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JOINT MEETING of the

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology and AUVA Research Center, *Vienna, Austria*

Oroboros Instruments and MitoFit Laboratory, *Innsbruck, Austria*

Institute of Surgical Research, *University of Szeged, Hungary*

Clinical Skills Centre, Faculty of Medicine, University of Szeged, Hungary

26-27 March 2018

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26-27 March 2018

Financial support:
EFOP-3.6.2-16-2017-00006 "LIVE LONGER"
COST Action CA15203 MitoEAGLE

SCIENTIFIC PROGRAM

DAY 1 | 2018-03-26 MONDAY

09:00-09:15 | WELCOME

Ferenc Bari, Dean, Faculty of Medicine, University of Szeged

Mihály Boros, Head of the Institute of Surgical Research, University of Szeged (15')

09:15-10:45 | SEPSIS AND SYSTEMIC INFLAMMATION

Chairs: Marcin F. Osuchowski, Susanne Drechsler

Marcin F. Osuchowski *(Vienna, Austria)*
Wiggers-Bernard Initiative - Update (30')

Susanne Drechsler *(Vienna, Austria)*
Effects of splenectomy in polytrauma and secondary sepsis (15')

József Kaszaki *(Szeged, Hungary)*
Therapeutic possibilities of microcirculatory and mitochondrial dysfunction in sepsis (15')

Attila Rutai *(Szeged, Hungary)*
Potential role of endothelin receptors in the therapy of experimental sepsis (15')

Szabolcs Tallósy *(Szeged, Hungary)*
Microbiological aspects of sepsis models in accordance with Sepsis 3 criteria (15')

10:45-11:00 | Coffee break

11:00-12:45 | CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

Chairs: Andrey V. Kozlov, Marietta Poles

Vladimir Jakovljevic *(Kragujevac, Serbia)*
Preconditioning by ion channel modulation in isolated rat heart (15')

Vladimir Zivkovic *(Kragujevac, Serbia)*
Effects of ischemic and preconditioning with proton pump inhibitors on functional recovery in isolated rat heart (15')

Jovana Bradic *(Kragujevac, Serbia)*
The effect of potassium-cyanide on isolated rat heart after short-term ischemia (15')

Andras Budai *(Budapest, Hungary)*
ALPPS: Bioenergetic maladaptations during induced liver regeneration (15')

Gabor Varga *(Debrecen, Hungary)*
Effect of early and delayed remote ischemic preconditioning on hemodynamic, hemorheological and microcirculatory parameters in a rat renal ischemia-reperfusion model (15')

Julia Jilge *(Vienna, Austria)*
Therapeutic efficiency of human adipose-derived stem cell secretome (ASC-Sec) in emergency setting: in vitro and vivo studies (15')

Andreia Luís *(Vienna, Austria)*
Endoplasmic Reticulum Stress and Unfolded Protein Response in shock and inflammation (15')

12:45-13:45 | Lunch break

13:45-14:15 | Oroboros innovations: O2k-FluoRespirometer and DatLab 7
Erich Gnaiger (Innsbruck, Austria) (30')

14:15-16:15 | BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES I.
Chairs: Erich Gnaiger, Beata Velika

Frédéric Bouillaud *(Paris, France)*
Sulfide and mitochondrial bioenergetics (30')

Andrey V. Kozlov *(Vienna, Austria)*
Alterations in nitric oxide homeostasis during traumatic brain injury, interplay with mitochondria and glutamate toxicity (30')

Andrea Müllebner *(Vienna, Austria)*
How do heme oxygenase and nitric oxide synthase regulate macrophage functions (15')

Dániel Érces *(Szeged, Hungary)*
Differentiation of pulmonary and mesenteric perfusion disorders from exhaled methane concentrations (15')

Gábor Bari *(Szeged, Hungary)*
Effect of methane inhalation in a large animal model of extracorporal perfusion (15')

Marietta Poles *(Szeged, Hungary)*
Effect of methane inhalation on nitrosative stress during mesenteric ischemia/reperfusion in rats (15')

16:15-16:30 | Coffee break

16:30-18:20 | BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES II.
Chairs: Frédéric Bouillaud, Ana Ledo

Erich Gnaiger *(Innsbruck, Austria)*
Competitive inhibition of respiration by nitric oxide and oxygen in cells with iNOS overexpression: A high-resolution respirometric study and kinetic model. (30')

László Juhász *(Szeged, Hungary)*
Inhibition of N-methyl-D-aspartate receptors improves polymicrobial sepsis-evoked mitochondrial dysfunction in rats. (15')

Eszter Tuboly *(Szeged, Hungary)*
Excessive alcohol consumption leads to non-microbial endogenous methane production (15')

Dániel Érces *(Szeged, Hungary)*
Effects of methane inhalation on platelet function in a large animal model of cardiac tamponade (10')

Petra Varga *(Szeged, Hungary)*
Newly discovered methane donor molecules with antioxidant potential (10')

18:20-19:20 | Dinner

19:20-20:30 | Project discussions

DAY 2 | 2018-03-27 TUESDAY | IOC127 Oroboros O2k WORKSHOP ON HYPOXIA

09:00-10:30 | MORNING SESSION ON MITOCHONDRIA

Chairs: László Tretter, Tímea Komlódi

Petr Pecina *(Prague, Chech Republic)*
Changes in oxygen kinetics between COX4i1 and COX4i2-containing COX (30')

László Tretter *(Budapest, Hungary)*
Bioenergetical approach of neurodegenerative diseases (30')

László Juhász *(Szeged, Hungary)*
$Ca^{(2+)}$N it be measured? High-resolution O2k-FluoRespirometric detection of extramitochondrial calcium movement (10')

Dávid Kurszán Jász *(Szeged, Hungary)*
Mitochondrial effects of methane gas treatment on rat cardiomyocytes subjected to simulated ischemia/reperfusion (10')

Eszter Tuboly *(Szeged, Hungary)*
Novel cardioprotective effects of methane in an ex vivo model (10')

10:30-10:45 | Coffee break

10:45-12:15 | Oroboros O2k WORKSHOP ON HYPOXIA I. - DEMO EXPERIMENT

András Mészáros *(Innsbruck, Austria* and *Szeged, Hungary)*
High-resolution mitochondrial oxygen kinetics in aerobic-anaerobic transitions (90')

12:15-13:00 | Lunch break

13:00-13:30 | Invited lecture on mitochondria

Christos Chinopoulos *(Budapest, Hungary)*
Interplay of respiratory components and mitochondrial diaphorases on redox state in anoxia (30')

13:30-14:30 | Oroboros O2k WORKSHOP ON HYPOXIA II.

Tímea Komlódi *(Innsbruck, Austria)*
Oxygen dependence of H_2O_2 production: application of High-Resolution FluoRespirometry with Amplex UltraRed(R) fluorescence. (60')

14:30-14:45 | Coffee break

14:45-16:15 | Oroboros O2k WORKSHOP ON HYPOXIA III.

Erich Gnaiger *(Innsbruck, Austria)*
Cellular respiration and mitochondrial oxygen kinetics: hypoxic steady-states and oxygen oscillations (90')

16:15-16:30 | Discussions

16:30-18:00 | COST ACTION MitoEAGLE
Chairs: Petr Pecina, Nina Krako

Ana Ledo *(Coimbra, Portugal)*
Age-dependent changes in the glutamate-nitric oxide pathway in the hippocampus of the triple transgenic model of Alzheimer's disease: implications on mitochondrial function (15')

Nina Krako *(Belgrade, Serbia)*
Investigation of subcellular mechanisms in insulin resistance models in hepatocytes and myocytes (15')

Beata Velika *(Kosice, Slovakia)*
The effect of short-term exposure to moderate altitude on respiration of peripheral-blood mononuclear cells (15')

Andrea Evinova *(Bratislava, Slovakia)*
Magnesium protects from calcium induced collapse of mitochondrial trans-membrane potential (Poster)

Kasja Pavlovic *(Belgrade, Serbia)*
C2C12 myoblasts as a cell model for studying the role of mitochondria in insulin resistance (Poster)

Ioana Z. Pavel *(Timisoara, Romania)*
The acute and chronic effects of a benzylamide derivative of maslinic acid in liver mitochondria isolated from mice with chemically induced skin carcinogenesis (Poster)

Conference abstracts

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SEPSIS AND SYSTEMIC INFLAMMATION

Minimum Quality Threshold in Pre-Clinical Sepsis Studies (MQTiPSS): an International Expert Consensus Initiative for Improvement of Animal Modeling in Sepsis.

Marcin F. Osuchowski

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in the AUVA Research Center, Vienna, Austria

Pre-clinical animal studies are mandatory before new treatments can be tested in clinical trials. However, their use in developing new therapies for sepsis has been controversial because of limitations of the models and inconsistencies with the clinical conditions. In consideration of the revised definition (Sepsis-3) and guidelines for clinical sepsis and septic shock, a Wiggers-Bernard Conference was held in Vienna in May 2017 titled: "*Pre-clinical Modeling in Sepsis: Exchanging Opinions and Forming Recommendations*". The goal of this conference was to identify limitations of pre-clinical models and to propose a set of guidelines, defined as the "*Minimum Quality Threshold in Pre-Clinical Sepsis Studies*" (MQTiPSS), to enhance translational value of the sepsis models. A total of 30 experts from 12 countries participated and were divided into 6 thematic Working Groups: 1) study design, 2) humane endpoints, 3) infection types, 4) organ failure/dysfunction, 5) critical fluid resuscitation and 6) antimicrobial therapy endpoints. Overall, the Wiggers-Bernard Conference participants reached consensus on 29 points; 20 at "recommendation" strength and 9 at "consideration" strength. The synopsis of the consensus is to-be-published as Executive Summary, while a detailed description of all approved MQTiPSS points is to-be-found in three full-length independent publications (Parts I-III). Each publication is built on two (related) Working Group themes and includes a narrative clarifying caveats and intricacies related to the accepted consensus points. We hope that these recommendations and considerations will serve to bring a level of standardization to pre-clinical models of sepsis and ultimately improve the translation of pre-clinical findings. These recommendations and considerations are proposed as "best practices" for animal models of sepsis that should be implemented.

SEPSIS AND SYSTEMIC INFLAMMATION

Effects of splenectomy on early immuno-inflammatory responses to trauma-hemorrhage and survival in secondary sepsis

S. Drechsler[1], P. Rademann[1,2], J. Zipperle[1], M. Jafarmadar[1], A. Klotz[1], S. Bahrami[1], M.F. Osuchowski[1]

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[2] Current Address: Center for Experimental Medicine, Medical Faculty, University of Cologne, Cologne, Germany

In trauma patients, mechanism and severity of injury are important factors influencing the susceptibility to secondary infections. The role of splenectomy in traumatic injuries is unclear, ranging from negative immunologic consequences to protective effects. We aimed to investigate the effects of splenectomy on polytrauma modeling and on survival in secondary sepsis. 12 weeks old, female BALB/c mice underwent either a 2-hit model consisting of a) polytrauma (TH, femur fracture and hemorrhagic shock with 40% blood loss) or b) polytrauma with splenectomy (TSH) (femur fracture, hemorrhagic shock with 30% blood loss of total blood volume and splenectomy) followed by cecal ligation and puncture (CLP) 48h later or c) CLP alone (without preceding trauma) to create polymicrobial abdominal sepsis. A separate set of mice underwent TSH or TH alone. We found that additional splenectomy increased the 28-day survival in secondary sepsis to 92%, while preceding TH lowered it to 46% compared to 62% survival in the CLP alone group ($p<0.05$). TSH induced stronger neutrophilia and lymphocytosis compared to TH mice. Moreover, TSH but not TH resulted in a rise of regulatory and cytotoxic T-cells ($p<0.05$) and significantly reduced the IL-6, CXCL-1 and MCP-1 release after CLP. Phagocytic capacity of leukocytes was increased after TSH but not TH. Additional splenectomy strongly modified the immuno-inflammatory responses after polytrauma and was life-saving after secondary septic insult. Based on our results we conclude that it is recommendable to do an end-effect verification of a polytrauma model before it is deemed and utilized as a clinically relevant experimental platform.

Supported by FWF Science Fund, Grant Number T707-B13

SEPSIS AND SYSTEMIC INFLAMMATION

Therapeutic possibilities of microcirculatory and mitochondrial dysfunction in sepsis

József Kaszaki, Andrea Szabó

Institute of Surgical Research, University of Szeged, Szeged, Hungary

Sepsis remains one of the leading causes of death at the intensive care units, because its specific therapeutic targets are still missing. Sepsis is not a “definitive disease”, but an extremely complex chain of inflammatory events, cellular reactions and circulatory abnormalities. The basic problem in sepsis is the discrepancy between oxygen delivery and oxygen consumption which can lead to irreversible oxygen extraction deficit and energy shortage. The cornerstone of acute care should be to prevent, assess and treat oxygen debt globally. We propose that causative factors and signs of oxygen deficit have to be examined and evaluated simultaneously on microcirculatory, cellular (endothelial) and subcellular (mitochondrial) levels in shock-affected organs by employing sufficiently long-term, clinically relevant experimental animal models. According to our current knowledge, the oxygen extraction deficit can originate either from an insufficient oxygen delivery to the cells, or the inability of the cells to utilize oxygen. On one side, the poorly functioning microvasculature with deterioration of the endothelial glycocalyx results in insufficient oxygen delivery to the tissue. On the other side, mitochondria are unable to use oxygen efficiently, and the switch to anaerobic pathways causes energy deficit and eventual cell death. These processes are interlinked and the host-pathogen interactions are finally leading to a combined microcirculatory and mitochondrial distress syndrome. The major (final) goal of our study is to find optimal, clinically applicable manoeuvres for microcirculatory recruitment and mitochondrial resuscitation to minimize the energy deficit of organs during the septic response by (1) pharmacological inactivation of targeted molecules of the inflammatory cascade; (2) treatment with kynurenic acid or synthetic kynurenic acid analogues, and (3) glycocalyx-dependent goal-directed fluid resuscitation strategies.

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SEPSIS AND SYSTEMIC INFLAMMATION

Potential role of endothelin receptors in the therapy of experimental sepsis

Attila Rutai, Roland Fejes, Szabolcs Tallósy, Marietta Zita Poles, László Juhász, Liliána Kiss, András Mészáros, Mihály Boros, József Kaszaki

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Introduction: The hypoxia-sensitive endothelin (ET) system plays important roles in flow regulation through vasoconstrictor ET-A and ET-B2 receptors and vasodilator ET-B1 receptors (ETAr; ETBr), respectively. Tissue hypoxia during the progression of sepsis is associated with an imbalance between the oxygen delivery and cellular oxygen consumption that might result in microcirculatory disturbances and mitochondrial dysfunction. Our aim was to investigate the possible influence of a selective ETAr antagonist and ETBr agonist treatment on oxygen dynamics, together with intestinal microcirculatory and mitochondrial respiration parameters in a clinically-relevant rat model of sepsis.
Methods: Anesthetized male rats were subjected to faecal peritonitis (n=32; 0.6 g/kg faeces ip) or sham-operation (n=8). Animals with confirmed sepsis were treated with sterile saline solution (0.9% NaCl2 iv; n=8), or received the ETAr antagonist ETR-p1/fl peptide (100 nmol/kg iv; n=8) or the ETBr agonist IRL-1620 (0.55 nmol/kg iv; n=8) treatments, 24 hr after peritonitis induction. Invasive hemodynamic monitoring (blood pressure, cardiac output measurements) and blood gas analyses were performed during a 150-min observational window. Intestinal microcirculatory parameters (perfusion rate, red blood cell velocity=RBCV) were investigated by orthogonal polarization spectral imaging technique. Complex I and complex II-linked (CI; CII,) mitochondrial respiration were evaluated in liver biopsies using high resolution respirometry. The mitochondrial respiratory function was determined by calculating the respiratory control ratio (RCR) and oxidative phosphorylation (OxPhos).
Results: The septic reaction was characterized by significant hypotension and elevated cardiac output, decreased microperfusion, oxygen extraction and reduced CI and CII-linked OxPhos and RCR values. The ETAr antagonist treatment significantly increased the RBCV and oxygen extraction and increased both the CII-OxPhos and CII-RCR values. The ETBr agonist treatment prevented the sepsis-induced hypotension and the decrease in oxygen extraction, and significantly increased the ileal microcirculatory parameters and the mitochondrial CII-OxPhos values.
Conclusion: The selective ETBr agonist countervailed the peritonitis-induced hypotension, while the ETAr antagonist maintained oxygen dynamics, thus a mixed ET receptor targeted treatment regime may offer hope for a simultaneous microcirculatory and mitochondrial resuscitation strategy in sepsis.

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SEPSIS AND SYSTEMIC INFLAMMATION

Microbiological aspects of sepsis models in accordance with Sepsis 3 criteria

Szabolcs P. Tallósy[1], Attila Rutai[1], László Juhász[1], Marietta Poles[1], Liliána Kiss[1], Roland Fejes[1], Edit Urbán[2], Dániel Érces[1], Mihály Boros[1], József Kaszaki[1]

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Introduction: According to the criteria of "Sepsis-3" consensus conference (Singer M, 2016), human sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. The related literature is exhaustive of several aspects of sepsis progression but the microbiological background is relatively less discussed. Our aim was to investigate and define the microbiological characteristics of peritonitis induced sepsis models from this aspect.
Material and Methods: Organ dysfunctions and microbiological profile were determined in peritonitis induced sepsis models (SPRD rats, n=22; Vietnamese minipigs n=18) by peritoneal instillation of polymicrobial fecal content (0.6 g/kg in both models). Invasive hemodynamic monitoring and blood gas analyses were performed in anaesthetized animals between 18-24 hrs of sepsis. Respiratory, cardiovascular and renal dysfunctions were assessed by the Sequential Organ Failure Assessment (SOFA) scoring systems. The initial concentration (by counting a total number of colony forming units) and composition of the microorganisms were compared with the samples taken from the abdominal fluid 20 hrs after sepsis induction. Microorganisms were isolated and identified with species selective media and MALDI-TOF mass spectrometry.
Results: Dose of the initial microorganism suspension causing sepsis was adjusted (rat model: 1.2×10^6 – 3.4×10^6 CFU; pig model: 4.95×10^6 – 3.37×10^7 CFU) and the lethal dose was over 4.8×10^6 CFU in rats. Two severity groups of sepsis (moderate and severe) were defined in case of pigs according to SOFA score. *Escherichia coli* and *Klebsiella pneumoniae* were the most common bacteria isolated from the fecal samples and from the abdominal fluid in both models. The concentration of microorganisms was lower (67%) in the abdominal fluid as compared to the initial suspension. Almost half of strains (46%) disappeared in the abdominal fluid sample and new strains (eg. Bacteroides sp., Staphylococcus sp.) were found in severe sepsis in both models.
Conclusion: No correlation was observed between the initial CFU and SOFA scores after 24 hrs of sepsis induction. Adjusting the concentration of the initial microorganism suspension can be an applicable method to meet Sepsis 3 criteria in these experimental models.

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CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

Preconditioning by ion channel modulation in isolated rat heart

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Aim of this study is to examine the cardioprotective effects of verapamil, amlodipine or nicorandil on ischemic-reperfusion injury of isolated rat heart and also to examine the role of oxidative stress. The hearts (n=48 total, 12 per group) were divided into four groups, one control and three experimental depending on acute administrated pharmacological agents (verapamil - 0,63 μmol/l, amlodipine - 0,1 μmol/l or nicorandil - 200 μmol/l). Rats were sacrificed and isolated hearts were perfused on a Langendorff apparatus. After stabilisation period, and 5 minutes long preconditioning by appropriate drug hearts were subjected to ischemia and reperfusion (30/60 minutes). During *ex vivo* protocol, the cardyodinamic parameters of myocardial function were continuously registered, while from coronary effluent we measured the oxidative stress parameters (TBARS, NO_2^-, O_2^-, H_2O_2). Our results clearly show that all administrated drugs improved functional recovery of ischemic heart and diminished all measured oxidative stress parameters. Examined ion channel modulators might be beneficial for preventing ischemic myocardial injury. Results of our study may be a starting point for further researchers which would fully clarify effect of these drugs on ischemic heart.

Key words: preconditioning, ion channel, isolated heart, rat

CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

Effects of ischemic and preconditioning with proton pump inhibitors on functional recovery in isolated rat heart

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Recent data indicate that in addition to ischemic preconditioning, the use of drugs from the group of proton pump inhibitors (PPI) can have a protective effect on ischemic myocardium. The aim of present study was to estimate the effects of ischemic and PPI preconditioning on the functional recovery of myocardium after global ischemia in a model of isolated rat heart. The rat hearts isolated from male Wistar albino rats were retrogradely perfused according to Langendorff technique at constant perfusion pressure of 70cm H_2O. After insertion the sensor in left ventricule parameters of cardiac function (dp/dt max, dp/dt min, SLVP, DLVP and HR) were continuously recorded. Coronary flow was measured by using flowmetric method. In control group, hearts were submitted to twenty-minute ischemia and thirty-minute reperfusion. Group with ischemic preconditioning was induced the two minute ischemia and four-minute reperfusion after it was induced twenty-minute ischemia and established thirty-minute reperfusion. In PPI groups, hearts were perfused with omeprazole, lansoprazole and pantoprazole in dose of 100μM/l during five-minute, and after that submitted to twenty-minute ischemia and thirty-minute reperfusion. Preconditioning with PPI has led to the better response of contractility and ishemic preconditioning lead to better recovery of heart rate and coronary flow in model of isolated rat heart. Both ischemic and PPI preconditioning may be powerful tools in preserving of cardiac function after ischemia. Deeper understanding of their effect requires future and more complex investigations.

Key words: ischemic preconditioning, proton pump inhibitors, isolated heart, rat

CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

The effect of potassium-cyanide on isolated rat heart after short term ischemia

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Pharmacological postconditioning (PostC) is proposed as a great and practicable strategy to limit the tissue damage due to ischemia-reperfusion (I/R) injury by application of different pharmacological agents during reperfusion. Therefore the aim of our study was to examine the effects of pharmacological PostC with potassium-cyanide (KCN) on functional recovery and oxidative stress of isolated rat heart subjected to I/R. Rats were divided into control and experimental group (n=10 for each group). The hearts of male *Wistar albino* rats were excised and retrogradely perfused according to the *Langendorff* technique at constant perfusion preassure of 70 cmH_2O. In control group after stabilization, hearts were subjected to global ischemia for 5 minutes, followed by reperfusion for 5 minutes. While in experimental group hearts underwent global ischemia lasting 5 minutes, and then submitted 5 minutes of reperfusion with 10 μmol/L KCN. The parameters of cardiac function including the maximum and minimum rate of pressure development, systolic and dyastolic left ventricular pressure and heart rate were continuously registered. Coronary flow was measured flowmetrically. Levels of superoxide anion radical, hydrogen peroxide, nitrites and index of lipid peroxidation (measured as thiobarbituric acid reactive substances) were determined spectrophotometrically in coronary venous effluent. Our results illustrated that application of KCN improved recovery of cardiac contractility and systolic and diastolic function and it was associated with the less production of pro-oxidants. These findings demonstrate that KCN exerted promising cardioprotective effects in acute myocardial infarction, partially mediated via attenuation of oxidative stress.

Key words: preconditioning, potassium-cyanide, isolated heart, rat

CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

ALPPS: Bioenergetic maladaptations during induced liver regeneration

Andras Budai[a], Gergo Horvath[b], Laszlo Tretter[b], Zsolt Radak[c], Erika Koltai[c], Zoltan Bori[c], Ferenc Torma[c], Akos Lukats[d], Pal Rohlich[d], Attila Szijarto[a], Andras Fulop[a]

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Associating Liver Partition and Portal vein Ligation for Staged hepatectomy (ALPPS) is a novel two-staged hepatectomy strategy, which induces immense regeneration in the remnant liver. However, the fast volume gain has the benefit for resectability, this technique presents alarmingly high mortality and morbidity rates. The aim of this study was to evaluate the background of this significant vulnerability of patients underwent ALPPS by assessing mitochondrial function, biogenesis and morphology during ALPSS induces liver regeneration.
Male Wistar rats (n=100) underwent portal vein ligation (PVL) or ALPPS. The animals were sacrificed at 0 (without operation) and 24, 48, 72 or 168 hours after the interventions. Regeneration rate was calculated and proliferation index was assessed. Mitochondrial O_2 consumption was evaluated using high-resolution oxygraphy and ATP production was measured by spectrophotometry. Mitochondrial biogenesis was evaluated by the western blot measurement of the protein levels of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) nuclear respiratory factor-1 and -2 (NRF1, NRF2) and mitochondrial transcription factor alpha (mTFA). In addition, mitochondrial morphology was evaluated by electron microscopy.
The regeneration rate and the Ki-67 index were significantly elevated in the ALPPS group compared to the PVL group. ($p<0,01$) In the ALPPS group mitochondrial state 3 and state 4 ATP production and oxygen consumption levels were significantly lower 48h after the interventions comparing to the PVL group. ($p<0,05$) Along with this, mitochondrial biogenesis was diminished, as PGC1-α and NFR1 levels were significantly decreased 48 and 72h after ALPPS compared to the PVL group. Besides significantly smaller mitochondria were present after ALPPS.
Conclusion: The deteriorations in mitochondrial function and biogenesis alongside with the fast and energy demanding cell proliferation can cause the energetic destabilization of the hepatocytes, which might be a significant factor in the higher vulnerability of patients underwent ALPPS.

CIRCULATORY SHOCK AND ISCHEMIA-REPERFUSION

Effect of early and delayed remote ischemic preconditioning on hemodynamic, hemorheological and microcirculatory parameters in a rat renal ischemia-reperfusion model

Gábor Varga, Souleiman Ghanem, Balázs Szabó, Kitti Nagy, Noémi Pál, Gábor Nadubinszky, Viktória Somogyi, Bence Tánczos, Ádám Deák, Katalin Pető, Norbert Németh

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Remote ischemic preconditioning (rIPC) is a known procedure for reducing renal ischemia-reperfusion (I/R) injury, but literature data are controversial. However, the optimal timing of preconditioning and its effect on hemorheology and microcirculation are still unclear. We aimed to investigate these issues.
In anaesthetised rats (permission number: 25/2016/UDCAW), after laparotomy left kidney preparation and left femoral artery cannulation were performed in the sham-operated group (n=7). In the I/R-group (n=7) a 45-minute left renal ischemia was carried out with microclip and reperfusion was monitored for 120 minutes. A tourniquet was applied around the right hind limb below the level of the inguinal ligament for 3x10 minutes (with 10-minute reperfusion periods), one hour (rIPC-1, n=7) or one day (rIPC-24, n=6) before the I/R. Blood samples were taken at the beginning of the operation and at the 30th, 60th and 120th minute of the reperfusion, for determining hematologic and hemorheological parameters (red blood cell (RBC) deformability and aggregation). By the end of the reperfusion acid-base parameters, metabolites and electrolyte concentrations were also measured. Arterial mean pressure, heart rate, respiratory rate, rectal temperature, surface temperature and microcirculation of the liver, kidneys and small intestine were tested.
The rIPC-1 group's blood pressure increased the most while giving the lowest pH and highest lactate values. In contrast to the rIPC-groups, during the reperfusion microcirculation increased in the I/R-group compared to the base values, and the highest leukocyte count and heart rate were also measured in this group. Deformability decreased in all ischemic groups, least in the rIPC-24 group, while the aggregation index elevated the most here.
Our results show that 45-minute renal I/R caused significant changes in all parameters. However, based on these parameters, it is not possible to determine which rIPC protocol is more effective in reducing the I/R injury in rats.

Efficacy of human adipose-derived stem cell secretome (ASC-Sec) in critical illness: in vitro and in vivo studies

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Recently we have shown that the systemic administration of rat ASC-Sec ameliorates the inflammatory reactions and the resulting organ damage caused by hemorrhagic traumatic shock (HTS) in rats (Ashmwe et. al. 2017).

The aim of this pilot study was to examine the efficacy of human ASC-Sec in the same model. In order to minimize in vivo experiments, efforts are made to establish an in vitro test system for quality control of human ASC-Sec.

Human ASC were isolated from liposuction material, characterized, cultured and the supernatant (ASC-Sec) was extracted after 24 hours.

In vivo; rats were subjected to HTS, receiving human ASC-Sec or vehicle 20 minutes after onset of reperfusion. Shock intensity, inflammatory response (IL-6, IL-10, MCP-1, GRO-α, Rantes, Leptin) and cell/organ injury were evaluated. HTS induced an inflammatory response e.g. reflected in an IL-10 increase, peaking at 24 hours in both groups with no difference. Circulating markers of cell injury (CK, LDH) and organ function (ALT, UREA, CREA) increased in both groups similarly up to 24 hours after shock.

In vitro; considering the well-known anti-inflammatory effects of stem cells, whole blood or isolated white blood cells stimulated with LPS/bacteria are tested to validate and optimize the biological activity of human ASC-Sec.

In contrast to the rat ASC-Sec, human ASC-Sec did not show any efficacy in rats. On-going studies aim to improve human ASC-Sec quality by preconditioning of the ASCs or an accumulation of the active components in the ASC-Sec.

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Sulfide and mitochondrial bioenergetics

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The three gasotransmitters described in mammals (NO, CO and H_2S) are inhibitors of the respiratory enzyme cytochrome oxidase. Complete inhibition takes place with low micromolar concentrations, as cyanide. There are potent H2S producers in nature: volcanoes and hydrothermal vents, anaerobic metabolism of sulfur in the seashore or swamps, and finally the microbiota inside the digestive tract. All could generate sulfide concentrations well above the toxic limit. Some bacteria use H_2S as an electron donor and this is true also for mitochondria. Hydrogen sulfide is metabolized by a sulfide quinone reductase (SQR) that feeds with electrons the mitochondrial respiratory chain. SQR enzyme is widely expressed and shows a high affinity for sulfide. Sulfide is thus a two-sided molecule: substrate or poison according to the concentration.

The primary product of sulfide oxidation is thiosulfate and this oxidation requires more oxygen than normal respiration. Therefore, sulfide oxidation increases mitochondrial/cellular oxygen consumption. For a given sulfide generation rate a cellular system would evolve towards two opposite endpoints: If sulfide oxidation exceeds sulfide production rate then respiration is maintained over time and sulfide concentration is maintained (driven to) low nM values. At the opposite, if sulfide flux exceeds, even marginally, SQR activity the initiation of inhibition of respiration results in a positive feedback that accelerates greatly sulfide accumulation and completion of respiration inhibition. With an experimental model the consequences on the apparent cellular affinity for oxygen were demonstrated.

BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Alterations in nitric oxide homeostasis during traumatic brain injury, interplay with mitochondria and glutamate toxicity

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Traumatic brain injury (TBI) is associated with the development of neuroinflammation, mitochondrial dysfunction, and glutamate toxicity. Here we show that up-regulation of key neuroinflammatory genes, nitric oxide synthase (iNOS), and tumor necrosis factor (TNF)-alpha-receptor by 4 h after TBI was accompanied by a decrease in mitochondrial respiration with glutamate but neither with pyruvate nor succinate in ipsilateral cortex, while after 3 d the rate of both succinate and glutamate (but not pyruvate) respirations were decreased in injured cortex. This suggests that at early phase after TBI electron transport through complex I is specifically impaired in injured cortex tissue possibly by reactive oxygen and nitrogen species derived from neuroinflammation and occurring simultaneously with mitochondrial dysfunction. The impairment of respiration with glutamate, but not with pyruvate, suggests that the defect is located in the enzymatic node of tricarboxylic acid (TCA) cycle providing electrons from glutamate to complex I (so called “glutamate node”) rather than in complex I itself. To localize the defect within TCA cycle, we determined the activities of major enzymes of the glutamate node (glutamate dehydrogenase, oxoglutarate dehydrogenase complex (OGDHC) and glutamine synthase) as well as activities of other enzymes critically involved in the transport of electrons to complex I (pyruvate dehydrogenase, malate dehydrogenase, and malic enzyme). We found that among all studied enzymes only OGDHC displayed remarkably decreased enzymatic activity. A subcutaneous bolus injection of thiamine, a coenzyme of OGDHC, prior to trauma recovered the activity of OGDHC as well as respiration with glutamate at 4 h, and also both glutamate and succinate respirations at 3 days. An increase in the activity of OGDHC caused by thiamine can be due to either posttranslational activation of OGDHC by thiamine or/and genetic upregulation of this enzyme. To test the latter assumption, we determined the mRNA of the OGDHC sub-units (E1k and E3). We did not find any difference in the expression of these genes either 4 hours or 3 days after TBI, suggesting that observed changes were exclusively due to posttranslational activation of OGDHC by thiamine. Our data suggest that OGDHC is the major site of damage to mitochondria upon TBI. Further studies are required to evaluate the pathological impact of this mitochondrial defect on clinical outcome after TBI and therapeutic potential of thiamine.

BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

How do heme oxygenase and nitric oxide synthase regulate macrophage functions

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Macrophages are cells of the innate immune system that populate every organ. They are required not only for defense against invading pathogens and tissue repair but also for maintenance of tissue and iron homeostasis. The two enzyme systems nitric oxide synthase (NOS) and heme oxygenase (HO), which generate the gaseous messenger nitric oxide (NO) and carbon monoxide (CO), are important for macrophage function. NOS is required for an efficient elimination of ingested pathogenic material. HO is the rate limiting enzyme in heme catabolism and essential for restoring homeostasis by limiting inflammation.

The aim of this study was to understand how NOS and HO contribute to the regulation of NADPH oxidase (NOX) activity and phagocytosis, key components of macrophage function, and whether mitochondria are involved.

J774A.1 macrophages were treated with hemin or vehicle and activity of NOS, HO, and NOX was inhibited using specific inhibitors. Reactive oxygen species (ROS) formation was determined by Amplex© red assay, mitochondrial function was assessed using extracellular flux analyzer (XFe96, Agilent), and phagocytosis was measured using fluorescein isothiocyanate-labeled bacteria. In addition, the fate of the ingested heme was analyzed using electron spin resonance spectroscopy.

We found that NOS activity does not directly affect phagocytosis, but inhibits mitochondrial respiration and stimulates NOX activity by triggering mitochondrial ROS production in a nitric oxide (NO) dependent manner. In contrast, HO activity neither clearly affects NOX activity nor mitochondrial function or phagocytosis. However, treatment of macrophages with hemin results in intracellular accumulation of ferrous heme. Heme accumulation compromises mitochondrial integrity by inducing an increased proton leak of the inner mitochondrial membrane and leads to an inhibition of phagocytosis. Both mitochondrial dysfunction and impaired phagocytosis were further aggravated upon inhibition of HO.

In conclusion our results suggest that effective degradation of heme by HO together with active NOS which amplifies NOX activity is essential for macrophages to exert their full biocidal potential.

BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Diagnostic differentiation of pulmonary and mesenteric perfusion disorders with detection of exhaled methane concentrations

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Mesenteric hypoperfusion frequently develops in patients admitted to intensive care units. Methane produced in the gastrointestinal tract can be present in the exhaled breath, thus methane in the breath (Ex-CH_4) may correlate with the mesenteric circulatory status and therefore this phenomenon may have diagnostic potential. However, concomitant damage of respiratory function can also influence Ex-CH_4 levels, thus our aim was to investigate this possibility in a comparative in vivo experimental protocol.

Anesthetized, ventilated male Sprague-Dawley rats received CH_4-enriched 0.9% saline (CH_4-NaCl; 10 ml/kg/h) inraluminally (into the intestinal lumen) or intravenously. Two series of experiments were carried out on 4-4 groups (n=6 each). Group 1 (*iv* administration) and Group 2 (intraluminal administration) underwent 45-min mesenteric ischemia (MI), in Groups 3 (*iv* administration) and 4 (intraluminal administration) the left pulmonary artery was occluded (PuI) for 5 min. In Study I CH_4-NaCl adm (intraluminally or *iv*) was given 10 min before the vascular occlusions, while in Study II the vascular occlusions preceded CH_4-NaCl administration. Photoacoustic laser spectroscopy was used for online Ex-CH_4 measurements; microcirculatory changes were recorded with intravital videomicroscopy (Cytocam-IDF, Braedius).

Study I: Intraluminal CH_4-NaCl significantly increased the Ex-CH_4. Induction of MI or PuI caused immediate Ex-CH_4 decreases, the values increased only after the restoration of the organ circulation. After *iv* CH_4-NaCl administration, the Ex-CH_4 increased before the vascular occlusions and decreased only after PuI.
Study II: Intraluminal CH_4-NaCl administration did not increase Ex-CH_4 until the end of MI. After *iv* administration the Ex-CH_4 elevated promptly. Intraluminal or *iv* CH_4-NaCl administration during PuI did not increase Ex-CH_4.

Dynamic detection of Ex-CH_4 levels can track the intestinal and pulmonary microcirculatory changes non-invasively and can differentiate between ischemia of the lungs and the bowels. The method can be suitable for the monitoring of mesenteric- or pulmonary circulation when the baseline Ex-CH_4 level is low.

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BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Effects of methane inhalation in a large animal model of extracorporeal perfusion

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Extracorporeal circulation (ECC) is frequently applied during cardiac surgical interventions but the technique has several unwanted complications. The large artificial surface of tube- and membrane components of ECC units activates the immune- and inflammatory cascade systems and the modulation of the developing systemic inflammatory response is a great challenge of cardiac surgery. The objectives of our study were to establish a clinically relevant large animal model of ECC and to examine the antiinflammatory efficacy of methane in this experimental set-up.

Anesthetized, ventilated minipigs were randomly allocated in two experimental groups (n=5 each). In both groups the same surgical interventions and central ECC cannulations were performed. After surgical preparations ECC was started and maintained for 120 min. In the post-ECC period the animals were monitored for further 180 min. In group 2 the animals received 2.5% methane-air mixture through the oxygen line of the oxygenator. During the post-ECC period circulatory support included norepinephrine (Arterenol) infusion. At the end of the experiments intestinal and heart tissue samples were taken for myeloperoxidase (MPO) and xanthine oxidoreductase (XOR enzyme activity measurements.

As a result of the ECC the MPO and XOR activities were increased in the ileum and the heart. In the methane-treated group MPO activity was decreased in the ileum, the XOR activity was reduced at both locations by the end of post-ECC period, and the need for norepinephrine support was also significantly lower (~30%) than in the control group without methane treatment.
This large animal model is suitable to investigate the clinical course of ECC and its concomitant inflammatory response. Our preliminary data demonstrate that exogenous methane treatment can be effective means to modulate the oxidative stress caused by ECC.

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BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Effect of methane inhalation on nitrosative stress during mesenteric ischemia/reperfusion in rats

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The aim was to test the hypothesis that inhaled methane may modulate the evolution of nitroxidation and the reaction of nitrergic neurons to transient gastrointestinal ischemia. The main components of the nitrosative stress reaction were characterized together with quantitative changes of the nitrergic myenteric neurons in adjacent intestinal segments in anesthetized rats (n=124) inhaling normoxic air with or without 2.2% methane. Serosal microcirculatory parameters (intravital videomicroscopy), the relative ratios of nitrergic neurons (immunohistochemistry), xanthine oxidoreductase (XOR) activities, local NO production (EPR technique), nitrite/nitrate (NOX), and nitrotyrosine levels were measured in the duodenum, ileum and colon after the occlusion of the superior mesenteric artery or after ischemia and reperfusion.
The intramural flow stopped completely only in the ileum the ischemic phase. The highest baseline XOR activity was measured in the duodenum, which increased further during ischemia ($p<0.05$). Here NO and nitrotyrosine levels increased while the NOX level and the nitrergic neuron ratio decreased (from 28.5% to 23.91%). Reperfusion uniformly elevated XOR activity and nitrotyrosine formation, with the highest level attained in the duodenum, where the nitrergic neuron ratio remained depressed These changes were eliminated in methane-treated animals, the XOR activity decreased in all intestinal segments, and the duodenal nitrergic neuron ratio was re-established to 30.6%.
The risk for nitrosative stress is highest in transiently hypoxic tissues with high endogenous XOR activities. Inhaled methane inhibits XOR thus reduces nitroxidation which leads to the protection of nitrergic neuron population.

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Competitive inhibition of respiration by nitric oxide and oxygen in cells with iNOS overexpression: A high-resolution respirometric study and kinetic model.

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Mitochondrial respiratory control by oxygen is modified by nitric oxide which, in turn, exerts a direct influence on intracellular oxygen levels as a result of the balance between oxygen supply and demand. High-resolution respirometry has been developed to resolve the kinetics of cellular and mitochondrial respiration in the micro- and nanomolar oxygen range (Oroboros O2k, Innsbruck, Austria) [1]. Whereas diffusion of NO from the chamber presents a frequently ignored problem when using perspex chambers and Teflon-coated magnets, high-resolution respirometry with glass chambers, PEEK stirrers, and PVDF stoppers minimizes both NO and O_2 diffusion.

NO production was stimulated by addition of arginine to HEK 293 cells stably expressing human iNOS [2]. NO levels measured in the cell culture medium in the O2k increased to 0.04 and up to 2.2 µM as a function of iNOS activity, but declined below 15 to 45 µM oxygen due to oxygen limitation of iNOS and predominance of NO degradation over NO production at low oxygen levels. Conventional models of linear competitive inhibition [3] or competitive and non-competitive inhibition of cytochrome *c* oxidase were inadequate to explain the complex interplay between oxygen and NO at pathophysiological concentrations. We developed, therefore, a kinetic model of hyperbolic competitive inhibition to describe the inhibition of cell respiration at the level of cytochrome *c* oxidase by varying NO and oxygen concentrations at ROUTINE respiration and physiological substrates in the intact cell [2]. Data from continuous traces of O_2 and NO in the low-O_2 range <60 µM were fitted by iterative adjustment of the parameters. The predictive power of the model was then tested in an independent series of experiments in the high-O_2 range up to 240 µM, when NO concentrations changed simultaneously but independently up to 2 µM. The complex response of cell respiration to dynamically changing O_2 and NO concentrations could be accurately predicted by the kinetic model without further adjustment of the parameters.

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BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Inhibition of N-methyl-D-aspartate receptors improves polymicrobial sepsis-evoked mitochondrial dysfunction in rats

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Introduction: Sepsis-related changes in oxygen dynamics and subsequent multi-organ failure are associated with mitochondrial dysfunction and depleted energy supplies. Recently, N-methyl-D-aspartate receptor (NMDA-R)-facilitated increase in the intracellular calcium (Ca^{2+}) level has been suggested as major regulator for mitochondrial Ca^{2+} uptake and Ca^{2+} overload. However, the relationship between the NMDA-R–linked Ca^{2+} entry and the function of the electron transport system is not fully elucidated. Thus our main goal was to investigate whether NMDA-receptor antagonists, the natural kynurenic acid (KYNA) and its synthetic analogue, SZR-72 affect the mitochondrial respiration in a rat model of peritonitis-induced sepsis (PS).
Materials and Methods: Fecal peritonitis was induced in male Sprague Dawley rats (n=18; 0.6 g/kg bw i.p.), the animals in the control group were treated with 0.9% saline (n=6, ip.). NMDA-R antagonists KYNA or SZR-72 (160 µmol/kg ip) were administered at 3h and 22 h after PS induction (n=6-6, each), then at 24h the rats were sacrificed for the assessment of cellular respiratory function in liver tissue samples. Mitochondrial Complex-I dependent (CI; stimulation with exogenous glutamate/malate+ADP) and Complex-II dependent (CII; stimulation with exogenous rotenone+succinate+ADP) oxygen consumption were assessed from liver homogenates using high-resolution respirometry (Oroboros O2k, Oroboros Instruments, Innsbruck, Austria).
Results: Both the glutamate/malate supported LEAK respiration and the succinate supported LEAK respiration decreased. Similarly, both CI and CII-driven OXPHOS exhibited significantly lower values in septic rats (40% and 35%, respectively) compared to the sham-operated control animals. However, the PS-induced decrease in the CII-linked substrate oxidation and the CII-linked OXPHOS capacity were markedly restored either by KYNA (72%) or SZR-72 (90%) treatments, while CI-linked mitochondrial respiratory function was only affected by SZR-72 administration. In addition, electron transport coupled to ATP synthesis evaluated by respiratory control ratio, was significantly higher in SZR-72-treated rats (RCR 30%).
Conclusion: Our results suggest that inhibition of NMDA receptors, perhaps through the regulation of intra-mitochondrial Ca^{2+} pool and/or by attenuating the overproduction of reactive oxygen species, may modulate mitochondrial respiration and can improve ADP utilization to produce ATP.

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BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Excessive alcohol consumption leads to non-microbial methane generation

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OBJECTIVE Various studies have established the possibility of non-bacterial methane (CH_4) generation in oxido-reductive stress conditions in plants and animals. Increased ethanol input is leading to oxido-reductive imbalance in eukaryotes, thus our aim was to provide evidence for the possibility of ethanol-induced methanogenesis in non-CH_4 producer humans, and to corroborate the *in vivo* relevance of this pathway in rodents.
MATERIALS AND METHODS Healthy volunteers consumed 1.15 g/kg/day alcohol for 4 days and the amount of exhaled CH_4 was recorded by high sensitivity photoacoustic spectroscopy. Additionally, Sprague-Dawley rats were allocated into control, 1.15 g/kg/day and 2.7 g/kg/day ethanol-consuming groups to detect the whole-body CH_4 emissions. The mitochondrial function (oxidative phosphorylation capacity) was measured in liver and hippocampus samples with high-resolution respirometry (Oroboros Instruments, Austria). The effects of mitochondria-targeted L-alpha-glycerylphosphorylcholine (GPC) effects were tested in further ethanol-fed group (n=6).
RESULTS Alcohol consumption was accompanied by significant CH_4 emissions in both human and rat series of experiments. 2.7 g/kg/day ethanol feeding reduced the oxidative phosphorylation capacity of rat liver mitochondria, while GPC significantly decreased the alcohol-induced CH_4 formation and hepatic mitochondrial dysfunction as well.
CONCLUSION These data demonstrate a potential for ethanol to influence human methanogenesis, and suggest a biomarker role for exhaled CH_4 in association with mitochondrial dysfunction.

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Effects of methane inhalation on platelet functions in a large animal model of cardiac tamponade

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The alteration of platelet functions frequently accompanies trauma- or hypoperfusion-induced organ dysfunctions and the mortality also correlates with platelet dysfunction (White et al, 2013). Exogenous methane can modulate the pathways involved in key events of inflammation (Boros et al, 2012), but the exact mechanism is still unmapped. Our aim was to investigate the possible effects of normoxic methane-air mixture inhalation on platelet reactivity in a pig model of circulatory shock.

Anesthetized, ventilated minipigs were randomly allotted into 3 groups. After thoracotomy, cardiac tamponade was induced by intrapericardial injection of heparinised blood, meanwhile the mean arterial pressure was kept between 40-45 mmHg for 60 min. Group 1 served as sham-operated control (n=6), groups 2 and 3 (n=7-7) inhaled 2.5% methane-normoxic air mixture or air only for 20 min. Macrohemodynamics were monitored for 240 min, platelet reactivity was regularly checked with impedance aggregometry (Multiplate Analyzer, Roche). In addition, platelet samples were examined with high-resolution respirometry (Oxygraph-2k) to analyze possible alterations of the mitochondrial electron transport chain.

Cardiac tamponade caused an approx. 20% decrease of MAP compared to the control values. The platelet count did not change significantly by the tamponade while the aggregation of thrombocytes decreased significantly after the addition of arachidonic acid (AUC=7±3), collagen (AUC=18±4) or ADP (AUC=17±6). Methane inhalation did not influence platelet counts, but the reactivity increased. In case of arachidonic acid (AUC=31±11), collagen (AUC=34±12) and ADP (AUC=38±4) substrates a significant changes were observed by the end of the observation period as compared to the tamponade-treated group. The mitochondrial oxygen consumption was increased as well, while the leak respiration was decreased.

These data demonstrate that a significantly decreased platelet dysfunction can effectively be modified by methane inhalation, and the results imply that the anti-inflammatory *effect of inhaled methane is linked to an influence on circulating thrombocytes as well.*

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BIOLOGICALLY ACTIVE GASES AND SMALL SIGNALLING MOLECULES

Methane-donor molecules with anti-oxidant potential

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Background: The anti-inflammatory properties of methane (CH_4) in ischemia/reperfusion injuries are established (Boros, 2012; Li, 2017) and *in vitro* studies demonstrated the role of organosulphur compounds in endogenous CH_4 formation (Althoff, 2014). We aimed to investigate the biological effects of a diet rich in organosulphur moieties with presumed methanogenic potential in a mouse model of oral alcohol intake-induced liver dysfunction.
Materials and methods: Hairless mice were fed with standard diet or 10% mustard-seed containing chow for 7 or 14 days (n=7 each). After this period the animals were allocated into control and 12% ethanol-consuming groups for further 7 days. The whole-body CH_4 emission was recorded using photoacoustic spectroscopy. Liver NADPH oxidase activity and the oxidative phosphorylation capacity (OXPHOS) were determined using high-resolution respirometry (Oroboros O2k, Innsbruck, Austria) to monitor the progress of oxidative stress. Repeated measurements of ANOVA and Kruskal-Wallis followed by Dunn's tests were used for statistical analysis.
Results: At day 1, both the 7- and 14-days diet resulted in prompt CH_4 generation after alcohol input ($p<0.001$ vs. control), which was followed by a decline. Without the diet, this effect appeared only from the fifth day of ethanol intake ($p<0.001$ vs. control) and it was kept up thereafter. Alcohol consumption led to a significant increase in the liver NADPH oxidase activity ($p<0.01$) which was remarkably reduced by the 14-days diet ($p<0.05$ vs. ethanol). The ethanol challenge did not modify the liver OXPHOS capacity.
Conclusion: Oral mustard seed feeding as pre-treatment caused an immediate endogenous CH_4 formation following ethanol intake. This CH_4 release was associated in time with a potentially protective response and contributed to a maintained oxido-reductive balance, however there was no direct effect on the liver mitochondrial function.

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MITOCHONDRIA

Changes in oxygen kinetics between COX4i1 and COX4i2-containing COX.

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Regulation of cytochrome c oxidase (COX), the terminal enzyme of electron transport chain, is realized through the alternating expression of subunits' isoform pairs; a process that is controlled in developmental, tissue, or environmental manner [1]. COX4 subunit, a crucial homeostatic response switch, is optimizing respiratory chain function according to oxygen-controlled expression of its isoforms COX4i1 and COX4i2 [2, 3]. However, the functional impact of the isoform switch for mammalian tissues and cells is not fully understood.

Employing CRISPR CAS9-10A paired nickase technology, we created HEK293-based cellular model with complete absence of subunit COX4. Double knock-out of both isoforms 1 and 2 of COX4 (COX4i1/4i2 KO) showed absence of majority of COX subunits resulting in total COX deficiency. Moreover, the levels of complex I subunits as well as the content of assembled complex were decreased in COX4i1/4i2 KO, while levels of complexes II, III, and V were not significantly changed. As expected, mitochondrial respiration was undetected in the COX4i1/4i2 KO cells.

COX4i1/4i2 KO were subsequently utilized as a platform for knock-in of COX4i1 and COX4i2 variants using stable overexpression of either variant from pcDNA 3.1 vector. Expression of both isoforms complemented the respiratory defect of COX4i1/4i2 KO. Respiration of digitonin-permeabilized cells in OXPHOS and ETC states, as well as COX capacity were not distinguishable between cells expressing either isoform 1 or 2 of subunit COX4. The oxygen kinetics in terms of p50 (partial pressure of oxygen at half-maximal respiration) was approximately two-fold increased in COX4i2 versus COX4i1 cells in the OXPHOS state. In ETC state, the difference in p50 between COX4 isoforms was partially attenuated.

Using this model, we further plan to investigate the ability of COX4 isoforms to serve as mitochondrial energy and redox sensors for regulation of ATP production and oxidative stress response during normoxia and hypoxia.

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MITOCHONDRIA

$Ca^{(2+)}$n it be measured? High-resolution FluoRespirometric detection of extramitochondrial calcium movement

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Introduction: The mitochondrion plays an essential role in controlling calcium (Ca^{2+}) homeostasis. However, physiological Ca^{2+} signalling can lead to deleterious processes in various disease states due to Ca^{2+} overload (e.g. ischaemia/reperfusion, sepsis); that can induce the opening of mitochondrial permeability transition pore (MPTP) and subsequent cell death. Our studies were aimed 1) to monitor simultaneously the extramitochondrial Ca^{2+} movement and oxygen flux (JO_2) through the electron transport system and 2) to assess whether the inhibition of MPTP affects mitochondrial Ca^{2+} influx/efflux using high-resolution FluoRespirometry (Oroboros O2k, Oroboros Instruments, Innsbruck, Austria).
Materials and Methods: Under ketamine and xylazine anaesthesia, SPRD rats (330-360 g; n=7) and SKH-1 mice (n=7; 20-30 g) were sacrificed then the left lateral liver lobe was immediately removed for homogenate preparation, or the whole liver was harvested for preparing isolated mitochondrial fraction. Samples were energized with complex I-specific substrates (10 mM glutamate and 2 mM malate) or a complex II substrate (10 mM succinate; after complex I blockade with 0.5 µM rotenone) in the presence or the absence of the MPTP inhibitor Cyclosporin A (1 µM). After reaching a stable respiration, Ca^{2+} movement was assessed by Calcium Green-5N fluorescent dye (2 µM) using Fluorescence-Sensor Blue (excitation 465 nm). Calcium influx was stimulated by $CaCl_2$ addition (50 µM). Finally, EGTA (1 mM) was used for the chelation of calcium.
Results: Exogenous $CaCl_2$ resulted in an abrupt elevation in Calcium Green-5N fluorescence intensity followed by a decrease (mitochondrial calcium uptake) with simultaneous elevation in oxygen consumption. This was followed by a rapid increase in the fluorescence signal (reflecting Ca^{2+} efflux), reaching a higher fluorescence (Ca^{2+}-efflux) than the initial $CaCl_2$-induced elevation. Chelation of Ca^{2+} with EGTA completely abolished the fluorescence of the indicator. After the pre-incubation with Cyclosporin A marked delay (approx ~15 min) in mitochondrial Ca^{2+} movement was observed, not only in isolated liver mitochondria but also in tissue homogenate.
Conclusion: Our results indicate that Calcium Green-5N is an applicable method for monitoring the Ca^{2+} flux of biological samples, in isolated mitochondria and tissue homogenates as well. Further experiments are required for the better understanding of MPTP-independent way of Ca^{2+} efflux and how pharmacological/genetic inhibition of mitochondrial Ca^{2+} transport affects the Ca^{2+} movement.

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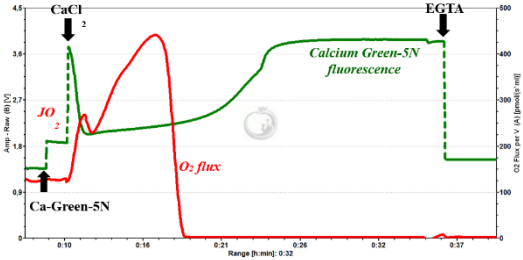

Figure 1. Simultaneous measurement of calcium- and O_2 flux in mouse liver homogenate

MITOCHONDRIA

Treatment of myocardial ischemia/reperfusion-induced mitochondrial dysfunction with methane gas *in vitro*

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Previous studies have demonstrated cytoprotective effects for methane (CH_4) in various models of ischemia-reperfusion (IR)-induced tissue damage (skin, liver, kidney, intestinal tract), but the exact mechanism and cellular targets are still unknown. We hypothesized that CH_4 can modify mitochondrial function and by this way can influence cell survival during hypoxia-reoxygenation conditions.
3-days-old cultured cardiomyocytes were subjected to 4 hr simulated ischemia and 2 hr reperfusion (sIR, n=6) with our without treatment with artificial air containing 2.2% CH_4 (sIR+CH_4, n=6). Normoxic groups served as controls (SH and SH+CH_4; n=6). Subsequently, mitochondrial functions were investigated with high (Oxygraph-2K, Oroboros, Austria), cytochrome c release was measured to detect the damage of the inner mitochondrial membrane. Cardiomyocyte survival was assessed by measuring lactate dehydrogenase activity, apoptosis was detected by TUNEL staining.
In response toCH_4 treatment, the baseline respiratory activity of cardiomyocytes significantly increased (from 21±8 pmol/ml/s to 35±15 pmol/ml/s), while the oxidative phosphorylation capacity doubled (from 64±18 pmol/ml/s to 116±24 pmol/ml/s) compared to the sIR group. In parallel, IR-induced cytochrome c release and cardiomyocyte apoptosis was significantly reduced in the CH_4-treated group.
Our experimental data confirm that mitochondria may be intracellular targets of CH_4. The results suggest thatCH_4 treatment can reduce I/R-induced cardiomyocyte damage.

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MITOCHONDRIA

Novel cardioprotective effect of methane in an *ex vivo* model

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Introduction: The anti-inflammatory properties of methane (CH_4) in ischemia/reperfusion injuries are established (Boros, 2012; Li, 2017) and we have recently provided evidence for the direct mitochondrial effect of exhaled CH_4 therapy in a clinically relevant rat model of liver ischemia/reperfusion (Strifler, 2016). Nevertheless, CH_4 inhalation has several limitation regarding its clinical application; we therefore aimed to investigate the effects of CH_4 dissolved into perfusion fluid. Principally, we tested the protective potential of methane-rich Krebs-Henseleit solution on the infarct size and on mitochondrial function in rat isolated-*perfused heart* according to *Langendorff,* under ischemia/reperfusion.
Methods: Hearts were harvested from 22 Sprague-Dawley rats and then were mounted on the Langendorff system. After stabilization for 15 min, a 30 min of global anoxia was applied and reperfusion was started with perfusing *Krebs-Henseleit buffer (n=11) or* CH_4 –enriched *Krebs-Henseleit buffer (n=11) for 60 (*n=3-3), or 120 min (n=8-8). After reperfusion was lasted, we determined the infarct size with triphenyltetrazolium chloride (TTC) staining and the activity of the mitochondrial electrontransport-chain complex I, II and the oxidative phosphorylation (OXPHOS) capacity of sample homogenates (Oroboros, Oxygraph-O2K, Innsbruck, Austria).
Results: In the methane-enriched *Krebs-Henseleit buffer hearts,* mitochondrial function was maintained and there was a strong tendency for a decrease in the infarct size after both 1 or 2 hours of reperfusion. Oxygen consumption rate of the homogenates were significantly lower in those samples which were perfused with methane-enriched *Krebs-Henseleit solution for 2 hours* ($p=0.0289$).
Conclusion: Methane-rich perfusion was ascertained to be protective in redeeming the function and structure of the heart against the ischemia/reperfusion challenge. The application of this solution have a promising therapeutic potential in the field of cardiac surgery.

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High-resolution mitochondrial oxygen kinetics in aerobic-anaerobic transitions

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Assessment of mitochondrial respiration at higher-than-physiological oxygen (O_2) concentrations has become a standard in biomedical research [1]. In contrast, the impact of mitochondrial O_2 kinetics on (patho)physiology is largely neglected in mitochondrial physiology. To facilitate further studies, we developed the automatized software module ***O2k**inetics*. Using this advanced tool, we re-evaluated the O_2 dependence of mitochondrial respiration in various respiratory states, experimental conditions, and in the presence of cytochrome *c* oxidase (CIV) inhibitors.

Mitochondria isolated from mouse brain, heart and liver were incubated at 25 °C or 37 °C in Oroboros O2k High-Resolution FluoRespirometers. Using substrate-uncoupler-inhibitor titration (SUIT) protocols with various fuel substrate combinations in OXPHOS-, LEAK- and ET-states, we investigated the effect of pathway and coupling control on mitochondrial p_{50} (O_2 partial pressure at half-maximum O_2 flux, J_{O2}). Kinetic data was obtained during aerobic-anaerobic transitions with high time-resolution at data sampling intervals of 0.2 s [2]. p_{50} values were calculated using the ***O2k**inetics* software for automatic O_2 calibration, correction for zero O_2 signal drift, instrumental background O_2 flux and exponential time constant of the polarographic oxygen sensor. Although J_{O2} of isolated mitochondria follows closely a monophasic hyperbolic function of p_{O2} in the O_2-dependent range (< 1.1 kPa or 10 µM O_2), biphasic curve fits were used to account for the frequently observed second, linear or hyperbolic component [3].

Depending on experimental temperature and respiratory state, p_{50} ranged from 0.006 to 0.07 kPa for NADH-linked LEAK respiration with glutamate&malate, $N(GM)_L$, and NADH-&succinate-linked OXPHOS capacity including GM and pyruvate, $NS(GMP)_P$, in agreement with and extending data published previously [4,5]. The p_{50} elevated with an increase from 25 °C to physiological temperature. In heart and liver mitochondria, the p_{50} was higher in OXPHOS- compared to LEAK-states, increasing proportionally with CIV turnover. Surprisingly, brain mitochondria did not follow this kinetic pattern in succinate-linked coupling control states. Upon inhibition of CIV by NO, p_{50} increased several-fold up to 0.15 kPa, $NS(GM)_P$, which was not reflected in J_{O2} at kinetic O_2 saturation.

In conclusion, determination of mitochondrial O_2 dependence has implications in a wide range of biomedical research topics, particularly in studies with biologically active gases (NO, hydrogen sulfide, carbon monoxide, methane) which directly or indirectly influence CIV. ***O2k**inetics* provides a fast and simple method for the detection of CIV impairment and extends standard OXPHOS analysis to the intracellular O_2 regime *in vivo*, reflecting low physiological O_2 concentrations or tissue hypoxia.

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Oxygen dependence of H_2O_2 production: application of High-Resolution FluoRespirometry with Amplex UltraRed® fluorescence.

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Ischemia-reperfusion injury (I/R) and hypoxia/reoxygenation are accompanied by elevated reactive oxygen species (ROS) production originating mainly from the mitochondria [1,2]. However, some publications suggest that I/R injury is followed by decreased ROS production [3]. Quantitative and accurate detection of ROS under hypoxic conditions remains challenging. One of the most frequently used methods for detection of H_2O_2 production is the Amplex UltraRed® and horseradish peroxidase (AmR) assay with several advantages and limitations. Results with the AmR assay strongly depend on the respiration medium, the O_2 concentration, and the duration of incubation.

Under normoxia, the partial pressure of oxygen (O_2) is around 20 kPa (~200 µM) in the ambient air but may decrease to < 1 kPa (~10 µM) in the vicinity of mitochondria [1]. Ambient oxygen levels are *in vitro* hyperoxic for the mitochondria. Therefore, we investigated the effect of various O_2 concentrations on O_2 flux and H_2O_2 flux in isolated mitochondria under well controlled conditions.

Measurements were carried out on mitochondria isolated from mouse brain and heart respiring with the combination of NADH-linked substrates glutamate plus malate and succinate in a closed-chamber respirometer combined with fluorimetry (Oroboros O2k High-Resolution FluoRespirometer). O_2 concentration was varied from 170 µM to 15-20 µM with injection of N_2 in the gas phase both in the LEAK- and OXPHOS-states. H_2O_2 production was determined by the AmR assay in MiR05 and KCl-based media. The H_2O_2 flux was corrected for chemical background as a function of O_2 concentration and for changes in the sensitivity of the assay.

Reducing the O_2 concentration to 20 µM did not compromise O_2 consumption both in MiR05 and KCl-based medium, which is in line with the high affinity of Complex IV for O_2 ($p_{50} \approx 0.03$-0.04 kPa in OXPHOS). The H_2O_2 flux, however, declined upon a decrease of the O_2 concentration from 170 to 20 µM in MiR05, and increased after re-oxygenation. This was observed in the LEAK-state but did not occur in the OXPHOS-state. In KCl-based medium the uncorrected H_2O_2 flux increased under hypoxia progressively after several hypoxia/reoxygenation cycles. This was an artefact due to the fluorescence background of the AmR assay in the absence of sample in KCl-based medium.

Taken together, when reducing the O_2 concentration to the physiological intracellular range *in vitro*, the H_2O_2 flux declines in a metabolic state-dependent manner in MiR05. The high photooxidation of the AmR assay causes an artefact in KCl-based medium under hypoxia, which must be avoided. In contrast, respiration was not affected by the

O_2 concentration down to 20 µM neither in brain nor in heart independent of respiration medium. Some controversies on hyperoxic versus hypoxic reductive oxidative stress may be resolved on the basis of a critical methodological evaluation of measurements of H_2O_2 production under hypoxia.

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Cellular respiration and mitochondrial oxygen kinetics: hypoxic steady-states and oxygen oscillations

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Studies of mitochondrial respiratory control by oxygen remain a methodological and conceptual challenge. A controversial issue relates to differences between oxygen kinetics at steady state conditions versus rapid aerobic-anoxic transitions. Using a combined open- and closed-systems approach, the oxygen kinetics of suspension cells of small cells (promyeloid cells 32D) was compared in the closed chamber of the Oroboros O2k (Innsbruck, Austria) with results at distinct hypoxic steady-states during continuous oxygen-injection of air-saturated aqueous medium (Oroboros Titration-Injection micropump TIP2k [1]). Oscillations of oxygen partial pressures, p_{O2}, were induced by oxygen titrations triggering the TIP2k by feedback control with the software DatLab. The apparent K_m (p_{50}) for oxygen was 0.2-0.4 µM O_2 (0.03 kPa) obtained from hyperbolic kinetics in aerobic-anaerobic transitions (37 °C). This agreed with the steady-state kinetics observed over up to 600 s of continuous oxygen injection, at constant levels of 0.4 µM down to 50 nM O_2, and oscillations in the hypoxic range. These results are comparable with the p_{50} for respiration of isolated mitochondria [1,2].
We investigated the relationship between cytochrome redox states and mitochondrial oxygen consumption at steady-state levels of hypoxia in mitochondria isolated from beef and mouse heart (BHimt and MHimt) [3]. A spectrophotometric system using visible light for the measurement of cytochrome redox states was combined with high-resolution respirometry. Monophasic hyperbolic relations were observed between ADP-stimulated oxygen consumption, J_{O2}, with NADH-linked substrate supply (OXPHOS capacity) and p_{O_2}. p_{50} (p_{O_2} at $0.5 \cdot J_{max}$) was 0.015±0.0004 and 0.021±0.003 kPa for BHimt and MHimt (25 °C). Redox states of cytochromes aa_3 and *c* were biphasic hyperbolic functions of p_{O_2}. The relation between cytochrome oxidation state and oxygen consumption revealed a separation of distinct phases from mild to severe and deep hypoxia. Under mild hypoxia, the steep slope of oxidation of cytochrome *c* when flux remains more stable represents a cushioning mechanism maintaining respiration high at the onset of hypoxia. Extending these studies by user-friendly software modules [4] provides a sensitive diagnostic tool for studies of mitochondrial dysfunction, and a quantitative reference for the functional evaluation of intracellular oxygen levels under environmental and physiological hypoxia.

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Age-dependent changes in the glutamate-nitric oxide pathway in the hippocampus of the triple transgenic model of Alzheimer's disease: implications on mitochondrial function

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Age-dependent changes in nitric oxide (•NO) concentration dynamics may play a significant role in both decaying synaptic and metabolic functions in Alzheimer´s disease (AD). This neuromodulator acts pre-synaptically to increase vesicle release and glutamatergic transmission and also regulates mitochondrial function. Under conditions of altered intracellular redox environment, •NO may react and produce reactive species such as peroxynitrite.

Here we performed a longitudinal study of the glutamate-•NO signaling axis in the hippocampus of the triple transgenic mouse model of AD (3xTgAD). We found that in the CA1 region, NMDAr-evoked •NO transients were significantly increased in young 3xTgAD mice, but age-dependent decay in signal strength was more pronounced in 3xTgAD as compared to non-transgenic groups. Evaluation of energy metabolism revealed age-dependent decrease in basal oxygen consumption rate, a general decrease in mitochondrial oxidative phosphorylation parameters and loss in mitochondrial sparing capacity in both genotypes. This prompted us to investigate whether •NO bioactivity may be shifted toward oxidative chemistry associated with neurotoxicity and indeed we observed age-dependent increase in 3-nitrotyrosine staining in the hippocampus, more pronounced in the 3xTgAD groups.

We conclude that the biphasic change in •NO dynamics in the hippocampus may, at an early age mitigate loss of synaptic strength, but ultimately is diverted towards increased oxidative damage, which may contribute to energetic crisis.

COST ACTION MitoEAGLE

Investigation of subcellular mechanisms in insulin resistance models in hepatocytes and myocytes

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Insulin resistance, which is based on an inadequate response of peripheral tissues to insulin (skeletal muscle, adipose tissue and liver) is the basis of prediabetes, type 2 diabetes and increased risk of developing diabetes complications in patients. It has been shown that molecular mechanisms such as mitochondrial dysfunction and endoplasmic reticulum stress play a major role in the development of insulin resistance. Recent studies focuse on the importance of the interaction of these organelles in the form of a functional and structural compartment called MAM – mitochondrial associated ER membranes. The goal of this research is to establish two different cell models of insulin resistance, using HuH7 hepatocyte and C2C12 skeletal muscle cell lines, and study the role of mitochondria and MAM in the conditions of *in vitro* insulin resistance (IR). In our models, insulin resistance was induced by chronic hyperinsulinemia. We observed a lower level of pAkt at Serine 473 in cells treated with chronic insulin treatment compared to controls. Mitochondrial morphology and contacts with ER were detected by confocal microscopy, and electron microscopy was used to visualise the spatial relationship of these membranes. Altereations in contacts of mitochondria and ER were observed in IR cells. In both cell models we have started measurements of mitochondrial function by high-resolution respirometry. Cell number per O2k chamber (*Oroboros, Austria*) was optimised, as well as digitonin concentration to successfully permeabilise HuH7 and C2C12 cells. These optimizations are necessary for correct application of Substrate-Uncoupler-Inhibitor Titration (SUIT) protocols that are relevant for the COST Action MitoEAGLE which aims at creating detailed SOPs for these measurements. Establishing these cell models of insulin resistance in hepatocytes and myocytes opens a possibility of comparative analysis that could clarify the potential tissue-specific mechanisms for regulation of insulin sensitivity that include mitochondria and MAM.

COST ACTION MitoEAGLE

The effect of short-term exopsure to moderate altitude on respiration of peripheral-blood mononuclear cells

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Peripheral blood mononuclear cells (PBMCs) provide a sensitive model for the study of mitochondrial respiratory function, potentially reflecting systemic changes happening in several organs in health and disease. Measurement of PBMC mitochondrial function provides a minimally invasive test well suited for large-scale diagnostic applications.

Mitochondrial respiratory function of PBMCs is influenced by many physiological factors, such as hormone levels, nutrition or infection, which lead to inter-individual variability. This study involved 8 healthy female volunteers (26.3 ± 5.7 years), on controlled diet and physical activity restricted to a light 1-hour walk per day to minimize variations between blood donors. 18 mL of whole blood was collected in the morning for four days at low altitude (575 m, Innsbruck, Austria), and for the following two days spent at moderate altitude of 2020 m (Kühtai, Austria). PBMCs were isolated from whole blood in Ficoll-Paque™ PLUS density medium and Leucosep tubes [1, 2]. Freshly isolated PBMCs ($3 \cdot 10^6$) were added into the 2-mL chambers of the O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, Austria) containing mitochondrial respiration medium MiR05-Kit (Oroboros Instruments, Innsbruck, Austria) with catalase at 37 °C. A coupling control&cell viability protocol was used [3].

The PBMC respiration was corrected for the contribution by contamination with platelets. The median of ROUTINE respiration and ET capacity of PBMCs at 575 m were 4.7 and 17.5 amol·s^{-1}·cell^{-1}, respectively, compared to 5.3 and 17.9 amol·s^{-1}·cell^{-1} during short-term exposure at 2020 m. There were no significant differences in respiration of PBMCs and flux control ratios were not influenced at moderate altitude. In conclusion, short-term moderate hypoxia can be exluded as a factor that can influence the results of diagnostic analyses of pathological mitochondrial dysfunction in PBMCs.

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COST ACTION MitoEAGLE

Magnesium protects from calcium induced collapse of mitochondrial trans-membrane potential.

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Mitochondria play an important role in intracellular Ca^{2+} signalling and cell death. Deregulation of mitochondrial Ca^{2+} homeostasis plays a key role in either necrotic or apoptotic cell death under several pathological conditions. In this study, we have investigated an effect of magnesium on calcium-induced depolarisation of succinate-driven mitochondrial trans-membrane potential ($\Delta\Psi_m$) that was measured at 25 °C following the safranine O fluorescence quenching at 540 nm (excitation) and 585 nm (emission).

Depending on the presence of Mg^{2+}, addition of Ca^{2+} to the suspension of isolated rat heart mitochondria induced either reversible depolarisation or irreversible collapse of $\Delta\Psi_m$. Irreversible collapse of $\Delta\Psi_m$, observed in the presence of 25 µmol/l Ca^{2+} and absence of Mg^{2+}, was insensitive to Ca^{2+} chelation, inhibition of Ca^{2+} uptake and increased efflux of Ca^{2+} from mitochondrial matrix. Based on these data, opening of mitochondrial permeability transition pore (mPTP) in a high-conductance mode is considered to be a major mechanism of the Ca^{2+}-induced irreversible collapse of $\Delta\Psi_m$. Involvement of mPTP in the process of Ca^{2+}-induced collapse of $\Delta\Psi_m$ was further supported by the protective effect of both cyclosporine A and ADP. On the contrary, reversible depolarisation of $\Delta\Psi_m$, observed in the presence of 125 µmol/l Ca^{2+} and 5 mmol/l Mg^{2+}, was sensitive to EGTA, ADP and inhibition of Ca^{2+} uptake as well as to increased efflux of Ca^{2+} from mitochondrial matrix. This may represent selective Ca^{2+}-dependent induction of a low-conductance mPTP pathway.

Presented results indicate important role of Mg^{2+} in the process of Ca^{2+} induced depolarisation of $\Delta\Psi_m$ mainly through discrimination between low- and high-conductance models of mPTP. Our results also indicate higher susceptibility of magnesium depleted cells to Ca^{2+}-induced and mitochondria-dependent necrosis.

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This work was supported by the Slovak Research and Development Agency under the contract No. APVV-16-0033.

COST ACTION MitoEAGLE

C2C12 myoblasts as a cell model for studying the role of mitochondria in insulin resistance

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Impairment of mitochondrial function has been linked to insulin resistance, but the precise role of mitochondria in the development of this state is not yet clearly understood. The goal of our research was to establish an *in vitro* model for studying cellular mechanisms of muscle insulin resistance. The cell line we used is C2C12 - mouse myoblasts that can be differentiated into multinuclear myotubes. In contrast to other published cell models of insulin resistance in muscle cells, induced mostly by palmitate exposure, we exposed differentiated C2C12 myotubes to chronic insulin. We followed the alterations of insulin sensitivity at the level of insulin signaling, by following the level of phosphorylation of protein kinase B by immunoblot. In order to establish if insulin sensitivity changes throughout the process of differentiation, and at which stage it is best to start with the insulin treatment, we studied the response to acute and chronic insulin stimulation at different stages of differentiation: from proliferative myoblasts to fully differentiated myotubes. Concentrations of insulin treatments used on muscle cells found in literature are very different, and in most cases a lot higher than the physiological concentrations found *in vivo*, so we also tested the response to insulin stimulation using a wide range of different insulin concentrations for acute and chronic treatments. In this *in vitro* IR model we have preliminary data on oxidative stress and mitochondrial physiology which has been obtained by high-resolution respirometry. Cell number per O2k chamber (Oroboros, Austria) was optimized, as well as digitonin concentration to successfully permeabilise that amount of C2C12 cells. These are preparative experiments necessary for Substrate-Uncoupler-Inhibitor Titration (SUIT) protocols relevant for the COST Action MitoEAGLE, aiming at defining common standards for measurements of mitochondrial function in cells. This study could reveal the role of mitochondria during cellular insulin resistance.

COST ACTION MitoEAGLE

The acute and chronic effects of a benzylamide derivative of maslinic acid in liver mitochondria isolated from mice with chemically induced skin carcinogenesis

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Background: Maslinic acid is a pentacyclic triterpenoid that has been systematically reported to elicit antitumor effects in several cancer cell lines. The present study performed in mouse isolated liver mitochondria was aimed to assess the effects of acute administration of a benzylamide derivative of maslinic acid ("EM2") and also its chronic effects in an experimental model of chemically induced carcinogenesis.
Materials & methods: Male SKH1 mice were randomly assigned to one of the following groups: NO TREATMENT group (mice with chemically-induced skin carcinogenesis - first 2 weeks - topical application of 7,12-dimetilbenzantracen solution (DMBA) 0.025% (once/week), followed by repeated applications of 12-a-13-ethyl-decanoilphorbol solution (TPA), and TREATED group (mice with chemically induced carcinogenesis plus the topical application of the EM2 1% hydrogel, twice per week) started after the appearance of papillomas. At the end of the experiment mice were sacrificed and liver mitochondria were isolated by differential centrifugations. Respiratory function was assessed by high-resolution respirometry in the presence of complex I and II substrates, according to the SUIT protocol. Respiratory rates (RR) in LEAK state (State 2, basal respiration), OXPHOS state (State 3, ADP-stimulated respiration), State 4 (ATP synthesis inhibition with oligomycin), and ETS capacity (uncoupled respiration - FCCP titrations) were assessed. In acute administration two concentrations of EM2 (5 and 10 μM) were tested in the oxygraph chamber.
Results: In chronic administration, EM2 elicited a significant antitumor local effect, the treated group showing fewer papillomas as compared to the untreated one. With respect to the mitochondrial respiration, a significant decrease in all respiratory parameters was found in the presence of both complex I (CI) substrates (glutamate/malate) and complex II (CII) substrate (succinate plus rotenone), respectively. In acute administration, 10μM EM2 (but not 5μM) induced a significant decrease in OXPHOS *vs.* CTRL for CI and CII-supported respiration. Moreover, in mitochondria respiring on succinate, EM2 (10 μM) elicited a generalized decrease in all other respiratory parameters (LEAK, State 4, and ETS) vs. CTRL. Addition of cytochrome c had no effect on respiration suggesting that EM2 does not affect the outer mitochondrial membrane integrity. Also, EM2 treatment elicited a significant decrease of H2O2 production following complex I activation, but not for complex II (Amplex Red assay).
Conclusions: In isolated liver mitochondria, acute application of EM2 elicited an overall, substrate-independent reduction in OXPHOS together with a substrate-dependent decrease in ROS production. In chronic administration, the compound had also an antitumor effect against the chemically-induced skin lesions.

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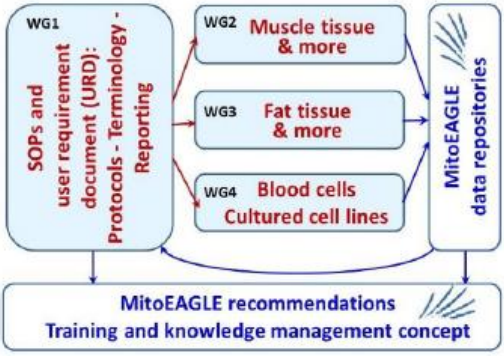
- Improve our knowledge on mitochondrial function in health and disease with regard to Evolution, Age, Gender, Lifestyle and Environment
- Interrelate studies across laboratories with the help of a MitoEAGLE data management system
- Provide standardized measures to link mitochondrial and physiological performance to understand the myriad of factors that play a role in mitochondrial physiology



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