Action BM1203

Working Groups Meeting
April 11-13, 2016 - Lisbon

Report on the Local Organizers Session (LOS)

Ana Fernandes (Universidade Lusófona, Lisbon) and Carlos Palmeira (Universidade de Coimbra)
Local organizers of the meeting and chairs of this session

The LOS was planned in order to address topics of scientific focus from different EU-ROS working groups, including sources of ROS, molecular mechanisms, pathophysiology, therapeutic approaches, and biomarkers. The summary of the talks is presented below:

Nitric oxide-mediated neurovascular coupling in healthy and diseased brain

João Laranjinha, Center for Neurosciences and Cell Biology, Portugal

Recently, Laranjinha's group has shown that, upon glutamatergic stimulation in vivo, neuronal-derived nitric oxide (·NO), bridges neuronal activation with a localized increased blood flow in ippocampus, thus supporting the role of ·NO as a direct mediator of neurovascular coupling. Now, these authors provided in vivo and real-time recordings supporting that during Alzheimer´s disease the impairment in neurovascular coupling is primarily due to cerebrovascular dysfunction (likely involving scavenging of ·NO by superoxide radical), rather than a dysfunctional signaling from neurons to blood vessels. Moreover, the bioactivity of ·NO in terms of neurovascular coupling may be redox modulated by nitrite and ascorbate.

Unacylated ghrelin (UnAG) a natural occurring antioxidant: a novel therapeutic option for diabetes-associated vascular complications

Maria Felice Brizzi, University of Turin, Italy

Brizzi's group has previously shown that the more abundant circulating form of ghrelin, desacyl-grelin (UnAG), exerts anti-oxidant effects on endothelial progenitor cells (EPCs). A proof of concept is provided by UnAG administration, which induces SOD-2 expression and improves mitochondrial dysfunction in muscles subjected to ischemia. UnAG is one of the ghrelin forms that is mainly produced in the stomach. UnAG differs from acylated ghrelin (AG) in term of its biological activity. Moreover, while both AG and UnAG are released into circulation, their ratio (AG:UnAG) varies and ranges from 1:2 to 1:9, while a relative excess of AG has interestingly been found in individuals suffering from insulin resistance connoted metabolic disorders. These observations, coupled with the role of glucose-mediated oxidative stress in vascular damage, have led this group to investigate and to present herein the clinical impact of UnAG administration on different mouse models of diabetes, with or without peripheral arterial disease. Moreover preliminary data on the impact of UnAG in a preclinical liver I/R model was also discussed.

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RNA oxidation, a novel mechanism for development complications in type 2 diabetes

Henrik Poulsen, Rigshospitalet University Hospital, Denmark

RNA undergoes significant oxidation and there are now convincing data suggesting that oxidation, and the consequent loss of integrity of RNA, is a mechanism for disease development. Poulsen's team recently showed that RNA oxidation, estimated by urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo), independently predicted mortality in a cohort of 1,381 treatment-naive patients with newly diagnosed type 2 diabetes. Furthermore, changes in 8-oxoGuo in patients with established and treated type 2 diabetes, during the first 6 years after diagnosis, are associated with mortality. These findings suggest that 8-oxoGuo could serve as a new clinical biomarker in diabetes. In addition, RNA oxidation may well be an important novel contributing mechanism for several diseases, including diabetes.

Antioxidant molecules in cardioprotection by preconditioning and postconditioning

Ioanna Andreadou, University of Athens, Greece

ROS constitute a double-sided coin. From one side, they exercise their deleterious effect on the heart but, on the other side, they confer a significant protection to the ischemic myocardium. Antioxidants provide controversial results in cardioprotection despite the fact that ROS are involved in ischemia–reperfusion injury. The full understanding of the oxidative mechanisms responsible for both the protection and the injury of myocardium will hopefully lead to the design of new clinical trials for the use of antioxidants in the cardiovascular system.

A possible new role for GAAP, a highly conserved Golgi ion channel, in ROS regulation

Guia Carrara, University of Cambridge, UK

Orthologues of the human Golgi anti-apoptotic protein (GAAP), share remarkable conservation in amino acid sequence, protein size, length and hydrophobicity profile throughout eukaryotes, prokaryotes and some poxviruses, suggesting a highly conserved ancestral structure and function. Since the discovery of its gene in 2002 in camelpox virus, this group has described several cellular functions and structural properties of GAAPs from varying origins. Within eukaryotes, GAAPs regulate Ca^{2+} levels and fluxes from the principal intracellular stores (Golgi and ER), confer resistance to a broad range of apoptotic stimuli, promote cell adhesion and migration via the activation of store-operated Ca^{2+} entry (SOCE) and importantly, these multi-transmembrane proteins were recently shown to form cation-selective ion channels, potentially forming the basis for the modulation of its diverse functions. In view of these functions as important hallmarks of cancer, a possible new role for GAAP in ROS regulation was investigated and suggests a significant effect of GAAP on overall basal ROS, in particular H_2O_2 levels, as well as enhancing the invasiveness of cells and the disregulation of MMP2 and 9 activity.

Oxidative lipidomics to unravel the radical induced oxidation of phospholipids

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Maria do Rosário Domingues, University of Aveiro, Portugal

Phospholipids are important components of cell membranes and biofluids, playing key roles in cell signalling. The presence of polyunsaturated fatty acid makes phospholipids prone to oxidation and oxidized phospholipids are known intermediaries of cellular and inflammatory events. The oxidized phospholipids, such as oxidized cardiolipin (CL) and oxidized phosphatidylserine, are major intervenients in programmed cell death. In particular, CL that is found almost exclusively in mitochondria, and their oxidized derivatives (OxCL) play an important regulatory role in loss of mitochondrial function and in apoptosis. In the recent years, mass spectrometry based approaches have been used for the study of the biological role of oxidized phospholipids. This Oxidative Lipidomics approach allowed detecting the accumulation of oxidized phospholipids, in oxidative stress conditions, associated with several inflammatory, cardiovascular, neurodegenerative and neurological diseases, such as the case of chronic stress model of Depression.

EU-ROS meeting Portugal April 11-13 session (U. Knaus) **Summary report WG1**

The session showed the breadth of ROS-related research in the EU-ROS COST action. Mathematical models of biological redox systems will aid in deciphering ROS-mediated signal flow in cells. Fernando Antunes, Portugal, presented a procedure that determines rate constants from typical redox signalling experiments by analysing H_2O_2 output with simple mathematical models. The methodology can be expanded to large-scale experiments, establishing the basis for redox kinetomics. Translating these models to patients is still challenging, thus biomonitoring oxidative stress is commonly used. Marcus Cooke, USA, gave an overview of biological matrices (blood, urine) in which oxidative damage to nucleic acids can be measured. A novel approach for collecting, storing and analysing very small volumes of blood was presented. Without any cell isolation step comet assays can be performed rapidly using redesigned, medium throughput equipment that leads to a ~90% decrease in slide handling time. Mass spectrometry remains the most sensitive path for 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) analysis, but a modified ELISA assay is gaining traction. In the future genome-wide assessment of DNA damage at a nucleotide resolution will likely be possible. Monitoring redox events in Gram-positive bacteria was the subject of Heike Antelmann's talk (Germany). Protein S-bacillithiolation, S-mycothiolation and other thiol oxidations are widespread redox modifications in bacteria under oxidative stress. For example, exposing *Mycobacterium smegmatis* to hypochlorite stress resulted in Cys modifications in over 700 proteins. The 65 S-mycothiolated proteins identified by shotgun-LC-MS/MS analysis were involved in diverse functions such as metabolism, protein translation, redox regulation and detoxification. Moving to mammalian ROS biology,

Michael Schrader, United Kingdom, outlined the cooperation between between peroxisomes and mitochondria, where both organelles cooperate in fatty acid β-oxidation and cellular ROS homeostasis. Peroxisomes are an important source of H_2O_2 which is generated during fatty acid beta-oxidation by acyl-CoA oxidases (ACOX). Mitochondria appear to be targets of changes in peroxisomal redox homeostasis, potentially contributing to disease development. Mitochondrial fatty acid beta-oxidation involves a peroxisomal acyl-CoA dehydrogenase (ACAD) in fungi and mammals. The presence of both ACOX and ACAD in peroxisomes may allow fatty acid beta-oxidation without the production of H_2O_2. Ines Castro, Portugal, outlined how the zebrafish as model organism can contribute to our understanding of aging. A hallmark of aging is telomere shortening. When telomeres get critically short, a DNA damage response (DDR) is triggered, which will lead to repair and cell proliferation or, if not successful, will cause either apoptosis or senescence. Pathways defining the outcome over time were interrogated using telomerase-mutant zebrafish. By 3 months of age, expression of p53 and p53-regulated genes (PUMA, PTEN) and apoptosis was increased in proliferative tissues derived from *tert-/-* mutant zebrafish, while by 6 months of age, senescence became the predominant pathway. At this stage the Akt/Foxo/mTOR pathway was activated and tissues showed accumulation of ROS, p38MAPK activation and p16/Ink4ab induction. Thus, DNA damage in combination with proliferative responses might be decisive for the cell fate transition from apoptosis to senescence.

Minutes WG2 session EU-ROS meeting Lisbon (A. Görlach)

The WG2 session at the Eu-ROS meeting in Lisbon focused on the role of ROS signaling in pathophysiological relevant signaling processes. The first talk of Prof. Tilman Grune, German Institute of Human Nutrition, Potsdam, Germany focused on the central role of protein aggregation and its relation to redox stress. He pointed out that disruption of the balance between protein synthesis and protein degradation results in the accumulation of modified proteins, which form high molecular weight aggregates, such as lipofuscin. They themselves can influence the metabolism of cells, in particular proteasomal activity and protein turnover. There is a preferential degradation of mildly oxidized proteins by the 20S proteasome, while aggregation and cross-linking of highly oxidized proteins that cannot be degraded by proteasome. At low oxidant exposures the proteolytic susceptibility of the substrate initially increases and the substrate is preferentially recognized and degraded by the proteasome. At higher oxidation states a decline in proteolytic susceptibility occurs, due to the formation of oxidized protein aggregates.

The second talk was from Prof. Antonio Cuadrado, Autonomous University of Madrid, Spain. He focused in his talk on the role of redox signaling in the regulation of neuronal stem cell fate. Low-to-moderate levels of reactive oxygen species supposedly are involved in different steps of neurogenesis via molecular pathways that have been decrypted only partially. Redox-sensitive molecules have been suggested to be involved in neuronal differentiation and stem cell fate, and putative mechanisms have been discussed.

In the third presentation, Dr. Flavia Radogna, from the Laboratoire de Biologie Moleculaire et Cellulaire du Cancer, Luxembourg shared extensive own data on the role of oxidative stress involvement in cell death induced by cardiac glycosides. Cardiac glycosides (CGs), prescribed to treat

cardiovascular alterations, display potent anti-cancer activities, although some cell types are resistant to this effect. It was discussed that the differential modulation of ROS and mitophagy by CG might be a main determinant of neuroblastoma resistance but possibly also other tumor types.

In the next talk, Prof. Malcom Jackson from University of Liverpool, UK, discussed the interesting role of ROS in skeletal muscle function. ROS appear to be important in the adaptation of skeletal muscle to contractile activity.

He intensively discussed the role of ROS in the loss of skeletal muscle fibres, atrophy of the remaining fibres and weakness in aging.

Although oxidative changes have been implicated in the mechanisms leading to age-related loss of muscle mass and in degeneration of the central nervous system, his findings could not demonstrate gross oxidative damage in sime nerves of old mice. His suggested that that an adaptation to increased oxidation occurs with minor changes in the oxidation of key cysteines that may contribute to defective redox signalling in the nerve.

Unofrtunately, due to an urgent disease conditions, Prof. Michel Toledano was unable to attend the meeting.

Thus, the last presentation was from Dr.Antonio Martinez Ruiz, Hospital de La Princesa, Spain. He presented very interesting data on the role of ROS signals in acute hypoxia. within this highly debated topic he suggested an important role of sodium/calcium exchange via NCLX in addition to mitochondrial complex I which could affect ROS signals under hypoxic conditions.

EU-ROS COST meeting Lisbon, April 11-13 2016. (T. Seredenina)

The WG3 session chaired by Tamara Seredenina from the University of Geneva, Switzerland, was dedicated to drugs and tools used in redox biology and medicine. The session contained six talks.

1. Višnja Stepanić from Rudjer Boskovic Institute, Croatia presented *in silico* portrayal of quercetin metabolites. She demonstrated how *in silico* approaches help to prioritize compounds and to increase the efficiency of the drug discovery process.
2. The meeting host Ana Fernandes from CBIOS-Universidade Lusófona, Portugal talked about the recent advanced in SOD mimics chemistry and their impact on adhesion and migration of doxorubicin-treated breast cancer cells.
3. The therapeutic potential of NOX4 is a subject of huge interest both in academic research and in pharma industry. In her talk Katrin Schröder from Goethe-University, Germany summarized the most recent knowledge and experimental evidence of the involvement of NOX4 in pathophysiology of atherosclerosis. The data from Schröder's group suggest that the H2O2-producing NADPH oxidase NOX4 is an endogenous anti-atherosclerotic enzyme. These findings are important for the future clinical trials and suggest that Nox4 inhibitors, currently under clinical evaluation, should be carefully monitored for cardiovascular side-effects.
4. A new first-in-class small molecule NOX2 inhibitor GSK2795039, developed by GSK and characterized at the University of Geneva was presented by Vincent Jaquet, University of Geneva, Switzerland. GSK2795039 inhibited both the formation of ROS and the utilization of the enzyme substrates, NADPH and oxygen, in a variety of semirecombinant cell-free and

cell-based NOX2 assays. It inhibited NOX2 in an NADPH competitive manner and was selective over other NOX isoforms, xanthine oxidase, and endothelial nitric oxide synthase enzymes. Following systemic administration in mice, GSK2795039 abolished the production of ROS by activated NOX2 enzyme in a paw inflammation model. Furthermore, GSK2795039 showed activity in a murine model of acute pancreatitis, reducing the levels of serum amylase triggered by systemic injection of cerulein. This talk gave an excellent example of a screening cascade to be used for successful discovery of NOX inhibitors.
5. Maria Monsalve, Autonomous Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Spain talked about the redox-dependent regulation of endothelial dynamics by PGC-1α. Her results show that PGC-1α control of hydrogen peroxide levels in endothelial cells plays a major role in the formation of stable interactions of endothelial cells with other cells and with the matrix. The presented data provide the rational for targeting redox signaling in metabolic disorders where microvascular complications are prevalent, such as diabetic retinopathy.
6. Another interesting multitarget compound was presented by Manuela Garcia Lopez, Instituto Teofilo Hernando, Universidad Autonoma de Madrid, Spain. ITH12674 is a hybrid of melatonin and sulforaphane with a dual drug-prodrug mechanism. The compound was protective in in vitro and ex vivo models of neurotoxicity and neuroinflammation. The presented evidence suggests that this compound could be useful for cerebral protection through the regulation of oxidative stress and neuroinflammation.

Workgroup 4 session, EU-ROS meeting, Lisbon, 12 April 2016-05-03 (P. Ghezzi)

The session on biomarkers saw the participation of Natasa Zarovni, from the SME Exosomic ins Siena., Italy. She gave a talk on the industrial perspective on the use of biomarkers and some down-to-earth consideration like the stoichiometry of exosomal biomarkers. Sebastian Steven discussed glucose transporters in sepsis and Arne Holmgren gave a very good lecture on the biological role of secreted enzymes, thioredoxins and glutaredoxins. Pietro Ghezzi concluded with some theoretical considerations of biomarkers been of different types, some mechanism based and some not, also based on the feedback from a collaborative publication on biomarkers from the EU-ROS group/

Some of these issues were brought up again during the final discussion in terms of inclusion of specific topics in a possible final publication.

WG5 session (chairs: S. Chłopicki and J. Zielonka)

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J. Zielonka **– Medical College of Wisconsin, USA**

Fluorescence spin trapping - a new method for sensitive detection of superoxide

A new approach has been proposed for the detection of superoxide radical anion by combination of the spin trapping of $O_2^{\bullet-}$ with fluorescence detection of the spin adduct using boronate-based fluorogenic probes. This approach is based on our earlier observation that boronic compounds can reduce superoxide adducts to cyclic nitrones into corresponding hydroxyl radical adducts, with concomitant oxidation of boronates to the corresponding phenols. We propose a combination of the DIPPMPO nitrone as a trap for superoxide with coumarin boronic acid as a probe for the superoxide adduct forming highly fluorescent product, 7-hydroxycoumarin. This method enables the detection of $O_2^{\bullet-}$ with high specificity and at least 10-fold higher sensitivity and higher throughput, as compared to conventional EPR spin trapping technique. The developed assay can be used to detect cell-free and extracellularily-released superoxide, with the parallel controls using superoxide dismutase (SOD) for confirmation of the identity of the oxidant. As a proof of concept the method has been applied to monitor the activity of NADPH oxidase-2 (Nox2) in differentiated HL60 cells and the effect of widely used Nox2 inhibitors has been tested.

M. Mojovic **- University of Belgrade, Serbia**

EPR spectroscopy in investigation of iron-sulfur cluster damage and BBB permeability in neural tissues of SOD1G93A ALS rat model

ALS, or amyotrophic lateral sclerosis, is a progressive neurodegenerative disease that affects nerve cells in the brain and the spinal cord. The reason for the occurrence ALS and the mechanism of its progression is still unknown. Now, the diagnosis is based on ALSFRS-R scores but there are still no confident biomarkers for early stage of the disease. It is assumed that developing of ALS is linked to the cell malfunctions caused by disturbed metabolism of reactive oxygen and nitrogen species which lead to motor neuron cell damage.

Since nervous tissue spontaneously generates and releases H_2O_2 into the CSF, it is of primary interest to understand the transitional metals metabolism of neural tissues. As, the redox activity of iron and other transitional metals is related to the presence of specific ligands, there was a need for using new experimental approach which could expose the existence of metal oxidation state and form. We used low temperature X-band EPR spectroscopy to investigate the state of Fe and other transitional metals in homogenates of brain tissue isolated from SOD1G93A transgenic ALS model and age-matched wild type (WT) rats. Our MRI studies showed the presence of iron deposits in the motor cortex of ALS patients, indicating the potential leakage of iron to CSF through compromised BBB. The idea was to use in vivo L-band EPR spectroscopy and a selection of spin-probes to demonstrate the increased BBB permeability in transgenic ALS rats.

V. Belousov **- Russian Academy of Science, Russia**

Redox Biosensors: state of the art and perspectives

Diffusion of H_2O_2 across cytoplasm is suggested to be restricted by cellular antioxidant enzymes. Now this H_2O_2 gradient was modelled within the living cell using D-amino acid oxidase (DAO)

localized to the nucleus. The gradient was visualized by combination of two HyPer probes located either to the nucleus and mitochondria or to the nucleus and cytoplasm (fused to keratin filaments). In both cases H_2O_2 generated by DAO was not able to cross a long distance within the cytoplasm and oxidized only HyPer next to the nucleus. However, inhibition of Trx system by auranofin completely eliminated the cytoplasmic barrier for H_2O_2 diffusion.

F. Peyrot **– Paris Descartes University and Paris Sorbonne University (ESPE), France**

Cyclin nitrone spin traps and nitroxides as probes for oxidative stress, from cellular to in vivo EPR studies

In the past, we showed that CD-DIPPMPO, a cyclodextrin-cyclic nitrone conjugate, is a superior spin trap for extracellular superoxide produced by PMA-stimulated RAW 264.7 macrophages [1,2]. Use of gas-permeable tubing enhances the sensitivity of detection on cell suspensions, while detection on adherent cells grown on microscope coverslips affords medium-throughput screening. Unfortunately, CD-DIPPMPO is not commercially available and cannot be used in vivo. Now, we turn to nitroxide spin probes for detection and mapping of oxidative stress by in vivo EPR. Probe synthesis and evaluation is underway.

[1] K. Abbas, M. Hardy, F. Poulhes, H. Karoui, P. Tordo, O. Ouari, et al., Detection of superoxide production in stimulated and unstimulated living cells using new cyclic nitrone spin traps, Free Radic. Biol. Med. 71 (2014) 281–290. doi:10.1916/j.freeradbiomed.2014.03.019.

[2] K. Abbas, M. Hardy, F. Poulhès, H. Karoui, P. Tordo, O. Ouari, et al., Medium-throughput ESR detection of superoxide production in undetached adherent cells using cyclic nitrone spin traps, Free Radic. Res. (2015) 1–7. doi:10.3109/10715762.2015.1045504.

P. Andjus **– University of Belgrade, Serbia**

Oxidative stress and microglia in ALS - the effect of ALS IgGs

An introduction was given on the hSOD1 G93A rat model of ALS and the different physico-chemical parameters of the oxidative stress. Emphasize was given on the role of microglia and their diverse cellular and subcellular characterization in the brain of the ALS model. Previously published results on the excitotoxic effect of patients' ALS IgGs on neurons and astrocytes in culture were presented as an introduction to the preliminary results on the efect of these IgGs on the microglial cell line BV2. Using fluorescent probes HyPer and SypHer a response was observed with 4/11 ALS IgGs demonstrating a rise in cytoplasmic peroxide and pH that deserve further investigation into a mutual mechanism.

EU-ROS COST meeting Lisbon, April 11-13 2016. (V. Jaquet)

WG6 session

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Chair: Vincent Jaquet University of Geneva, Switzerland. This session consisted of three presentations focusing on grants and translational aspects of research in oxidative stress.

1. *Some personal perspectives on the translation of basic redox biology into commercial applications*, Paul Winyard, University of Exeter Medical School, UK. Paul Wyniard initiated a discussion about the benefits and limitations of filing patents from academic institutions. He focused on his own experience and presented his own studies which generated patents. They consist of measurements of oxidative modifications, which have a diagnostic value in inflammatory conditions, such as rheumatoid arthritis as well as circulating markers indicating of pathogenic bacteria.

2. *Redox Biology in Medicine: a new COST application*, Fernando Antunes, University of Lisbon, Portugal. Dr Antunes presented a novel project for a COST action on redox medicine. He presented different working groups and insisted on the interdisciplinary, inter-european and translational aspects of this new action. The proposal will be submitted on April 25th.

3. *Funding Opportunities for European Researchers*, Sheila Vidal, Instituto Gulbenkian de Ciência, Portugal. Sheila Vidal presented grant opportunities for basic research. Most researchers are funded by national science foundations, but many opportunities exist: They include private foundations (many are from the US) and from Horizon2020. She gave of tips to write an excellent grant proposal and encourage the attendance to submit and resubmit proposals to improve chances of success.