Available online at www.sciencedirect.com
SCIENCE DIRECT
Experimental Gerontology 41 (2006) 103–109
Experimental Gerontology
www.elsevier.com/locate/expgero

Techniques in Aging Research

High-resolution respirometry–a modern tool in aging research

E. Hütter [a], H. Unterluggauer [a], A. Garedew [b], P. Jansen-Dürr [a], E. Gnaiger [b,c,*]

[a] Department of Molecular Cell Biology, Institute for Biomedical Aging Research of the Austrian Academy of Sciences, Rennweg 10, Innsbruck, Austria
[b] Oroboros Inst, Schöpfstr 18, 6020 Innsbruck, Austria
[c] Innsbruck Medical University, Department of Transplant Surgery, D.Swarovski Research Laboratory, Innrain 6/6, Innsbruck, Austria

Received 13 June 2005; received in revised form 13 September 2005; accepted 20 September 2005
Available online 23 November 2005

Abstract

Alterations in mitochondrial function are believed to play a major role in aging processes in many species, including fungi and animals, and increased oxidative stress is considered a major consequence of altered mitochondrial function. In support of this theory, a lot of correlative evidence has been collected, suggesting that changes in mitochondrial DNA accumulate with age in certain tissues. Furthermore, genetic experiments from lower eukaryotic model organisms, indicate a strong correlative link between increased resistance to oxidative stress and an extended lifespan; in addition, limited experimental evidence suggests that the inhibition of mitochondrial function by selected pharmacologically active compounds can extend lifespan in certain species. However, changes in mitochondrial function may affect aging in a different way in various tissues, and a clear statement about the role of mitochondrial deterioration during physiological aging is missing for most if not all species. At this point, respirometric analyses of mitochondrial function provide a tool to study age-associated changes in mitochondrial respiratory chain function and mitochondrial ATP production within living cells and isolated mitochondria. In the recent years, new instruments have been developed, which allow for an unprecedented high-resolution respirometry, which enables us to determine many parameters of mitochondrial function in routine assays using small samples of biological material. It is conceivable that this technology will become an important tool for all those, who are interested in experimentally addressing the mitochondrial theory of aging. In this article, we provide a synopsis of traditional respirometry and the advances of modern high-resolution respirometry, and discuss how future applications of this technology to recently established experimental models in aging research may provide exciting new insights into the role of mitochondria in the aging process.

Keywords: Mitochondria aging

'However, one approach that is underutilized in whole-cell bioenergetics, and that is accessible as long as cells can be obtained in suspension, is the oxygen electrode, which can obtain more precise information on the bioenergetic status of the in situ mitochondria than more 'high-tech' approaches such as fluorescent monitoring of $\Delta\psi_m$' (Nicholls and Ferguson, 2002).

1. Introduction

The free radical theory of aging (Harman, 1956) implicates molecular damage caused by reactive oxygen species (ROS) as a major cause of the aging processes. Based on the initial hypothesis by Harman, the mitochondrial theory of aging (for recent review, seen Kowald, 2001; Wallace, 2001) has been developed, linking alterations in mitochondrial function to the aging process. This concept is supported by substantial correlative evidence and is based on the idea of a vicious cycle, in which somatic mutations of mitochondrial DNA (mtDNA) engenders respiratory chain dysfunction, enhancing the production of ROS. In turn, this is proposed to result in the accumulation of further mtDNA mutations and other types of macromolecular damage in the mitochondria (for recent review, see Jacobs, 2003; see Fig. 1). Sporadic loss of mitochondrial function with aging, based on in situ assays of cytochrome *c* oxidase (COX), has indeed been observed in individual cells of rodent and mammalian tissues (Cottrell et al., 2001), and loss of function was correlated to the occurrence of mtDNA mutations, at least in some cases. In numerous non-reproductive tissues of many species, mitochondrial genes (like nuclear genes) accumulate mutations as the animals age (Vijg, 2000). However, rarely all mitochondria (or even their majority) within a given cell are inactivated, which would be required to deplete a cell from mitochondrial function such as in ragged-red fibres. In this respect, it has been

* Corresponding author. Address: Oroboros Inst, Schöpfstr 18, 6020 Innsbruck, Austria
E-mail address: erich.gnaiger@uibk.ac.at (E. Gnaiger).

0531-5565/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.exger.2005.09.011

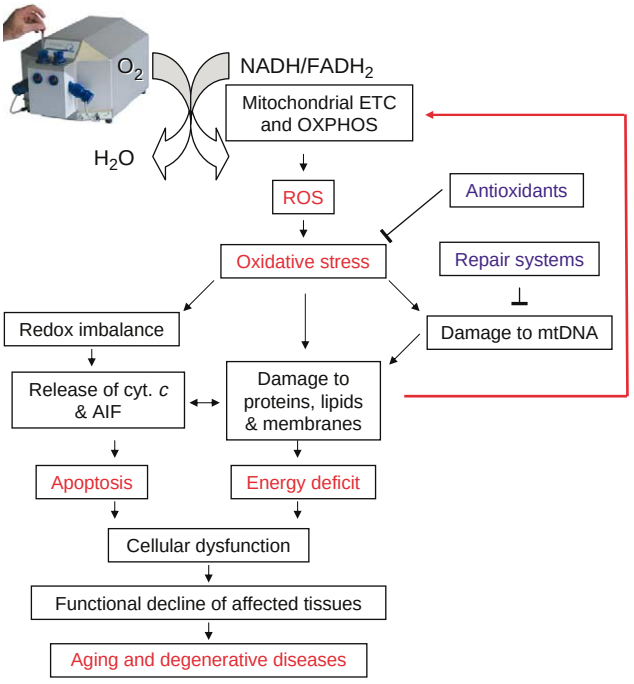

Fig. 1. The mitochondrial theory of aging and potential contributions from high-resolution respirometry. The mitochondrial theory of aging links alterations in mitochondrial function to aging process. This concept is based on the idea of a vicious cycle, in which accidental ROS production by mitochondria (and probably other sources) induces various kinds of damage to mitochondria, including somatic mutations of mitochondrial DNA (mtDNA), and oxidative demage to proteins, lipids and membranes. This induces respiratory chain dysfunction, enhancing the production of ROS, which then results in the accumulation of further damage and more severe mitochondrial dysfunction. With the accumulation of defects over time, cellular dysfunction ensues which can be caused by energy deficit and/or apoptosis; this leads to functional decline of tissues and finally to aging and age-related diseases. A critical prediction of the mitochondrial theory of aging that remains to be fully validated, would be that the overall physiological performance of mitochondria, which can best be monitored by high-resolution repirometry of intact tissue, becomes limiting with aging.

argued that phenotype manifestation of a mitochondrial genetic defect, such as in certain genetic diseases, occurs only when a threshold level is exceeded, and this phenomenon has been named the 'phenotypic threshold effect' (for review, see Rossignol et al., 2003). Apparently, these findings do not prove a general functional impairment of mitochondria in aging mammalian tissues and do not establish a cause-effect relationship between mitochondrial damage and the aging process.

A functional role of mitochondria and ROS in aging has also been investigated in lower eukaryotes that are frequently used as model organisms for aging research. Increased replicative longevity in *Saccharomyces cerevisiae*, due to caloric restriction, has been linked to enhanced mitochondrial respiratory activity (Lin et al., 2002), which was found to decrease the rate of mitochondrial ROS production (Barros et al., 2004). In the nematode *Caenorhabitis elegans*, lifespan can be extended by inhibiting mitochondrial electron transport, either through genetic manipulations (SiRNA mediated gene silencing and the introduction of specific mutations, respectively,) or the addition of drugs (e.g. antimycin A). Available experimental evidence suggests that mitochondrial activity during early development but not during adulthood triggers regulatory mechanisms which establish respiratory activity per se bears the inherent potential to restrict lifespan, somehow supporting the hypothesis that the 'rate of living' is inversely correlated with lifespan, at least in these model organisms. Whereas recent experimental data suggest that various long-lived mutants indeed display distinct alterations in respiration rate and other metabolic features (Braeckman et al., 2002), the situation seems to be quite complex and more work will be required to clearly establish the role of mitochondrial activity for aging in *C. elegans* (reviewed by Houthoofd et al., 2005).

Using a mouse model expressing a mutant mtDNA polymerase, it was shown recently that increased levels of point mutations and deletions of mtDNA, resulting from the transgenic defective enzyme, can indeed cause a reduction of lifespan and premature onset of aging-related phenotypes (Trifunovic et al., 2004). The observation suggests that damaged yet active mitochondria can cause aging in a mouse model. Moreover, recent observations suggest that transgenic overexpression of catalase in mitochondria extends lifespan of the mouse (Schriner et al., 2005), further supporting the view that ROS are major contributors to aging also in mammals. However, the questions remains if mitochondrial function does indeed deteriorate during physiological aging in any species, and if so, which tissues are concerned.

As mentioned above, a key questions in the field concerns the impact of aging on mitochondrial function and, more precisely, on the functional integrity of the respiratory chain including ATP production, versus defects leading to deregulated ROS production. The existing data provide some information about age-trends concerning the integrity of the mtDNA. What would be required in addition is an in vivo assessment, within living tissue, of mitochondrial function during aging. This could be achieved either by in vivo C/P NMR approaches to study mitochondrial phosphorylation in vivo (Petersen et al., 2003) or, to overcome some limitations inherent to this methodology, by respirometric analyses of small biopsies, which are now possible with the new instruments (see below).

Studies carried out with isolated mitochondria (e.g. from experimental animals of different age) have the advantage that all potentially confounding factors from the cellular environment can be excluded and 'pure' mitochondrial properties can be studied. However, it is now clear that there is intense cross-talk and interdependency between mitochondria and the rest of the cell (exemplified by the retrograde response; for review, see Butow and Avadhani, 2004), which affects the activity of both mitochondria and other cellular constituents. In this article, we will discuss the scientific requirements for appropriate measurements of mitochondrial activity in intact cells and the technological solutions to the problem that have been developed for measurements on limited amounts of cultured cells. We will also demonstrate the use of advanced high-resolution respirometry with the Oxygraph-2k (see below) to

determine mitochondrial function in human endothelial cells, a model for vascular aging that is well established in our laboratory (Hampel et al., 2004; Unterluggauer et al., 2003; Wagner et al., 2001).

2. Monitoring of mitochondrial function by respirometry: traditional approaches and recent developments

2.1. Principles of respirometric measurements

The principle of respirometry in a closed chamber involves the determination of changes in oxygen concentration, usually starting at ambient concentration of 21% oxygen. As the biological sample consumes oxygen, the concentration is lowered. Plotting oxygen concentration against the elapsed time provides an estimate of oxygen consumption. 50 years ago, Chance and Williams (Chance and Williams, 1955) published one of the key papers leading the way to modern methods for measuring oxidative phospharylation in isolated mitochondria, replacing the previously common manometric and volumetric techniques by polarographic recording of oxygen concentration over short periods of time. Using a vibrating platinum microelectrode, the 1 cm^3 mitochondrial suspension in the cuvette was not closed against the air, with oxygen backdiffusion amounting to 100 nmol s^{-1} when half of the oxygen dissolved at air saturation was used up. Nevertheless, the principle of a closed chamber was applied, with high mitochondrial concentrations leading to oxygen depletion within 120–200 s (corresponding to respiratory fluxes of 800–2000 nmol s^{-1} cm^{-3}, i.e. more than 1 million-fold above the resolution of our modern instruments). Since then, traces of oxygen concentration over time (oxygraphy) have been presented to illustrate the linear slopes obtained after additions of sample and various chemicals. Such oxygen traces yield limited information on the sensitivity and accuracy of measuring respiratory rate (for example, see the traces shown in Fig. 2A). Modern instruments, therefore, allow to perform on-line calculation of respiration as the negative time-derivative of oxygen concentration.

Ideally, the decline of oxygen concentration in a closed respiration chamber is strictly due to the chemical oxygen consuming reactions. In practice, such an ideally closed system is difficult to achieve. With progressive oxygen depletion diffusion gradients increase and 'backdiffusion' of oxygen into the medium may distort the results. Even recent publications (N'Guessan et al., 2004) report correction of respiration by a constant decline of oxygen measured initially at high oxygen concentration. This is erroneously thought of as a correction declines at low oxygen and becomes even negative, due to backdiffusion of oxygen in to the chamber (Gnaiger, 2001; Gnaiger et al., 1995).

2.2. New technology; high-resolution respirometry

Modern trends in mitochondrial physiology and respiratory pathology set advanced standards with respect to high-resolution rspirometry of isolated mitochondria, cultured cells, tissue preparations and human biopsies. Small changes in cellular respiration, minor alterations in respiratory control ratios, and subtle differences in respiratory effects of inhibitors may indicate significant mitochondrial defects, reflecting injuries of mitochondrial proteins or membranes, defects of mtDNA, or alterations in mitochondrial signalling cascades. The high resolution and accuracy requried to meet these challenges is not provided by conventional approaches, which had to be replaced by a new concept now known as high-resolution respirometry (Gnaiger, 2001, 2003; Gnaiger et al., 2000). Thus 10 years ago, a new standard for the measurement of mitochondrial and cellular respiration was established with the Oroboros Oygraph (Gnaiger et al., 1995; see Fig. 3), based on highly sensitive electrodes, a new integrated design, minimization of oxygen diffusion, and advanced software that allows the instantaneous on-line recording of oxygen consumption rates (see Fig. 2B).

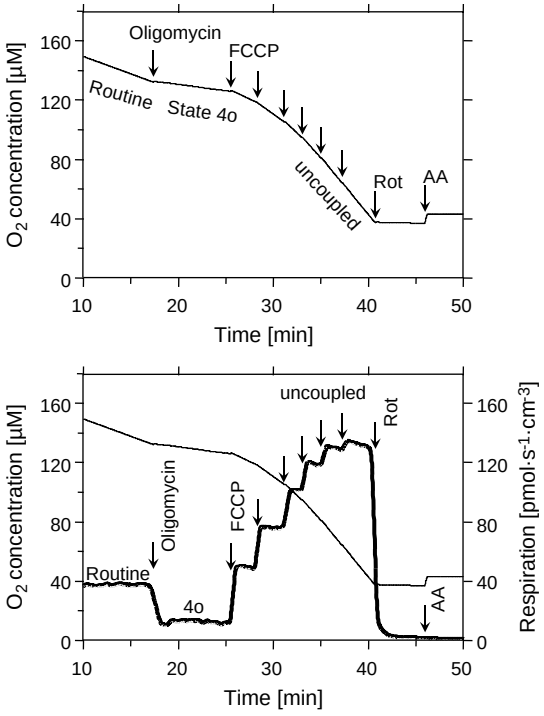

Fig. 2. Respirometric traces according to traditional representation of oxygen concentration as a function of time (panel A)versus high-resolution respirometric display of oxygen concentration and respiration as a function of time (panel B). The representative experiment with senescent HUVEC (0.44 million cells per ml) illustrates the regime of a phosphorylation control titration, starting with rountine respiration (time is shown after addition of cells into the 2 ml chamber of the OROBOROS Oxygraph-2k) in culture medium, oligomycin inhibition of ATP synthase, stepwise uncoupling by an FCCP titration (increments of 0.5 µM FCCP up to maximum stimulation of uncoupled respiration, and inhibition by rotenone and antimycin A. Arrows indicte times of titration. In the trace for respiration (thick line), disturbances by the titrations (e.g. increase of oxygen concentration after titration of antimycin A dissolved in ethanol) have been eliminated by interpolation. High-resolution requires on-line calculation of respiration as the negative time-derivative of oxygen concentration, which is immediately evident when evaluating the FCCP titrations in panels A and B. Respiration is automatically corrected for contributions of the polarographic oxygen sensor and of oxygen diffusion to total apparent respiration.

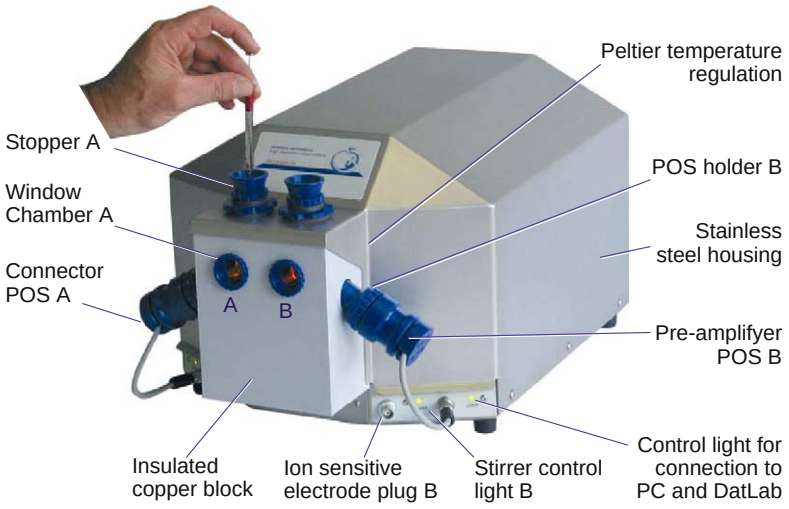

Fig. 3. The OROBOROS Oxygraph-2k for high-resolution respirometry, The picture provides a view of the oxygraph, a benchtop microrespirometer which contains two independent 2 ml glass chambers (A, B) inserted into a copper block which is maintained at constant temperature by electronic temperature regulation. Merely between 200,00 and 2 million cells are required for a diagnostic analysis. Oxygen concentration is recorded continuously by polarographic oxygen sensors (POS) in each chamber and the data are on-line processed to calculated oxygen consumption of intact cells, permeabilized tissues or isolated mitochondria (© 2005 by OROBOROS INSTRUMENTS®, reproduced with permission).

The following features distinguish high-resolution respirometry from conventional oxygraphs. Chamber design and materials, the oxygen sensor, and electronics (including Peltier temperature regulation with stability better than ± 0.001 °C) yield a high long-term signal stability and low noise of the oxygen signal, as a basis for on-line calculation of oxygen flux (negative time derivative of oxygen concentration) at sufficient time resolution (compare Fig. 2B). In high-resolution respirometry, oxygen flux is background-corrected on-line as a continuous function of oxygen concentration. Standardized calibration procedures of the oxygen signal, response time of the sensor, and instrumental or chemical background effects provide an experimental basis for high accuracy. At low respiratory flux per volume, the oxygen capacity of the system provides sufficient time for evaluation of slow approaches of the biological sample to a steady state, and for applocation of complex titration regimes in intact or permeabilized cells and tissues (see, example Hutter et al., 2004; Renner et al., 2003; Kuznetsov et al., 2004).

3. Analysis of mitochondrial function in senescent human endothelial cells by high resolution respirometry

To illustrate the application of high resolution respirometry in intact cells, we demonstrate in the following section a simple diagnostic approach to characterize mitochondrial activity in senescent human endothelial cells, which are widely used as a model for aging of the human vascular system. For additional applications and further experimental details, the reader is referred to previous work using high-resolution respirometry with cultured human cells (Hutter et al., 2004; Renner et al., 2003).

3.1. Experimental procedures

3.1.1. Cell culture

Endothelial cells were isolated from human umbilical veins as described (Jaffe et al., 1973) and cultured in Endothelial Cell Basal Medium (Cambrex BioScience, Verviers), containing hEGF (0.1%), Hydrocortisone (0.1%), GA-1000 (0.1%), BBE (0.4%), FBS (2%). The cells were subcultured by trypsinization with trypsin-EDTA (Gibco Life Technologies, Vienna, Austria), seeded on cell culture dishes coated with 0.2% gelatine and grown in an atmosphere of 5% CO_2 at 37 °C. Cells were passaged at a ratio of 1:5 at regular intervals. At later passages, the splitting ratio was gradually reduced to 1:3 and 1:2, respectively. Cells were passaged such that the monolayers never exceeded 70–80% confluency. Population doublings (PDL) were estimated using the following equation: $n = (\log 10F - \log 10I)/0.301$ (with $n =$ population doublings, $F =$ number of cells at the end of one passage, $I =$ number of cells that were seeded at the beginning of one passage). After roughly 65 population doublings, the cells reached growth arrest and senescence (Wagner et al., 2001); the senescent status was confirmed by staining for senescence-associated beta galactosidase (Dimri et al., 1995).

3.1.2. Respirometric analyses

Cellular oxygen consumption was measured by high-resolution respirometry with the OROBOROS Oxygraph-2k in a standard configuration, with 2 ml volume of the two chambers, at 37 °C, and 750 rpm stirrer speed. The software DatLab 4 (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition (2 s time intervals) and analysis, including two-point calibrations of the OROBO-POS polarographic oxygen sensors, and on-line calculation of the time derivative

of oxygen concentration. Respiration was automatically corrected for contributions of the polarographic oxygen sensor and of oxygen back-diffusion to total apparent respiration. The correction is a linear function of experimental oxygen concentration, and declined from 1.4 to -0.9 pmol O_2 s^{-1} cm^{-3} in the experimental oxygen range shown in Fig. 2. The procedure used for background correction has been described in detail by (Gnaiger, 2001). Without this routinely applied background correction, the error for oligomycin-inhibited respiration would amount to 10%, and the RCR ratio (see below) would be calculated at 9.7 instead of 10.7. In small-volume and leaky Perspex chambers which are sometimes still in use today, back-diffusion of oxygen increases by an order of magnitude. If back-diffusion would be -10 pmol O_2 s^{-1} cm^{-3} (which is a modest figure for low-resolution small-volume respirometers), the oxygen concentration at 40 μM would increase after inhibition of respiration by rotenone, corresponding to a negative flux (apparent oxygen 'production') of -7 pmol O_2 s^{-1} cm^{-3}.

3.2. Rationale of the respirometric regime

A simple phosphorylation control titration regime was performed, to obtain several defined states of mitochondrial respiration in intact cells. In classical experiments with isolated mitochondria (Chance and Williams, 1955; see also: Nicholls and Ferguson (2002)), respiratory states are induced by sequential titrations into the oxygraph chamber, starting with addition of mitochondria into the phosphate-containing mitochondrial medium (state 1), addition of substrates for electron input into a specific respiratory complex (state 2: respiration remains slow, mainly compensating for the passive proton leak and inner membrane ion channels such as uncoupling proteins or the permeability transition pore), and finally titration of a low but saturating concentration of ADP (state 3: respiration is activated by the back-flow of protons into the matrix through the ATP synthase and the concomitant partial drop of the electrochemical proton gradient across the inner mitochondrial membrane). After exhaustion of ADP which is phosphorylated to ATP, respiration returns to a resting level (state 4: elevated above state 2 if ATPase activity recycles ATP to ADP).

The plasma membrane of intact cells prevents the access to mitochondria of various susbstrates such as succinate and ADP. Even without cell membrane permeabilization or isolation of mitochondria, however, defined respiratory states can be induced in intact cells by application of specific membrane-permeable inhibitors and uncouplers, Analogous to ADP limitation of respiration in state 4, inhibition of ATP synthase (complex V) by oligomycin arrests mitochondrial respiration at a minimum level. Oxygen flux measured in this resting state reflects (i) proton leak (uncoupled respiration at maximum mitochondrial membrane potential, which is the main component), (ii) proton or electron slip (decoupled respiration which includes electrons diverted away towards ROS production), (iii) cation cycling (Ca^{2+}, K^+), and importantly in intact cells (iv) all non-mitochondrial and mitochondrial oxidase activities other than cytochrome *c* oxidase which consume oxygen and partially contribute to ROS production. The addition of uncouplers (protonophores, such as carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone, FCCP, or dinitrophenole, DNP) induces a state of maximum respiration (uncoupled state). Uncouplers dissipate the mitochondrial membrane potential and consequently activate the electron transport chain to recycle protons from the matrix side across the inner mitochondrail membrane. The release of mitochondrial respiratory control by the phosphorylation system in the uncoupled state, compared to the maximum inhibition of respiration achieved through blocking ATP sysnthesis by oligomycin, therefore, leads to information on potential respiratory control by coupling as expressed by the respiratory control ratio, RCR (ratio of respiration in the uncoupled state over respiration in the presence of oligomycin; Gnaiger, 2001). Specific segments of the respiratory chain or the P/O ratio, however, cannot easily be analyzed in intact cells, hence isolated mitochondria or permeabilized cells are preferable for that purpose. The particular advantage of studying intact cells is the quantification of respiration in the physiologically controlled state (routine or endogenous respiration), the availability of substrates at physiological concentrations, and exclusion of potential artefacts of mitochondrial isolation (Hofhaus et al., 1996). The ratio of respiration at state 3u over routine or endogenous respiration is conventionally distinguished from the RCR as the uncoupling control ratio, UCR (Steinlechner-Maran et al., 1996; Villani et al., 1998).

3.3. Experimental results

The experiment started with routine respiration, which is defined as respiration in cell culture medium without additional substrates or effectors. After observing steady-state respiratory flux in the time interval between 15–30 min after closing the chamber, ATP-synthase was inhibited with oligomycin (1 μg/ml), followed by uncoupling of oxidative phosphorylation by stepwise titration of FCCP up to optimum concentrations in the range of 1.5–3 μM, for maximum stimulation of flux. Importantly, higher concentrations of uncoupler exert an inhibitory effect on cellular respiration (Steinlechner-Maran et al., 1996). Finally, respiration was inhibited by sequential addition of rotenone at 0.5 μM (to test for the effect of inhibiting complex I activity) and antimycin A at 2.5 μM (inhibiting complex III). This titration protocol was completed within 90 min. At high cell concentrations, intermittent re-aerations are necessary to avoid oxygen limitation of respiration. In Fig. 2A, the recordings of the oxygraph are shown, from which oxygen consumption rates were calculated, as shown in Fig. 2B. Uncoupled respiration amounted to 293 ± 14 pmol O_2 s^{-1} 10^{-6} cells (mean ± SD; $n = 3$) in senescent cells. The corresponding RCR ratio amounted to 7.8 ± 2.9 in senescent HUVEC, indicating a high coupling state of mitochondria in senescent endothelial cells. The UCR, in turn, was 2.9 ± 0.5, indicating a high excess capacity of the electron transport chain over the controlled state

of routine respiration in senescent HUVEC. This UCR corresponds well with uncoupling control ratios of 2.5–3.0, as obtained by high-resolution respirometry in young HUVEC (Steinlechner-Maran et al., 1996, 1997), in transformed endothelial EA.hyb 926 cells (Stadlmann et al., 2002), diploid human fibroblasts (Hutter et al., 2004) and lymphoblastoma cells (Renner et al., 2003). Taken together, the approach described here provides a suitable experimental protocol to assess mitochondrial function in cell-culture-based model systems for human aging, and our results suggest that the respiratory capacity of senescent human endothelial cells does not limit the bioenergetic function of mitochondria in these cells.

4. Conclusions and future developments

High resolution in respirometric analysis is required in particular for (a) analysis of pathological effects resulting in reduced respiration (apoptosis; mitochondrial and metabolic diseases, aging, ischemia-reperfusion injury; oxidative stress); (b) human biopsies with limited amount of sample (genetic and acquired mitochondrial defects, exercise); (c) cell cultures with limited number of cells, and mutants with diminished respiratory capacity; (e) chemical oxidation rates and antioxidant capacities; and (f) respiration at low, physiological intracellular oxygen levels and oxygen kinetics. A 'phosphorylation control titration' (Hutter et al., 2004) with very low concentrations of cultured fibroblasts illustrates the scope of high-resolution respirometry. Some controversies on mitochondrial respiratory function related to aging (for a review see Rasmussen et al., 2003) may be resolved by adhering to the rigorous standard set by the concept and methodology of high-resolution respirometry (Hutter et al., 2004). In the near future, additional applications of the current devices are conceivable, such as new possibilities to measure simultaneously different parameters relevant for monitoring OXPHOS in vivo, in addition to respiration. These include for instance the use of an optic fiber inserted inside the oxygraph to record the fluorescent signals of specific probes allowing to measure mitochondrial membrane potential (JC1, TMRM;) matrix pH (GFP pH) and matrix redox state (GFP redox) etc. These and other portential extensions of high-resolution respirometry will set the stage for a better understanding of mitochondrial function, also in the context of aging research.

Using cellulare in vitro senescence as model system for human aging, we have recently shown that partial uncoupling of the respiratory chain can be observed in senescent human fibroblasts (Hutter et al., 2004), and others have reported a significant decline in the efficiency of oxidative phosphorylation in human fibroblasts derived from the skin of elderly donors (Greco et al., 2003). Along with the correlative data on mitochondrial alterations during aging summarized in section 1, these results raise the possibility that mitochondrial damage may be an important contributing factor for human aging, although our present results with human endothelial cells suggest that the situation may be more complex. In any way, more work will be required to clarify these points. For example, it will be increasingly important to determine mitochondrial function directly in human biopsies from various tissues. Accordingly, a recent publication presented biochemical data from fresh biopsies suggesting a decline of mitochondrial function in human skeletal muscle (Short et al., 2005). However, the study by Short et al. did not directly address the activities of the respiratory chain complexes, a task that can now be approached by high-resolution respirometry, since with the Oxygraph-2k merely 2 mg of biopsies are required per experiment (Kuznetsov et al., 2004; Kuznetsov et al., 2002). It is conceivable that high resolution respirometry will also be applicable to address the role of mitochondrial function in aging of lower eukaryotic model organisms such as *C. elegans*, where a large body of substantial evidence suggests a key role for mitochondria and/or oxidative stress in lifepan determination.

References

Barros, M.H., Bandy, B., Tahara, E.B., Kowaltowski, A.J., 2004. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in Saccharomyces cerevisiae. J. Biol. Chem. 279, 49883–49888.

Braeckman, B.P., Houthoofd, K., Vanfleteren, J.R., 2002. Assessing metabolic activity in aging Caenorhabditis elegans: concepts and controversies. Aging Cell 1, 82–88.

Butow, R.A., Avadhani, N.G., 2004. Mitochondrial signaling: the retrograde response. Mol. Cell 14, 1–15.

Chance, B., Williams, G.R., 1955. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. J. Biol. Chem. 217, 383–393.

Cottrell, D.A., Blakely, E.L., Johnson, M.A., Borthwick, G.M., Ince, P.I., Turnbull, D.M., 2001. Mitochondrial DNA mutations in disease and ageing. Novart. Fnd Symp. 235, 234–243.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Smith, J., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl Acad. Sci. USA 92, 9363–9367.

Gnaiger, E., 2001. Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphyosphate supply. Respir. Physiol. 128, 277–297.

Gnaiger, E., 2003. Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. Adv. Exp. Med. Biol. 543, 39–56.

Gnaiger, E., Steinlechner-Maran, R., Mendez, G., Eberl, T., Margreiter, R., 1995. Control of mitochondrial and cellular respiration by oxygen. J. Bioenerg. Biomembr. 27, 583–596.

Gnaiger, E., Mendez, G., Hand, S.C., 2000. High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc. Natl Acad. Sci. USA 97, 110880–111085.

Greco, M., Villani, G., Mazzucchelli, F., Bresolin, N., Papa, S., Attardi, G., 2003. Marked aging-related decline in efficiency of oxidative phosphorylation in human skin fibroblasts. FASEB J. 17, 1706–1708.

Hampel, B., Malisan, F., Niederegger, H., Testi, R., Jansen-Durr, P., 2004. Differential regulation of apoptotic cell death in senescent human cells. Exp Gerontol 39, 1713–1721.

Harman, D., 1956. Aging a theory based on free radical and radiation chemistry. J Gerontol 11, 298–300.

Hofhaus, G., Shakeley, R.M., Attardi, G., 1996. Use of polarography to detect respiration defects in cell cultures. Method Enzymol. 264, 476–483.

Houthoofd, K., Fidalgo, M.A., Hoogewijs, D., Braeckman, B.P., Lenaerts, I., Brys, K., Matthijssens, F., De Vreese, A., Van Dygen, S., Munoz, M.J., Vanfleteren, J.R., 2005. Metabolism, physiology and stress defense in three aging INs/IGF-1 mutants of the nematode Caenorhabdities elegans. Aging Cell 4, 87–95.

Hutter, E., Renner, K., Pfister, G., Stockl, P., Jansen-Durr, P., Gnaiger, E., 2004. Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts. Biochem. J. 380, 919–928.

Jacobs, H.T., 2003. The mitochondrial theory of aging: dead or alive? Aging Cell 2, 11–17.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745–2756.

Kowald, A., 2001. The mitochondrial theory of aging. Biol. Signal Recept. 10, 162–175.

Kuznetsov, A.B., Strobl, D., Ruttmann, E., Konigsrainer, A., Margreiter, R., Gnaiger, E., 2002. Evaluation of mitochondrial respiratory function in small biopsies of liver. Anal. Biochem. 305, 186–194.

Kuznetsov, A.B., Schneeberger, S., Seiler, R., Brandacher, G., Mark, W., Steurer, W., Saks, V., Usson, Y., Margreiter, R., Gnaiger, E., 2004. Mitochondrial defects and heterogeneous cytochrome c release after cardiac cold ischemia and reperfusion. Am. J. Physiol. Heart Circ. Physiol. 286, H1633–H1641.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R., Guarente, L., 2002. Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature 418, 344–348.

N'Guessan, B., Zoll, J., Ribera, F., Ponsot, E., Lampert, E., Ventura-Clapier, R., Veksler, V., Mettauer, B., 2004. Evaluation of quantitative and qualitative aspects of mitochondrial function in human skeletal and cardiac muscles. Mol. Cell Biochem. 256–257, 267–280.

Nicholls, D.G., Ferguson, S.J., 2002. Bioenergetics 3, Academic Press, London, pp 287.

Petersen, K.F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D.L., DiPietro, L., Cline, G.W., Shulman, G.I., 2003. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science 300, 1140–1142.

Rasmussen, U.F., Krustrupp, P., Kjaer, M., Rasmussen, H.N., 2003. Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. Exp. Gerontol. 38, 877–886.

Renner, K., Amberger, A.A., Konwalinka, G., Kofler, R., Gnaiger, E., 2003. Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. Biochim. Biophys. Acta 1642, 115–123.

Rossignol, R., Faustin, B., Rocher, C., Malgat, M., Mazat, J.P., Letellier, T., 2003. Mitochondrial threshold effects. Biochem. J. 308, 1909–1911.

Schriner, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun, P.E., Ladiges, W., Wolf, N., Van Remmen, H., et al., 2005. Estension of murine lifespan by overexpression of catalase targeted to mitochondria. Science 308, 1909–1911.

Short, K.R., Bigelow, M.L., Kahl, J., Singh, R., Coenen-Schimke, J., Raghavakaimal, S., Nair, K.S., 2005. Decline in skeletal mscle mitochondrial function with aging in humans. Proc. Natl Acad. Sci. USA 102, 5618–5623.

Stadlmann, S., Rieger, G., Amberger, A., Kuznetsov, A.V., Margreiter, R., Gnaiger, E., 2002. H2O2-mediated oxidative stress versus cold ischemia-reperfusion: mitochondrial respiratory defects in cultured human endothelial cells. Transplantation 74, 1800–1803.

Steinlechner-Maran, R., Eberl, T., Kunc, M., Margreiter, R., Gnaiger, E., 1996. Oxygen dependence of respiration in coupled and uncoupled endothelial cells. Am. J. Physiol. 271, C2053–C2061.

Steinlechner-Maran, R., Eberl, T., Kunc, M., Schrocksnader, H., Margreiter, R., Gnaiger, E., 1997. Respiratory defect as an early event in preservation-reoxygenation injury of endothelial cells. Transplantation 63, 136–142.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooy, Y.M., Gidlof, S., Oldfors, A., Wibom, R., et al., 2004. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417–423.

Unterluggauer, H., Hampel, B., Zwerschke, W., Jansen-Durr, P., 2003. Senscence-associated cell death of human endothelial cells: the role of oxidative stress. Exp. Gerontol. 38, 1149–1160.

Vijg, J., 2000. Somatic mutations and aging; a re-evaluation. Mutat. Res. 273, 31829–31836.

Villani, G., Greco, M., Papa, S., Attardi, G., 1998. Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. J. Biol. Chem. 273, 31829–31836.

Wagner, M., Hampel, B., Bernhard, D., Hala, M., Zwerschke, W., Jansen-Durr, P., 2001. Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp. Gerontol. 36, 1327–1347.

Wallace, D.C., 2001. A mitochondrial paradigm for degenerative diseases and ageing. Novart. Fnd Symp. 235, 247–263.