

Mitochondrial Techniques Workshop

Tuesday 12 – Wednesday 13 September 2017
University College London, UK

C_fMR

Organised by

**Michael Duchen, Sean Davidson &
Gyorgy Szabadkai**

**UCL Consortium for Mitochondrial Research
University College London, UK**



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Welcome to this Mitochondrial Techniques Workshop

We are very excited to host this workshop, and hope that we will deliver to match your expectations. Experimental exploration of mitochondrial behaviour requires a specialised set of experimental skills and the theoretical understanding to interpret the data properly.

Our goal in this short workshop is to give an overview of key approaches that will help in that task.

We hope that the experience will be enriching, enjoyable and entertaining.

We thank The Physiological Society, the Biochemical Society and British Pharmacological Society.

Above all, we thank the members of the Duchen/Szabadkai labs and other colleagues who have given a great deal of their time to help with practical aspects of organisation, far beyond the call of duty, and who will be available throughout the workshop to help you in whatever way they can.

We thank Gauri Bhosale, Nicoletta Plotegher, Bobby Bentham, Will Kotiadis, Tom Blacker, Kate Hawkins, Federico Zambon, Tânia Soares, Amandine Marecahl, Siân Culley, Chris Kay, Johann Klare, Marthe Ludtmann, and Sam Ranasinghe for their time and hard work in supporting the lab based demonstrations.

Most importantly, the workshop is for you – if you have questions, please ask; if you have comments – please tell us.

And thanks to you all for coming!

With our very best wishes

Michael Duchen, Sean Davidson and Gyorgy Szabadkai

Programme

Tuesday, 12 September

- 10.30 Registration and workshop selection
- 12.00 Lunch and posters
- 13.30 Welcome and housekeeping
Michael Duchen, University College London, UK
- 13.40 Introduction to the mitochondrial respiratory chain and energy coupling
Peter Rich, University College London, UK
- 14.30 Laboratory workshop sessions (see pages 6–7)**
- 16.30 Break
- 17.00 Respirometry – interrogating the respiratory chain
David Nicholls, Buck Institute for Research on Aging, USA
- 17.45 Reception and posters
- 19.00 Reception ends
- 20.00 Dinner
Bellaria, 71 Great Titchfield St, Fitzrovia, London W1W 6RB

Wednesday, 13 September

- 8.30 Reception desk opens
- 9.00 Super-resolution imaging
Siân Culley, University College London, UK
- 9.30 Using mitochondrial targeted probes and fluorescence imaging to interrogate mitochondrial function and dysfunction
Gyorgy Szabadkai, University College London, UK
- 10.00 Laboratory workshop sessions (see pages 6–7)**
- 12.00 Lunch and posters
- 13.00 EM and tomography
Anwen Bullen, University College London, UK

Programme

Oral communication session 1

- 13.30 001 **Role of CLIC proteins in the regulation of mitochondrial function in pulmonary hypertension**
Mai Alzaydi, Imperial College London, UK
- 13.45 006 **The mitochondrial Ca²⁺ uniporter (MCUa) is required for glucose-stimulated mitochondrial Ca²⁺ uptake and insulin secretion in mouse pancreatic beta cells**
Elizabeth Haythorne, University of Oxford, UK
- 14.00 007 **Oscillations in mitochondrial ROS production during the early cell cycles in *Xenopus* embryos**
Javier Iglesias-Gonzalez, University of Manchester, UK

14.15 Laboratory workshop sessions (see pages 6-7)

16.15 Break

Oral communication session 2

- 16.45 010 **Exploring the role of Kynurenine 3-monooxygenase (KMO) in mitochondrial dynamics**
Daniel Maddison, University of Leicester, UK
- 17.00 011 **Dynamin related protein-1 inhibition drives megamitochondria formation and protects from alcohol-induced liver toxicity**
Elena Palma, Institute of Hepatology- Foundation for Liver Research, UK
- 17.15 012 **Preventing glyoxalase-1 (Glo-I) downregulation attenuates mitochondria dysfunction in hearts of diabetic rats**
Jaipaul Singh, University of Central Lancashire, UK
- 17.30 **Using *Drosophila* as a model organism to study roles of mitochondria in physiology and disease**
Alex Whitworth, MRC MBU Cambridge, UK
- 18.00 Workshop ends

Laboratory workshop sessions

You can register for up to three workshop sessions from the list below. Sign up sheets will be available at the registration desk. There is no more than ten people per group and allocation is on a first come, first served basis.

Confocal: Uv-vis

This will be a basic introduction to principles of fluorescence imaging and laser scanning confocal imaging using UV based illumination to image NADH and visible light to image mitochondrial form and to measure membrane potential.

Led by Michael Duchen, University College London, UK

Confocal: 2p-FLIM – TB

This will be an introduction to the principles and applications of two photon imaging and the use of fluorescence lifetime imaging (FLIM) to extract further information about the chemical status of NADH and NADPH in cells.

Led by Thomas Blacker, University College London, UK

Confocal: Zeiss 880 with airyscan

This will introduce basic principles of confocal laser scanning microscopy to explore mitochondrial form and function on a machine equipped with airyscan near superresolution imaging. If there is sufficient demand we can also run confocal imaging session on a Zeiss 700 again to introduce basic principles of confocal imaging of mitochondrial form and function, but without the UV.

Led by Marthe Ludtmann, University College London, UK

Superresolution microscopy

Siân Culley will be giving a talk about her work with superresolution imaging and these sessions will provide an opportunity to see these principles put into practice, pushing the boundaries of optical resolution.

Led by Siân Culley, University College London, UK

Laboratory workshop sessions

Seahorse

We will operate two parallel sessions to demonstrate the operation of the 'Seahorse' multichannel instrument for measurement of respiratory rate and its modulation by a range of compounds to assess mitochondrial respiratory capacity and operation.

Led by Nicoletta Plotegher and Tânia Soares, University College London, and Nick Howe, Product Specialist, Seahorse Bioscience, UK

EPR

This will introduce the principles and operation of paramagnetic resonance spectroscopy to assess generation of free radicals using spin traps.

Led by Chris Kay, University College London, UK and Johann Klare, Universität Osnabrück, Germany

UV/visible and FTIR spectroscopy

This will introduce the use of UV/visible and infrared spectroscopy to gain qualitative, quantitative and structural information from the components of the mitochondrial respiratory chain.

Led by Amandine Marechal, University College London, UK

High throughput imaging 'Image Xpress'

This will explore the application of a high throughput fluorescence imaging platform to explore changes in mitochondrial function for screening applications, emphasising measurements of mitochondrial nucleoid numbers to follow changes in biogenesis, measurements of potential and morphology.

Led by Gyorgy Szabadkai, University College London, UK

Oroboros respirometry

This is an introduction to the use of the Oroboros Oxygraph 2k and associated fluorescence module for high resolution respirometry measurements from cell populations in suspension.

Led by Will Kotiadis, University College London, UK

Abstracts

001

Role of CLIC proteins in the regulation of mitochondrial function in pulmonary hypertension

Mai Alzaydi, Imperial College London, UK

Mai Alzaydi, Vahitha Abdul-Salam and Beata Wojciak-Stothard

Pulmonary arterial hypertension (PAH) is a severe disease characterised by extensive remodelling of intrapulmonary arteries and right heart hypertrophy [1]. PAH bears many hallmarks of cancer, including apoptosis resistance, increased cell proliferation, mitochondrial dysfunction i.e. dysmorphic, hyperpolarized mitochondria, mitochondrial fission, and metabolic switch from mitochondrial oxidative phosphorylation to cytoplasmic glycolysis [2]. We have identified intracellular chloride channel proteins (CLICs) as new mediators of vascular dysfunction in PAH. CLIC1 and CLIC4 are highly expressed in animal and human PAH lung and are elevated in plasma of PAH patients [3]. CLIC proteins are stress-response proteins that show glutaredoxin activity and play a role in membrane trafficking, cytoskeletal function, apoptosis, cell cycle control, angiogenesis, and cell differentiation. CLIC1 and CLIC4 localize in the outer mitochondrial membrane, whereas CLIC5 is present in the inner mitochondrial membrane [4, 5]. We have shown that CLIC4 associates with dynamin, a protein homologous to DRP1 and proteins regulating vesicular trafficking and cytoskeletal function, including clathrin, tubulin and Arp2,3 complex.

Here we overexpressed CLIC1, CLIC4 and CLIC5 via adenoviral gene transfer and studied their effect on mitochondrial distribution and function in human primary pulmonary endothelial cells (HPAECs). Results show that CLIC1 but not CLIC4 or CLIC5 induced mitochondrial fission and dramatically reduced expression of mitofusin 2 (MFN2). In contrast, CLIC5 increased mitochondrial fusion and the formation of mitochondrial networks in HPAECs. CLIC1- and CLIC5-induced effects were accompanied by reorganization of microtubule cytoskeleton. CLIC1 and CLIC5 also altered expression of mitochondrial proteins, including HSP60 and pyruvate dehydrogenase.

In summary, our results suggest that CLIC proteins may play a contributory role in the mitochondrial dysfunction in PAH lung.

Abstracts

002

RKIP protects heart mitochondria in a β -adrenergic receptor-dependent manner

Theresa Brand, Leibniz-Institut für Analytische Wissenschaften -ISAS- e.V., Germany

Theresa Brand¹, Nilgün Gedik², Gerd Heusch², Petra Kleinbongard², Kristina Lorenz^{1,3}

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It is well known that the Raf kinase inhibitor protein (RKIP) regulates Raf and G-protein coupled receptor signaling dependent on its phosphorylation status. RKIP phosphorylated at serine 153 inhibits G-protein coupled receptor kinase 2 (GRK2), thereby inhibiting β -adrenergic receptor (β AR) desensitization and thus enhancing β AR downstream signaling. In line with this, mice with cardiac specific overexpression of RKIP (RKIP-tg) showed a hypercontractile phenotype and are protected from heart failure. β 1AR were identified as the major driver of RKIP-mediated increase in cardiac force. Surprisingly RKIP-mediated hypercontractility is well tolerated since β 1AR-mediated adverse effects are prevented by simultaneous activation of β 2AR.

As a persistent increase in contractility leads to a higher cardiac energy demand, we hypothesized that mitochondria as the cellular engines may be affected by RKIP. Indeed, electron microscopy images of cardiac sections reveal significant morphological differences between RKIP-tg, RKIP knockout (RKIP-KO) and wild type (WT) mice: in RKIP-tg, the percentage of tissue occupied by mitochondria was significantly higher than in WT (41.2 ± 6.8 vs. $33.4 \pm 9.4\%$ means \pm S.E.M., ANOVA, $p < 0.05$, $n = 3$ mice and at least 15 left ventricular sections/mouse; male mice at the age of 8 weeks) and occurred in highly ordered rows between the myofilaments; in RKIP-knockout (RKIP-KO) mice the inter-mitochondrial network and the structure of the cristae were disrupted. The overall mitochondrial arrangement and morphology in RKIP-KO hearts was further damaged in response to chronic pressure overload (induced by transverse aortic constriction for 3 weeks in 8 week old mice, $n = 3$ mice and at least 15 left ventricular sections were analyzed/mouse) whereas the mitochondrial integrity was preserved in RKIP-tg hearts compared to WT. Functional analysis hints to a critical role of RKIP also for mitochondrial function as mitochondria derived from RKIP-KO hearts show a lower oxygen consumption measured as ADP-stimulated complex I respiration compared to controls ($n = 6$; 85.8 ± 21.3 vs. 123.2 ± 32.4 nmol/min/mg; means \pm S.E.M., Student's t-test, paired, $p < 0.05$). Since (i) these experiments suggest a connection between RKIP and mitochondria, (ii) the protective effects of RKIP are mediated via the β 2AR and (iii) β 2AR activation and mitochondrial biogenesis are thought to be linked, we analyzed mitochondrial respiratory chain proteins in the absence and presence of β 2AR. In fact RKIP-overexpression increases the expression level of mitochondrial respiratory chain proteins as e.g. NDUFB1 only in the presence of β 2AR analyzed by western blots ($n = 5$).

Abstracts

In summary, β 2AR mediated cardiac protection by RKIP involves not only the prevention of hyperphosphorylated proarrhythmic β AR downstream targets – as previously published (Schmid et al., Nat Med. 2015) – but also the protection of mitochondria.

003

Parkin protects against mitochondrial and neuronal dysfunction in a drosophila model of Huntington's Disease

Susanna Campesan, University of Leicester, UK

Susanna Campesan, Victoria E. Cotton, Charalambos P. Kyriacou and Flaviano Giorgini.
Department of Genetics, University of Leicester, UK

Metabolic dysfunction has long been recognised as part of the pathology in Huntington's Disease (HD). Decreased glucose metabolism and increased lactate concentrations in HD brains suggest mitochondrial involvement. Inhibition of the mitochondrial respiratory complex II results in neuropathology and clinical features resembling HD in several animal models, and post-mortem studies of HD brains show deficiencies of complexes II, III and IV.

In this study we used a *Drosophila* model of HD to study mitochondrial and neuronal dysfunction. Moreover we investigated the overexpression of Parkin, an E3 ubiquitin ligase which is involved in mitochondria quality control, and causes early onset Parkinson disease when mutated.

We performed high resolution respirometry in HD flies. We also measured the mitochondrial mass using the citrate synthase assay and electron microscopy and overexpressed fly Parkin to investigate whether this could offer any protection against HD phenotypes.

High resolution respirometry of HD fly muscle revealed mitochondrial dysfunction resulting in a high leak state and reduction of oxphos capacity. HD flies also showed increased mitochondrial mass compared to controls. Overexpression of Parkin robustly rescued all these phenotypes. Pan-neuronal expression of Parkin was also strongly neuroprotective and significantly increased lifespan.

In conclusion, overexpression of Parkin results in rescue of all fly HD related phenotypes highlighting the importance of mitochondrial function and quality control in this disease.

004

Studying mitochondrial bioenergetics in ghrelin-mediated neuroprotection

Maria Carla Carisi', Swansea University, UK

Carisi MC, Rees DJ, Morgan AH, Davies JS.
Medical School, Swansea University (UK).

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterised by progressive memory loss and cognitive decline. Calorie restriction (CR) is known to prevent cognitive deficits in mouse models of AD and in aged humans. Our group showed that CR-mediated neuroprotection, neurogenesis and memory are dependent on the hormone ghrelin, produced in the stomach in response to changes in the body's metabolic status. Acylated-ghrelin (AG) activates Growth Hormone Secretagogue Receptor-1a (GHSR) in several areas of the brain. In the arcuate nucleus of the hypothalamus, AG regulates food intake, adiposity and insulin secretion during CR, through AMP-activated kinase (AMPK). In the hippocampus, AG affects neurogenesis and synaptic density, increases long-term potentiation and enhances learning and memory^{1,2}.

Recent evidences showed that ghrelin activity involves mitochondrial signalling. Cells treated with serum from calorie-restricted rats, displayed reduced mitochondrial membrane potential (MMP) and ROS production, increased mitochondrial biogenesis and bioenergetics capacity³. Neurons pre-treated with ghrelin exhibit increased resistance to rotenone-induced toxicity and reduced cytochrome C release⁴. Increased ghrelin levels activate mitochondrial respiration, increase mitochondrial number, and contribute to fatty acids oxidation and ROS clearance⁵. Preliminary data from our group, in in-vitro and in-vivo models of Parkinson's Disease, showed that AG attenuates rotenone-induced dopamine neurone loss in nutrient restricted media; induces phosphorylation of AMPK and Acetyl-CoA carboxylase, a fatty acid biosynthesis regulator; promotes MMP and partially prevents rotenone-induced mitochondrial fragmentation (unpublished).

Our hypothesis is that AG-mediated neuroprotection may occur through regulation of mitochondrial energetic metabolism and fusion/fission balance. To investigate this, human neural stem cells (ReN VM, Merk Millipore) will be differentiated to mature neurones and treated for 5 days with AG before being incubated for 24h with amyloid-beta(1-42) oligomers (A β Os) - a peptide known to induce neuronal toxicity and mitochondrial dysfunction in AD patients¹. To assess mitochondrial respiration and bioenergetics we will use an Agilent Seahorse XF system and MMP and mitochondrial fusion/fission will be quantified using confocal microscopy (Mitohealth assay, Life Tech). Finally, using post-mortem human brain tissue (Brains for Dementia Research), we will determine GHSR expression in hippocampus and entorhinal cortex in AD and whether this correlates with expression of mitochondrial proteins.

Abstracts

1. Santos, V. V. et al. Acyl ghrelin improves cognition, synaptic plasticity deficits and neuroinflammation following amyloid beta (Ab1-40) administration in mice. *J. Neuroendocrinol.* 29, (2017).
2. Bayliss, J. A. & Andrews, Z. B. Ghrelin is the metabolic link connecting calorie restriction to neuroprotection. *Neural Regen. Res.* 11, 1228–9 (2016).
3. López-Lluch, G. et al. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc. Natl. Acad. Sci. U. S. A.* 103, 1768–73 (2006).
4. Yu, J., Xu, H., Shen, X. & Jiang, H. Ghrelin protects MES23.5 cells against rotenone via inhibiting mitochondrial dysfunction and apoptosis. *Neuropeptides* 56, 69–74 (2016).
5. Andrews, Z. B. et al. Ghrelin Promotes and Protects Nigrostriatal Dopamine Function via a UCP2-Dependent Mitochondrial Mechanism. *J. Neurosci.* 29, (2009).

Abstracts

005

Integrated analysis for the development of a mitochondrial metabolic model in *Saccharomyces cerevisiae*

Francesca Di Bartolomeo, Chalmers University of Technology, Sweden

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As semi-autonomous organelles that supply the cell with energy, mitochondria are considered the centre of many essential metabolic processes. Together with the study of their biochemical properties, there has been a growing interest regarding the role of mitochondria in the development of many degenerative diseases. Furthermore, cutting-edge investigations also consider mitochondria as a tool in metabolic engineering (1).

In this study, we apply a comprehensive way to look at the mitochondrial metabolism of *Saccharomyces cerevisiae*, in order to understand how mitochondrial and cellular metabolism interacts with each other in response to different metabolic conditions. To address this point, a quantitative proteomic study of mitochondria is integrated with an in-depth computational metabolic modeling and metabolic flux analysis, creating a unique model that improves our understanding of mitochondria metabolism.

The core experiment of this research is characterised by the recently developed live-cell proteomics. This method employs an engineered ascorbate peroxidase called APEX2, targeted to a particular compartment(s), to obtain a spatially resolved quantitative proteomic mitochondrial maps of *S. cerevisiae*'s cells grown on different carbon sources and at specific time points (2). Upon incubation with H₂O₂ and of biotin-phenol (BP), APEX2 catalyses the conversion of BP to biotin-phenoxyl radicals. These radicals are covalently attached to all the proteins situated at <20 nm from APEX2 in 1 minute, meaning that with this method it is possible to obtain a complete protein map of the selected cellular compartments with high spatial and temporal specificity (2).

(1) Avalos JL, Fink GR, Stephanopoulos G. (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nature Biotech.* 31:335-341.

(2) Hung V, Udeshi ND, Lam SS, Loh KH, Cox KJ, Pedram K, Carr SA, Ting AY. (2016) Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nat. Prot.* 11:456-475.

Abstracts

006

The mitochondrial Ca²⁺ uniporter (MCUa) is required for glucose-stimulated mitochondrial Ca²⁺ uptake and insulin secretion in mouse pancreatic beta cells

Elizabeth Haythorne, University of Oxford, UK

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Background and aims: Oxidative metabolism is central to the stimulation of insulin secretion by glucose. Whether mitochondrial Ca²⁺ uptake in pancreatic β -cells potentiates this process, however, remains unclear. MCUa is believed to form the main route of entry for Ca²⁺ into the mitochondria. To test this hypothesis, we have generated β -cell specific MCUa knockout mice and assessed the impact of blocking mitochondrial Ca²⁺ uptake on β -cell function and whole body glucose homeostasis.

Materials and Methods: C57BL6 mice bearing MCUa alleles with floxP sites flanking exons 11 and 12 were bred with mice bearing Cre recombinase inserted at the Ins1 locus (Ins1Cre). This strategy allowed highly β -cell selective deletion of both MCUa splice variants (KO). Mice bearing floxed MCUa alleles but lacking Cre recombinase were used as littermate controls (WT). Intraperitoneal glucose (1 g/kg) tolerance was measured every four weeks from eight weeks of age, and insulin sensitivity (0.75U/kg) was determined at 8-10 weeks of age. Glucose-stimulated insulin secretion (3 to 17 mM) was examined in vitro during perfusion at 37 °C in Krebs-bicarbonate medium. Adenovirus-mediated delivery of a targeted recombinant Ca²⁺ probe, R-GECO, and Fura-Red, were used respectively to record mitochondrial and cytosolic free Ca²⁺ changes in dissociated β -cells during fluorescence microscopy (Olympus IX81 microscope, 40x objective).

Results: Glucose (3 vs 17 mM)-stimulated mitochondrial Ca²⁺ uptake was markedly reduced in KO vs WT mice (AUC: WT, 24.06 ± 0.67 vs KO, 22.12 ± 0.42, p<0.05, n= 5, 3 mice per genotype) while the cytosolic Ca²⁺ responses were unaffected. Glucose (3 vs 17 mM)-stimulated insulin secretion was also impaired from isolated islets of KO mice in comparison to littermate controls (AUC: WT, 0.035 ± 0.001 vs KO, 0.017 ± 0.005, p<0.05, n= 3-4, 3 mice per genotype). Paradoxically, male KO mice displayed significantly improved glucose tolerance compared to WT mice at eight weeks of age (AUC: WT, 1329 ± 47.78 vs KO, 1113 ± 46.2 mmol/L*min, p<0.01, n=11-15). However, this difference normalised from twelve weeks of age. Body weight, fed and fasting glycaemia and insulin sensitivity did not differ significantly between genotypes.

Abstracts

Conclusions: MCUa is crucial in pancreatic β -cells to allow glucose-stimulated Ca^{2+} uptake by mitochondria and normal glucose-stimulated insulin secretion. These findings thus support the view that increases in mitochondrial matrix Ca^{2+} are required for a full activation of oxidative metabolism, the generation of increases in ATP/ADP and potentially of other mitochondrially-derived coupling factors in response to stimulation with glucose. The apparent compensatory mechanisms which allow maintained or improved insulin production in MCUa KO mouse *in vivo* remain to be established.

007

Oscillations in mitochondrial ROS production during the early cell cycles in *Xenopus* embryos

Javier Iglesias-Gonzalez, University of Manchester, UK

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The ability to repair and regenerate tissues is an essential process for the survivability and development of the organisms. Amphibians excel on these processes and are invaluable models to study the molecular and cellular mechanisms underlying scar free wound healing and tissue regeneration. Among these, we have used the frog, *Xenopus*, as an animal model to study the role of reactive oxygen species (ROS) during the early embryonic development and appendage regeneration. Both embryonic development and tissue repair/regeneration require cell proliferation, which relies on the synchronized mechanisms that regulate the cell cycle [1]. The mitochondrion is the powerhouse of the cell but it is also involved in other processes such as cellular signaling and calcium buffering. However, the roles of mtROS during early vertebrate development have remained largely unknown. For this reason, our main aim is to understand how the mitochondria, metabolism and ROS are regulated during early development and tissue regeneration. We have recently shown using transgenic *Xenopus* frog embryos expressing the genetically encoded ROS indicator HyPer that mtROS is increased after fertilization and that it oscillates during each cell division. When we exposed the embryos to mitochondrial inhibitors we observed that complex II is the primary source of ROS *in vivo* and that the inhibitors differentially affect the oscillatory patterns of ROS production. Furthermore, in order to identify the source of mtROS in the electron transfer system, we performed a study of mitochondrial function in a cell-free system (i.e. egg extract) combining high-resolution respirometry, hydrogen peroxide production and membrane potential [2]. Our study reveals that the succinate dehydrogenase complex (CII), specifically the flavoprotein in the SdhA subunit, is the major source of mtROS when the mitochondria are fuelled by succinate. Also, we have found that the calcium acts upstream of ROS production from the mitochondria. Finally, we have discovered that the ATP levels also oscillate during the cell cycle and our results suggest that cellular metabolism alternates between aerobic glycolysis (Warburg Effect) and OXPHOS in association with the cell cycle. Our results highlight an entanglement between calcium, metabolism and ROS but further work is required to understand how these processes are related to the cell cycle and its relevance for the early development and tissue regeneration.

008

Mitochondrial function is negatively impacted by ER stress in obesity

Laura Jackisch, University of Warwick, UK

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Cellular damage in the ER and mitochondria are known to contribute to the pathology of obesity and associated comorbidities. This damage may occur in part as a consequence of ER-mitochondria cross talk in conditions of nutrient excess such as obesity. To date insight into this dynamic relationship is not well characterised in human adipocytes. Therefore this study investigated whether the induction of ER stress contributes to the development of mitochondrial inefficiency in human adipocytes.

Human differentiated adipocytes from 1) Chub-S7 cell line and 2) primary lean and obese abdominal subcutaneous (AbdSC) adipocytes, were treated with Tunicamycin (Tn) to induce ER stress over time. Key parameters of mitochondrial function were assessed, including oxygen consumption rate (OCR), mitochondrial membrane potential (MMP), ATP concentration fission and fusion proteins, mitochondrial dynamics and number.

Induction of ER stress led to a 26% ($P \leq 0.001$) increase in OCR in Chub-S7 cells in a concentration dependent manner. This increase in OCR also corresponded to diminished ATP production (26%↓; $P \leq 0.001$) and impaired MMP (32%↓; $P \leq 0.0001$), highlighting the formation of inefficient mitochondria due to ER stress. Morphological analysis via confocal microscopy also revealed a reduction mitochondrial elongation (16%↓; $P \leq 0.05$) and cellular area occupied by mitochondria (28%↓; $P \leq 0.05$). Additionally, Drp1, a key protein in mitochondrial fission, significantly increased ($p < 0.001$), likely stimulating mitochondrial fragmentation as seen with the confocal analysis. Furthermore, AbdSc adipocytes from lean subjects mirrored the Chub-S7 cellular response with a 21% ($p \leq 0.01$) rise in OCR by Tn treatment. In contrast, mitochondria from obese subjects displayed 33% ($p \leq 0.001$) lower basal respiration than their lean counterparts and were not responsive to Tn, demonstrating significantly impaired respiratory function.

In summary, these human data suggest that adipocyte mitochondrial inefficiency is impacted by ER stress and exacerbated in obesity. Together these findings indicate the important relationship between the ER and mitochondria as a mechanism to contribute to obesity mediated T2DM.

Abstracts

009

Miro proteins are required for priming mitochondria for Pink1 induced Parkin translocation

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Dzamilja Safiulina - Estonia

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Miriam Hickey - Estonia

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It has been shown that Parkin, along with Pink1, regulates mitochondrial trafficking and quarantines damaged mitochondria by severing their connection to the microtubule network via Miro1, a key component of the motor complex that anchors kinesin to the mitochondrial surface. It has been proposed that Pink1 phosphorylates Miro1 leading to proteasomal degradation of Miro1 in a Parkin-dependent manner. Our aim was to investigate this interaction in more details in neuronal cells.

We first tested whether Miro proteins are involved in PINK1 overexpression-induced Parkin translocation to mitochondria. Results demonstrate that shRNAs against both Miro isoforms suppressed significantly Pink1-induced Parkin translocation to mitochondria in PC6 cells. Miro shRNAs also suppressed oligomycin/antimycin induced Parkin translocation to depolarised mitochondria. We next attempted to rescue the effect of Miro shRNAs by overexpressing partially shRNA insensitive Miro-1. To our surprise Miro-1 overexpression alone induced significant Parkin translocation to mitochondria. This translocation, however, had different pattern compared to oligomycin-antimycin treatment - or PINK1 overexpression induced Parkin translocation. Compared with later two conditions, where majority of cytosolic Parkin was translocated to dot like mitochondria, the Miro1 induced translocation was significantly weaker and also the structures it translocated sustained their rod shaped structures. Miro1 overexpression did not induce mitochondrial depolarisation, on the contrary it led to slight hyperpolarisation, and Parkin translocated into healthy, polarized mitochondria.

To test whether Miro1 induced Parkin translocation to mitochondria requires PINK1 we first suppressed endogenous PINK1 using specific shRNA. Similarly, in cells transfected with scrambled shRNA, Miro1 overexpression induced Parkin translocation. We repeated similar experiment in MEFs prepared from Pink1 deficient mouse.

We next tested whether Miro1 induced Parkin translocation requires E3 ligase activity of Parkin. However, ligase dead Parkin mutants T240R as well as C431N translocated to mitochondria as efficiently as wt Parkin when Miro1 was overexpressed. Altogether these experiments suggest that Miro1 overexpression induced Parkin translocation to mitochondria is independent of Pink1, it probably functions before Parkin binds to mitochondrial substrates. Results allow us to speculate that Miro-s are required for priming mitochondria for Pink1 induced Parkin translocation.

Abstracts

010

Exploring the role of kynurenine 3-monooxygenase (kmo) in mitochondrial dynamics

Daniel Maddison, University of Leicester, UK

Daniel Maddison, Susanna Campesan, Charalambos Kyriacou, Flaviano Giorgini
Department of Genetics, University of Leicester, United Kingdom

Background: The enzyme kynurenine 3-monooxygenase (KMO) functions at a critical branch-point in the kynurenine pathway (KP) of tryptophan degradation, which has been strongly implicated in a number of human diseases. Pharmacological inhibition of KMO activity restores the imbalance in levels of neuroactive KP metabolites observed in several neurodegenerative diseases and is thus a promising therapeutic candidate. KMO is localised to the mitochondrial outer membrane, but no mitochondrial function for this protein has yet been described.

Aims: To elucidate the mitochondrial role of KMO through the study of mitochondrial properties in KMO-deficient *Drosophila melanogaster*.

Methods: High resolution respirometry was performed on *Drosophila* whole body homogenates using the Oroboros Oxygraph-2k system. Mitochondrial morphology of *Drosophila* eye tissue was observed by transmission electron microscopy and in *Drosophila* S2 cells by confocal microscopy. Citrate synthase activity was employed as a measure of mitochondrial content.

Results: KMO-deficient *Drosophila* exhibit complex I-linked respiration defects. Flies and cells lacking KMO have elongated mitochondria and increased citrate synthase activity, indicative of increased mitochondrial content. Feeding flies 3-hydroxykynurenine - the product of KMO enzymatic activity - restores the metabolite to physiological levels, but does not rescue defects in respiration. Furthermore, the KMO encoding gene *cinnabar* genetically interacts with both *pink1* and *parkin* - familial Parkinson's disease associated genes - which play a crucial role in mitophagy, the autophagic clearance of damaged mitochondria from the cell.

Conclusions: These results indicate that KMO plays a role in mitochondrial quality control independent from its enzymatic function in the KP. Future work will address the mechanism(s) of action by detailed investigation of the interactions between KMO and the factors underlying regulation of mitochondrial dynamics.

Abstracts

011

Dynamin related protein-1 inhibition drives megamitochondria formation and protects from alcohol-induced liver toxicity

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Background:

The detection of megamitochondria in liver biopsies from patients has been recognised as a specific histological feature and hallmark of Alcoholic Liver Disease (ALD) since the 1970s. Recent clinical observations have associated this manifestation with a more favourable outcome in approximately 500 patients with ALD. However, the mechanisms driving megamitochondria formation and their impact on cellular viability remain unknown. In order to maintain their functionality and to respond to the changing needs of the cell, mitochondria undergo cycles of fusion and fission. The equilibrium between these events determines the mitochondrial shape, which is strictly regulated by the activity of mitochondria-shaping proteins (MSP).

Methods:

The effects of ethanol (EtOH) on mitochondrial shape and MSP were studied in hepatoma cells (VL-17A, 3/14 days with EtOH), human precision cut liver slices (PCLS, 24/72h with EtOH) and in a cohort of patients with ALD. EtOH toxicity was evaluated by FACS, ATP quantification and mitochondrial function. Changes in mitochondrial shape and megamitochondria formation were assessed by confocal and electron microscopy; MSP expression/activation was analysed by Western blot or RT-PCR.

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Results:

In the current study, we describe the morphological changes in the hepatic mitochondria in response to alcohol toxicity. Exacerbated mitochondrial fragmentation, megamitochondria appearance and upregulation of the fragmentation pathway were consistently detected in vitro (VL-17A) and ex vivo (PCLS) and in 55 patients with ALD. The master fission MSP Dynamin-related protein-1 (Drp-1) was the main mediator driving these mitochondrial alterations and its inactivation stimulated megamitochondria formation. This manifestation was associated with improvements in cellular viability during alcohol exposure and protection from alcohol mediated toxicity.

Conclusions:

This study describes the impact of alcohol on mitochondrial remodelling and presents experimental evidence to confirm the protective effect of megamitochondria during alcohol-induced liver injury. The inactivation of Drp-1, with formation of megamitochondria represents a novel therapeutic strategy for ALD, in order to directly reduce ethanol driven hepatotoxicity.

012

Preventing glyoxalase-1 (Glo-I) downregulation attenuates mitochondria dysfunction in hearts of diabetic rats

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Diabetic heart failure (dHF) is an established cause of morbidity and mortality in individuals with diabetes mellitus (DM). Supra-physiologic production/flux of the alpha-oxoaldehyde methylglyoxal (MG) has emerged as likely candidate since it can promote mitochondrial dysfunction, a known cause of dHF. In this study, a custom-designed adeno-associated virus in conjunction with echocardiography, electron, atomic force and electro-paramagnetic resonance spectroscopies, live cell imaging, Western blot analyses and high performance liquid chromatography was used to determine if preventing supra-physiologic MG in DM rat hearts is mitochondria and cardio-protective. After eight weeks of streptozotocin-induced DM, E:A ratio was increased and % fractional shortening and ejection fraction were reduced in rats, indicative of dHF. In ultrathin ventricular sections from DM hearts, SSM were no longer thread-like but disjointed from each other and migrated away from the plasma membrane (~5 nm). Purified SSM from DM hearts were also smaller (850 ± 20 nm, compared to 1090 ± 25 nm for control) with more compact, lamelliform cristae (21 ± 4 nm compared to 28 ± 3 nm for control), and generated ~2.5X higher basal reactive oxygen species (ROS)/μg mitochondria. Isolated SSM and SSM in ventricular tissues also contained single and double membraned mitochondrial-derived vesicles, suggesting extrusion of oxidized cargoes.

Abstracts

Isolated SSM also contained ~50% less connexin 43 and superoxide dismutase-1 (SOD-1), and ~ 2X more dynamin-1-like protein (Drp-1) and mitofusin II (mfn-2). NDUF2, 3 and 10 contained ~ 2-3X more MG adduct on them compared to controls. Preventing Glo-I downregulation in DM rats by administering an AAV2/9 containing Glo-I driven by the endothelin-1 promoter one week after the onset of DM, attenuated impairments in E:A ratio, % fractional shortening and ejection fraction later in the disease (7-8 weeks). It also attenuated changes in structure and function of SSM induced by DM. These data show for the first time that supra-physiologic MG flux is an underlying cause of SSM dysregulation in hearts of DM rats. They also show that attenuating supra-physiologic MG flux by preventing Glo-I downregulation is sufficient to blunt SSM dysregulation and HF in DM.

013

Assessment of mitochondrial function following 7 days exposure of human bronchial epithelial cells with total particulate matter from a modified-risk tobacco product versus cigarettes

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Mitochondrial dysfunction caused by cigarette smoking is involved in driving the oxidative stress-induced physiology in airway diseases. Reduction of harmful and potentially harmful constituents (HPHC) by heating rather than combusting tobacco could reduce the mitochondrial changes that contribute to oxidative stress and cell damage. We evaluated mitochondrial function in human bronchial epithelial cells (BEAS-2B) following a 7 days exposure to total particulate matter (TPM) from the aerosol of a modified-risk tobacco product (MRTP), in comparison with TPM from the 3R4F reference cigarette.

We found that 3R4F TPM has the strongest inhibitory effect on both basal and maximal oxygen consumption rate measured in intact BEAS-2B cells compared to TPM from an MRTP after 7 days of exposure. Moreover, we have found that alterations in the oxidative phosphorylation (OXPHOS) are accompanied by increased mitochondrial superoxide levels as well as by increased level of oxidatively damaged proteins in cells treated with 3R4F TPM or a 20-fold higher concentration of MRTP TPM, while cytosolic ROS levels revealed no differences between studied compounds. Taken together, these results indicate that 3R4F TPM has a stronger effect on OXPHOS and manifestation of oxidative stress in comparison with TPM from an MRTP. Reduction of HPHC by heating rather than combusting tobacco would be a sound strategy to reduce mitochondrial dysfunction and oxidative stress-related diseases associated with smoking combustible tobacco products.



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