

## Session 11: Mitochondria and Aging

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

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

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

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

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

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

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

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

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

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

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

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

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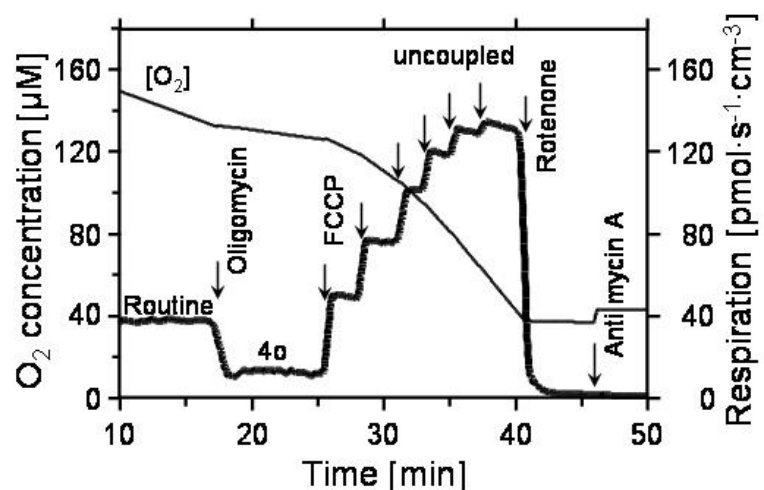
### **11-04. Mitochondrial respiratory control and oxygen dependence of ROS production in aging endothelial cells.**

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Experiments utilizing MitoSox, a dye specific for mitochondrial superoxide radical anions revealed that IRA at physiologically relevant doses leads to an at least threefold increase in O<sub>2</sub><sup>·-</sup>. We have found that IRA induced MMP-1 expression in primary human fibroblasts can be abrogated by mitochondrial respiratory chain inhibitors. This effect was specific since UV-A or UV-B induced MMP-1 expression was not inhibited. In addition we were able to demonstrate, that mitochondria depleted (rho<sup>-</sup>) cells do not show IRA-induced MMP-1 upregulation in contrast to corresponding rho<sup>+</sup> cells. UV-A and UV-B induced MMP-1 induction was not altered by absence of mitochondria.

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

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

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

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

