Introduction

Mitochondria are multifunctional organelles derived from a bacterial ancestor that established a symbiotic relationship with another cell early in the evolution of living things. Every cell in the human body has hundreds or thousands of energy-producing mitochondria. These organelles participate in a range of cellular processes such as energy production, proliferation, cell death and aging. They form a dynamic network that efficiently delivers energy to all parts of the cell. They also undergo fission or fusion, depending on the cell’s energy requirements. The past few decades have seen the discovery of diseases in which mitochondria are specifically involved. Mitochondria are now considered to be key players in aging, cellular homeostasis, cancer as well as neurodegenerative disorders.

These complex organelles are also influenced by environmental factors. This 2005 LVMH Recherche Symposium is dedicated to various aspects of mitochondria.

Mitochondria are a key element for human life and harbor proteins that can be released into the cytosol, where they govern programmed cell death and are the guardians of the cell homeostasis. Dysfunction of mitochondria plays a key role in the development of several cancers and most of the anti-cancer drugs in use today cause genetic changes during the process of apoptotic cell removal.

Mitochondrial DNA comes only from the mother’s egg and can be used to trace maternal ancestry without the complicating effects of the mixing of genes from both parents. The mitochondria genome is relatively small and contains few genes. There is now good evidence that the expression of mitochondria genes can be modulated by nuclear genes and new data indicate that there is cross talk between mitochondria genes and nuclear genes. An accumulation of mutagenic oxidative mitochondrial DNA lesions including the formation of 8-oxoguanine is involved in the development of mitochondrial dysfunction in aging and in aging-associated disorders. The mitochondrial genome is more sensitive to reactive oxygen species than the nuclear genome. An age-dependant decline in mitochondria DNA repair has been recently linked to a decline in 8-oxoG DNA glycosylase activity (OGG1) and an accumulation of an endonuclease (APE1), which removes abasic sites in mitochondria.
The mitochondrial respiratory cycle is based on the transfer of electrons from NADH or succinate to molecular oxygen. This electron transfer is accompanied by the release of protons from the mitochondrial matrix into the intermembrane space. The resulting uneven distribution of protons generates a pH gradient and a transmembrane electrical potential that is often used as an indicator of cell viability or of mitochondrial function. Any break in the electron transport chain interferes with ATP production and hence disrupts the cell energy supply. Mitochondria function diminishes with aging, leading to an insufficient energy production for optimal cell function while more toxic oxygen species are generated. Due to aging, some cells lose their oxidative phosphorylation capability and survive thanks to a plasma membrane redox system that reduces oxygen at the cell surface and export a large number of electrons; under such circumstances, the only available electron acceptor is oxygen, highly toxic superoxide anions are formed in large amount, leading to lipid peroxidation and accelerated aging. Oxidative stress can result in the inactivation of mitochondrial matrix proteins and in the accumulation of oxidized proteins as it is observed during aging.

UV light and environmental stresses induce mutations in the mitochondrial genome. Basal epidermal skin cells are extremely sensitive to stresses; under reactive oxygen species stress, their mitochondria potential is dissipated and the protease caspase-8 is activated opening the route to apoptosis. With aging, apoptosis induced by stresses decreased probably in relation with a defective phosphorylation of p53 (a tumor suppressor), and a decrease in the activation of Bax (an apoptosis regulator). Based on centenarian mitochondria studies, high longevity could be related to modulation of the p53 activity and to the maintenance of an efficient autophagy to eliminate damaged mitochondria.

This LVMH Recherche symposium is a powerful affirmation of our belief in the importance of mitochondria as a leading research topic for the future. I would like to thank all the contributors for presenting their original research in this symposium. The many and varied participants from centers in Europe and abroad is a clear evidence that this is a important and fascinating research field that encourages exchanges between scientists working in widely different area.

Frédéric Bonté, Ph.D.
Scientific Manager, LVMH Recherche (Groupe Louis Vuitton—Moët Hennessy)
e-mail: fredericbonte@research.lvmh-pc.com

The Complexity of Mitochondria

Mitochondria 2005 Progress and Challenges

Immo E. Scheffler
Division of Biology, Molecular Biology Section, University of California, San Diego, La Jolla, CA 92093-0322, USA
e-mail: ischeffler@ucsd.edu

Mitochondria represent a prime example of our understanding of biological function related to structure and organization at the level of a subcellular organelle. They were recognized ~50 years ago as the site of cellular respiration, and thus dubbed the
“powerhouse of the cell”. We now have a very detailed understanding of how the free energy from the oxidation of carbohydrates is converted to ATP, the “energy currency” of the cell. ATP hydrolysis is the primary driving force for almost all biological work. P. Mitchell’s chemiosmotic hypothesis is one of the milestones of 20th century biology, and it proposes a detailed mechanism for oxidative phosphorylation involving membrane complexes, electron transport, proton pumps, and a proton-driven molecular motor for ATP synthesis. Many of the relevant protein complexes are now known at atomic resolution, and their integration and function in the inner mitochondrial membrane provide convincing support for the Mitchell hypothesis.

Mitochondria have their own small genome (~16.5 kb in mammals), and this finding gave the impetus for a large number of studies devoted to the evolutionary origin of mitochondria. It is now widely accepted that mitochondria are derived from a bacterial ancestor that established a symbiotic relationship with another cell at one of the earliest stages of the evolution of eukaryotes. Over the course of evolution the mitochondrion lost most but not all of the genes of the proto-bacterium, either by transfer to the nucleus of the eukaryotic cell, or by complete loss of redundant genes. The eukaryotic cell gained a new mechanism for abstracting energy from metabolites in the environment by using oxygen as the ultimate electron acceptor.

The discovery of mtDNA had obvious implications: it must be replicated, it must be transcribed, and machinery must exist within the mitochondria to translate mRNA into proteins. Thirteen proteins are made inside mitochondria. Replication, transcription and translation are mechanisms that resemble well-understood nuclear and cytosolic processes, but differ in some details that challenge us still today.

A mitochondrion contains ~800–1000 different proteins in various amounts, depending on the tissue. The vast majority has to be imported. The import and targeting of the various proteins to the outer and inner membranes, and to two compartments (matrix and inter-membrane space) requires elaborate machinery that has been elucidated in great detail.

Only a fraction of these proteins (~100) are directly associated with respiration and oxidative phosphorylation. A large number are enzymes functioning in a variety of metabolic pathways. One of the fundamental insights from the earliest studies of mitochondria was that metabolism in a eukaryotic cell is highly compartmentalized. As examples one can cite the Krebs cycle (tricarboxylic acid cycle) and the oxidation of the common fatty acids which take place exclusively in mitochondria. The Krebs cycle has been referred to as the “hub of metabolism”, giving mitochondria a central role in all of metabolism. The import and export of various metabolites across the mitochondrial inner membrane requires a large variety of specific transporters. Other transporters are responsible for the transport of various ions, with mitochondria serving as a major reservoir or buffer for calcium ions.

The participation of mitochondria in determining the life and fate of a cell is much broader than recognized only a couple of decades ago. The most dramatic breakthrough came from the discovery that mitochondria harbor proteins that can be released to the cytosol where they play a key role in the mechanism of programmed cell death (apoptosis). These proteins normally reside in the inter-membrane space, but mechanisms can be induced to permeabilize the outer membrane for the release of larger molecules, and these in turn have targets in the cytosol to initiate enzyme cascades. Therefore, the focus continues to be on understanding the signals received by mitochondria from the cytosol, and on the mechanism of release of signals from mitochondria.
Mitochondrial activity controls the redox state in the cell, and under extreme conditions there is oxidative stress with multiple consequences at the level of gene expression, metabolism, cell cycle progression and survival. In many ways they are integrators of information, linking the energy status of the cell to its function and behavior. It is clear that mitochondria are a major source of reactive oxygen species (ROS). At some level ROS have normal regulatory functions with a variety of potential targets, but in excess they are clearly pathogenic. Scavenging enzymes for the removal of ROS in mitochondria and in the cytosol maintain a delicate balance.

A new category of genetic diseases has emerged in the past two decades: mitochondrial diseases. In light of the many essential proteins present in mitochondria the term should be restricted to pathologies resulting from partial defects in the bioenergetic functions of mitochondria. Of particular theoretical interest are diseases due to mutations in the mitochondrial genome. The pattern of inheritance is distinctly non-mendelian, making genetic counseling a challenge. The mutations can occur in heteroplasmic or in homoplasmic individuals. In heteroplasmic individuals the mutations can be severe, but a fraction of the ~1,000 mtDNAs per cell are wild type, and the ratio may be related to the severity of the pathological symptoms. Homoplasmic individuals carry the mutation in all mtDNAs, but the mutations must be missense mutations or leaky, to allow the expression of partially functional proteins, rRNAs, or tRNAs. Thus, the range and severity of symptoms associated with mitochondrial diseases is highly variable, and their diagnosis can be testing. Nuclear mutations with an effect on the capacity for oxidative phosphorylation are also being identified; they can directly affect the activity of the electron transport chain or ATP synthase, or they can affect the maintenance/replication of mtDNA, or the expression of the mitochondrial genes. As might be expected, partial deficiencies lead to pathologies primarily in tissues such as the nervous system and muscle where the demand for ATP is particularly high.

A characteristic aspect of many mitochondrial diseases is their delayed onset. The delayed onset has prompted the consideration of the role of mitochondrial deficiencies in various well-known neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, diabetes mellitus, ALS, cancer, and others. The picture is still cloudy. Mitochondrial involvement is strongly indicated but perhaps not as a primary event.

Finally, there are many new studies suggesting that the phenomenon of aging is strongly influenced by mechanisms associated with mitochondria. In the simplest view, ROS produced by mitochondria act as mutagens on the mitochondrial genome, leading to an accumulation of mutations in mitochondrial DNA and hence, over a lifetime, to a deterioration of the output from the “powerhouse”. In support of these ideas, the lifespan of various experimental organisms has been extended by raising the activity of ROS-scavenging enzymes. Alternatively, the lifespan of mice has been significantly reduced by reducing the scavenging activity, or by expressing an error-prone mitochondrial DNA polymerase in genetically engineered animals.

**General References**


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Interaction Between Mitochondrial and Nuclear DNA

Pierre L. Roubertoux

Génomique Fonctionnelle, Comportements et pathologies (Plasticité et Physio-Pathologie de la Motricité) UMR 6196 CNRS-Université de la Méditerranée 31 Chemin Joseph-Aiguier, 13402 Marseille cedex 20, France
e-mail: rouber@dpm.cnrs-mrs.fr

Mammalian mitochondrial genome has been implicated in a wide set of pathologies including chronic progressive ophthalmoplegia, lactic acidosis with stroke like episodes, myoclonus epilepsy with ragged red fibers encephalopathies and Kear–Sayre disease, among others (Wallace 1999). Implication of mitochondrial genome in ageing has been recently demonstrated (Hirai et al. 2001) and its contribution to neuro-degenerative disorders has been suspected, in Parkinson disease, particularly. Several lines of evidence suggest also the possible role of mitochondrial genome in psychiatric disorders (Kato 2001). Two recent studies, with mice, demonstrate that the role of mitochondrial genome was not limited to pathologies but that mitochondrial DNA polymorphism contributes to the inter-individual differences that occur in typically developing individuals. Body weight, sensorial and motor rate of development, motor behavior, cognitive impairment and in brain structures are modulated by mitochondrial DNA polymorphisms (Totsuka et al. 2001; Roubertoux et al. 2003).

Mitochondrial DNA encodes 13 subunits and specifies 22 tRNAs and 2 rRNAs (Schon, 2000). The implication of the 13 subunits has been demonstrated in respiratory chain. Mitochondrial DNA is exclusively maternally transmitted with probably some exceptions in invertebrates, in interspecies crosses, in mice and, may be in some abnormal cases in human (Kaneda et al. 2001). The point is that mitochondrial genome is of reduced size and that it encompasses few genes. How this reduced number of genes could be involved in a so large number of phenotypes? We suggest here that the polyvalent effect of mitochondrial gene could result from epistatic effect with nuclear genes. Three main arguments are considered. The first argument derives from the study of the transmission of “mitochondrial disorders”. The transmission of the disorders implicating the mitochondrial genome does not tally with the mitochondrial DNA transmission. The pedigrees indicate that the diseases do not follow the maternal transmission that should be expected from mitochondrial inheritance. A second argument derives from the functional study of mitochondria. A mitochondrion uses between 500 and 1,200 proteins whereas it produces no more than 13 proteins. The nuclear genome produces the proteins that are necessary to mitochondrion functioning, development and maintenance. These particularities have been used for deciphering the physiological functions of mitochondrion. Gene targeting of the genes implicated in proteins necessary for mitochondrial physiology results in a vast set of dysfunctions including embryo implantation, heart disorders or accelerated ageing. A third argument is the differential expression of mitochondrial genes. The number of mitochondria vary
according to the cells, from 10,000 in liver up to 70,000 in neuron (Zenisek and Matthews 2001). The effect of a mutation reaching a DNA gene is subsequently tissue dependent (Johnson et al. 2001). We present here experimental evidence indicating that mt genes have different expression in different brain tissues and that a mt gene has different expression, in the same structure when the nuclear genes vary.

If the modulation of mt gene expression by nuclear genes is well-documented, little is known about a retrograde pathway from mt genes to nuclear gene expression. Several studies seem to support the hypothesis of variation of nuclear gene expression (SOD1, APP) according to mt haplotypes, in human.

We address the question of a retrograde pathway from mt genes to nuclear genes in mice by analyzing congenic strains for mtDNA. We selected NZB/BLN and CBA/H mice that carry mtDNA from different origins (Yonekawa et al. 1988) and we performed repeated backcrosses during more than 30 generations (Roubertoux et al. 2003). We present here the development of the quartet of congenic strains, controls for mtDNA cross-transfer and for the isogenicity of the nuclear background. Preliminary results with micro-arrays show that the expression of more than 2,000 nuclear genes was modified by mtDNA cross-transfer.

The findings show that mtDNA which, in most cases, is of unknown origin, may have unpredictable effects. Cloning therefore might generate phenotypes which differ from those expected from the nuclear genotype. Before generating tissues for clone transplanting or using additional mtDNA for in assisted reproduction, fundamental research on the effects of mtDNA is clearly needed.

References


The Plasma Membrane Redox System: Pro-aging and Anti-aging Roles

Aubrey D. N. J. de Grey

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK
e-mail: ag24@gen.cam.ac.uk

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During aging, a small number of cells in various tissues become functionally anaerobic, as a result of the clonal expansion of mitochondrial mutations which eliminate the synthesis of proteins necessary for oxidative phosphorylation (OXPHOS) (de Grey 1997); the contribution of these cells to aging remains controversial (de Grey, 2004). Since the only way to keep OXPHOS-negative mammalian cells alive in culture is to add grossly supraphysiological concentrations of electron acceptors (typically pyruvate) to the medium (King and Attardi 1989), it is paradoxical that such cells can survive in vivo. I proposed in 1998 (de Grey 1998) that they do so by using molecular oxygen as an electron acceptor, reducing it to water just as OXPHOS does but doing so at the plasma membrane rather than at the mitochondrion: this has become known as the reductive hotspot hypothesis (RHH). The basis for this proposal is that the plasma membrane of all cells harbours a transmembrane electron transport capacity termed the plasma membrane redox system (PMRS), whereby cell-impermeant electron acceptors such as ferricyanide are reduced using cytosolic NAD(P)H as electron source (Crane et al. 1985). This system may have a role in cellular metabolism far exceeding that suggested by the so far modest amount of research interest that it has attracted.

Mechanism of Cell Survival without OXPHOS

The reason why OXPHOS-negative cells need pyruvate to survive in vitro is that they cannot otherwise “balance the electron books”. They can generate ATP by glycolysis, and if the pyruvate that this generates is then reduced to lactate and excreted there is no net oxidation or reduction of NAD(P)H. However, the remainder of cellular metabolism evidently requires a net production of electrons. Normally these electrons would be consumed at Complex I and eventually transferred to oxygen at Complex IV, but when OXPHOS is absent this cannot occur. Exogenous pyruvate is thus presumed, in the standard interpretation, to be taken up by such cells and reduced along with the pyruvate derived from glycolysis, thereby relieving the cell of its excess electrons. In vivo, the only electron acceptor likely to be available in adequate amounts is molecular oxygen, and RHH proposes that, since no cytosolic NADH oxidase exists, rather than being reduced in the cytosol oxygen is reduced at the cell surface by the PMRS.

At least in vivo, however, there is evidence that more is going on. OXPHOS-negative cells can be identified histochemically, and they generally exhibit up-regulation of the TCA cycle enzyme succinate dehydrogenase (SDH) (Moraes et al. 1992). This challenges the model just described, in which pyruvate is not imported into mitochondria and the TCA cycle is inactive on account of the unavailability of a destination for the electrons it would produce. However, if cytosolic NADH could be reduced using intramitochondrial NADH and then reoxidised (by either lactate dehydrogenase or the PMRS), the TCA cycle could proceed, just so long as the rate of cytosolic NADH oxidation is sufficient. This has innumerable benefits for the cell, in view of the central role of the TCA cycle in such processes as amino acid synthesis, and it also means the cell can make ATP from fatty acid oxidation and twice as much from glucose oxidation as otherwise, since succinyl CoA dehydrogenase creates ATP (via GTP). Thus, I proposed (de Grey 1998) that cells do just that: they achieve it by reversing the normal direction of flow of the malate/aspartate and glycerophosphate shuttles, presumably by altering the redox state of the NAD pools in the mitochondrial and/or cytosolic compartments.
Pro-aging Consequences of OXPHOS-free Survival via the PMRS

This has potentially disastrous consequences during aging. If OXPHOS-negative cells survive by exporting electrons at a high rate, and if the only available electron acceptor is oxygen, there may be a risk of one-electron reduction of extracellular oxygen to superoxide, just as occurs at the mitochondrion when the respiratory chain is not flowing smoothly. This is potentially highly toxic, because it can initiate peroxidation of lipids in circulating material, particularly low-density lipoprotein (LDL). Lipid peroxidation chain reactions can thus greatly amplify the damage done by rare OXPHOS-negative cells, since contaminated LDL may be internalised by OXPHOS-positive cells and raise their oxidative stress (de Grey 2002). The PMRS's structure is still poorly understood (de Grey 2003), but electron transport is known to be accompanied by superstoichiometric proton export, i.e. proton pumping (Sun et al. 1995), so superoxide production is distinctly plausible. Superoxide is also produced at high levels when the PMRS is stimulated with extracellular NADH (O'Donnell and Azzi 1996). This could explain why the redox state of the plasma shifts throughout life to a progressively more oxidised state as measured by the cysteine/cystine and GSH/GSSG couples (Jones et al. 2002).

Anti-aging Consequences of OXPHOS Modulation via the PMRS

One may ask why the PMRS is so ubiquitous: after all, in cells with intact OXPHOS the cytosolic redox state should be under control. Possibly the mitochondrial shuttles are not responsive enough to changes in cytosolic redox state and the PMRS acts as an additional safety valve. Interestingly, however, one possible example of this is in the anti-aging response of many animals, including rodents, to caloric restriction (CR) (Rae 2004). A decade ago it was reported that CR induces a dramatic reduction in the activity of mitochondrial Complex I in skeletal muscle but does not affect Complex II (Desai et al. 1996). This is consistent with a milder adjustment of compartment-specific redox states, enough to reverse the malate/aspartate shuttle but not the glycerophosphate shuttle, with the consequences for the cytosolic NAD pool again being offset by upregulation of the PMRS. Since the throughput of the PMRS would be raised only mildly, superoxide generation there might not occur; however, since Complex I is the major superoxide producer in the mitochondrial respiratory chain, there could be a marked suppression of mitochondrial (and hence total) superoxide production, with obvious consequences for aging (de Grey 2001).

References


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Mitochondria and the Environment

UV and Environmental Stress: Influence on the Mitochondrial Genome

Jean Krutmann

M.D. Institut für Umweltmedizinische Forschung (IUF) at the Heinrich-Heine University gGmbH, D-40225 Duesseldorf, Germany

Photoaged skin is characterized by increased mutations of the mitochondrial genome (Berneburg et al. 1997). Intraindividual comparison studies have revealed that the so-called common deletion, a 4977 base pair deletion of mtDNA, is increased up to 10-fold in photoaged skin, as compared with sunprotected skin of the same individuals. The amount of the common deletion in human skin does not correlate with chronological aging, and it has therefore been proposed that mtDNA mutations such as the common deletion represent molecular markers for photoaging. In support of this concept it was shown that repetitive, sublethal exposure to UVA radiation at doses acquired during a regular summer holiday induces mutations of mtDNA in cultured primary human dermal fibroblasts in a singlet oxygen-dependent fashion (Berneburg et al. 2005). Even more important, in-vivo studies have revealed that repetitive 3-times daily exposure of previously unirradiated buttock skin for a total of two weeks to physiological doses of UVA radiation leads to an approximately 40% increase in the levels of the common deletion in the dermal, but not epidermal compartment of irradiated skin (Berneburg et al. 1999). Furthermore, it was shown that, once induced, these mutations persist for at least 16 months in UV-exposed skin. Interestingly, in a number of individuals, the levels of the common deletion in irradiated skin continued to increase with a magnitude up to 32-fold. It has been postulated for the normal aging process as well as for photoaging that the induction of ROS generates mtDNA mutations, in turn leading to a defective respiratory chain and, in a vicious cycle, inducing even more ROS and subsequently allowing mtDNA mutagenesis independent of the inducing agent (Berneburg et al. 1999). It is the characteristic of vicious cycles that they evolve at ever increasing speeds. Thus, the increase of the common deletion up to levels of 32-fold, independent of UV
exposure, may represent the first in vivo evidence for the presence of such a vicious cycle in general and in human skin in particular.

The mechanisms by which generation of mtDNA mutations by UVA exposure translates into the morphologic alterations observed in photoaging of human skin are currently being unraveled. Recent studies demonstrate that UVA radiation-induced mtDNA mutagenesis is of functional relevance in primary human dermal fibroblasts and apparently has molecular consequences suggestive of a causative role of mtDNA mutations in photoaging of human skin as well (Berneburg et al. 2000). Accordingly, induction of the common deletion in human skin fibroblasts is paralleled by a measurable decrease of oxygen consumption, mitochondrial membrane potential and ATP content as well as an increase of MMP-1, while TIMP remains unaltered, an imbalance that is known to be involved in photoaging of human skin. These observations suggest a link not only between mutations of mtDNA and cellular energy metabolism, but also between mtDNA mutagenesis, energy metabolism and a fibroblast gene expression profile that would functionally correlate with increased matrix degradation and thus premature skin aging.

References


Repair of Oxidative Damage in Mitochondrial Genome

Bartosz Szczesny, Ranajoy Chattopadhyay, Tapas K. Hazra, Kishor K. Bhakat, Tadahide Izumi, Lee R. Wiederhold, Istvan Boldogh, and Sankar Mitra
Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX 77555, USA

A significant number of human diseases have etiologic linkage to mutations and damage in the mitochondrial (mt) genome which is more susceptible to reactive oxygen species (ROS) than the nuclear genome. Mammalian mitochondria are the predominant source of endogenous ROS, primarily superoxide anion radical (O2•−). Furthermore, any initial damage to the mt genome has a spiraling effect due to increased ROS generation which in turn causes more oxidative damage to mt genomes. These damages are repaired via the DNA base excision repair (BER) process. BER is initiated by a DNA glycosylase which excises damaged bases, and by AP-endonuclease (APE) which removes abasic (AP) sites and 3′ blocking groups at strand breaks induced by ROS. Decline in cellular functions is responsible for the aging syndrome whose underlying causes are not completely clear. However, it is widely accepted that chronic oxidative stress is a major contributor to the
aging process. Our goal is to explore if and how age-dependent decline in repair of oxidative damage in the mt genome contributes to decline in cellular functions.

We have investigated age-dependent modulation in the activities of 8-oxoguanine (8-oxoG) DNA glycosylase (OGG1), primary responsible for repair of 8-oxoG, a major ROS product, and of mtAPE. The same nuclear gene encodes nuclear OGG1-α and mt OGG1-β via alternative splicing. In attempting to explain the paradox of age-dependent increase in both 8-oxoG level in the mt genome and OGG1-β level in mitochondria, we examined mt translocation of OGG1-β. Translocation of a protein into mt matrix generally requires a N-terminal mitochondrial targeting sequence (MTS), which is cleaved in the mt matrix. We clearly showed that in old mouse tissues most OGG1-β is not in the matrix, but remains stuck on the mt membrane. Based on this and similar results with UDG, which repairs uracil (generated by oxidative deamination of cytosine), we postulate that repair deficiency in mitochondria of old animals is due to age-dependent decline in mt import (Szczesny et al. 2003).

The origin of mammalian mtAPE was not clear until our recent studies showed that it is derived from APE1, the sole APE in mammalian cells, as a result of cleavage of 33 N-terminal amino acid (aa) residues by a mitochondrial membrane/endoplasmic reticulum (ER)-associated, unidentified serine protease (Szczesny et al. 2003; Chattopadhay et al. (submitted). These results have led to a model that APE1 is often cytosolic, but is translocated to the nucleus following oxidative stress due to the presence of NLS (Ramana et al. 1998; Jackson et al. 2005). However, oxidative stress, which activates the Ser protease, cleaves off the NLS in a fraction of APE1 molecules for mt targeting of the truncated polypeptide, presumably due to the presence of an internal MTS. Analysis of age-dependent modulation in intracellular distribution of APE activity in liver and heart of BALB/c mice showed that, unlike OGG1 and UDG, both mitochondria and nuclei of old mouse tissues have higher APE level relative to those from young mice, associated with corresponding decrease in the level of cytosolic enzyme. These results are consistent with the scenario that age-dependent chronic oxidative stress induces nuclear and mt translocation of APE (Szczesny and Mitra 2005). However, in spite of the signal for similar mt targeting of OGG1, the defect in the import machinery prevents its accumulation inside the mt matrix. A different import mechanism may thus be responsible for mt accumulation of APE1.

APE1, unlike OGG1, is essential for development of mouse embryos, and no APE1 null cells could be generated. We established mouse embryo fibroblasts which lack endogenous APE1 alleles but express the human APE1 transgene. Deletion of the transgene induces apoptosis which could be prevented by simultaneous expression of wild type but not repair-deficient APE1 mutant (Izumi et al. 2005). Because AP sites and oxidized bases in the nuclear genome could be alternatively repaired via an APE1-independent pathway (Wiederhold et al. 2004), we propose that the mt APE generated from APE1 is essential for survival. (Research supported by U.S. Public Health Science grants P01 AG 021830, R01 ES08457, and R01 CA53791.)

References


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Mitochondrial Protein Degradation in Ageing and Oxidative Stress

Bertrand Friguet

Université Denis Diderot—Paris 7, Laboratoire de Biologie et Biochimie Cellulaire du Vieillissement, 2 place Jussieu, 75005 Paris, France
e-mail: bfriguet@paris7.jussieu.fr

Mitochondria are the major source of intracellular reactive oxygen species, the production of which increases with age and they are also one of the main targets for reactive oxygen species induced damage. The deleterious effect of reactive oxygen species can be responsible for the impairment of mitochondrial function observed in various situations such as oxidative stress and ageing. Oxidative damage to proteins is a hallmark of the ageing process and protein maintenance (i.e. degradation and repair) has been recognized as an important factor for cellular homeostasis and survival while protein maintenance failure is considered as a critical component of the ageing process. Mitochondrial matrix proteins are sensitive to oxidative inactivation and oxidized proteins are known to accumulate during ageing. The ATP-stimulated mitochondrial Lon protease is a highly conserved protease found in prokaryotes and the mitochondrial compartment of eukaryotes and is believed to play an important role in the degradation of oxidized mitochondrial matrix proteins. In addition, mammalian Lon has been shown to display chaperone properties and to specifically bind sequences of human mitochondrial DNA and RNA, as well as to interact with mitochondrial DNA polymerase γ and the twinkle helicase (Liu et al. 2004). Concerning oxidized protein degradation in the mitochondrial matrix, Kelvin Davies and coworkers have shown that the oxidant-sensitive Krebs cycle enzyme aconitase, when oxidatively damaged, is degraded by the Lon protease in an ATP-stimulated fashion (Bota and Davies 2002). In the same study, treatment with anti sense oligonucleotides in WI-38 human lung fibroblasts resulted in decreased Lon protease content and activity while causing an accumulation of oxidatively modified aconitase. More recently, they have also shown that down-regulation of the human Lon protease results in the impairment of mitochondrial structure and function and causes cell death, with the majority of cells undergoing caspase 3 activation and apoptosis within 4 days (Bota et al. 2005). Electron microscopy performed on Lon-deficient cells revealed aberrant mitochondrial morphology and the presence electron dense inclusion bodies in the mitochondrial matrix thought to be caused by aggregated proteins.

Concerning the mitochondrial oxidatively modified protein status and the fate of the Lon protease during ageing, we have reported that an age-related accumulation of altered (i.e. oxidized and glycoxidized) proteins occurs in the liver mitochondrial matrix.
of rats and that the ATP-stimulated proteolytic activity decreases considerably in 27-month-old rats, whereas no concomitant changes in the levels of Lon protein expression occur in the liver (Bakala et al. 2003). This decline is associated with a decrease in the activity of mitochondrial aconitase, an essential Krebs’ cycle enzyme. Contrary to what we observed in the liver and what was previously observed in mouse skeletal muscle where ageing was associated with a decrease in the levels of Lon gene and protein expression (Lee et al. 1999; Bota et al. 2002), 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 (Delaval et al. 2004). 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. These results indicate that matrix proteins such as the critical enzymes aconitase and Lon protease are getting inactivated with ageing and that the effects of ageing on these enzymes vary from one organ to another.

Using transgenic mice under-expressing mitochondrial superoxide dismutase as a model of oxidatively challenged animals, Bota et al. (2002) reported a decreased Lon protease protein level and this Lon deficiency was associated with increased levels of oxidized proteins. More recently, Luke Szweda and coworkers have evidenced that during in vivo cardiac ischemia-reperfusion, ATP-stimulated protease activity similar to Lon protease activity was increased during early reperfusion followed by a time-dependent reduction in activity back to the control level (Bulteau et al. 2005). This modulation in proteolytic activity paralleled an increase and subsequent decrease in the level of oxidized protein while a role for pro-oxidants induced activation of ATP-dependent protease activity was supported by in vitro experiments. Indeed, when challenged with low concentrations of hydrogen peroxide, ATP-dependent protease activity was stimulated. These results suggest that reperfusion-induced prooxidant production regulates mitochondrial ATP-dependent proteolytic activity, an event that would be expected to reduce irreversible damage to the mitochondria.

Taken together, these data underscores the important role of the mitochondrial Lon protease as a major contributor in both mitochondrial protein maintenance and cellular redox homeostasis through regulated degradation of oxidatively modified proteins.

References

Homeostasis of the Human Epidermis: Central Role of Mitochondria

T. Zuliani, M. Dumas, and M. H. Ratinaud*
LVMH Recherche, Saint Jean de Braye, France, * EA-3842, Homéostasie Cellulaire & Pathologies, Université de Limoges, France

The outermost layer of the human skin, the epidermis, is a dynamic organ. Its active renewal is due to keratinocytes, the major cell type. Keratinocyte stem cells in the basal cell layer give rise to the proliferative transit amplifying cells that undergo terminal differentiation in the suprabasal cell layers to become corneocytes (Watt 2001). Apoptosis is a physiologic controlled cell death, essential for organ homeostasis and maintenance by eliminating unwanted or damaged cells. It is active in the epidermis, which is chronically exposed to environmental stress (Murphy et al. 2002). Among them, ultraviolet radiation (UVR) is the main cause of neoplastic development and photoaging. These harmful effects of UVR are produced directly or through the generation of reactive oxygen species, including hydrogen peroxide (H$_2$O$_2$), which is generated by nearly all sources of oxygen radicals and oxidative stress.

Mitochondria plays a key role in apoptosis releasing pro-apoptotic factors, such as cytochrome c, Smac-Diablo, AIF (apoptosis-inducing factor) and pro-caspases (cysteine-containing aspartate-specific proteases), from its inter-membrane space into the cytoplasm. Cytochrome c binds to Apaf-1 (apoptosis protease activating factor 1) and activates caspase-9, which then activates caspase-3 (Li et al. 1997). Smac/Diabolo binds to XIAP (X chromosome inhibitor of apoptosis protein) and prevents it inhibiting caspase activation (Du et al. 2000). AIF translocates to the nucleus and induces chromatin condensation and DNA fragmentation through a caspase-independent pathway (Susin et al. 1999). The mitochondrial transmembrane potential ($\Delta\Psi_{mt}$) is also dissipated in the early stage of apoptosis, implicating a megapore, the PTPC (permeability transition pore complex) composed of proteins of the two mitochondrial membranes (Marzo et al. 1998). All these early events are regulated by pro-apoptotic proteins such as Bax and Bak and anti-apoptotic proteins like Bcl-2 and Bcl-x$_L$ and their ratio and relocalisation in the mitochondrial membrane determine whether or not a cell enters apoptosis (Marzo et al. 1998). In addition to the mitochondrial intrinsic signalling pathway, there is also an extrinsic route that involves the death receptors. These receptors belong to the TNFR (tumor necrosis factor receptor) family containing a cytoplasmic death domain that binds the ubiquitous protein FADD (Fas-associated death domain) that can, in turn recruit procaspase-8 (Kischkel et al. 1995). Autocatalytic activation of caspase-8 induces the production of the executive proteases caspase-3, caspase-6 and capase-7, and finally apoptosis.

In cultured human keratinocytes, the signalling pathway of H$_2$O$_2$-induced apoptosis includes dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_{mt}$) without any change in the permeability of the plasma membrane, and large increases in the activities of caspase-8, capase-9 and capase-3. Thus both the extrinsic route (caspase-8 and death receptors) and the intrinsic route (caspase-9 and mitochondrial loop) are activated in undifferentiated keratinocytes (Zuliani et al. 2003).
Double staining studies with JC-1 (to analyse ΔΨmt) and TOTO-3 (to measure plasma membrane integrity) revealed that isolated β1-integrin positive basal epidermal cells are more sensitive to H2O2-induced apoptosis (JC-1\textsuperscript{low}/TOTO-3\textsuperscript{high}) than are suprabasal differentiated cells. This sensitivity of basal cells to apoptosis was confirmed by studies on H2O2-treated human skin biopsies where caspase-3 activity and cells with single strand DNA breaks were mainly located in the β1-integrin positive cells of the basal epidermis. (Zuliani et al. 2005a).

The age of the skin affects dramatically the in vivo apoptotic response of the epidermis to full solar spectrum irradiation. There were fewer cells with active caspase-3 and single strand DNA breaks per square millimeter of epidermis in skin from older donors than in skin from younger ones. This global decline in apoptosis suggests that damaged keratinocytes persist for longer in the epidermis of older people following UV irradiation and may thus foster the development of neoplastic cells and the loss of cell and tissue function. Nevertheless, the high percentage of cells entering apoptosis in the basal layer, more than 1/3 of the total apoptotic cells, confirms that apoptosis is a key repair mechanism in this germinative layer and occurs more frequently here than in the suprabasal layers. In some ways, it could also account for the gradual reduction in the number of epidermal cell with age due to repeated acute exposure to UV and/or oxidative stress. This hypothesis is reinforced by the observation that the proportion of apoptotic cells in the basal layer was slightly but significantly increased in the epidermis of older donors.

The number of cycling Ki67-positive cells in the epidermis decreases with age. Cell growth stops after irradiation to allow cells to repair damage or to enter apoptosis. This is most marked in the skin of young donors, but can be absent from the skin of older people, indicating cell cycle dysregulation suggesting the implication of p53. The p53 exerts an anti-neoplastic activity by blocking the cell cycle, by increasing the activities of DNA repair enzymes, the release of cytochrome c from mitochondria and apoptosis (Chipuk et al. 2004). The protein dramatically increases in the epidermis 24 h after full spectrum UV irradiation, but the increases were the same in the skin samples from young and old subjects. However immunostaining showed that it was not as extensively phosphorylated at Ser-15 in older skin samples as in young skin samples, and this could contribute to cell cycle dysregulation and reduced apoptosis.

P53 regulates the apoptotic machinery by activating the transcription of Bax. It can also directly activate Bax to permeabilise the mitochondria, so allowing an uninterrupted pathway (Chipuk et al. 2004). With a selected anti-Bax antibody in vivo we found a strong perinuclear and punctiform staining in epidermal cells 24 h after UV irradiation, which fits well with the relocalisation of Bax in the mitochondria during apoptosis. More over, double labelling indicated that these Bax positive cells were also caspase-3 positive (Zuliani et al. 2005b). Bax was less strongly activated in skin from older donors than in skin from younger ones, which explains part of the lost apoptosis. We also detected 3 Bax transcripts in normal human undifferentiated keratinocytes in addition to Bax α: the two already characterized β and γ variants and a new one λ. (Zuliani et al. 2005). The λ splice variant is specific to basal keratinocytes, suggesting a role in the regulation of apoptosis in this compartment and in the greater sensitivity of basal cells to apoptosis but its pro- or anti-apoptotic role has now to be clarified.

These data show that apoptosis is active in the epidermis after acute exposure to UV irradiation and that it is a differentiation-dependant process that occurs most frequently in the basal germinative layer where stem cells and transit amplifying cells reside. The apoptotic response to UV stress declines with the age in the epidermis, probably due to...
dysregulation of the cell cycle, defective p53 phosphorylation and Bax activation implicating the mitochondrial pathway. These events constitute key targets and provide new perspectives for maintaining cells and tissue homeostasis in the skin of older people.

References


Are Mitochondria a Key Step to Longevity? Studies on Centenarians

Stefano Salvioli, Miriam Capri, Daniela Monti, Aurelia Santoro, Cristiana Barbi, and Claudio Franceschi

Department of Experimental Pathology, via S. Giacomo 12 - 40126 Bologna, Italy
e-mail: stefano.salvioli2@unibo.it

Mitochondria in Aging and Longevity

In the last years mitochondria have attracted considerable attention because of their role in aging and in a number of major age-associated diseases, such as cancer, diabetes, and Parkinson's disease.

These organelles are emerging as crucial modulator for a series of biological activities which depend not only on ATP availability, but also on signalling pathways that are integrated at mitochondrial level. This flow of information from mitochondria to the nucleus has been termed in yeasts “retrograde response” (1). An increasing amount of evidences indicates that likely this kind of response also exists in mammals and humans (2).

Giving the importance of mitochondria in most of the biological processes of the cells, we wondered if a possible key determinant of longevity could lie at the level of mitochondrial function. It is well known that mitochondria can play a critical role in aging since they are the major source and the most proximal target of reactive oxygen
species, but also because they regulate stress response and apoptosis. Recent literature indicates that, in response to stress, a variety of molecules translocate to and localize in mitochondria, among which there are p53 (3) and p66\textsuperscript{Shc} (4). These molecules are likely to interact with each other, in order to mediate mitochondria/nucleus cross-talk and to regulate apoptosis. We surmised that an integration of signals in multi-molecular complexes can occur at mitochondrial level (5).

The Centenarians as a Model of Longevity in Humans

Centenarians are exceptionally long living subjects, who escaped or delayed the above reported age-associated diseases, and thus they are considered as the best example of successful aging (6). Is it possible that mitochondria from centenarians have some peculiar difference with respect to those of non long living subjects? Do centenarians become “centenarians” because of their mitochondria?

Mitochondria, Apoptosis and p53

In previous studies we described a decreased tendency to apoptosis after 2′-deoxy-D-Ribose (dRib)-induced oxidative stress in peripheral blood mononuclear cells (PBMC) from old people and centenarians with respect to PBMC from young people. This different susceptibility to apoptosis was associated with a decreased tendency to lose mitochondrial membrane potential (MMP), that is an index of well preserved mitochondrial functionality. Intracellular bcl-2 levels were similar in all age classes (7,8). These data have been reproduced also in dermal fibroblasts and surprisingly we found that the decreased susceptibility to apoptosis was correlated to a common C372G polymorphism of p53 gene. This polymorphism determines an arginine (Arg) to proline (Pro) aminoacidic substitution at codon 72 (9). The Arg variant of p53 has a greater apoptotic potential than the Pro one (10), and in our hands this difference was evident as the age of the donors increased, being negligible in young subjects and much more evident in old people and centenarians (11). Our data suggested that this could be at least in part explained by the fact that, with respect to p53Pro variant, p53Arg variant is much more localized in mitochondria, where it can act as an apoptosis regulator (11). These data point out an important role of mitochondria in modulating the apoptotic signal of p53 and indicate that this phenomenon is highly age-dependent.

Mitochondrial DNA (mtDNA) and Longevity

The role of mtDNA in longevity has been rather neglected since some years ago, as most of the mitochondrial proteins are encoded by nuclear genes and mtDNA codes for a very limited number of genes. However, owing to lack of histones, poor DNA repair, and vicinity to ROS, mtDNA mutations accumulate with age much faster than in nuclear DNA, leading to mitochondrial dysfunction and possibly increased ROS production (12). In centenarians, most of the mutations found in the mtDNA are known to be disease-associated mutations, even though in centenarians there is no clinical evidence of such diseases (13). Due to the low number of subjects studied until now, these results must be confirmed and we are at present starting a more extensive study aimed to the resequencing of the complete mtDNA molecule in an unprecedented number of centenarians. A notable exception to this finding is the C150T transition found in the
D-loop of mtDNA. This mutation causes a remodeling of one mtDNA replication origin from position 151 to position 149 and is found at higher frequency in centenarians with respect to young people (17% vs 3%) and it is thus considered as associated to longevity (14). This study has been recently confirmed in ethnically different populations (15).

Beside acquired, somatic mutations, the mtDNA is characterised by the presence of natural, germ-line inherited variants that define several different types of mtDNA, indicated as “haplogroups”. In the Caucasian population, there are at least 9 different haplogroups that have long been considered as biochemically neutral, while recently it has been reported that a correlation of haplogroup J with longevity is present (16). In particular, this haplogroup has been found to be more represented among centenarians with respect to young people in European populations (Northern Italians, Irish and Finns), but not in Southern Italians (17). Likely it is not by coincidence that the C150T mutation seems to be associated with J2 mtDNA subhaplogroups (15). Further, haplogroup J can interact with nuclear genes by influencing their penetrance. This is the case for example of the epsilon4 allele of APOE gene, a well known risk factor for sporadic Alzheimer’s disease. Some mtDNA haplogroups (K and U) seem to neutralize the harmful effect of the APOE epsilon4 allele, lowering the epsilon4 odds ratio from statistically significant (3.77 for APOE4 carriers vs non carriers) to non-significant values, while haplogroup J increased this OR to 9.693 (18). We hypothesize that these figures are likely the first example of a broader interaction between nuclear and mitochondrial genetic variants.

A Possible Role for Autophagy in Longevity

Finally, it is possible that the proper elimination of damaged mitochondria is a crucial event for attaining longevity. Mitochondria are mainly degraded through autophagic processes, and autophagy is observed to decline with age. Among the proteins involved in autophagy there is Akt, which is involved also in IGF-1 signalling pathway, whose alteration leads to longevity in animal models (19). Moreover, autophagy is induced by caloric restriction, the best known strategy to prolonge lifespan. Consequently, it has been hypothesized that the maintenance of an efficient autophagic capacity can be important in longevity as it ensures of the elimination of dysfunctional mitochondria (20). This may depend on the proper expression of targeting proteins such as Uth1p, needed for autophagic degradation of mitochondria (21) in cells of aged subjects. Incoming studies are needed to test these intriguing hypothesis.

References