and transcription and is a bidirectional origin of replication [3] and produces a polyC tract.

We have modeled the 16189 variant of mild mitochondrial dysfunction in mice by administering the mitochondrial inhibitor, zidovudine (AZT). This is a viral reverse transcriptase inhibitor used for the treatment of HIV infection. One prominent side effect of AZT is mtDNA depletion as a result of inhibition of the mitochondrial gamma polymerase. We used four groups of mice: control (C), on either AZT (AZT), a low protein diet (LPD), or a combination of both (AZTLPD).

We have previously shown that AZT reduces mtDNA copy number in liver of offspring (P=0.021). LPD decreased birthweight and litter size (P=0.012 and 0.01 respectively). A combination of AZT with LPD decreased birthweight and litter size and increased fasting glucose and insulin compared with untreated controls. Our preliminary data shows that LPD in combination with AZT significantly increases placental size (P=0.039). We are now investigating this further by exploring placental histology and gene expression.

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9-08A. Role of mitochondrial dysfunction in mechanism of reversible metabolic depression of over-wintering lamprey liver during pre-spawning migration.

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We have conducted an investigation with the aim to clarify mechanism(s) of metabolic depression observed in poikilothermic animals during over-wintering period. In this study we have elucidated the role of mitochondria in reversible metabolic depression of hepatocytes of Baltic lamprey (*Lampetra fluviatilis L.)* taking place in the last year of its life cycle. It is known that in autumn the lamprey migrates from the Gulf of Finland to the Neva River (North-Western Russia) and switches off the exogenous feeding during all period of pre-spawning migration. Using isolated mitochondria as a model, we have revealed clear-cut seasonal variations of the main bioenergetical parameters of the lamprey liver. These changes indicate that the metabolic depression observed during the last winter of the lamprey's life cycle is mediated by prolonged reversible mitochondrial dysfunction. The dysfunction is found to manifest itself in: (1) the very low activity of mitochondrial respiratory chain, especially of its complex I, (2) low oxidative phosphorylation, (3) decreased content of mitochondrial adenine nucleotides, (4) high level of reduced pyridine nucleotides, and (5) leaky mitochondrial membranes. The sharp activation of oxidation and phosphorylation in the lamprey liver mitochondria followed by spawning and death of the animal is observed in spring. The possible causes of the phenomenon and its difference from that taking place under oxidative stress are discussed. An amazing analogy between some molecular mechanisms underlying the metabolic depression in lamprey liver cells and those in cells of patients suffering from mitochondrial encephalomyopathies, neurogenerative diseases, sepsis, poisoning, and cancerogenesis is revealed [1-3].

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9-09A. Effect of thallium (I) ions on isolated rat liver mitochondria in the presence of nonactin.

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It is known that the inner mitochondrial membrane is lightly permeable to Tl^+ ions [1]. Transport of Tl^+ in energized mitochondria occurs via the electrophoretic uniport mechanism [2,3]. It was found that nonactin being ionophore stimulates transport of Tl^+ in mitochondrial matrix [3]. However, the mechanisms of effect of nonactin on mitochondria are not studied completely.

Effects of Tl^+ on isolated rat liver mitochondria were studied. Both uptake of $^{204}Tl^+$ in the presence of various nonactin concentrations in a medium and action of Tl^+-nonactin complex on uptake of $^{137}Cs^+$ with valinomycin were tested in succinate-energized mitochondria. Besides, transport of Tl^+ in mitochondria was evaluated from swelling of mitochondria incubated in the $TlNO_3$ or Tl-acetate media. Action of Tl-acetate and nonactin on mitochondrial enzymes containing active SH-groups was estimated from their effects on the state 3, state 4, or 2,4-dinitrophenol uncoupled respiration of mitochondria.

It was found that uptake of $^{204}Tl^+$ by energized mitochondria was enhanced by an increase of nonactin concentration in the incubation medium. On the other hand, uptake of $^{137}Cs^+$ by energized mitochondria was markedly decreased in the presence of Tl^+ with nonactin in the medium. Nonactin accelerated swelling of nonenergized mitochondria in the$TlNO_3$ medium. At the same time, contraction of mitochondria after their succinate energization was markedly retarded in the presence of nonactin. In the medium containing 25-50 mM Tl-acetate, swelling of mitochondria before and after succinate administration and mitochondrial contraction after oxygen depletion were accelerated in experiments with nonactin. The state 4 respiration of mitochondria in the Tl-acetate medium was 2.5-fold increased in the presence of nonactin. The state 3 or 2,4-dinitrophenol uncoupled mitochondrial respiration was not affected by nonactin. The problems of nonactin effects on active and passive transport of Tl^+ across the inner mitochondrial membrane and importance of the transmembrane potential ($\Delta\psi_m$) on the transport processes are discussed [4].

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Session 10: Mitochondria and Degenerative Diseases. II. Patients and Human Cell Lines

10-01. Oxidative capacity, lipotoxicity and mitochondrial function in type 2 diabetes mellitus.

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Recent evidence points towards decreased oxidative capacity and mitochondrial aberrations as a major contributor to the development of insulin resistance and type 2 diabetes mellitus.

Type 2 diabetes mellitus is accompanied by accumulation of fatty acids in non-adipose tissues. In metabolically active tissues, such as skeletal muscle, fatty acids are prone to so-called oxidative damage: in addition to producing energy, mitochondria are also a major source of reactive oxygen species, and the latter can lead to lipid peroxidation. Especially the mitochondrial matrix, which contains DNA, RNA and numerous enzymes necessary for substrate oxidation, is sensitive to peroxides-induced oxidative damage and needs to be protected against the formation and accumulation of lipids and lipid peroxides.

One of the proteins thought to be involved in the protection of lipid-induced mitochondrial damage is the mitochondrial uncoupling protein 3 (UCP3). We and others have observed that the regulation pattern of UCP3 closely parallels changes in fatty acid metabolism: UCP3 is up regulated under conditions of an abundant fatty acid supply to the mitochondria and is down regulated when fatty acid oxidation is increased or plasma FFA levels are lowered. Under conditions where fatty acid delivery mismatched oxidative capacity, the surplus of fatty acids may reach the mitochondrial matrix and be responsible for the formation of lipid peroxides, leading to mitochondrial damage. Because UCP3 is able to export fatty acid anions across the mitochondrial membrane away from the matrix and is activated by 4-hydroxynonenal, a byproduct in lipid peroxidation [1], we have postulated the hypothesis that UCP3 is an exporter of fatty acid anions with the function to protect the mitochondrial matrix against lipid accumulation and lipid peroxidation-induced mitochondrial damage [2,3].

Interestingly, type 2 diabetic patients are characterized by a 50 % reduction in UCP3 protein content [4], and are also characterized by smaller and damaged mitochondria [5] and increased levels of lipid peroxidation [6]. Thiazolidinedione treatment and life style interventions, known to improve muscular insulin sensitivity, restore UCP3 levels in type 2 diabetic patients (unpublished observations), although the effect on mitochondrial function and lipid peroxidation is yet unknown. In addition, UCP3 protein content is already reduced in the pre-diabetic state of impaired glucose tolerance (IGT). These observations suggest that low levels of UCP3 may ultimately lead to increased levels of lipid peroxidation and lipid-induced mitochondrial damage [2], which can be of relevance in the etiology of diabetes.

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10-02. Dissipating excess energy in the liver is a potential treatment strategy for the metabolic syndrome.

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Much data indicates that lowering of plasma triglyceride levels by hypolipidemic agents is caused by a shift in the liver metabolism, toward peroxisome proliferator activated receptor (PPAR) α regulated fatty acid catabolism in mitochondria. Feeding rats with tetradecylthioacetic acid (TTA) leads to hypolipidemia possibly by increased channeling of fatty acids to mitochondrial fatty acid oxidation at the expense of triacylglyceride synthesis [1]. Our results suggest that a TTA-induced increase in hepatic fatty acid oxidation and ketogenesis drains fatty acids from blood and extrahepatic tissues and that this contributes significantly to the beneficial effects of TTA on fat mass accumulation and peripheral insulin sensitivity [2]. These effects are associated with altered energy state parameters of the liver at the tissue-, cellular, and mitochondrial level [3]. The hepatic phosphate potential, energy charge, and respiratory control coefficients were lowered, while rates of oxygen uptake and oxidation of pyridine nucleotide redox pairs. This is compatible with uncoupling of mitochondria due to increased proton conductance of the inner membrane. Thus, uncoupling activity of TTA was confirmed by measuring the proton electrochemical potential. The data suggested that TTA influences expression and/or activity of electrogenic ion transport systems in the mitochondrial membrane. A candidate protein is uncoupling protein 2 (UCP2) whose mRNA expression was induced after TTA treatment in rats as well as in wild type and PPARα-deficient mice. TTA also activates the other PPARs (e.g. PPARδ), and this may compensate for the deficiency of PPARα.

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10-03. Endothelial mitochondria exhibit an initial target of hyperglycemia.

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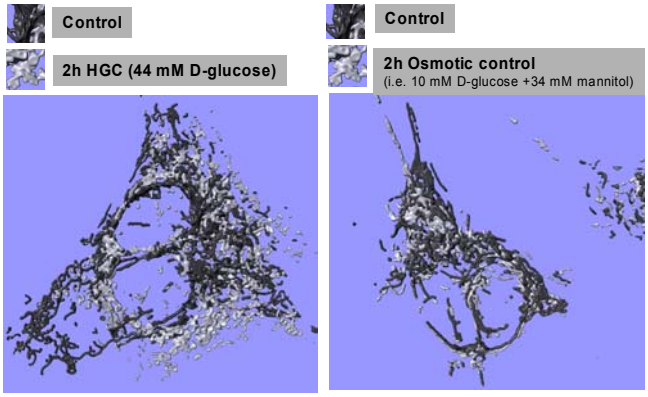
Diabetes mellitus is associated with vascular disorders that are thought to be causally linked to endothelial dysfunction initiated by elevated high D-glucose, glycated (lipo-) proteins and advanced glycation end products [1]. However, the molecular mechanisms responsible for the development of endothelial malfunction in human diabetes is only



fractionally clarified. Recent findings suggest that endothelial mitochondria may represent a causal link between high blood glucose levels and the appearance of vascular diseases [2]. We showed that under hyperglycaemic conditions (HGC) endothelial mitochondrial shape changed from a mainly tubular, highly interconnected network towards multiple, isolated singular structures within several hours. This striking and fast alteration in mitochondrial architecture was accompanied by enhanced mitochondrial free radical formation and a prolonged mitochondrial Ca^{2+} accumulation upon cell stimulation with an IP_3 generating agonist. Notably, the changes in mitochondrial structure by HGC could not be correlated with altered cytosolic Ca^{2+} signaling, while cytosolic Ca^{2+} signaling under HGC was normalized with antimycin A, an inhibitor of the respiratory chain. These data suggest that although mitochondrial structure greatly changed during HGC, alterations in cytosolic Ca^{2+} signalling are more likely due to the enhanced energy status/metabolism of the mitochondria. As endothelial Ca^{2+} signalling represents a key regulator for many endothelial vascular functions [3], mitochondria related alterations of endothelial Ca^{2+} homeostasis may support the development of vascular diseases in human diabetes. In addition, the balance between the metabolic stimulation of mitochondria and their free radical production during HGC may represent the turning point of either cell adaptation response or the initiation of fatal pathways is decided in diabetes.

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10-04. Complex I and physiopathology of mitochondrial oxygen metabolism.

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Conditions leading to arrest of cell replication are associated with decline of the activity of complex I of the respiratory chain and increased level of ROS in a variety of cell cultures *in vivo*. Short term (60 min) activation of the cAMP cascade by cholera toxin results in marked enhancement of the rotenone sensitive NADHubiquinone oxidoreductase activity of complex I, a rate limiting step of the respiratory chain, and disappearance of ROS from the cells. Experiments on fibroblast cultures from patients with pathological mutations of nuclear genes of complex I were also carried out.

The results showed that mutations in the NDUFS4 gene (18 kDa subunit), causing complete suppression of complex I NADHubiquinone oxidoreductase activity, prevented ROS formation in the fibroblast cultures. Mutation in the NDUFS1 gene (75 kDa, FeSprotein) causing severe depression of the NADHubiquinone oxidoreductase activity of

complex I, was associated with production ROS, reversed by activation of the cAMP cascade.

10-05. Two components in pathogenic mechanism of mitochondrial ATPase deficiency: Energy deprivation and ROS production.

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Isolated defects of mitochondrial ATP-synthase (ATPase) due to diminished biosynthesis of the enzyme represent a new class of severe mitochondrial diseases, characterized by selective, 70-90 % decrease in cellular content of ATPase [1,2]. The primary cause is a mutation in nuclear gene(s) affecting the initial stage of enzyme biogenesis - assembly of the F_1 catalytic part of the enzyme [1,3]. Our studies of cellular respiration and ATP synthesis in fibroblasts from several unrelated patients with ATPase deficiency showed that the content of the enzyme is insufficient, or without any reserve capacity to maintain basal mitochondrial energy provision. The cells exhibited altered discharge of mitochondrial membrane potential $\Delta\psi_m$ (cytofluorometry with TMRM), which was increased at state 4 and especially at state 3-ADP. ATPase-deficient cells further showed several-fold increase in mitochondrial ROS production analyzed by CM-H_2DCFDA fluorescence (using fluorometry or confocal microscopy) that was fully abolished by uncoupler FCCP. Activated ROS production was associated with a variable increase of mitochondrial superoxide dismutase (MnSOD), and small changes in cellular content of glutathione, but inhibition of glutathione synthase with buthionine sulphoximide (BSO) significantly activated ROS production. Aurovertin and oligomycin titration studies and replacement of glucose by galactose in culture medium demonstrated a marked decrease in viability of ATPase-deficient cells when they depend on mitochondrial oxidative metabolism rather than on glycolysis.

Our studies demonstrate that altered discharge and high levels of $\Delta\psi_m$ activate ROS production in ATPase-deficient cells. The resulting oxidative stress can be counteracted by activity of MnSOD and glutathione levels, however, activated mitochondrial ROS production is lethal in patient cells.

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10-06. Trolox restores aberrant mitochondrial morphology and improves the assembly and activity of complex I in patients with an isolated deficiency.

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Mutations in nuclear-encoded subunits of NADH:ubiquinone oxidoreductase (complex I) cause complex I deficiency (OMIM 252010), which is associated with a broad spectrum

of clinical presentations. Currently, the cellular consequences of these disorders remain elusive and rational treatment strategies are lacking. We recently demonstrated that mitochondrial complexity is increased upon inhibition of complex I [1] and linearly correlated with residual complex I activity in patient cells [2]. This finding suggests that an increase in mitochondrial complexity might reduce complex I deficiency. Here, we investigated this idea in a collection of 13 patients carrying mutations in nuclear-encoded subunits of complex I (NDUFV1, NDUFS1, NDUFS2, NDUFS4, NDUFS7 or NDUFS8). Cluster analysis highlighted two classes of patients within this cohort displaying a relatively low (class I: 33±3 % of lowest control) or high (class II: 65±3 %) residual complex I activity. Mitochondrial complexity was significantly lower in class I (68±4 % of control) than in class II (115±3 %) and not related to the activity of complex III and IV. Under resting conditions, patient cells displayed a normal cytosolic calcium homeostasis but an increased rate of ROS generation. The latter was fully normalized by treatment with the vitaminic antioxidant Trolox. Both in vehicle and Trolox-treated patient cells complex I activity and assembly were linearly ($y=x$) correlated. This suggests that complex I, once fully assembled, displays normal catalytic activity. In class I, treatment with Trolox fully restored mitochondrial complexity and enhanced the expression and activity of complex I by 200 %. In class II, Trolox did not affect mitochondrial complexity and increased complex I expression and activity by 50 %. Such restoration was induced by treatment with the mitochondria-targeted antioxidant mitoquinone (MitoQ). We conclude that nuclear-encoded mutations in complex I affect the assembly of the complex but, once assembled, not its catalytic activity. The reduced expression of complex I results in enhanced levels of ROS that contribute to the cellular phenotype by affecting mitochondrial complexity and complex I expression/activity. The effectiveness of Trolox might indicate a potential beneficial role in the treatment of patients.

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10-07. Cytochrome *c* oxidase assembly defects.

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Mutations in SURF1, the cytochrome *c* oxidase (COX)-specific assembly factor, are the primary cause of Leigh syndrome associated with COX deficiency (LS^{COX}), one of the most frequent mitochondrial disorders with fatal consequences [1]. The Surf1p absence in LS^{COX} patient fibroblasts leads to severe reduction of the COX holoenzyme content and accumulation of COX assembly intermediates. Our previous studies showed that such deficiency manifests in decreased stability of the enzyme, impaired proton-pumping ability of COX, and decreased affinity of the enzyme for oxygen [2,3]. We suggested that these functional changes were due to altered properties of the COX assembly intermediates [2,3].

Recently, we extended our studies in the SURF1 knock-out mice model of LS^{COX} [4]. In mitochondria isolated from tissues of homozygous SURF1 -/- mice we observed similar functional alterations as in patient fibroblasts, e.g. decreased affinity of COX for oxygen and release of the enzyme downregulation. These changes, however, were not accompanied by the accumulation of COX assembly intermediates, only severe reduction of the holoenzyme content was found. Using high-resolution BN-PAGE and 2D-PAGE

techniques and the SURF1 knock-out mice model, we aim to further resolve whether the functional manifestations of the COX assembly defect are due to (i) accumulation of intermediates with altered functional properties; (ii) changes in subunit composition of the COX holoenzyme that has not been so far recognized due to low resolution of BN-PAGE, i. e. absence of one or several small nuclear-encoded subunits; and (iii) decreased content of otherwise structuraly and functionaly normal COX.

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10-08. Mitochondrial alterations during apoptosis induced by a thia fatty acid in leukemia cells.

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Some fatty acids and derivatives are known to induce cell death in cancer cells. Mitochondria may have important roles in the death process. The modified fatty acid tetradecylthioacetic acid (TTA) has in multiple studies been demonstrated to have anticancer properties [1, 2]. The objective in this study was to investigate the underlying mechanism behind TTA-induced apoptosis in IPC-81 leukemia cells. The first signs of apoptotic morphology were seen after 12 hours. Overexpression of the antiapoptotic protein Bcl-2 partially blocked the induction of apoptosis. Mitochondrial release of cytochrome *c* could be detected less than one hour after TTA administration. This was equally evident in Bcl-2 overexpressing cells, and in cells overexpressing the inducible cAMP early repressor (ICER), which are resistant to cAMP indcuced apoptosis. Intriguingly, caspase-3 activation in TTA-treated cells occurred in parallel with the appearance of apoptotic morphology, i.e. several hours after the release of cytochrome *c*. The broad-spectrum caspase inhibitor zVAD-fmk did not block induction of apoptosis; however, it appeared to suppress nuclear fragmentation. In addition to early cytochrome *c* release, the importance of mitochondria in TTA-induced apoptosis was further substantiated by depolarisation of the mitochondrial membrane potential ($\Delta\psi$). Finally, we observed an early depletion of mitochondrial glutathione and a reduction in the percentage of non-oxidized glutathione (GSH). This supports the understanding that the mitochondrial level of glutathione may be of importance early in the apoptotic process. It is concluded that TTA, as a modified fatty acid, seems to cause apoptosis possibly through a direct effect on mitochondria [3]. This type of compounds might therefore be candidates for new mitochondrion-targeting drugs that overcome apoptosis resistance.

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10-09. Mitochondrial derangement induced by PPAR-ligands in HEP-G2 cell lines. Pathophysiological and pharmacotoxicological implications.

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Thiazolidinediones, fibrate derivative drugs, have emerged as a treatment option in the pharmacotherapy of type 2 diabetes mellitus. Some drugs belonging to this class, however, are characterized by an intriguing pharmacotoxicological profile. The first thiazolidinedione drug, troglitazone, was rapidly withdrawn from the market because of liver toxicity. Moreover, also the 'newer' glitazones have been reported to provoke a dangerous hepatotoxicity [1]. Recently the American Heart Association and the American Diabetes Association contraindicated thiazolidinediones in patients at risk for heart failure. Our recent studies showed that some fibrates (clofibric acid, gemfibrozil and bezafibrate) and a thiazolidinedione derivative, ciglitazone, are all capable of inhibiting NADH cytochrome c reductase activity of the mitochondrial electron transfer chain in human myeloid and human muscular cell lines [1].

All these drugs are considered well-known PPARs (Peroxisome Proliferator Activated Receptors) ligands and their pharmacological activities are classically reported as consequences of their capacity to bind and activate these particular class of nuclear receptors [2]. In particular fibrates, interacting with subtype alpha of PPARs, seem mainly to control the transcription of genes encoding key enzymes related to fatty acid catabolism, and thereby are used as hypolipidemic-hypotrygligeridemic drugs while fibrate derivatives thiazolidinediones, interacting mainly with PPAR subtype gamma, seem to act as insulin sensitizers and because of this they have been introduced in therapeutic protocols of type 2 diabetes mellitus.

At present, clinical data about a moderate activity of glitazones as hypoglycemic agents together with unwanted noxious effects (episodes of acute liver insufficiency and/or heart failure) seem to confirm that the pharmacotoxicological profiles of these PPAR ligands cannot be fully ascribed only to receptor activation. Mitochondria may represent secondary but still important targets of these drugs and this aspect can not be underestimated in all its implications anymore. In fact, these organelles, characterized by a typical physical and chemical matrix *milieu* ($\Delta\psi_m$ and ΔpH), may accumulate some amphipatic xenobiotics like fibrates and glitazones at concentrations much higher than those measured in blood [3].

PPAR-ligands impairing NADH oxidation at the level of Complex I of the mitochondrial respiratory chain induce cells to respond to the energy demand by a series of metabolic shortcuts (i.e., stimulation of glycolysis and/or β-oxidation) which could explain some aspects of their pharmacological and toxicological profiles. In our opinion, a right emphasis on the interaction between synthetic PPAR-ligands and mitochondria is fundamental in order to reduce, in genetically or pathologically predisposed patients, further dangerous side effects.

Considering the role of liver in the pathophysiology of type 2 diabetes and its demonstrated vulnerability to the toxic effects of glitazones, the scope of this investigation was the characterization of the metabolic and toxicological effects of different PPAR-ligands in a human liver cell line.

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10-10. Cancer cell growth can be affected by different mitochondrial DNAs from subjects without cancer.

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The role of mitochondrial dysfunction in carcinogenesis has been shown in previous studies [1,2]. Most of the previous studies used pathogenic mutant mitochondrial DNA (mtDNA) to examine the effect of mtDNA on carcinogenesis. In this study, we made trans-mitochondrial hybdrids (cybrids) containing 143B osteosarcoma cell nucleus and mtDNAs from platelets of normal subjects (n = 24) who did not have cancer at the time of study. Since mtDNA haplogroup D has been reported to be associated with longevity in Japanese [3], we included hapolgroup D with other common Asian-specific haplogroups. The mtDNAs used in this study were haplogroup A, B, D, and F (n = 6 in each haplogroup). We compared cell counts at 72 h after seeding same number of cells and found that the cybrids harboring haplogroup D mtDNAs grew at the lowest rate (P<0.05 compared to 143B rho+ parent cell). When comparing individual clones, we observed that cybrid A1 showed the highest growth rate and cybrids D1, D3, D6 showed the lowest rate. The thymidine uptake rate was higher in cybrid A1. By FACS analysis using propidium iodide staining, we found that the cells of sub-G1 phase were less than 40% in cybrid A1 and more than >50 % of cybrids D1, D3, and D6 remained at sub-G1 phase. There was no difference in the rate of apoptosis. In prostate cancer, it has been shown that reactive oxygen species (ROS) contributes as cell growth stimuli [1]. However, we could not find any difference among cybrids A1, D1, D3, and D6, although 143B rho+ cells showed significantly increased ROS levels. By sequencing of whole mtDNA, we found that cybrid A1 has Met490Thr mutation in COI gene and Thr127Ala mutation in ND5 gene. Among them, Thr127Ala is highly conserved amino acid across all species. Thus, it could be responsible for the functional difference of cybrid A1. From above results, we conclude that cancer cell growth is affected by different mtDNAs from subjects without clinically evident cancer and subjects harboring mtDNA haplogroup D could live longer by avoiding carcinogenesis.

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10-11. Importance of the mitochondrial genetic background in the mtDNA mutations expression: Implication in mitochondrial diseases.

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The mitochondrial genetic diseases can be caused by more than 50 mtDNA mutations and 200 rearrangements. It has been already observed that several distinct mtDNA mutations can product the same disease and reciprocally several diseases can be related to the same mutation. These observations highlight the problem of the variability of the expression of these mutations. Several hypotheses such as differences in the "threshold effect", or heteroplasmy levels have been proposed to explain this variability. However, differences in the mitochondrial genetic background is another hypothesis, but this notion is difficult to investigate with traditional genetic approaches.

The recent progress in molecular anthropology has lead to the possibility of the definition of monophyletic groups of mitochondrial DNA. Indeed, the analysis of mtDNA sequences has shown a high degree of homogeneity among European populations with 99% of European mtDNAs fall into one of ten haplogroups (H, I, J, K, M, T, U, V, W or X). These "haplogroups" can be a useful tool for such genetic/epidemiology study. For the moment, only few studies are published on the relationships between haplogroups and mitochondrial diseases and concern few of these pathologies. In addition, these works often concern a small number of patients, which do not allow a clear statistical analysis.

Three laboratories of Bordeaux[1,2,3] in collaboration with the French network of the mitochondrial diseases work to define the haplogroups of most of 500 French patients and to highlight the importance of the mitochondrial genetic background in the mtDNA mutations expression.

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10-12. A novel approach for rapid screening of mitochondrial D310 polymorphism.

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Mitochondrial DNA alterations have been suspected to play an important role in the development and progression of cancer. Several mutations have been identified in a wide variety of human tumors including breast, colorectal, ovarian, gastric, hepatic and esophageal cancers as well as hematological malignancies [1]. D-loop region of the mtDNA is the most potent accumulation site for many of these mutations and numerous polymorphisms have also been reported in this region. The sequence alterations of this region may contribute to altered replication or transcription properties.

Recently, Sanches-Cespedes and colleagues [2] have identified a polyC mononucleotide repeat located between 303 and 315 nucleotides within the D-loop region as a mitochondrial hot spot of deletion or insertion mutations. This region is part of the conserved sequence block (CSB) II and consists of a stretch of cytosines interrupted by a thymine nucleotide (CCCCCCCTCCCCC). Although the number of the cytosine residues at the first stretch of polyC is accepted as 7-C (GeneBank NC_001807), it is highly polymorphic ranging between 6-C to 9-C [3,4]. It is still questionable if there is any correlation between the number of the cytosine residues and development and progression of the cancer. Typically, time and money consuming methods such as sequencing and radioactivity based gel electrophoresis are required in order to evaluate this polymorphism among individuals. Also, gel electrophoresis remains ineffective unless confirmed with sequencing and these limidations are especially obvious with studying large populations.

In this study we established a restriction fragment length polymorphism (RFLP) assay for the first step rapid screening of the individuals if they carrying 7-C at their mitochondrial D310 region. We tested a total of 141 tissue samples including normal and cancerous tissues of 25 breast and 25 colorectal cancer patients and 41 blood samples of healty individuals. By using this simple approach, 41 % of the studied samples were found that have 7-C in their mtDNA D310 region without need for sequencing and/or radioactive labelling. Furthermore, we compared the cases and normal samples for their RFLP status and found a statistically significant difference between colorectal cancer samples and healty individuals.

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10-13. Reduced skeletal muscle mitochondrial O_2 flux capacity in type 2 diabetes.

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Hyperglycemia in insulin-resistant type 2 diabetes is associated with mitochondrial dysfunction characterized by diminished mitochondrial oxidative capacity and proton uncoupling, elevated membrane potential, increased ROS production and impaired lipid metabolism. While mitochondria are considered a central locus of altered metabolic pathways leading to pathogenic processes in type 2 diabetes, the mechanisms underlying these factors remains to be elucidated. Evidence for reduced oxidative capacity of skeletal muscle in diabetes is based on markers of oxidative enzyme levels and gene expression defects, yet there is a paucity of data reporting direct measures of mitochondrial O_2 flux capacity in intact cells. In this study, O_2 flux capacity of permeabilized muscle fibers from biopsies of the quadriceps in healthy humans (n=5) and patients with type 2 diabetes (n=7) was measured at 37 °C using high resolution respirometry (OROBOROS Oxygraph-2k). Oxygen flux was expressed per mg muscle fresh weight. Diabetic subject characteristics were; age = 62.2±2 years; body mass index = 32.9±2; fasting glucose = 157±13.7 mg/dl; lactate = 13±0.1 mmol/l.

In healthy controls and diabetics respectively, ADP-stimulated state-3 respiration with complex I substrate (glutamate) was 43±4 vs. 33±2 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$, and state-3 O_2 flux with parallel electron input from complex I+II (glutamate+succinate) was 87±8 vs. 70±3 pmol$\cdot s^{-1} \cdot mg^{-1}$. Further increases in flux capacity were observed with uncoupling by FCCP, but were lower in type 2 diabetics (106±12 vs. 84±2 pmol$\cdot s^{-1} \cdot mg^{-1}$). Subsequent fluxes with rotenone were 73±9 vs. 58±3 allowing for an estimation of individual fluxes through complex I and II. The findings demonstrate serial blunting of state-3 O_2 flux with electron flux through either complex I or II, and a similar reduction with parallel electron input through both complexes. Furthermore, on the basis of similar uncoupled responses relative to state 3 (glutamate+succinate) in both healthy (1.22) and diabetic subjects (1.2), the results reflect an attenuation of mitochondrial oxidative capacity in skeletal muscle of type 2 diabetic patients indicative of an impaired electron transport capacity.

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Session 11: Mitochondria and Aging

11-01. Molecular networks affecting mitochondrial functions and lifespan in the fungal aging model *Podospora anserina.*

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Podospora anserina is a filamentous fungus with a limited lifespan. Experimental research of the last several decades unravelled that mitochondria play a crucial role in the control of lifespan [1]. This is documented by a number of different mutations and genetic manipulations which affect different pathways and lead to an increase in lifespan. Overall it was found that manipulations resulting in (i) a decreased production of mitochondrial reactive oxygen species (ROS), (ii) an induction of ROS scavengers, and (iii) an increase of the stability of the mitochondrial DNA are efficient in lifespan extension. Other pathways may also be of significance.

The characterization of one long-lived mutant, grisea, was previously found to affect cellular copper homeostasis [2]. This mutant is a copper-uptake deficiency mutant because the gene for the high affinity copper transporter, PaCTR3, is not expressed [3]. Consequently, cellular copper levels are low and cytochrome c oxidase (COX) assembly is greatly impaired. As a kind of a retrograde response, an alternative, iron-dependent oxidase is induced. Respiration via this pathway generates less ROS. In addition, the mitochondrial DNA (mtDNA) which is destabilized efficiently during aging of the short lived wild-type is stabilized in the mutant. Since copper does effect also other pathways (e.g. Cu/Zn SOD, tyrosinase), we generated a strain in which copper to the respiratory chain is specifically interrupted. A gene, *PaCox17*, coding for a copper chaperone involved in the delivery of copper to COX, was deleted in the wild-type strain of *P. anserina*. The corresponding strain respires via the alternative pathway, is characterized by an altered profile of superoxide dismutases, a stabilized mtDNA and a lifespan that is dramatically increased [4].

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11-02. Mitochondrial functions in yeast aging and apoptosis.

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Yeast mother cell-specific aging is increasingly studied as a model for replicative aging of human cells. Our aim is to clarify the relationship between oxidative stress, apoptosis and the aging process. The transcriptome of senescent wild type yeast mother cells isolated by elutriation centrifugation was studied and compared with the transcriptome of apoptotic yeast cells of cdc48 TS as well as orc2-1 TS mutant strains. A large overlap of the old cell and apoptotic cell transcripts was revealed, comprising the functional categories DNA repair, oxidative stress defense and mitochondrial functions. Several of

the genes identified in this way were deleted and tested for oxidative stress resistance and longevity. Deletion of genes coding for mitochondrial ribosomal proteins generally led to oxidative stress sensitivity, but we found one example that caused resistance and a marked increase in lifespan. We are presently testing co-segregation of these phenotypes in meiotic tetrads and are analyzing the mechanism which is at work in this longevity mutant.

Recently we started to investigate the yeast member of the highly conserved eukaryotic gene family, TCTP. The TCTP protein shuttles to the mitochondria when the cells are stressed with a mild oxidative treatment which induces apoptosis. Our findings so far indicate that yeast mother cell-specific aging involves an apoptotic process and that mitochondria play a functional role in this process.

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11-03. Yeast as a model for investigating mitochondrial oxidative damage.

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The mitochondrial respiratory chain is an important source of ROS (reactive oxygen species), which cause protein damage, lipid peroxidation and mitochondrial DNA (mtDNA) mutations. This oxidative stress has been implicated in fundamental processes such as ageing, degenerative diseases and apoptosis. However many aspects of ROS production by mitochondria and its physiological implications for cell death and ageing are unknown. We are studying how ROS production contributes to mitochondrial dysfunction, in particular how levels of oxidative stress correlate with mtDNA damage and fixation of mutations. These processes are being investigated in the budding yeast *Saccharomyces cerevisiae*, which is an excellent eukaryotic model amenable to genetic manipulation and extensive colony scoring.

Superoxide ($O_2^{\bullet-}$) is the proximal ROS produced by the mitochondrial respiratory chain via single-electron reduction of O_2, and is therefore of primary interest and significance to the study of oxidative damage. As little was previously known about ROS production by yeast mitochondria, it was first necessary to understand the sources of ROS in yeast, before focusing on the genetic consequences. We therefore optimised a range of biochemical assays for $O_2^{\bullet-}$ measurement, to establish the sites, topology and magnitude of $O_2^{\bullet-}$ production by the yeast respiratory chain. Levels of $O_2^{\bullet-}$ were inferred from assaying: the rate of aconitase inactivation (an enzyme of the Krebs cycle, located in the mitochondrial matrix which contains an iron-sulphur cluster at its active site, susceptible to attack by $O_2^{\bullet-}$) [1], coelenterazine chemiluminescence (a compound involved in the bioluminescence of marine organisms, which reacts specifically with $O_2^{\bullet-}$ *in vitro* leading to quantifiable light emission) [2], and hydrogen peroxide efflux (a more stable conversion product of $O_2^{\bullet-}$, which is able to diffuse across biological membranes and can be detected with fluorometric probes).

The above techniques have being applied to isolated yeast mitochondria, energised with various respiratory substrates, and incubated with compounds such respiratory

inhibitors, redox cycler and uncoupler. From these experiments we have identified the conditions of $O_2^{\bullet-}$ production by yeast mitochondria, which provides support for current studies into the relationship between ROS production and oxidative damage to mitochondria, with particular focus on mtDNA. We are exploring various methods to monitor levels of mtDNA damage and mutations rates, including quantitative PCR [3] and HPLC detection of 8-hydroxydeoxyguanosine (8OHdG), a DNA lesion formed by attack with the hydroxyl radical (•OH) and widely used as a biomarker for oxidative damage [4]. This research aims to clarify how ROS production leads to mtDNA damage.

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11-04. Mitochondrial respiratory control and oxygen dependence of ROS production in aging endothelial cells.

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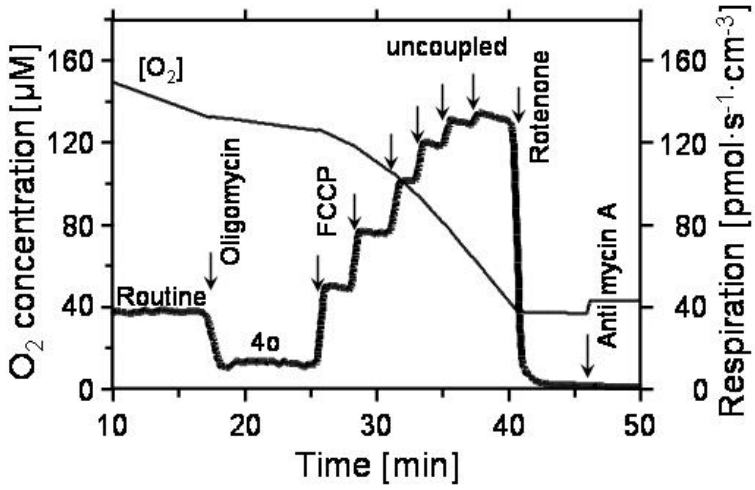
Oxidative stress is a major determinant of cellular aging. Mitochondria are frequently considered as the major source of ROS production, but the relative contributions and oxygen dependence of mitochondrial and non-mitochondrial ROS remain controversial. In contrast to low oxygen levels in the mitochondrial microenvironment of many tissues, isolated mitochondria are routinely exposed to air-level oxygen concentrations [1,2]. Using human umbilical vein endothelial cells (HUVEC) as an in vitro model of cellular aging [3], the present study was aimed at the evaluation of mitochondrial integrity and quantitative assessment of ROS production as a function of oxygen concentration.

Cellular oxygen consumption was measured by high-resolution respirometry with the OROBOROS Oxygraph-2k [1,4]. In contrast to results obtained in senescent fibroblasts [5], mitochondrial respiratory capacity and coupling of oxidative phosphorylation were preserved in senescent HUVEC, as shown by oligomycin inhibition and FCCP stimulation in intact cells (Fig. 1), and by substrate/inhibitor titrations after permeabilization with digitonin. Respiration through cytochrome *c* oxidase constituted a hyperbolic high-affinity component of oxygen kinetics in the low-oxygen range (up to 10 µM), independent of age. Enhanced oxidative stress was observed in aged endothelial cells by staining with the oxidant sensitive dye dihydroethidium. In direct agreement, a linear component of oxygen kinetics was pronounced in senescent cells suspended in culture medium at increasing oxygen levels. Hypoxia-induced down-regulation of ATP turnover could be excluded by performing oxygen kinetics of not only coupled, but also uncoupled respiration, which also showed linear oxygen dependence in the high oxygen range in senescent HUVEC. This indicates



predominantly non-mitochondrial sources of ROS production. While the quantitative importance of several non-mitochondrial oxidases was excluded by application of specific inhibitors, the enzyme family of NADPH oxidases is being studied as one of the potential sources of ROS in senescent HUVEC.

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11-05. Mitochondrial protein modification and degradation by the Lon protease in rat liver and heart.

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Mitochondrial matrix proteins are sensitive to oxidative inactivation, and oxidized proteins are known to accumulate during ageing. The Lon protease is believed to play an important role in the degradation of oxidized matrix proteins such as oxidized aconitase. We previously reported that an age-related accumulation of altered (i. e. oxidized and glycoxidized) proteins occurs in the liver matrix of rats and that the ATP-stimulated proteolytic activity, referred as to Lon-like protease activity, decreases considerably in 27 month-old rats, whereas no concomitant changes in the levels of Lon protein expression occur in the liver [1]. This decline is associated with a decrease in the activity of aconitase, an essential Krebs'cycle enzyme.

Contrary to what we observed in the liver, the ATP-stimulated protease activity was found to remain constant in the heart mitochondrial matrix during ageing, and the levels of expression of the Lon protease increased in the older animals in comparison with the younger ones. Although the ATP-stimulated protease activity remained practically the same in the heart of older animals as in younger ones, a decrease in the level of aconitase activity was still observed [2]. These results indicate that matrix proteins such as the critical enzymes aconitase and Lon protease are inactivated with ageing and that the effects of ageing vary from one organ to another.

Furthermore, analysis of glycoxidized protein pattern, monitored by western blotting with anti-carboxymethyllysine antibodies after a two-dimensional electrophoresis, revealed that only a restricted set of proteins were glycoxidized with age in rat liver mitochondrial matrix. Using LC-MS/MS analysis, we have identified proteins that are implicated in the urea cycle, especially glutamate dehydrogenase. *In vitro* assay of the glutamate dehydrogenase activity incubated with the glycating reagent methylglyoxal showed both an inactivation of the enzyme and alteration of its allosteric properties. These results suggest a role for glycoxidative modifications in the age-related dysfunction of mitochondria and indicate that carboxymethylated glutamate dehydrogenase could be used as a bio-marker of cellular aging.

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11-06. The mitochondrial DNA content of preimplantation embryos can be altered by environmental stress.

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Recent experiments have shown that the preimplantation period of mouse development may be particularly prone to environmental stress with adverse long-term consequences, however the molecular mechanisms of this process are unknown [1]. mtDNA provides a plausible vector for transmission of very early environmental stress since damaged mtDNA may remain dormant for many generations until some additional cellular stress increases the level of damaged mtDNA and causes cellular dysfunction (2). However the pre-implantation phase was thought to be relatively immune to environmental stress since mtDNA replication was not thought to occur until after D6.5 of development. We have recently shown that mtDNA can be manipulated in vitro during pre-implantation development [3] and we show here that similar changes can be induced by an abnormal maternal diet which is known to have adverse long-term effects on offspring. 5 week old female MF1 mice were fed either a control C (18 % casein), or a LPD (9 % casein) for 10 days prior to mating. After mating as judged by the appearance of a vaginal plug, pregnant females were fed either C (n=18), or LPD (n=18) for a further 3.5 days. Females were killed by cervical dislocation at day 1 and day 3.5 and germinal vesical stage oocytes (GV), fertilised eggs (FE) and blastocyst stage embryos were isolated and snap frozen and stored at –80 °C prior to analyses of mtDNA content. For *in-vitro* studies staged embryos, GV, unfertilized eggs (UF), FE 2,4,8 cell and blastocyst (B) stage embryos were isolated from 5 week old female MF1 mice control fed animals, and transferred to KSOM media and cultured for 4 h in in the absence or presence of 25 μm azodo deoxycytidine (AZT) an specific inhibitor of mtDNA polymerase. Both *in vivo* and *in vitro* treatments produced embryos with reduced mtDNA content, indicating a short window of mtDNA synthesis and degradation immediately after fertilisation. These findings have important repercussions for all embryo based technologies.

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11-07. Mitochondrial DNA replication and mitochondrial activity in mammals.

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Mitochondria are the main intracellular source and immediate target of reactive oxygen species (ROS) which are continually generated as byproducts of aerobic metabolism in mammalian cells. Cumulative damage to mtDNA is implicated in the aging process and in the progression of such common diseases as diabetes, cancer and heart failure.

It has been observed that increase in the mtDNA copy number is associated with elevated oxidative stress in the aging tissues like brain and skeletal muscle. MtDNA copy number is increased at the late passage of diploid human fibroblasts. The increase in mtDNA with aging is proposed as a feedback response that could compensate the accumulation of defective mitochondria and is also associated to increased intracellular levels of ROS [1]. We studied the effect of ROS on mtDNA replication using two-dimensional agarose gel electrophoresis of replication intermediates (RIs) [2].

We analyzed the pattern of RIs with DNA digestions which allow the detection of intact bubble arcs in mouse mtDNA in isolated mitochondria from mouse liver and in primary mouse fibroblasts treated with different mitochondrial drugs. We found that increased ROS production by rotenone and antimycin A treatment directly affects mitochondrial replication activity, decreasing the percentage of intact bubbles while DNP increases mtDNA replication. These data suggest that interfering with mitochondrial electron transfer, altering mitochondrial membrane potential and modifying ROS production affect directly mtDNA replication. Since our data suggest an inverse relationship between mitochondrial activity and mtDNA replication we investigated if there is any effect at structural levels. We used confocal microscopy to detect mtDNA with anti-mtSSB (mitochondrial single strand binding proteins) and mitochondrial electron transfer complexes with anti-COX IV. With this approach we found that mitochondria show a different localization for DNA and the respiratory chain complexes suggesting a specific sub-mitochondrial compartmentalization whose organization may be crucial for mitochondrial physiology.

Supported by F.I.R.C.

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11-08. Mitochondrial protection using Ginkgo biloba extract (*EGb 761*) *in vitro* and *in vivo* using several animal models.

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During aging mitochondrial abnormalities occur assisting the development of neurodegenerative diseases including Alzheimer's disease (AD). Ginkgo biloba extract (EGb 761) is known to aid in the prevention of age-associated mitochondrial changes [1]. A decrease in mitochondrial membrane potential is a key element indicating mitochondrial dysfunction. Previous studies in our labs showed the protective effect of EGb 761 on mitochondrial membrane potential *in vitro* using PC 12 cells [2]. In this study we extended our *in vitro* results by using brain dissociated cells prepared from NMRI mice at the age of 3 months. The EGb 761 extract showed significant protection on mitochondrial membrane potential in concentrations as low as 0.1 mg/ml. Moreover we investigated the effect of EGb 761 *in vivo* using transgenic (tg) mice bearing mutant amyloid precursor protein (Swedish and London mutation) and non-tg littermate control mice at the age of 3 months. TgAPP mice exhibit onset of amyloid beta plaques at an age of 6 months, but intracellular amyloid beta load is already seen at an age of 3 months. The treated mice received 100 mg/kg EGb 761 in 0.9% NaCl solution while the untreated controls received 0.9% NaCl solution only per os daily for 14 consecutive days. The mitochondrial membrane potential was measured in dissociated brain cells prepared from treated and untreated control groups after initiating mitochondrial damage by sodium nitroprusside (SNP), hydrogen peroxide (H_2O_2) and amyloid beta peptide. EGb 761 showed a protective effect on mitochondrial membrane potential in both animal models in comparison to the untreated controls. In conclusion our results indicate the protective effects of EGb 761 on the mitochondria both *in vitro* and *in vivo* using several different animal models.

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11-09. Maternal diet 'programs' late onset mitochondrial dysfunction in renal tissue of male offspring.

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Reduced intrauterine protein availability followed by adequate nutrition during weaning and adulthood has been shown to influence the adult health of the offspring [1]. We have developed a model of protein deprivation and catch up growth, which demonstrates reduced longevity [2] and increased rates of telomere attrition [1]. We hypothesise that dysfunctional mitochondria could be a contributory factor in these observations [3]. The activities of key mitochondrial enzymes together with ubiquinone (CoQ9 & CoQ10) concentrations were measured in kidney at various ages after birth.

Wistar rats were fed a control diet (C) 20 % protein during pregnancy and lactation, after weaning the offspring were fed a chow diet, until tissue collection, or a recuperated diet (R) 8 % protein during pregnancy, 20 % protein during lactation and a chow diet until tissue collection. Citrate synthase, mitochondrial enzyme complex I to IV activities and ubiquinone were measured in the cortex and medulla regions of the kidney at 3 months and 12 months, whole kidney was analysed for the 22 day samples. Results were normalised using protein content of the sample.

Our results show statistically significant ($P<0.05$) mitochondrial enzyme activity decreases in the 12 month old male offspring in both cortex and medulla regions of the kidney, and a significant reduction in CoQ9 in the cortex, both these alterations precede significant differences in telomere length [2]. Results at earlier stages (22 days/3 months) showed negligible effects of maternal diet.

We conclude that intrauterine protein restriction and catch up growth causes mitochondrial abnormalities in adult offspring. This could contribute to deficiencies in energy production or increases in oxidative stress leading to telomere attrition and premature death.

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11-10. Modulation of mitochondrial function by the IGF signalling pathway in human cells.

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The free radical theory of aging predicts that oxygen radicals and highly active compounds derived therefrom play a major role in aging processes by causing macromolecular damage to nucleic acids, proteins and lipids. This theory implies that an increased metabolic rate would lead to increased mitochondrial activity; increased production of reactive oxygen species caused by this change would speed up the aging process. This assumption is based on a large body of correlative evidence. However, experimental proof is still missing. From experiments with lower eukaryotes it is known that the metabolic rate and also the rate of aging are tightly controlled by the IGF / insulin signal transduction pathway [1]. Further, it could be shown that Akt accumulates in mitochondria after stimulation with IGF-I or insulin [2]. Together, these findings would imply that mitochondrial activity might be influenced by insulin/IGF signalling, a

hypothesis that has not been tested experimentally so far. Therefore we have established an experimental system to determine the influence of IGF-I dependent signalling on mitochondrial function, by high-resolution respirometry (OROBOROS Oxygraph-2k). We used DU145 prostate cancer cells, where the IGF signal transduction pathway is intact, to address the influence of IGF receptor activation on mitochondrial function. These experiments revealed that indeed mitochondrial function is regulated by IGF signalling, and this finding is independent of IGF effects on cell cycle progression. Collectively these data establish a regulatory cross-talk between these major pathways implicated in controlling the rate of aging.

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11-11. Mitochondrial dysfunction in brain neurodegenerative disorders: ageing and chronic cerebral hypoperfusion.

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Decreases in mitochondrial respiratory chain complex activities have been implicated in neurodegenerative disorders such as Alzheimer`s disease. There are two basic factors for the development of neurodegeneration – advanced aging and chronic cerebral hypoperfusion [1]. The objective of our study was to evaluate brain energy metabolism in the aged (15-16 months old) Wistar rat model of chronic cerebral hypoperfusion accomplished by the occlusion of the brachiocephalic trunk and left common carotid artery (three-vessel occlusion, 3-VO) [2]. The forward rate constant of creatine kinase (*k*for) was studied in vivo by saturation transfer ^{31}P magnetic resonance spectroscopy on SISCO 4.7 T imaging spectrometer [3]. Oxygen consumption of isolated brain mitochondria was measured with a Gilson 5/6 oxygraph using a Clark oxygen electrode and sodium glutamate as substrate. Dynamic measurements of ^{31}P MRS saturation transfer showed statistically significant decrease in forward rate constant of creatine kinase measured 10 weeks after 3-VO as compared to the control group of aged rats (TAB 1). There were no significant changes in basal (QO2S4) and ADP-stimulated (QO2S3) mitochondrial oxygen uptake, however, calculated ATP production (OPR) and coefficient of oxidative phosphorylation (ADP:O) were significantly decreased 10 weeks after 3-VO. Our experiments revealed that significant reduction of in vivo measured forward rate constant of creatine kinase correlates with the significant decrease of the coefficient (ADP:O) and the rate (OPR) of the oxidative phosphorylation measured in isolated brain mitochondria. Thus, ^{31}P MRS technique can be used as preventive noninvasive measure for the detection of mitochondrial bioenergetics in the aged and hypoperfused brain.

Table 1. Forward rate constant, *k*for [PCr=>ATP] and parameters of oxidative phosphorylation before (control) and 10 weeks after three-vessel occlusion (3-VO) in the brain of aged rats. **P*<0.05, ***P*<0.01.

Parameter	Control (*n*=8)	3-VO (*n*=6)
K_{for} [s^{-1}]	0.30 ± 0.04	0.20 ± 0.01**
QO2S3 [nAtO.mg $prot^{-1}.min^{-1}$]	52.97 ± 2.40	51.35 ± 1.19
QO2S4 [nAtO.mg $prot^{-1}.min^{-1}$]	16.72 ± 0.85	17.13 ± 0.91
RCI [S3 S4]	3.32 ± 0.16	3.03 ± 0.15
ADP:O [nmol ADP.$nAtO^{-1}$]	2.51 ± 0.08	2.21 ± 0.03**
OPR [nmol ATP.mg $prot^{-1}.min^{-1}$]	132.19 ± 6.48	113.35 ± 4.14*

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11-12. A role for mitochondria in infrared A radiation-induced intracellular signalling.

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Infrared-A-radiation (IRA; 760 – 1440 nm) is the major component of natural sunlight and significantly contributes to extrinsic skin ageing. A major hallmark of extrinsic skin ageing is a loss of collagen fibres which results from overexpression of matrixmetalloproteinase-1 (MMP-1). We have previously shown that IRA radiation is a potent inductor of MMP-1 expression in human dermal fibroblasts. Its effect was shown to be mediated by IRA-induced activation of ERK1/2. Since MAPKinase signalling can be induced by oxidative stress, we aimed to identify whether IRA leads to the formation of ROS, and if yes, whether this would be of functional relevance for IRA-induced gene expression.

Experiments utilizing Mitosox, a dye specific for mitochondrial superoxide radical anions revealed that IRA at physiologically relevant doses leads to an at least threefold increase in $O2^{\cdot}$-. We have found that IRA induced MMP-1 expression in primary human fibroblasts can be abrogated by mitochondrial respiratory chain inhibitors. This effect was specific since UV-A or UV-B induced MMP-1 expression was not inhibited. In addition we were able to demonstrate, that mitochondria depleted (rho^{-}) cells do not show IRA-induced MMP-1 upregulation in contrast to corresponding rho^{+} cells. UV-A and UV-B induced MMP-1 induction was not altered by absence of mitochondria.

Taken together our studies for the first time demonstrate that IRA induced gene expression involves retrograde mitochondrial signalling, which seems to be mediated by superoxide radical anions leaking from the respiratory chain.

11-13A. Increased sensitivity of MPT-pore opening in old rat heart.

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Alterations in the sensitivity of mitochondrial permeability transition pore (MPTP) opening are important for the prevention of aging-induced chronic oxidative stress. Two indices of MPTP opening were used in this study: (i) the swelling-induced decrease in optical density (λ=520 nm) of isolated mitochondria and (ii) the release of a mitochondrial factor (MF, λ=230-260 nm), which is the marker of MPTP-opening. Some inducers of MPTP-opening $CaCl_2$ (10^{-7}-10^{-4} M), phenylarsine oxide (PAO, 10^{-8}-10^{-4} M) and t-butyl-hydroperoxide (t-BuOOH, 10^{-9}-10^{-3} M) caused more swelling of mitochondria isolated from hearts of old than from adult rats. Mitochondria from old rat hearts released some MF in the absence of MPTP inducers. The release of MF by $CaCl_2$ (10^{-7} M) and PAO (10^{-9} M) was significantly higher from mitochondria isolated from old than from those from adult rat hearts. Thus mitochondria isolated from old rat hearts possess an increased sensitivity towards $CaCl_2$, PAO and t-BuOOH. It was accompanied by an increase in hydrogen peroxide (23.75±3.93 pmol/mg protein) and hydroxyl radical production [3.69±0.81 $\Delta E \cdot 10^2$/(30 min·mg protein)] in old in comparison to adult rat hearts [5.56±0.47 pmol/mg protein and 1.18±0.22 $\Delta E \cdot 10^2$/(30 min·mg protein),

respectively] and by an increase m-RNA of proapoptotic Bax protein. The classical inhibitor of MPTP opening cyclosporin A (10^{-5} M) inhibited mitochondrial swelling and MF release completely, whereas this inhibitory effect was incomplete in mitochondria from old rat hearts. The antioxidant melatonin (10^{-5} M) prevented t-BuOOH-induced mitochondrial swelling completely in adult and in old rat heart mitochondria. Thus mitochondria from old hearts are more sensitive to inducers of MPTP opening as a result of aging-induced chronic oxidative stress. These results may be useful for treatment of mitochondrial dysfunction caused by aging.

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