MiP*summer* 2009

3rd MiP*summer* School on **Mitochondrial Physiology**

June 17-23, 2009 **Baton Rouge, Louisiana, USA**

Final Program

Local Organizers	**Steve Hand** (Baton Rouge, LA) **Michael Menze** (Baton Rouge, LA)
Scientific board	**Erich Gnaiger** (Innsbruck, AT) **Vilma Borutaite** (Kaunas, LT) **Guy C Brown** (Cambridge, UK) **Steven C Hand** (Baton Rouge, US) **Charles Hoppel** (Cleveland, US) **Rodrigue Rossignol** (Bordeaux, FR) **Eiji Takahasi** (Yamagata, JP)

MiP*summer* Partners:

MiP*summer* Cooperating Institutions

Austria	Medical University of Innsbruck, Department of General and Transplant Surgery, D. Swarovski Research Laboratory, Innsbruck, Austria. Contact: *Erich Gnaiger*
Belgium	Equine Clinic, Faculty of Veterinary Medicine, University of Liège, Sart Tilman, Liège, Belgium. Contact: *Dominique-Marie Votion*
France	Université Victor Segalen Bordeaux 2, INSERM U688, Bordeaux. Contact: *Rodrigue Rossignol*
Germany	St.-Georgen-Platz 6, MiPNet OROBOROS INSTRUMENTS, D-93047 Regensburg, Regensburg, Germany. Contact: *Kathrin Renner-Sattler*
Lithuania	Institute for Biomedical Research, Kaunas University of Medicine, Contact: *Vilma Borutaite*
USA	Case Western Reserve University School of Medicine, Department of Pharmacology, Center for Mitochondrial Diseases, Cleveland, Ohio. Contact: *Charles Hoppel*
	Louisiana State University, Department of Biological Sciences, Baton Rouge. Contacts: *Steven C. Hand, Michael A. Menze*
	Louisiana State University System, Pennington Biomedical Research Center, Baton Rouge. Contact: *Eric Ravussin*

Program Overview

Wednesday, June 17

Afternoon	Registration and check-in at Lod Cook Hotel (LSU Campus).
6:00-9:00 pm	Casual wine and cheese welcome reception at Lod Cook Alumni Center, Sheldon Conference Room, first floor (in front of Lod Cook Hotel).

Thursday, June 18

6:30-08:30	Breakfast.
8:50-12:30	Morning lectures: A101 Life Sciences Bldg. (Lecture Hall), with coffee break.
12:30-2:00 pm	Lunch break.
2:00-7:00 pm	Afternoon lectures, with coffee break; poster flash presentations, poster session, discussions. A101 Life Sciences Bldg. (Lecture Hall and lobby).
9:00 pm	MiP*board* Meeting.

Friday, June 19

6:30-08:30	Breakfast.
8:45	Depart by Bus from Lod Cook Hotel.
9:00-12:00	**MiP*summer* Excursion to Alligator Bayou**.
12:30-2:00 pm	Back on campus, lunch break.
2:00-6:00 pm	Afternoon lectures, with coffee break; poster flash presentations, poster session, discussion. A101 Life Sciences Bldg. (Lecture Hall and lobby).

8:30-10:00 pm	Discussion, Special Interest Groups.
Saturday, June 20	
6:30-08:30	Breakfast.
8:30-12:00	Morning lectures: A101 Life Sciences Bldg. (Lecture Hall), with coffee break.
12:00-1:30 pm	Lunch break.
1:30-3:45 pm	Afternoon lectures, with coffee break. A101 Life Sciences Bldg. (Lecture Hall).
4:30 pm	Bus departs Lod Cook Hotel for **MiP*summer* Excursion: Mississippi R. Levee Walk and Talk** (downtown Baton Rouge).
5:00-7:45 pm	Refreshments and Dinner (on your own, downtown; suggestions provided for restaurants).
8:00 pm	Bus departs promptly from downtown in front of The Shaw Center for the Arts, returning to Lod Cook Hotel.
9:00-10:00 pm	Discussion, Special Interest Groups.
Sunday, June 21	
6:30-08:30	Breakfast.
8:30-12:00	Morning lectures: A101 Life Sciences Bldg. (Lecture Hall), with coffee break.
12:00-2:00 pm	Lunch break.
2:00-5:45 pm	Afternoon lectures/demonstrations, with coffee break; poster flash presentations, discussion. A101 Life Sciences Bldg. (Lecture Hall).
8:30 pm	**After dinner MiP*summer* Lecture** by **Charles Hoppel** (Case Western Reserve Medical School, USA). "Inner mitochondrial membrane and outer space". A101 Life Sciences Bldg. (Lecture Hall).
Monday, June 22	
6:30-08:30	Breakfast.
8:30-12:30	Morning lectures: A101 Life Sciences Bldg. (Lecture Hall), with coffee break.
12:30-2:00 pm	Lunch break.
2:00-5:00 pm	10 min walk from Lod Cook Hotel to **MiP*summer* Excursion: Canoeing/Kayaking site on University Lake**; also Walk and Talk along University Lake.
5:30-6:15 pm	Poster flash presentations, discussion.
6:15-6:30 pm	Questions and suggestions by participants; MiP*summer* evaluation.
8:00-12:00 pm	**MiPsummer 2009 Dinner (buffet style) and Party.** Lod Cook Alumni Center, Cook Conference Room, first floor (in front of Lod Cook Hotel).
Tuesday, June 23	Morning departures to airport.

See you at UMDF-MiP, Washington D.C. – June 24-27, 2009
http://www.umdf.org/site/pp.aspx?c=piKYL1PHLtF&b=4864979

MiP*summer* 2009

The program of the 3rd MiP*summer* School on "Mitochondrial Physiology" provides a balance between introductions into the basic concepts, advanced methodological and scientific approaches, and specific applications, with a focus on mitochondrial functions linked to the general theme of MiP with basic scientific and biomedical perspectives.

1 - Thursday, June 18

8:50 Summer school strategies and the program – the MiP perspective. Steve Hand, Michael Menze.

Mitochondrial respiratory capacity and respiratory control.

Co-Chairs: Vilma Borutaite (LT), Steve Hand (US)

1-01L 9:00-10:30 **Erich Gnaiger** (Innsbruck, AT) Mitochondrial Respiratory Physiology: OXPHOS, LEAK, ETS, and seven instigations to change.

10:30-11:00 Coffee break.

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

Lunch.

1-03L 2:00-3:30 pm **Erich Gnaiger** (Innsbruck, AT) The design of protocols on substrate control and coupling control of OXPHOS capacity: Mitochondrial diversity from mouse to man.

3:30-4:00 pm Coffee Break.

Flash presentations of posters (10 min + 5 min discussion)*:*

1-04P 4:00-4:15 pm **Zuzana Sumbalová** (Bratislava, SK) Postnatal changes in rat brain energy metabolism and mitochondrial antioxidants content – a preliminary study.

1-05P 4:15-4:30 pm **María Isabel Hernández-Alvarez** (Barcelona, ES) Measurement of mitochondria oxidative capacity in rat hepatoma FAO cells.

1-06P 4:30-4:45 pm **Fredrik Sjövall** (Lund, SE) Mitochondrial respiration in human platelets.

1-07P 4:45-5:00 pm **Jessica Segalés** (Barcelona, ES) Measurement of oxygen consumption in C2C12 myotubes.

5:00-7:00 pm **Poster session**

Dinner.

9:00 pm MiP*board* Meeting: MiP2009 and beyond (Life Sciences Building Annex, A610).

2 - Friday, June 19

8:30-8:45 Depart by Bus from Lod Cook Hotel.

9:00-12:00 **MiP*summer* Excursion to Alligator Bayou.**

12:30-2:00 pm Back on campus, lunch break.

Membrane potential and protein import

Co-Chairs: Erich Gnaiger (AT), Rodrigue Rossignol (FR)

2-01L 2:00-3:30 pm **Vilma Borutaite** (Kaunas, LT) Mitochondrial membrane potential: what can it tell about health of cells?

2-02L 3:30-4:30 pm **Steve Hand** (Baton Rouge, US) Protein targeting and import into mitochondria: The process, its conserved nature, and potential for mitochondrial stabilization.

4:30-5:00 pm Coffee break.

Flash presentations of posters (10 min + 5 min discussion)***:***

2-03P 5:00-5:15 pm **Vigdis Aas** (Oslo, NO) Chronic hyperglycemia reduces myotube mitochondrial function without changing mitochondrial gene expression or mitochondrial content.

2-04P 5:15-5:30 pm **Nicolas Pichaud** **(**Rimouski, CA) Thermal sensitivity of the mitochondrial metabolism in two distinct mitotypes of *Drosophila simulans*.

2-05P 5:30-5:45 pm **Douglas Crawford** (Miami, US) The importance of individual variation: NDUF expression.

2-06P 5:45-6:00 pm **Ellen Friday** (Shreveport, US) Mitochondrial Complex I regulates ammoniagenesis in pig kidney proximal tubule-like LLC-PK1 cells.

Dinner.

8:30-10:00 pm **Discussion, Special Interest Groups.**

3 - Saturday, June 20

Mitochondrial Structure and Molecular Physiology

Co-Chairs: Charles Hoppel (US), Kathrin Renner-Sattler (DE)

3-01L 8:30-10:00 **Rodrigue Rossignol** (Bordeaux, FR) Ultrastructure of the mitochondrion and its bearing on function and bioenergetics.

10:00-10:30 Coffee

3-02L 10:30-12:00 **Steve Hand** (Baton Rouge, US) Mitochondria and apoptosis: Energy-limited states and signaling for cell death.

12:00-1:30 Lunch

3-03L 1:30-3:00 pm **Vilma Borutaite** (Kaunas, LT) Nitric oxide, mitochondrial permeability transition and cell death.

3-04L 3:00-3:45 pm **Michael Menze** (Baton Rouge, LA) Mitochondrial calcium import, signaling, and ER cross-talk.

4:30 pm Bus departs Lod Cook Hotel for **MiPsummer Excursion: Mississippi River Levee Walk and Talk** [downtown Baton Rouge; The Shaw Center for the Arts (100 Lafayette St.), drop off point].

5:00-7:45 pm Refreshments and Dinner (on your own, downtown; suggestions for restaurants will be provided), Levee walk.

8:00 pm Bus departs promptly from downtown in front of The Shaw Center for the Arts (100 Lafayette St.), returning to Lod Cook Hotel.

9:00-10:00 pm **Discussion, Special Interest Groups.**

4 - Sunday, June 21

Heart and Muscle Mitochondria; Clinical Perspectives

Co-Chairs: Vilma Borutaite (LT), Michael Menze (US)

4-01L 8:30-9:45 **Charles Hoppel** (Cleveland, US) Integrated mitochondrial function and the clinical utility of polarographic analysis.

4-02L 9:45-10:45 **Dominique-Marie Votion** (Liège, BE) High-resolution respirometry to assess muscle mitochondrial respiratory function in horses: potential applications in sport and myopathic horses.

10:45-11:15 Coffee break.

4-03L 11:15-12:00 **Charles Hoppel** (Cleveland, US) Ischemia-reperfusion injury in heart mitochondria.

Lunch.

Practical Approaches and Instrumentation in Mitochondrial Physiology

Co-Chairs: Rodrigue Rossignol (FR), Dominique Votion (BE)

4-04L 2:00-2:45 pm **Dominique-Marie Votion** (Liège, BE), **Dominik Pesta** (Innsbruck, AT) and **Erich Gnaiger** (Innsbruck, AT) Preparation and application of permeabilized muscle fibers for respiration studies.

4-05D 2:45-4:00 pm **Kathrin Renner-Sattler** (Regensburg, DE) and **Dominik Pesta** (Innsbruck, AT) High-resolution respirometry of permeabilized fibers with the OROBOROS Oxygraph-2k.

4:00-4:30 pm Coffee break.

4-06L 4:30-5:45 pm **Min Wu** (Billerica, US) Real-time monitoring of cellular bioenergetics in a microplate using the Extracellular Flux Analyzer.

Dinner.

4-07S 8:30 pm **After dinner MiP*summer* Special Lecture** by **Charles Hoppel** (Case Western Reserve Medical School, USA). "Inner mitochondrial membrane and outer space".

5 - Monday, June 22

Metabolic Remodelling: Impacts of Calorie Restriction, Fitness, and Warburg/Crabtree Effect

Co-Chairs: Steve Hand (US), Charles Hoppel (US)

5-01L 8:30-10:00 **Eric Ravussin** (Baton Rouge, USA) Calorie restriction and mitochondrial biogenesis.

5-02L 10:00-10:30 **Erich Gnaiger** (Innsbruck, AT) Fewer mitochondria - *mitochondrial 'fever'*: Mitochondrial physiology and declining OXPHOS capacity in sedentary and dietary life styles.

10:30-11:00 Coffee

5-03L 11:00-12:30 **Rodrigue Rossignol** (Bordeaux, FR) Mitochondria: From bioenergetics to metabolic regulation of carcinogenesis.

Lunch.

2:00-5:00 pm 'Walk and Talk' from Lod Cook Hotel to **MiP*summer* Excursion: Canoeing on University Lake** (map provided). Walking time is approx. 30 min to the park where canoes will be available.

Flash presentations of posters (10 min + 5 min discussion)***:***

5-04P 5:30-5:45 pm **Yuvraj Patil** (Baton Rouge, US) Pyruvate dehydrogenase complex in *Artemia franciscana* embryos: A possible contributor to metabolic depression during diapause.

5-05P 5:45-6:00 pm **Sheila R. Costford** (Baton Rouge, US) Skeletal muscle NAMPT is induced by exercise and correlates with mitochondrial content.

5-06P 6:00-6:15 pm **Sudip Bajpeyi** (Baton Rouge, US) No relationship between skeletal muscle mitochondrial function and insulin resistance in type 2 diabetes.

6:15-6:30 pm Comments and evaluation by participants regarding MiP*summer* School 2009.

8:00-12:00 pm **MiPsummer 2009 Dinner (buffet style; wine and beer service) and Party.** Lod Cook Alumni Center, Cook Conference Room, first floor (in front of Lod Cook Hotel).

MiP*summer* 2009 Abstracts

Day 1: Mitochondrial respiratory capacity and respiratory control.

1-01L Mitochondrial Respiratory Physiology: OXPHOS, LEAK, ETS, and seven instigations to change.

Erich Gnaiger

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Mitochondrial respiratory physiology continues a tradition of quantitative bioenergetics with a focus on the control of oxidative phosphorylation (OXPHOS) in cells and tissues. Form the perspective of mitochondrial physiology (MiP), seven changes are proposed to introduce fundamental principles of mitochondrial respiratory physiology, extending bioenergetics to an interdisciplinary approach for understanding mitochondrial function *in vivo* in states of health and disease.

Consideration of units (Inst. 1) may appear to be trivial, but standardization helps in practice.[1,2] Changes in protocols (Inst. 2 and 3) have far-reaching consequences for understanding mitochondrial respiratory control, and for building a comparative data base on mitochondrial function and dysfunction in MiP.[3] Clarification of terminology (Inst. 4 and 5) lags behind conceptual developments, but is fundamentally important in teaching and interdisciplinary scientific communication. Some terms may become established in science to the extent of fixation of an erroneous concept which requires correction (Inst. 6). Measurement of the dynamics and control of respiration requires not only high accuracy of determination of oxygen consumption, but needs fundamental consideration on expressing respiratory fluxes (per mg mitochondrial protein or mg tissue), flows (per cell) or rates (normalization).

How much time would you spend on reading a paper that beings with: "DPN-linked oxidation was measured in bioblasts"? Resistance to change the terminology from bioblasts (chondriosomes, chromidia, chondriokonts, eclectosomes, histomeres, microsomes, plastosomes, polioplamsa, vibrioden)[4] to mitochondria, or from DPN to NADH is unknown today, but comparable changes are about to take place continuously in science, including MiP. Transitions in nomenclature reflect conceptual developments and sometimes indicate a paradigm shift.

Inst. 1. µg.atoms of oxygen·min^{-1}: Expressing mitochondrial oxygen consumption in terms of 'natoms O' is conceptually based on the fact that oxidation of reducing equivalents (NADH, $FADH_2$) is stoichiometrically related to two electrons (equivalent to 0.5 O_2). In the Q-cycle, however, electron transport is switched from a two-electron to a one-electron transfer mechanism through Complex III (CIII) and cytochrome *c*. In CIV (cytochrome *c* oxidase), finally, molecular oxygen, O_2, is reduced to water. Mitochondrial respiration, cell respiration, tissue respiration, and respiration of an entire organism (e.g. $V_{O2,max}$) should be expressed in identical SI units: mol O_2·s^{-1} (pmol, nmol etc.).

- Convert respiration (395 µg.atoms.min^{-1}) to SI units [nmol O_2·s^{-1}].

Inst. 2. Measured at 25 °C: Manometric techniques (Warburg apparatus) require good temperature control and were used extensively at 37 °C for respirometry with mammalian cells up to the middle of the last century, and later occasionally with isolated mitochondria. Spectrophotometers and Clark-type oxygraphs had comparatively poor or no temperature control and thus measurements were performed at room temperature. This conceptually trivial (technically less simple) reason is largely forgotten but a convention is continued, and mammalian mitochondrial respiration is still measured at 25 °C or 30 °C in 'modern' bioenergetics. MiP requires functional measurements at physiological temperature. A Q_{10} of 2 (multiplication factor for an increase by 10 °C) is frequently assumed, but can be considered as an approximation only.

- Convert 395 µg.atoms.min^{-1} to SI units and 37 °C, assuming a Q_{10} of 2 (the corresponding multiplication factor for a temperature change from 25 °C to 37 °C is 2.30). What is the multiplication factor for measurements at 30 °C and a Q_{10} of 2?.

Inst. 3. Pyruvate+Malate or Succinate+Rotenone, PM or S(Rot): To analyze site-specific H^+:e and ADP:O ratios, segments of the electron transport system are separated into linear pathways by using either NADH-linked substrates (PM or glutamate+malate, GM) or the classical S(Rot) combination. Convergent electron flow to the Q-junction from PM+S or GM+S (PMS, GMS, or PMGS) simultaneously through CI and CII (convergent CI+II e-input), however, yields an increased respiratory capacity.[3] Convergent CI+II e-input corresponds to full operation of the tricarboxylic acid (TCA) cycle and provides the relevant basis for quantifying enzymatic thresholds and excess capacities of individual steps of OXPHOS. Multiple substrate-uncoupler-inhibitor titration protocols and advanced OXPHOS flux control analysis extend the diagnostic potential of mitochondrial physiology in health and disease.

- Convert 395 µg.atoms.min^{-1} measured with PM to SI units, 37 °C and to physiological capacity, considering that respiratory capacit through CI is limited to 0.64 of convergent CI+II e-input.

Inst. 4. Electron transport chain: Electron transport 'chain' (ETC) will be one of the most frequently used misnomers at MiP*summer* 2009 and other mitochondrial meetings in 2009. This misnomer of the year should be changed to electron transport system (ETS), considering that – very different from the arrangement of sequential links in a chain – electron flow converges at the Q-junction from various branches, including CI and CII from the TCA cycle, glycerophosphate dehydrogenase from glycolysis, and electron transferring flavoprotein from β-oxidation of fatty acids.[5]

Inst. 5. State 2, 3 and 4: The terminology on respiratory states was introduced for describing sequential steps in a protocol: State 2 is induced by addition of ADP to mitochondria incubated without external substrates to assure complete oxidation of endogenous substrates; State 3 is active respiration after addition of substrates; and State 4 is resting respiration after ADP is phosphorylated to a maximum ATP/ADP ratio.[6] Later this termonology became confused, and State 2 with added substrate but without ADP was considered to be comparable to State 4.[7] Clarification is obtained by changing (*a*) from technical jargon to a terminology with functional explanations of coupling states:[3] LEAK, *L*: non-coupled resting respiration, compensating for proton leak, slip and cation cycling in the absence of ADP or inorganic phosphate; OXPHOS, *P*: coupled active respiration, in the presence of ADP and inorganic substrate; ETS, *E*: uncoupled maximum respiration, in the presence of optimum concentrations of uncoupler; ROX: residual oxygen consumption, in the absence of substrates (the final phase of the original State 2) or after inhibition of electron transport from substrates to oxygen. (*b*) Coupling states require complementary definition of substrate(s) supplied, and sufficient information must be provided on inhibitors, uncouplers, ions and any effectors of mitochondrial respiration. Mitochondrial coupling states *L*, *P* and *E* should be corrected for ROX. The term State 3u (ETS, State *E*) might suggest that the uncoupled state (e.g. in intact cells) provides an alternative for the measurement of OXPHOS capacity (*P*; State 3). ETS capacity, however, can be equal to or much higher than OXPHOS capacity, depending on the degree of limitation of OXPHOS by the capacity of the phosphorylation system. This limitation is expressed by the *P/E* coupling control ratio. *P/E* is close to unity in mouse skeletal muscle, where the phosphorylation system does not limit oxygen flux in State *P*. In human skeletal muscle, however, the low *P/E* ratio of 0.7 – 0.8 for convergent CI+II e-input indicates a significant limitation by the phosphorylation system.[3]

- Convert 395 µg.atoms.min^{-1} measured with PM in State *P* to SI units, 37 °C and to ETS capacity with covergent CI+II e-input, considering that the CI/CI+II substrate control ratio (OXPHOS) is 0.64, and the *P/E* ratio (CI+II) is 0.71.

Inst. 6. Respiratory control ratio, RCR: The RCR (State 3/State 4; *P/L* flux ratio) was introduced as a sensitive index for the quality of a preparation of isolated mitochondria.[6] Whereas this represents a perfectly valid approach, application of the RCR as an index of coupling of electron transport to phosphorylation is based on a misconception. Uncoupling as expressed by the relative LEAK oxygen flux (*L/P* ratio) is overestimated, if OXPHOS capacity is kinetically limited. In mitochondria with kinetic limitation of OXPHOS by the phosphorylation system (*P/E* <1.0; see Inst. 5), the *L/E* ratio is lower compared to the *L/P* ratio, and hence the degree of uncoupling is less than suggested by the conventional RCR.[3]

- Convert the RCR(CI+II) of 6.7, as measured in human skeletal muscle at 25 °C, to the *L/P* ratio, calculate the corresponding *L/E* ratio and the relative error by which the conventional RCR overestimates the degree of uncoupling. What are the theoretical minimum and maximum values of the RCR? What are the theoretical minimum and maximum values of the *L/E* ratio?

Inst. 7. Rate of respiration: Most respiratory studies of isolated mitochondrial express OXPHOS capacity per mg mitochondrial protein (P_{mt}). Whereas important information is obtained from such investigations of mitochondrial quality, changes in mitochondrial density constitute a complementary mechanism for alterations of tissue-OXPHOS capacity, which is of primary physiological (training) and pathological (type 2 diabetes; obesity) consequence. Respiration of permeabilized muscle fibers yields tissue-OXPHOS capacity directly [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ wet weight]. With isolated mitochondria, a mitochondrial marker (e.g. citrate synthase activity, CS) must be quantified in the original tissue and in the final preparation, to determine the mitochondrial yield (µg P_{mt} isolated per mg tissue wet weight). Then mt-OXPHOS capacity [nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ P_{mt}] can be converted to tissue-OXPHOS capacity [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ wet weight]. Alternatively, tissue-OXPHOS capacity measured in permeabilized fibers or intact cells can be expressed per mt-marker (e.g. CS or mtDNA), to separate the effects of mt-quantity and mt-quality on tissue-OXPHOS capacity. Methodologically, the most sensitive expressions of mitochondrial quality are flux control ratios, *FCR*, obtained by normalization of flux for a common metabolic reference state (e.g. ETS with CI+II). Similarly, coupling control ratios and substrate control ratios provide functional indexes of mitochondrial quality. Taken together, the effects of mitochondrial quality and quantity are integrated in the measurement of tissue-OXPHOS capacity, which is directly related to tissue function and thus has the highest clinical significance of pathological or therapeutic change.[4] Mass-specific oxygen flux, J_{O2} [pmol$\cdot s^{-1} \cdot mg^{-1}$ wet weight], must be distinguished from the extensive (per system) quantity of oxygen flow, I_{O2},[2] obtained in respirometry with cultured cells [pmol$\cdot s^{-1} \cdot 10^{-6}$ cells]. Average cell size may change in populations of cultured cells. Thus an additional distinction is required between effects on oxygen flow of mitochondrial quality, density and cell size or total mt-amount per cell.[8]

- Convert 395 µg.atoms.min^{-1} measured with PM in State *P* to SI units and 37 °C, considering that the CI/CI+II substrate control ratio (OXPHOS) is 0.64, and the mitochondrial yield is 10.5 µg $P_{mt} \cdot mg^{-1}$ tissue wet weight.

Readings:

1. Mills I, Cvitas T, Homann K, Kallay N, Kuchitsu K (1993) *IUPAC. Quantities, Units and Symbols in Physical Chemistry*. 2nd ed, Blackwell, Oxford.
2. Gnaiger E (1993) Nonequilibrium thermodynamics of energy transformations. *Pure & Appl. Chem.* 65: 1983-2002.
3. Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell Biol.* doi:10.1016/j.biocel.2009.03.013

4. Lane N (2005) *Power, Sex, Suicide. Mitochondria and the Measing of Life*. Oxford Univ. Press. 354 pp.
5. Gnaiger E, ed (2009) *Mitochondrial Pathways and Respiratory Control.* OROBOROS MiPNet Publications, Innsbruck, 2nd ed. www.oroboros.at/index.php?o2k-protocols
6. Chance B, Williams GR (1955). Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. *J. Biol. Chem.* 217: 429-438.
7. Nicholls DG, Ferguson SJ (2002) *Bioenergetics 3*. Academic Press, London. 287 pp.
8. Renner K, Amberger A, Konwalinka G, Gnaiger E (2003) Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. *Biochim. Biophys. Acta* 1642: 115-123.

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

Charles Hoppel

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1-03L **The design of protocols on substrate control and coupling control of OXPHOS capacity: Mitochondrial diversity from mouse to man.**

Hélène Lemieux, Erich Gnaiger

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Modern advances in the diagnosis of mitochondrial dysfunction make it possible for clinical laboratories to perform routine analyses of OXPHOS defects in cardiac tissues of patients undergoing heart surgery. On the other hand, a single mouse heart provides sufficient tissue for monitoring mitochondrial function by high-resolution respirometry. Limited amounts of tissue and limited potential for sample throughput require optimum design of multiple substrate-uncoupler-inhibitor-titration protocols. Diagnostic protocols are evaluated with the specific aim of assessment of maximum OXPHOS capacities and elucidation of major elements of respiratory control, with particular emphasis on the additive effect of convergent Complex I+II electron input at the Q-junction.[1]

In human heart mitochondria, ADP-stimulated flux through Complex I was only 0.4 of ETS capacity, indicating a strong limitation by the phosphorylation system, in striking contrast to the flux control pattern observed in murine animal models. In mouse heart, OXPHOS capacity (coupled) and ETS capacity (uncoupled) were nearly identical, indicating a matching of the capacity of the phosphorylation system and electron transport system, in contrast to the excess ETS capacity in the human heart. Similarly, mouse skeletal muscle[2] and human skeletal muscle[3] differed with respect to the control exerted by the phosphorylation system. The functional implications of these differences on OXPHOS flux control patterns remain to be analyzed further, and have to be considered in applications of animal models.

In human and mouse heart and skeletal muscle, maximum ETS capacity was observed with a combination of NADH-related substrates plus succinate, supporting convergent electron input through Complexes I and II simultaneously into the Q-cycle. Differences between human and mouse mitochondria were also apparent in a comparison of human fibroblasts and a mouse pro-myeloid 32D cell line. These functional differences are important for evaluation of biochemical thresholds and enzymatic defects, but are generally masked by applications of conventional bioenergetics protocols. This unexpected variability of mitochondrial respiratory function is revealed only when analyzed in terms of OXPHOS flux control patterns on the basis of physiological substrate combinations.[1]

Readings:

1. Gnaiger E, ed (2009) *Mitochondrial Pathways and Respiratory Control.* OROBOROS MiPNet Publications, Innsbruck, 2nd ed.
2. Aragonés J *et al* (2008) Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nature Genetics* 40: 170-180.
3. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsøe R, Dela F (2007) Patients with Type 2 Diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* 50: 790-796.

1-04P Postnatal changes in rat brain energy metabolism and mitochondrial antioxidants content – a preliminary study.

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Perinatal asphyxia is a major cause of mortality and morbidity of newborn infants. Thus, elucidation of mechanisms underlying susceptibility of the neonatal brain to hypoxia-ischemia is of high importance for neonatology practice. Our preliminary data suggested that maturity of neonatal brain energy metabolism may account for its vulnerability to hypoxic-ischemic insult. In the present study, we therefore examined the postnatal developmental changes of brain energy metabolism in Wistar rats, starting with newborn rats, at their age of 4 and 14 postnatal days (PD), up to their weaning (PD21), adolescence (PD28) and adulthood (PD100). In isolated brain mitochondria, the activities of respiratory chain (RC) complexes were measured with an oxygen Clark-type sensor, and concentrations of main lipophilic antioxidants α-tocopherol, coenzyme Q9 (CoQ9) and CoQ10 were determined by HPLC [1]. In addition, NAD(H) and NADP(H) concentrations in the forebrain were determined by enzymatic methods [2]. The mortality rate of newborn rats exposed to 10-min anoxia increased gradually with age and reached 3.4% in PD4 and 84.2% in PD14. The activities of RC complexes increased with age and reached the maximal levels at different age: for complex I (CI) in PD21, complex II (CII) in PD 28, and complex IV in PD14. Activities of CI and CII then decreased slightly by PD100 (85% of the value of PD28). Concentrations of α-tocopherol, CoQ9 and CoQ10 in brain mitochondria also increased with age reaching a steady-state level at PD21, similarly as did NAD and NADH in brain tissue. Further studies are needed to reveal key determinants of susceptibility of neonates to brain hypoxia-ischemia and their possible relation to the maturity of brain energy metabolism. *This work was supported by grant VEGA 2/0083/09.*

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1-05P Measurement of mitochondria oxidative capacity in rat hepatoma FAO cells.

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The ATP is mainly generated by the mitochondria via oxidative phosphorylation. To determine the respiratory capacity of all, increasing concentrations of uncoupling agents such as FCCP are used. The use of uncoupling agents abolishes the obligatory linkage between the respiratory chain and the phosphorylation system used to generate ATP. Similarly, characterization of respiration in cells requires the use of inhibitors of respiratory complexes (I-IV) of the mitochondrial ATP-synthase.

In this work we have evaluated the response of rat hepatoma FAO cells to FCCP and the optimal concentrations of inhibitors to measure its oxidative capacity, under basal conditions or after stimulation with dexamethasone (2μM, 24h). To do this we have used the OROBOROS *Oxygraphh 2-k* and the XF24 *Extracellular Flux Analyzer* (Seahorse Bioscience).

We found that in our conditions was necessary to seed 150,000 cells per well in a 24 multi well X24 *Extracellular analyzer* plate and 500,000 cells per chamber in the OROBOROS respirometer. The optimal concentration of FCCP set up was 1.5μM. We also evaluate the effects of inhibitors of the respiratory chain using oligomycin (Complex V inhibitor), rotenone (Complex I inhibitor) and Antimycin A (Complex III inhibitor) and found that the optimal concentration were 1μg/ml, 2μM and 2μM respectively.

The values of oxygen consumption rate (OCR) expressed in pmol*s^{-1}*mg $protein^{-1}$ in routine, oligomycin and FCCP injection with OROBOROS system were 26.99, 11.63 and 38.79 respectively. Values obtained with the X24 Analyzer were 273.27, 73.51 and 362.09 for the same parameters.

The FAO cells routine flux control ratio (Routine/FCCP) found was of 0.69 in OROBOROS respirometer and 0.75 in X24 *Analyzer*; the LEAK flux control ratio (oligomycin/FCCP) was 0.29 OROBOROS and 0.20 in X24 and the net routine flux control ratio (routine-oligomycin)/FCCP) were 0.39 and 0.55, respectively. The absolute oxygen consumption values differ depending on the instrument used in FAO cells. However, the flux control ratios are relative similar in both cases.

To examine the effect of dexamethasone in these cells we used the X24 *Analyzer*. After 24 h dexamethasone treatment we found that the OCR was higher in the treated cells along the respirometry protocol, showing a statistically significant difference in all parameters.

In conclusion, our data indicate that dexamethasone treatment shows higher oxygen consumption and suggests stimulation of mitochondrial biogenesis.

1-06P Mitochondrial respiration in human platelets.

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In order to analyze human mitochondrial respiration tissue samples must be obtained. Muscle biopsies are probably the most common source but have drawbacks as being invasive and not suited for repetitive measurements. We have therefore developed a protocol of analyzing mitochondrial respiration in human platelets which are easily accessible via blood sampling. Blood, 10-20 ml, was taken from healthy blood donors in EDTA vacutainer vials. Each sample was centrifuged twice to produce close to cell free plasma and a platelet pellet that was resuspended in 1-2 ml of plasma. Mitochondrial respiration was determined in an Oroboros O2k high-resolution oxygraph at a platelet concentration of 100-200 x 10^6/ml. Oxidative phosphorylation (oxphos) was analyzed in intact platelets suspended in either PBS with glucose 5 mM or in plasma. Two oxphos protocols were used; in the first (oxphos1o), oligomycin-induced state 4 (State 4o) preceded FCCP titration to maximal uncoupled state 3 respiration (State 3u). In the second (oxphos2), State 3u was titrated directly from routine respiration. A substrate-uncoupler-inhibitor titration (SUIT) protocol (see titration sequence below) was used to evaluate individual complexes as well as convergent respiration in digitonin-permeabilised platelets suspended in mitochondrial respiration medium (MIR05). Results from 10 healthy blood donors are tabulated below. Routine respiration in intact cells did not differ between the three suspending buffers. Uncoupling control ratios, (UCR, State 3u/Routine) were higher in non-oligomycin treated cells, 1.92 in plasma, 1.66 in PBSGlucose, compared to oligomycin treated, 1.26 in plasma and 1.23 in PBSGlucose. This could possibly be explained by a direct inhibition by oligomycin or reflect the difference between maximal respiration with active ATP production and uncoupling vs. uncoupling without a functioning complex V. UCR in permeabilised cells was even higher 2.9, and could be attributed to the unlimited supply of exogenous substrates feeding into the Krebs cycle. In the SUIT protocol State 3u and convergent state 3 respiration (Jo2, M+P+ADP+G+S) were similar, giving a respiratory control ratio (RCR, State 3u or state 3/State 4o) of 7. This indicates that phosphorylation is not rate-limiting under these conditions. The RCRs in oxphos varied between buffers and were, if corrected for non-mitochondrial respiration, PBSGlucose 13 and plasma 21. The non-corrected values were less variable: in SUIT 6, PBSGlucose 7 and plasma 5. In conclusion, platelets are an easy obtainable human source of viable mitochondria and seem to be well suited for studying mitochondrial respiratory function using the O2k oxygraph. The technique could be applied to both basal mitochondrial research as well as in the clinical setting.

	Routine	State3u	State 4o	Jo2,M+P	Jo2,M P+ADP	Jo2,M+P+ADP G	Jo2,M+P+ADP+G+S
MIR05	14 ± 4	40 ± 8	5.5 ± 1.1	10 ± 2	24 ± 5	27 ± 6	38 ± 8
Plasma (oxphos1o)	15 ± 4	19 ± 3	0.9 ± 0.8				
Plasma (oxphos2)	13 ± 2	25 ± 4					
PBSGlucose (oxphos1o)	13 ± 2	16 ± 4	1.2 ± 0.4				
PBSGlucose (oxphos2)	12 ± 3	20 ± 4					

Data shown as mean pmol $O_2 \cdot s^{-1} \cdot 10^{-8}$ platelets ± SD, corrected for non-mitochondrial respiration. JO_2 = oxygen flux, Ama = antimycin A, As = ascorbate, Dig = digitonin, G = glutamate, M = malate, o = oligomycin, P = pyruvate, Rot = rotenone, S = succinate, Tm = TMPD
The titration sequences were as follows:
SUIT: Routine, Dig+M+P, ADP, G, S, o, FCCP, Rot, Ama, As, Tm
oxphos1o: Routine, o, FCCP, Rot, Ama
oxphos2: Routine, FCCP, Rot, Ama

1-07P **Measurement of oxygen consumption in C2C12 myotubes.**

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Mitochondrial dysfunction is central to the pathophysiology of cancer, neurodegenerative and cardiovascular diseases, and it has been reported in skeletal muscle of obese subjects and in type 2 diabetic patients. In the present work we have studied the respiration of a murine skeletal muscle cell line (C2C12 myotubes). For this purpose we have used the *OROBOROS Oxygraph-2k* and the *XF24 Extracellular Flux Analyzer* (*Seahorse Bioscience)* that allow us to measure the oxygen consumption rate (OCR) of the cells. In both cases respiration of intact cells was measured under routine conditions (*R*), inhibition by oligomycin (reflecting the proton leak, *L*), uncoupling to maximum flux with FCCP (*E*) and inhibition by rotenone and antimycin A (indicating the non-mitochondrial respiration, *nmt*).

For the *OROBOROS Oxygraph-2k* experiments we used 600,000 cells per chamber in 2ml of DMEM culture medium. The oxygen consumption rate (nmol·min^{-1}·10^{-6}cells) showed by cells in routine, oligomycin-inhibited, uncoupled and rotenone-antimycin-inhibited states was 5.18; 1.58; 19.61 and 0.53 respectively. The routine flux control ratio, R/E, was 0.24 and the LEAK flux control ratio, L/E, was 0.05. This indicates tight coupling of OXPHOS and a very large respiratory excess capacity over routine respiration in this cellular model. The net routine flux control ratio, netR=(R-L)/E was 0.19, indicating that 19% of electron transport system capacity was activated for ATP production.

Using the *XF24 Analyzer* we seed 15,000 cells per well and the OCR obtained in the different respiratory states was 24.3; 7.7; 104.2 and 5.47 nmol·min^{-1}·10^{-6}cells. The routine flux control ratio, R/E, was 0.20; the LEAK flux control ratio, L/E, was 0.03 and the net routine flux control ratio, netR=(R-L)/E was 0.17.

In conclusion, the absolute oxygen consumption values differ depending on the instrument used in C2C12 myotubes. However, the flux control ratios are very similar in both cases. The *XF24 Analyzer* presents some advantages when working with myotubes, the principal one is the use of fewer cells and that the myotubes do not lose their morphology as occurs when they are in suspension.

Day 2: Membrane potential and protein import.

2-01L Mitochondrial membrane potential: what can it tell about health of cells?

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One of vitally important functions of mitochondria is supply of energy to the cell in a form of ATP by the process of oxidative phosphorylation. The system of oxidative phosphorylation consists of two main components: the respiratory chain generating electrochemical proton gradient (protonmotive force) and ATP synthase which uses that gradient to synthesize ATP. Protonmotive force is a central intermediate in bioenergetics as it drives the synthesis of ATP, ion transport across mitochondrial membrane, is involved in proton leak-mediated heat production, etc. Like any other electrochemical potential, protonmotive force has two components: (i) an electrical term - membrane potential across mitochondrial inner membrane, (ii) a concentration term - ΔpH. Mitochondrial membrane potential is an important indicator of mitochondrial function. It reflects: (i) activity of the respiratory chain; (ii) intactness of mitochondrial inner membrane, (iii) activity of phosphorylating system. In this lecture we will discuss the methods for measurement of mitochondrial membrane potential and several examples of how these methods can be applied to detect the sites of action of various pathological factors within the system of oxidative phosphorylation.

Reading:

Brown, G.C. and Cooper C.E., eds. (1995) *Bioenergetics. A practical approach.* Oxford University Press.

2-02L Protein targeting and import into mitochondria: The process, its conserved nature, and potential for mitochondrial stabilization.

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

The translocase of the outer mitochondrial membrane (TOM) is a central entry gate for practically all nuclear-encoded mitochondrial proteins. After recruitment to and passage through TOM, precursor proteins follow one of three major pathways. (a) Preproteins with a presequence (those of this type destined for the matrix) are transferred to the translocase of the inner membrane (TIM23 complex; channel forming). TIM23 cooperates with mt Hsp70, which represents the core of the presequence translocase-associated motor (PAM). (b) Precursors of hydrophobic proteins of the inner membrane (e.g., the ANT) utilize chaperone-like proteins (Tim9, Tim10, Tim12) and the insertion machinery of the inner membrane (TIM22 complex; the 'carrier protein' translocase). c) Precursor proteins with complicated topologies (e.g., porin, Tom40) are first imported via the TOM complex to the intermembrane space, then via small Tims (9, 10) are passed on to the SAM Complex (sorting and assembly machinery) for insertion into the outer membrane. Other smaller proteins with cysteine motifs are aided in their targeting to the intermembrane space by the mitochondrial import and assembly machinery (MIA; main component Mia40), which promotes the formation of intramolecular disulfides and initiates the mature conformation of these proteins.

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

Supported by NIH grant 2-RO1-DK046270-14A1 and donation from the William Wright Family Foundation.

Readings:

1. Bolender N et al (2008) Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Reports* 9: 42-49.
2. Menze, M.A.,Boswell, L., Toner, M., and S.C. Hand (2009) Occurrence of mitochondria-targeted LEA gene in animals increases organelle resistance to water stress. *J. Biol. Chem.* 284(16): 10714–10719.

3. Neupert W., Herrmann J.M. (2007) Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76: 723-749.

2-03P Chronic hyperglycemia reduces myotube mitochondrial function without changing mitochondrial gene expression or mitochondrial content.

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We have previously shown that chronic hyperglycemia (exposure to 20 mM glucose for 4 days, HG) impairs glucose and lipid metabolism in human myotubes [1]. Insulin-stimulated glucose uptake and glycogen synthesis were reduced, whereas *de novo* lipogenesis and triacylglycerol synthesis were increased after HG-treatment. We hypothesized that HG might reduce energy consumption and increase energy storage because of impaired mitochondrial function. Simultaneous accumulation and oxidation of glucose or oleic acid (OA) were then determined with a multi-well assay [2]. Human myotubes exposed to HG were incubated with U-^{14}C-glucose (1 µCi/ml, 100 µM) or 1-^{14}C-oleic acid (1 µCi/ml, 100 µM) for 4 hours and complete substrate oxidation to CO_2 was measured. Glucose oxidation was reduced by 48 (± 9) % (p=0.07) and oleic acid oxidation by 29 (± 4) % (p=0.16) after HG-treatment (n=4). However, the maximum oxidative capacity assessed as substrate oxidation in presence of the mitochondrial uncoupler dinitrophenol (DNP, 100 µM) showed that glucose oxidation was severely compromised by HG, reduced by 53 (± 9) % (p<0.001), whereas there was no significant effect on OA oxidation. Measurements of total cell ATP concentrations showed that hyperglycemia reduced ATP concentrations by 25 (± 9) % (p=0.025) and preliminary results imply an increased production of lactate. Expression of some genes involved in mitochondrial functions, as SCD-1, ACC, CYC1. CPT-1B, CPT-1A, PDK-4, UCP-2 and UCP-3 assessed by real time RT-PCR, were not changed by hyperglycemia. Neither was the total cell mitochondrial content measured as the ratio of mitochondrial DNA to nuclear DNA. These results suggest an impaired mitochondrial function in cells exposed to hyperglycemia, without any reduction in mitochondrial number or gene expression levels. Addition of Na-lactate (1 or 10 mM) to myotubes reduced glucose oxidation to the same extent as HG, by 26 (± 17) % (p=0.19) and 44 (± 8) % (p=0.04), respectively. A similar effect was observed at low concentrations of lactic acid (0.1 and 0.2 mM), suggesting that the effect of HG might be mediated by an increased lactate production.

In conclusion, exposure of myotubes to chronic hyperglycemia impairs mitochondrial function without changing mitochondrial gene expression or mitochondrial content. Possibly the effect of hyperglycemia is mediated by an increased lactate production.

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2. Wensaas AW, Rustan AC, Lövstedt K, Kull B, Wikström S, Drevon AC, Hallén S (2007) Cell-based multiwell assay for the detection of substrate accumulation and oxidation. *J. Lipid Res.* 48: 961-967.

2-04P Thermal sensitivity of the mitochondrial metabolism in two distinct mitotypes of *Drosophila simulans*.

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Temperature is a pervasive environmental factor that affect bioenergetic of most ectothermic organisms. This thermal sensitivity of bioenergetic is partly modulated by the impact of temperature on mitochondria. We can therefore suspect that mitochondria could be adapted to different thermal regimes partly through adjustment of mitochondrial DNA encoded peptides.

We then compared the thermal sensitivity of mitochondrial functions from two populations of fruit fly (*Drosophila simulans*) with divergent mitochondrial haplotypes (Si1 and Si2). Mitochondrial respiration rate was measured in isolated mitochondria for Complex I (with pyruvate+malate+L-proline as substrates), for Complex III (with sn glycerol-3-phosphate), for uncoupled respiration (with FCCP) and for oxidative phosphorylation (ADP/O ratios) at four different temperatures (12, 18, 24 and 28 °C). We also measured the thermal sensitivity of citrate synthase, Complex IV (COX) activity as well as that of excess capacity of COX at maximum convergent pathway flux (with pyruvate+malate+proline+glycerol-3-phosphate) using inhibitors titration experiments. Finally, activities of enzymes associated to oxidative stress (catalase and aconitase) and levels of thiobarbituric reactive substances were measured.

We observed significant differences between mitotypes in the rates of substrate oxidation with Si2 being less affected by temperature. We also noticed higher state 4 of respiration for Si2 but only at intermediate temperatures (18 and 24 °C). In parallel to these result the ADP/O ratio was less affected by temperature in Si2. Finally COX excess does not vary between both mitotypes and appeared to be much higher at low temperature suggesting a switch of control from ETS to upstream dehydrogenase process or ADP phosphorylation at low temperature. These results could help to explain the much wider range of distribution of Si2 populations and support the adaptive value of mitochondrial DNA.

2-05P The importance of individual variation: NDUF expression.

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A fundamental scientific goal is to understand the biological importance of individual variation because variations among individuals affect human health and disease. For example, mutations in NADH dehydrogenase (NDUF; Complex 1 of electron transport system) are associated with heterogeneity of diseases: early childhood death, cardiomyopathies, or the inability to exercise. This heterogeneity in the response is not well understood but is most likely explained by interactions among metabolic loci.

To better understand the role of variation, we have examined patterns of NADH dehydrogenase mRNA expression and examined how correlative patterns of gene expression were related to variation in cardiac metabolism. Differential expression of NADH dehydrogenase subunits is associated with the variation in cardiac metabolism, yet these patterns are complex. This complexity has two causes: one – metabolic gene expression within the oxidative phosphorylation pathway and within enzyme complexes are both negatively and positively correlated; two – the patterns of mRNA expression that explain differences in metabolism differ among groups of individuals. These patterns suggest a complexity in how gene expression affects Complex 1 function and how this relates to cardiac metabolism.

2-06P Mitochondrial Complex I regulates ammoniagenesis in pig kidney proximal tubule-like LLC-PK1 cells.

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The hallmark of metabolic acidosis (MA) in humans is an increase in renal ammonium production from the amino nitrogen of glutamine (Gln) resulting in 2 (amide and amino nitrogens) NH_4^+ released per 1Gln metabolized. We utilized the NH_4^+ /Gln ratio (ranging 1 to 2=0-100% Complex I activation) to study the mechanism of Complex I regulation in situ mitochondria of the proximal tubule model LLC-PK1 cell line. Since both MA (pH 6.8) and troglitazone activate extracellular response kinase (ERK), we tested whether Complex I activation was regulated via MAPKK. Confluent cells were exposed to DMEM pH 7.4, DMEM pH 6.8 or DMEM pH 7.4 plus 25uM TRO for 18h after which Complex I activation was assessed by the NH_4^+/Gln ratio (NH_4^+determined by microdiffusion and Gln by HPLC respectively) and compared affects on Complex I activation to the mitochondrial membrane potential (Ψm) monitored using TRME and FAC analysis. Both MA and TRO increased the NH_4^+/Gln consistent with increased Complex I activation (1.0 to 1.22±0.08 and 1.50±0.10 for MA and for TRO respectively<0.05) as a result of increased ammonium production (1,355±215 vs. 2,217±431vs 1,785±195nmol/mg respectively, both p<0.05). Both MA and TRO caused Ψm to decrease (23 and 12% for MA, p<0.05, and TRO, p<0.10, respectively). Preventing ERK activation using MEKK inhibitors UO126 (10uM) and PD98059 (30uM) eliminated MA and TRO-induced Complex I activation (1.22±0.08 to 1.03 and 1.50±0.10 to 1.07 for MA and TRO respectively) as a consequence of reduced ammoniagenesis; Ψm was unchanged. These findings are consonant with mitochondrial function regulated by ERK activation in response to alterations in homeostasis (MA) selective for a physiological substrate and expressed at a discrete site in the electron transport chain.

Day 3: Mitochondrial Structure and Molecular Physiology.

3-01L Ultrastructure of the mitochondrion and its bearing on function and bioenergetics.

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

Reading:

Benard G, Rossignol R. (2008) Ultrastructure of the mitochondrion and its bearing on function and bioenergetics. *Antioxid Redox Signal.* 10(8): 1313-1342.

3-02L Mitochondria and apoptosis: Energy-limited states and signaling for cell death.

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

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

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

Supported by NIH grant 2-RO1-DK046270-14A1.

Readings:

1. Hand, S.C. and M.A. Menze (2008) Mitochondria in energy-limited states: Mechanisms that blunt the signaling of cell death. *J. Exp. Biol.* 211: 1829-1840.
2. Menze, M.A. and S.C. Hand (2007) Caspase Activity during cell stasis: Avoidance of apoptosis in an invertebrate extremophile, *Artemia franciscana*. *Amer. J. Physiol.* 292: R2039–R2047.

3-03L **Nitric oxide, mitochondrial permeability transition and cell death.**

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Nitric oxide (NO) and related reactive nitrogen species have several effects on mitochondria that may impact cell death and survival of cells. NO can cause rapid but reversible inhibition of mitochondrial respiration which may synergize with hypoxia to induce cell death. NO and reactive nitrogen species may cause production of reactive oxygen species which may have signalling role in the cells or may induce cell death. Reactive nitrogen species also activate mitochondrial permeability transition pore leading to release of cytochrome c, depletion of ATP and necrotic cell death due to energy deficiency. Permeability transition pore-related release of cytochrome c may cause caspase activation and apoptotic cell death when glycolytic activity in cells is high enough to maintain ATP. Recently we and others have found that low concentrations of NO may activate signalling pathways involving activation of protein kinase G and leading to increased resistance of mitochondria to opening of permeability transition pore. This may have important protective effects in heart or brain ischaemia/reperfusion and other pathologies.

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3-04L **Mitochondrial calcium import, signaling, and ER cross-talk.**

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Dynamic regulation of cytosolic calcium is fundamental to survival and function of virtually all eukaryotic cells. The role of calcium as a second messenger depends on the maintenance of a free cellular concentration $[Ca^{2+}]_c$ that is normally in the range of 0.05-0.5 µM. Transport systems in at least three membranes (e.g. inner mitochondrial, plasma, and ER) regulate $[Ca^{2+}]_c$ and allow for a balance between uptake and efflux. Calcium uptake by the mitochondrion is driven by the large negative potential ($\Delta\Psi_m$) across the inner membrane. The mitochondrial uniporter is the primary influx pathway for Ca^{2+} into the matrix of energized mitochondria. The Ca^{2+} uptake activity of the transporter is defined by the value of the membrane potential and is inhibited by divalent cations (e.g., Mg^{2+}, Mn^{2+}) and protons. Furthermore, Ca^{2+} uptake is highly sensitive to inhibition by the polycationic dye ruthenium red but the molecular nature of the uniporter remains unknown. Extrusion of calcium is under control of sodium dependent (Na^+/Ca^{2+} exchanger) and independent (H^+/Ca^{2+}) efflux pathways resulting in a constant cycling of the ion across the inner membrane. At a membrane potential of $\Delta\Psi_m$ = -150 mV the Nernst Equation predicts an equilibrium free calcium gradient between cytoplasm and matrix of 10^5. Net uptake into the matrix occurs if the cytoplasmic calcium concentration exceeds the 'set-point' for calcium ($[Ca^{2+}]_c \sim 0.5$ µM) at witch Ca^{2+} uptake and efflux are equal and opposite.

The ability of mitochondria to accumulate calcium is spectacular and can reach amounts equivalent to a total concentration ($[Ca^{2+}]^{tot}_m$, free plus bound) approaching 1 mol/l. Mitochondrial calcium uptake parallels the uptake of P_i and the formation of an osmotically inactive calcium-phosphate gel that buffers the free calcium concentration in the matrix. Under steady-state physiological conditions, free matrix calcium $[Ca^{2+}]_m$ is approximately 0.05-2 µM and serves as a direct regulator of mitochondrial function. Three matrix enzymes (pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase) can be activated by $[Ca^{2+}]_m$ in the range of 0.1-1 µM. As a consequence, elevated $[Ca^{2+}]_m$ can increase citric acid cycle activity, NAD^+ reduction and electron transport thereby stabilizing protonmotive force when cellular ATP turnover is high. However, $[Ca^{2+}]^{tot}_m$ of mammalian mitochondria has an upper limit. When exceeded, $[Ca^{2+}]_m$ rises to a value that promotes opening of the mitochondrial permeability transition pore (MPTP) and causes mitochondrial swelling, outer membrane rupture, and the release of proapoptotic factors.

Compared to mitochondrial values, the intraluminal free calcium concentration of the ER $[Ca^{2+}]_{ER}$ is set rather high (100-800 µM). Mitochondria and the ER maintain a local Ca^{2+} communication pathway that allows highly effective Ca^{2+} transfer between the two organelles. The area of the contact sites between both organelles is estimated to constitute 5-20% of the total mitochondrial surface. The main route of Ca^{2+} release from the ER is through the IP_3 receptor (IP_3R) and direct coupling between IP_3R and the voltage dependent anion channel (VDAC) in the outer mitochondrial membrane by the human chaperone grp75 has been observed in rat liver and HeLa cells. Ca^{2+} that is released through the IP_3R can thereby immediately travel through VDAC and uniporter into the mitochondrial matrix and increase respiratory capacity. The mitochondrion exciting calcium is taken back to the ER by the Sarco/Endo-plasmic Reticulum Ca^{2+} ATPase (SERCA). Calcium itself regulates IP_3R activity in a biphasic manner. Low concentrations activate the channel where high concentrations inhibit release. This negative feedback regulation prevents excessive calcium exposure of the mitochondrion. During apoptosis the integrity of the outer mitochondrial membrane can be compromised by the action of proapoptotic Bcl-2 family members or by opening of the MPTP. Both pathways ultimately lead to release of proapoptotic proteins including cytochrome c (cyt-*c*). Cyt-*c* binds to IP_3R and reduces the inhibitory action of high calcium concentrations on IP_3R calcium release. The reduced negative feedback enhances calcium release from the ER and causes an increase in the population of mitochondria that undergo opening of the MPTP. This feed-forward behavior can lead to a lethal inter-organelle cross talk. [Supported by NIH grant 2 RO1 DK04670-14A1]

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3. Nicholls DG (2005) Mitochondria and calcium signaling. *Cell Calcium* 38: 311-317.

Day 4: Heart and Muscle Mitochondria; Clinical Perspectives. Practical Approaches and Instrumentation in Mitochondrial Physiology.

4-01L Integrated mitochondrial function and the clinical utility of polarographic analysis.

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4-02L High-resolution respirometry to assess muscle mitochondrial respiratory function in horses: potential applications in sport and myopathic horses.

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Horses are outstanding athletes who have been bred over time to a wide variety of utilisations. Depending on their breed and training program, they may excel in different types of disciplines requiring endurance and/ or speed aptitude(s). Endurance horses may race 160 km at a mean speed of 20 km/h, Thoroughbred race horses run at high speed (~ 60 km/h) over distances up to 5000 meters and Quarter Horses sprint for a quarter of a mile (400 m) at a speed above 80 km/h. Several physiological adaptations contribute to explain the superior athletic capacity of horses as compared to most other animals of similar body size. These adaptations have improved the maximal aerobic capacity of equine which shares, with the pronghorn antelope, the largest maximal oxygen uptake (VO_{2max}) per unit body mass.

The VO_{2max} is an important variable that correlates with athletic performance in horses. The VO_{2max} integrates all components of the respiratory cascade (from inspired air to O_2 consumption at the level of mitochondria) but cardiac output is the principal determinant of VO_{2max}. Indeed, VO_{2max} is O_2-supply limited because the mitochondrial oxidative capacity largely exceeds the capacity of the respiratory and cardiovascular systems to deliver O_2. The muscle (like the cardiovascular system) is highly adaptable and responds to training by improving the arterial-venous O_2 extraction owing to several adaptations such as increasing the density of the capillary bed, increasing the mitochondrial density and intramyocyte oxidative enzymes. Thus, training contributes to increase the aerobic capacity of the *muscle* with subsequent improvement of O_2 utilization at maximal (*i.e.* at VO_{2max}) and submaximal exercise. Increased aerobic capacity of the *muscle* tightens metabolic control which reduces glycolysis and glycogen utilization. Muscle glycogen reserves as an anaerobic energetic fuel are important to sprint at the end of a race (even in endurance horses) and to prevent fatigue.

Surgical biopsy has contributed to define the histological, histochemical and biochemical properties of equine skeletal muscle. Significant variations in muscle fibre composition and metabolism are observed among breeds and types of horses known to have a predisposition for specific disciplines. For example, among endurance horses, the better performers have higher percentages of slow-twitch fibers, higher activities of oxidative enzymes, higher lipid oxidation capacity and lower percentages of fast-twitch fibres. Despite the determining role of mitochondria in the metabolic properties of muscle, there are only anecdotic studies on equine mitochondrial function. Our study aims at determining muscle oxidative phosphorylation (*OXPHOS*) capacity in trained and untrained horses using a minimally invasive technique. We have recently introduced the muscle microbiopsy technique combined with high-resolution respirometry (HRR) to better understand exercise physiology and potentially identify *OXPHOS* defects in myopathic horses and poor performers.

Microbiopsies in the *triceps brachii* and *gluteus medius* were performed with a 14 G biopsy needle that enables the sampling of 20 to 40 mg of muscle. The specimens were prepared according to the OROBOROS Oxygraph-2k manual and mitochondrial respiration was measured by HRR with the Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria) at 37 °C, using 1 to 4 mg wet weight of saponin-permeabilized muscle fibers. Multiple substrate-uncoupler-inhibitor titration (SUIT) protocols were devised to determine the *OXPHOS* capacity per muscle mass ($J_{O2,P}$) following electron input at different levels of the electron transport system (ETS). Firstly, electrons enter into the ETS at the level of Complex I (CI) using NADH-linked substrates such as glutamate+malate (GM) or pyruvate+malate (PM) with subsequent addition of G. At the second step, convergent electron flow through Complexes I and II (CI+II) into the Q-junction is obtained following addition of succinate (S) oxidized at the level of Complex II (CII) with reduction of FAD into $FADH_2$ (with subsequent electron flow to the Q-junction). By stepwise addition of an uncoupler (u) such as FCCP, the SUIT protocols inform about the coupling control from *OXPHOS* to *ETS* capacity (*i.e.*, $J_{O2,P}$ to $J_{O2,E}$). Electron input into the Q-junction through CII alone is subsequently obtained by addition of rotenone (Rot) which prevents the transfer of electrons from CI to the Q-junction. Finally, residual oxygen consumption (*ROX*) which is subtracted from the $J_{O2,P}$ and $J_{O2,E}$ recorded on-line is obtained by addition of antimycin A (Ama) or myxothiazol (Myx) to block electron transfer to Complex III (CIII). Addition of cytochrome *c* (*c*) after adding CI-substrates tests the integrity of the outer mitochondrial membrane. In additional experiments, it was checked that CII-respiration (S+Rot) was not significantly influenced by prior uncoupling.The different SUIT protocols may be summarised as follows:

e_N+M+G_{+D}+*c*+S_{+u}+(Rot)
e_N+P+M_{+D}+*c*+G+S_{+u}+(Rot)
e_N+S+$(Rot)_{+u}$

where (e) are endogenous substrates, N means no added adenylates, and D is ADP.

These SUIT protocols were applied to a wide variety of healthy horses trained for different disciplines (jumping, eventing, dressage, endurance) as well as to untrained animals. It was also used to follow the effect of endurance training on *OXPHOS* capacity in endurance horses. Results show that $J_{O2,P}$ and $J_{O2,E}$ per muscle mass significantly increase after endurance training. Together with an increased oxidative capacity, there were specific modifications in the substrate control ratios. The question remains whether HRR might be developed towards a new approach for the prediction of performance in sport horses.

Acquisition of reference values with healthy horses enables application of the SUIT protocols for the detection of *OXPHOS* defects in horses with clinical and subclinical myopathies. Respirometric *OXPHOS* analysis on pathological cases will be presented and discussed. [The authors gratefully acknowledge OROBOROS INSTRUMENTS (Innsbruck, Austria) and the '*Ministère de l'Agriculture et de la Ruralité de la Région Wallonne*' of Belgium for supporting this work.]

Readings:

Gnaiger E. (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell Biol.* doi:10.1016/ j.biocel.2009.03.013.

Lemieux H., Votion D.M., Gnaiger E. *(*2007) Mitochondrial respiration in permeabilized muscle fibers: needle biopsy from horse skeletal muscle. *Mitochondr. Physiol. Network* 12.23: 1-4.

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4-03L Ischemia-reperfusion injury in heart mitochondria.

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4-04L Preparation and application of permeabilized muscle fibers for respiration studies.

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This presentation of permeabilized muscle fibers consists of two parts. (1) Practical aspects of fiber preparation, following the group of Saks et al.[1] Application of permeabilized muscle fibers and high-resolution respirometry offer a sensitive diagnostic test of mitochondrial dysfunction in small biopsy specimens of muscle or limited amounts of cultured cells.[2] By using these techniques in conjunction with multiple substrate-uncoupler-inhibitor titration (SUIT) protocols, respirometric studies of human and animal tissue biopsies improve our fundamental understanding of mitochondrial respiratory control and the pathophysiology of mitochondrial myopathies.[3,4] (2) We critically review the theoretical arguments advanced in favour of application in respirometry of permeabilized muscle fibers (Pfi) or permeabilized cells (Pc; collectively permeabilized tissues and cells, Ptic) versus isolated mitochondria (Imt).

1. Preparation of permeabilized muscle fibers

The relaxing and biopsy preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 (for details, see www.oroboros.at/index.php?protocols). Remove all connecting tissue using a pair of sharp scissors and forceps. Cut the muscle lengthwise into small samples. These are placed into 1 ml BIOPS onto a petri dish on ice, where individual fibre bundles are separated with two pairs of sharp forceps. As soon as possible, the sample is gently blotted, and subsamples may possibly be separated for additional assays (histochemical/morphological; spectrophotometric; mtDNA). The tissue sample for respirometry is transferred immediately into ice-cold BIOPS. In this specially designed preservation solution, the sample can be stored for several hours up to days at 0 °C, depending on the source of muscle tissue (e.g. mouse skeletal muscle for at least six hours; human skeletal muscle for up to 24 h;[5] horse skeletal muscle[3] up to several days).

The degree of separation of the tissue may be evaluated by observing a change from red to pale colouring of the separated fiber bundles. The fiber bundles are placed into 2 ml of icecold BIOPS, and 20 µl of saponin stock solution (5 mg/ml) are added (final concentration is 50 µg/ml). Shake by gentle agitation in the cold room (on ice) for 30 min. Transfer the fiber bundles into 2 ml of ice cold mitochondrial respiration medium (MiR05)[6] and shake by gentle agitation for 10 min in the cold room (on ice). Weight measurements are made after permeabilization, eliminating variations in water contents due to osmotic stress, allowing for elimination of connective tissue during mechanical separation before weight measurement, and for partitioning of subsamples of similar wet weight. Before adding the tissue into the respiration chamber, take wet weight measurements of several loosely connected fiber bundles of c. 1-3 mg wet weight (skeletal muscle) or 0.5-2 mg wet weight (mammalian heart). On another petri dish on ice, separate droplets of MiR05 are prepared, into which the sample is transferred immediately after reading the wet weight.

2. Permeabilized muscle fibers versus isolated mitochondria – or intact cells

Whereas isolated mitochondria (Imt) remain one of the gold-standards in studies of bioenergetics and mitochondrial physiology, permeabilized tissues and permeabilized cells (Ptic) or intact cells are alternative or complementary models with several advantages. But their limitations with respect to Imt have to be considered, too, for optimum experimental design and critical evaluation of results. (i) Ptic needs less tissue or fewer cells than Imt. With high-resolution respirometry, only 1 mg wet weight of cardiac fibers is required per experimental test, in a 2 ml chamber at 37 °C, or 0.3 million cells (fibroblasts, endothelial cells). (ii) Optimization of isolation of mitochondria may be significantly more time-consuming compared to the optimization of Ptic preparation. (iii) All types of mitochondria are experimentally accessible in Ptic, whereas Imt preparations allow for the separation of different mitochondrial subpopulations (advantage), or it has been argued (but probably never shown experimentally) that Imt may involve the selective loss of damaged mitochondria. (iv) Intact cells do generally not provide a model amenable for assessment of maximum OXPHOS capacity.

Advantages of isolated mitochondria (Imt) include (i) Imt preparation is required for separation and study of different mitochondrial subpopulations. (ii) The homogeneous suspension of Imt yields a representative avarage for the tissue sample, and fewer replica are required compared to averaging over heterogenous subsamples of fibers. (iii) The oxygen dependence of respiration in permeabilized muscle fibers increases by two orders of magnitude, due to oxygen diffusion to the mitochondria in the small unperfused fiber bundle.[7] Imt and small isolated cells with insignificant effects of limitation by oxygen diffusion provide, therefore, the only choice for the study of mitochondrial oxygen kinetics.[7] (iv) ADP has to be added to Ptic at high concentrations to assess OXPHOS capacity, due to diffusion restriction and since the outer membrane may exert a barrier function different from Imt.[1]

Readings:

1. Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS (1998) Permeabilised cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol. Cell. Biochem.* 184: 81-100.
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4-05D High-resolution respirometry of permeabilized fibers with the OROBOROS Oxygraph-2k.

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The OROBOROS Oxygraph-2k provides a unique high-resolution approach to the monitoring of cellular and mitochondrial respiratory function. World-wide, the 'O2k' is established as the leading instrument in mitochondrial respiratory physiology. High-resolution respirometry (HRR) is superior in routine applications, and is dramatically different to conventional polarography or new semi-quantitative approaches. In addition, HRR has opened up a new dimension for quantitative analysis of low respiratory activities and low oxygen levels. Diagnostic substrate-uncoupler-inhibitor-titration protocols are applied with as little as 2 mg wet weight of permeabilized muscle fibers obtained from a small needle biopsy, or 0.5 million cultured cells such as fibroblasts. The most important biomedical applications include evaluation of mitochondrial and metabolic defects, ischemia-reperfusion injury, oxidative stress, apoptosis, degenerative diseases and aging.

High-resolution respirometry (HRR) with the OROBOROS Oxygraph-2k is an integrative systems approach to polarographic measurement of oxygen dynamics. O_2 concentration and respiration (oxygen flux) are displayed on-line and simultaneously by the DatLab software. The mechanics, critical selection of materials, and state-of-the-art electronics provide the basis for high signal stability of an optimized polarographic oxygen sensor, in an electronically controlled thermal environment with outstanding temperature stability. Calibrations and instrumental controls follow standardized HRR procedures, with highly automatic on-line analysis.

Recent developments lead to a modular extension of the Oxygraph-2k to the MultiSensor-O2k for simultaneous application of O_2 and NO sensors and ion selective electrodes (pH for evaluation of proton fluxes, TPP^+ for mitochondrial membrane potential, Ca^{2+}), and optical methods (cytochrome spectra; spectrofluorimetry). Scientific support by the OROBOROS O2k-Team is complementary to the approach of high-resolution respirometry. Find information on the newest experimental HRR protocols, or search through more than 250 publications on HRR on www.oroboros.at. We invite you to join our O2k-Course for a practical demonstration experiment on high-resolution respirometry. You are welcome in our growing *MiPNet* – the Mitochondrial Physiology Network.

4-06L Real-time monitoring of cellular bioenergetics in a microplate using the Extracellular Flux Analyzer.

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Dysregulation of energy homeostasis in cells is a fundamental process that is associated with aging and age-related diseases such as cancer, diabetes and neurodegeneration. For example, a high rate of conversion of glucose to lactic acid in the presence of oxygen, known as the Warburg effect, is the hallmark of aggressive tumors cells. Mitochondrial oxidative phosphorylation and glycolysis are the two primary pathways to generate ATP to meet cellular energy demand. It is important to understand the two energy producing processes of cells in health and disease states.

The Seahorse XF analyzer measures oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of living cells in a microplate by monitoring the changes in the levels of oxygen and pH in the extracellular medium in equilibrium with the cells. A small volume is temporally formed around the cells during the measurement by lowering the piston-like sensor probe into the well of a plate. Oxygen consumption rate of cells is proportional to mitochondrial respiration. In an open system, ECAR is primarily lactic acid production rate of cells and is an indicator of aerobic glycolysis. Hence, the XF analyzer made it easy to rapidly and simultaneously monitor both the cellular respiration and glycolysis rates of living cells in a microplate without using large quantities of cells and/or detaching them from the plates as required by traditional methods. The Microplates are in either 24 or 96 wells format, depending on the system. The plate-based assays broaden the range of biological samples from adherent cells to isolated mitochondria and tissue samples.

The XF sensor cartridge is designed with an integrated reagent delivery system that can be loaded with up to 4 port of compounds, and released into each cell sample at programmed times within a single assay. This feature enables interrogation of cellular metabolic activities in real-time with energy substrates such as glucose, glutamine and fatty acid and/or metabolic perturbation agents such as oligomycin and 2-deoxyglucos

In this presentation, I will focus on the use of XF Analyzer to 1) monitor cellular bioenergetics and dynamic interplay of OXPHOS and glycolysis pathways; 2) determine bioenergetic function (or dysfunction) of mitochondria; 3) investigate signals that regulate cellular bioenergetics; 4) interrogate metabolic activities in the cell using substrates such as glucose, glutamine and fatty acid and pharmacological agents and make inference of intermediate metabolism; 5) evaluate metabolic effect of pharmaceutical agents.

Reading:

Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. (2007) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am. J. Physiol. Cell. Physiol.* 292(1): C125-136.

4-07S Inner mitochondrial membrane and outer space.

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Day 5: Metabolic Remodelling: Impacts of Calorie Restriction, Fitness, and Warburg/Crabtree Effect.

5-01L Calorie restriction and mitochondrial biogenesis.

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The focus of my presentation will be on research involving long-term calorie restriction (CR) to prevent or delay the incidence of the metabolic syndrome with age. I will concentrate mostly on the surprising phenomenon of mitochondrial biogenesis in a condition in which energy demand is reduced. However, in this abstract, I am providing most of the physiological and psychological/behavioral results obtained in our first phase study of caloric restriction in non obese people. This will allow me to concentrate more during the presentation on my topic in this course, i.e. "Calorie restriction and mitochondrial biogenesis".

Prolonged CR has been shown to extend median and maximal lifespan in a variety of lower species (yeast, worms, fish, rats, and mice). Mechanisms of this lifespan extension by CR are not fully elucidated, but possibly involve alterations in energy metabolism, oxidative damage, insulin sensitivity, and functional changes in neuroendocrine systems. Ongoing studies of CR in humans now makes it possible to identify changes in "biomarkers of aging" to unravel some of the mechanisms of its anti-aging phenomenon. Analyses from controlled human trials involving long-term CR will allow investigators to link observed alterations from body composition down to changes in molecular pathways and gene expression, with their possible effects on the metabolic syndrome and aging.

In a recent study at our Center, 48 (36.8±1.0y), overweight (BMI 27.8±0.7kg/m^2) participants were randomized to four groups for 6-months; *Control*: energy intake at 100% of energy requirements; *CR*: 25% calorie restriction; *CR+EX*: 12.5% CR plus 12.5% increase in energy expenditure by structured exercise; *LCD*: low calorie diet (890kcal/d) until 15% weight reduction followed by weight maintenance. Body composition (DXA), total daily energy expenditure (TDEE) over 14-days by doubly labeled water (DLW), 24-h sedentary energy expenditure by a respiratory chamber and activity related energy activity (AREE) were measured after 3 (M3) and 6 (M6) months of intervention. Weight changes at M6 were −1.0±1.1% (Control), −10.4±0.9% (CR), −10.0±0.8% (CR+EX) and -13.9±0.8% (LCD). At M3, absolute TDEE was significantly reduced in CR (-454±76 kcal/d) and LCD (-633±66 kcal/d) but not in CR+EX or controls. At M6 the reduction in TDEE remained lower than baseline in CR (-316±118 kcal/d) and LCD (-389±124 kcal/d) but reached significance only when CR and LCD were combined. In this combined groups (CR/LCD), TDEE adjusted for body composition, was significantly lower by -431±51 and -240±83 kcal/d at M3 and M6, respectively, indicating a clear metabolic adaptation. Similarly, after adjustment for changes in body composition, sedentary 24h energy expenditure (respiratory chamber) was decreased in CR (-135±42 kcal/d), CREX (-117±52 kcal/d) and LCD (-125±35 kcal/d) i.e. approximately 6-7% more than expected on the basis of the loss in fat-free mass and fat mass. Importantly, physical activity (TDEE adjusted for sleeping metabolic rate) was significantly reduced from baseline at month 3 but tended to return towards baseline at month 6. We therefore showed for the first time in free-living conditions that CR results in a metabolic adaptation and a behavioral adaptation with decreased physical activity levels. These data also indicate how CR may causes large inter-individual variability in the rates of weight loss even in fully adherent individuals and how exercise may influence weight loss and weight loss maintenance. Some data from the weight loss registry will be briefly presented.

A summary of the physiological and psychological/behavioral responses to six months of calorie restriction in humans from the Pennington CALERIE randomized clinical trial are summarized in the Table below.

PHYSIOLOGICAL RESPONSES		PSYCHOLOGICAL / BEHAVIORAL RESPONSES
Body Composition ↓ Fat mass ↓ Fat-free mass ↓ Abdominal fat (visceral and subcutaneous) ↓ Abdominal fat cell size ↓ Intra-hepatic lipid content ↔ Intra-myocellular lipid content		**Development of eating disorder symptoms** ↓ Disinhibition ↓ Binge eating ↓ Concern about body size and shape ↔ Fear of fatness ↔ Purgative behavior
Diabetes Risk Factors ↓ Insulin sensitivity (not significant) ↓ Acute insulin response to glucose		**Depressed mood** ↓ MAEDS Depression scale ↔ Beck Depression Inventory II
Cardiovascular disease risk ↓ 10-year risk ↓ Blood pressure ↑ HDL-C ↓ Triacylglycerol (TG) ↓ Factor VIIc ↔ Fibrinogen, homocysteine, endothelial function		**Subjective feelings of hunger** ↓ Eating Inventory, Perceived Hunger Scale

Biomarkers of Longevity ↓ Fasting insulin ↓ Core body temperature ↔ DHEA-S		**Quality of life** ↑ Physical functioning ↔ Vitality
Energy Expenditure ↓ 24-h sedentary energy expenditure Metabolic adaptation for 24-h energy expenditure ↓ Sleeping metabolic rate (SMR) Metabolic adaptation for SMR		**Cognitive Performance** ↔ Verbal memory ↔ Short-term memory and retention ↔ Visual perception and memory ↔ Attention/concentration
Endocrinology ↓ T3 ↓ T4 ↔ GH ↔ IGF-1 ↓ Ghrelin		
Physical Activity ↓ Physical activity level (TDEE adjusted for SMR) ↔ Spontaneous physical activity		

Interestingly and relevant to this course, CR increased muscle expression of genes involved in mitochondrial biogenesis including PGC1-α, mtTFA, endothelial nitric oxide, SIRT1 and PARL. In parallel, mitochondrial content increased by 35±5% in this group. This study therefore supports the "rate of living" theory of aging mediated by a potential improvement in mitochondrial function associated with decreased DNA damage.

My presentation will mostly concentrate on this latter aspect of "Calorie restriction and mitochondrial biogenesis".

Readings:

1. Civitarese, A.E., Carling, S., Heilbronn, L.K., Hulver, M.H., Ukropcova, B., Deutsch, W.A., Smith, S.R., and E. Ravussin for the CALERIE Pennington Team (2007) Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Medicine* 4(3): 1-10.
2. Civitarese, A.E. and E. Ravussin (2008) Minireview: Mitochondrial energetics and insulin resistance. *Endocrinology* 149(3): 950–954.

5-02L **Fewer mitochondria - *mitochondrial 'fever'*: Mitochondrial physiology and declining OXPHOS capacity in sedentary and dietary life styles.**

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A tight relationship is described between mitochondrial respiratory capacity of human skeletal muscle and physical fitness, which quantifies the decline of respiratory function as the result of a sedentary life style in the progression towards obesity.[1] Tissue-OXPHOS capacity is the capacity of oxidative phosphorylation in skeletal muscle, which is the product of mitochondrial density and respiratory intensity (structure times function; i.e. mitochondrial marker per tissue mass times OXPHOS capacity per mitochondrial marker). Tissue-OXPHOS capacity per unit wet weight [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$] is measured directly in permeabilized muscle fibers, and high-resolution respirometry provides a routine approach under physiological conditions (37 °C; Complex I+II substrate combination)[1] with minimal amounts of tissue biopsy (1 to 3 mg wet weight per assay).[2]

In healthy subjects varying from athletic to sedentary life styles, tissue-OXPHOS capacity of vastus lateralis increases linearly with maximum aerobic ergometric performance ($V_{O2,max}$) and declines steeply with body mass index (BMI=body mass per body height squared [kg/m^2]) in the range of 180 to 60 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$. The tissue-OXPHOS/BMI relationship spans from world-class endurance athletes and physically active subjects (normal BMI 20-25), overweight individuals (BMI 25-30) with predominantly sedentary life style, to obese patients who are qualified as healthy controls in studies of type 2 diabetes (BMI >30). Total muscle tissue is unchanged or increases rather than decreases with higher BMI, whereas over-proportionally reduced mitochondrial density per muscle mass explains the loss of aerobic ergometric performance in the sedentary life style and development of obesity. Mitochondrial quality (coupling and OXPHOS capacity per mitochondrial marker) is largely maintained, does not appear to be a primary defect contributing to obesity as a risk factor for development of type 2 diabetes, and specific mitochondrial injuries accumulate perhaps as a consequence of reduced mitochondrial density and correspondingly low mitochondrial turnover.

Based on the tissue-OXPHOS/BMI relationship and integrating known mechanisms responsible for dysregulation of mitochondrial biosynthesis under conditions of chronic low-grade inflammation, low mitochondrial density is a primary risk factor related to a wide range of degenerative diseases, including type 2 diabetes. The health benefits are emphasized of maintaining mucle mitochondrial density high, particularly with progressive age, as achieved by a physically active and nutritionally normal life style.

This abstract will be presented at: UMDF Mitochondrial Medicine and MiP2009; June 23-26, Washington DC, USA.

1. Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell Biol.* doi:10.1016/j.biocel.2009.03.013
2. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsøe R, Dela F (2007) Patients with Type 2 Diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* 50: 790-796.

5-03L Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis.

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In this lecture, we will discuss the metabolic remodelling of cancer cells and the subsequent metabolic signaling in tumors, specifically the various metabolites that participate in the regulation of gene expression in cancer cells. In particular, pyruvate, oxaloacetate, succinate and fumarate, four mitochondrial metabolites, activate genes relevant for tumor progression. When the balance between glycolysis and oxidative phosphorylation is altered, these metabolites accumulate in the cytoplasm and regulate the activity of the Hypoxia Inducible Factor 1alpha (HIF-1alpha). HIF is one of the main factors that orchestrate the metabolic switch observed during oncogenesis. There is also an important role for lactate, fructose 1-6 bisphosphate or citrate that leads to the diversion of glucose metabolites to anabolism. In addition reactive oxygen species, which are produced by the respiratory chain, could serve as an endogenous source of DNA-damaging agents to promote genetic instability. Accordingly, several mitochondrial DNA mutations were reported in tumors, and the construction of cybrids recently demonstrated their role in the control of tumor progression.

Reading:

Bellance N, Lestienne P., Rossignol R. (2009) Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. *Front Biosci.* 14: 4015-4034.

5-04P **Pyruvate dehydrogenase complex in *Artemia franciscana* embryos: A possible contributor to metabolic depression during diapause.**

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Diapause embryos of the brine shrimp, *Artemia franciscana*, are characterized by a low metabolic rate. Diapause is a developmentally-programmed state of ontogenetic and metabolic arrest that occurs under normoxia in fully-hydrated embryos. Based on our previous data, we hypothesized that metabolic arrest is due to a combination of at least two mechanisms, namely substrate limitation to the mitochondrion and reversible inhibition of oxidative phosphorylation. We measured metabolic intermediates in metabolically-active (control) embryos and in diapause embryos. A significant reduction in glucose-6-phosphate was evident in diapause (0.22 ± 0.02 nmol/mg embryo protein; mean ± SE, n = 6) versus active (1.85 ± 0.53 nmol/mg; n = 6) embryos. Values for glucose in diapause (5.98 ± 1.8 nmol/mg, n = 6) and active (3.58 ± 2.09 nmol/mg, n = 6) embryos allow the calculation of product/substrate ratios (0.037 ± 0.011 diapause vs. 0.52 ± 0.34 active; significantly different, $p<0.05$), which indicate inhibition at the hexokinase (HK) step, suggest a reduced glycolytic flux, and support substrate limitation to the mitochondrion in diapause. Pyruvate is lower in diapause (0.24 ± 0.02 nmol/mg, n = 3) than in active (0.46 ± 0.05 nmol/mg, n = 6) embryos, but not as low as anticipated, despite being downstream of the glycolytic inhibition at HK. Measurements of acetyl CoA concentrations reveal lower values during diapause (0.0075 ± 0.0009 nmol/mg protein; n = 3) versus active (control) embryos (0.0193 ± 0.0001; n = 6). The resulting product/substrate ratios (0.0133 ± 0.002, diapause; 0.0419 ± 0.003, active; significantly different, $p<0.005$) suggest a modest negative crossover point at PDH and enzyme inhibition during diapause. Preliminary Western blots probed with antibody against phosphorylated PDH (E1 subunit; phosphorylated at site 1, serine 293) suggest there may be greater phosphorylation during diapause. Mammalian PDH phosphorylated at site 1 has been shown to exhibit inhibition by over 90%. Kinetic characterization of *A. franciscana* PDH is underway. At this point our data indicate a restriction of carbon availability to mitochondria during diapause. Reversible inhibition of oxidative phosphorylation, as suggested from respiration rates of cell lysates (data not shown), has not been ruled out. [Supported by NIH grant 2 RO1 DK04670-14A1]

5-05P **Skeletal muscle NAMPT is induced by exercise and correlates with mitochondrial content.**

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In mammals, intracellular nicotinamide phosphoribosyltransferase (iNAMPT) is responsible for the first and rate-limiting step in the conversion of nicotinamide to nicotinamide adenine dinucleotide (NAD^+). NAD^+ is an obligate co-substrate for mammalian sirtuin 1 (SIRT1), a deacetylase which activates peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) in skeletal muscle, which in turn is known to activate mitochondrial biogenesis. Previous studies have demonstrated that AMP-activated protein kinase (AMPK) is required to increase NAMPT activity during glucose deprivation and that AMPK activation enhances SIRT1 activity as well as subsequent deacetylation of PGC-1α. Given that AMPK and mitochondrial biogenesis are activated by exercise, we hypothesized that exercise would increase NAMPT mRNA and protein, as a potential mechanism leading to increased mitochondrial content in muscle. If true, NAMPT may be a useful target for increasing mitochondrial content in individuals who have type 2 diabetes mellitus (T2DM) where defects in mitochondrial biogenesis have been demonstrated. Our initial objective was to

establish an association between NAMPT protein expression and mitochondrial content in human subjects. Athletes had ~2-fold higher skeletal muscle NAMPT protein expression compared to sedentary subjects ($P < 0.05$). NAMPT protein correlated with mitochondrial content ($R^2 = 0.28$, $P < 0.01$), MRS-measured maximal ATP synthesis ($R^2 = 0.37$, $P = 0.002$), and VO_2max ($R^2 = 0.63$, $P < 0.0001$). Consistent with this result, NAMPT protein increased by 127% in sedentary non-obese subjects after 3 weeks of exercise training ($P < 0.01$). To establish whether this was a direct or indirect effect of exercise on muscle, we treated primary human myotubes *in vitro* with forskolin and ionomycin to activate the cAMP and calcium signaling pathways, which are activated in skeletal muscle by exercise *in vivo*. Treatment with this 'exercise cocktail' resulted in ~3-fold higher NAMPT protein content ($P < 0.001$) and ~50% higher mitochondrial content ($P < 0.05$). Overall, results indicate that skeletal muscle NAMPT expression is induced in response to exercise, NAMPT correlates with mitochondrial content, and the stimulation of calcium/cAMP signaling pathways is sufficient to increase NAMPT protein content in skeletal muscle. These results suggest that NAMPT might contribute to the mitochondrial biogenesis observed in response to exercise. Future studies will focus on the regulation of NAMPT expression and activity.

5-06P No relationship between skeletal muscle mitochondrial function and insulin resistance in type 2 diabetes.

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Objective: Numerous clinical studies have shown that populations with insulin resistance have reduced mitochondrial content in skeletal muscle. The objective of this study was to determine the role of mitochondrial content and function on the variation in insulin sensitivity observed within in a population of patients with T2DM.

Research Design and Methods: Fifty eight participants (8 athletes, 17 healthy controls without (FH-) and 7 with (FH+) family history of diabetes, 4 obese and 21 patients with T2DM) enrolled in a cross-sectional design. Mitochondrial function was measured non-invasively using ^{31}P MRS of the vastus lateralis. Maximal ATP synthetic rate (ATP_{max}) was determined from the rate of phospho creatine (PCr) recovery after short-term isometric exercise.

Results: Consistent with the literature ATP_{max} was lower in T2DM and higher in athletes as compared to healthy sedentary FH- controls (athletes 1.01 $\pm$ 0.2; control FH- 0.7 $\pm$ 0.2; control FH+ 0.6 $\pm$ 0.1; obese 0.6 $\pm$ 0.1; T2DM 0.5 $\pm$ 0.2 mM ATP/s; ANOVA $p < 0.0001$). Insulin sensitivity (GDR) measured by euglycemic hyperinsulinemic ($80mIU/m^2/min$) clamp was also reduced in T2DM (athletes 12.0 $\pm$ 3.2, control FH- 7.8 $\pm$ 2.2; control FH+ 6.8 $\pm$ 3.5; obese 3.1 $\pm$ 1.0; T2DM 3.4 $\pm$ 1.6 mg/kg EMBS//min; ANOVA $p < 0.0001$). ATPmax measure was correlated with other measures of skeletal muscle mitochondrial content and function such as maximal aerobic capacity (VO2 peak), percent of type-I fibers and total OXPHOS protein content (Complex I to V), used as a marker of mitochondrial content. Unexpectedly, there was a broad range of ATP_{max} within the T2DM population (range 0.23 – 0.79 mM ATP/s) where 52% of subjects with T2DM had ATP_{max} values that were within the range observed in lean healthy sedentary controls. In addition 24% of the T2DM subjects overlapped with the endurance trained athletes (range 0.65 – 1.27 mM ATP/s). Given the apparently 'normal' ATP_{max} in some patients with T2DM we explored the relationship between ATP_{max} and M-value within the T2DM population. In contrast to the positive correlation between ATP_{max} and M-value in the population as a whole ($r^2 = 0.35$; $p <0.0001$) or in non diabetic population ($r^2=0.26$; $p=0.004$), there was no correlation between ATP_{max} and M-value in the patients with T2DM ($r^2 = 0.004$; $p = 0.79$). When we divided patients with T2DM into 2 groups, those with normal and those with low ATP_{max} there were no differences in M-value ($p = 0.24$). Similarly, high vs. low M-value patients showed no differences in ATP_{max} ($p=0.52$).

Conclusions: This data casts doubt on the role of reduced mitochondrial mass and mitochondrial dysfunction as a key component of the insulin resistance seen in T2DM.

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