Minireview

Flow cytometry of isolated mitochondria during development and under some pathological conditions

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Abstract Mitochondria play an essential role in the generation of the energy needed for eukaryotic cell life and in the release of molecules involved in initiation of cell death. Here we review the changes in isolated mitochondrial fluorescent populations as distinguished by flow cytometry during postnatal development and their regulation by thyroid hormones and catecholamines. The use of flow cytometry in the study of mitochondrial changes occurring under hypothyroidism, alcohol abuse and aging is also reviewed.

Key words: Mitochondrion; Flow cytometry

1. Introduction

Extensive biochemical studies have shown that mitochondria undergo major functional and structural changes during development, which are related, to a large extent, to adaptation to postnatal life [1,2]. Moreover, mitochondrial activities are not only involved in but are also dependent on the overall cell physiology, the cell cycle position, differentiation stage, hormone stimulation, pathological status and drug effects (for review see [3]). Under specific circumstances (i.e. during fusion of myoblasts), the analysis of mitochondrial activities should more likely be performed on intact cells, although frequently, these type of measurements are complex. Stainings with membrane potential sensitive fluorescent probes (i.e. rhodamine-123 (Rh-123), 3,3′-dihexylocarbocyanine iodide (DiOC6(3)), safranine O) have been extensively used for the study of mitochondria in intact cells by either flow cytometry or image analysis (for review see [4]). However, fluorescence variations observed in a cell are the result of the equilibrium in the distribution of the probe among the different cell compartments including the extracellular medium; though overall fluorescence due to mitochondrial uptake of lipophilic cations depends on membrane potential, it also reflects mitochondrial mass and activity [5]. Therefore, for the specific characterization of mitochondria, the analysis of dye-associated fluorescence in isolated organelles submitted to various metabolic conditions, provides unique information. Here we review the contribution of flow cytometry to the study of isolated mitochondria in both normal development and under different pathological conditions (Table 1).

2. Changes in mitochondrial populations during perinatal development

The acquisition of fully developed mitochondria after birth is an important homeostatic mechanism that enables newborn mammals to successfully adapt to extra-uterine life in which oxygen is freely available. It has been clearly demonstrated that during the perinatal period, rat liver mitochondria undergo many significant changes with respect to their structure and function. This process includes mitochondrial proliferation [6,7] whereby the number of mitochondria are increased and organelle differentiation achieved through an exclusive mechanism during which pre-existing RNA messengers are promptly translated in order to cope with the adaptation to the extra-uterine life [2,8–11].

Since mitochondrial proliferation is a continuous process during the entire period of normal life (for review see [1]), the pattern of induction of mitochondrial enzymatic activities after birth [12,13] has been explained by the proliferation of the organelle in these circumstances [6,7,14]. It should be mentioned that unlike differentiation, proliferation is controlled at the transcriptional level of protein synthesis [15]. On the other hand, mitochondrial differentiation occurs very rapidly after birth, within the first postnatal hour in the rat [1,2,9,16,17]. Under the differentiation process, pre-existing mitochondria acquire ultrastructural, molecular and functional characteristics that define organelle function. The most striking changes are related to the increase of the respiratory control ratio (RCR) which takes place immediately after birth [9,16,18]. These changes are accounted for by the increase of cytochromes *a*+*a*3, *b*, *c*+*c*1 [19], respiratory complexes [2,9] and synthesis of β-subunit of the F1 part of H^+-ATP synthase [20] in a process that is particularly regulated at the transductional level [11]. In addition, such functional changes are associated with an enhancement in the resistance of mitochondrial membranes to osmotic pressure [8,17,18], suggesting that achievement of mitochondrial oxidative function is associated with structural changes in mitochondrial membranes.

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Abbreviations: RCR, respiratory control ratio; Rh-123, rhodamine-123; ROS, reactive oxygen species; PTPC, permeability transition pore complex

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Table 1
Analysis of isolated mitochondria by flow cytometry: review of the literature

Cell type	Biological properties analyzed	Fluorescence probe	Reference
Potato tuber	Mitochondrial subpopulations based on Con-A and/or WGA binding sites	Con-A FITC, WGA-FITC	[79]
Mouse liver	Membrane potential under several metabolic conditions	Rh-123	[80]
Rat liver	Effects of high protein diet on membrane potential	Rh-123	[81]
Rat liver	Mitochondrial subpopulations based on their Rh-123 uptake and light scatter	Rh-123	[27]
Rat and mouse liver	Relationship between membrane potential and structural, metabolic and functional heterogeneity of mitochondria	Rh-123, safranine O, DiOC6(3)	[82]
Rat liver	Developmental changes in mitochondrial subpopulations	Rh-123	[28]
Potato tuber	Effects of membrane potential modulators	Rh-123	[83]
Rat hepatocyte (primary culture)	Changes in Rh-123-stained mitochondrial populations during cell cycle	Rh-123	[29]
Rat liver	Postnatal changes in mitochondrial populations induced by protein synthesis inhibitors	Rh-123	[16]
Rat liver	Effects of EtOH intake in the distribution of mitochondrial populations and their membrane potential	Rh-123	[50]
Rat liver	Effects of hypothyroidism on postnatal changes of mitochondrial populations	Rh-123	[43]
Rat liver	Changes in mitochondrial populations at different energy states	Rh-123	[30]
Rat hepatocyte	Age-associated mitochondria peroxide generation and size	Dihydrorhodamine-123	[67]
Rat hepatocyte (primary culture)	Analysis of Ca^{2+} mobilization in mitochondria	Fluo3-AM	[34]
Jurkat and FL5.12 cell lines	Quantitative evaluation of mitochondrial membrane potential and size	Rh-123	[77]
Rat and mouse liver	Quantitative evaluation of mitochondrial membrane potential and size	DiOC6(3)	[78]
Human fibroblast	Sorting of mitochondria for analysis of mDNA	10 *N*-nonyl-acridine orange	[76]

WGA: Wheat germ-agglutinin. Con-A: Concanavalin A. DiOC6(3): dihexlocarbocyanine iodide

Adenine nucleotides are known to promote significant ultrastructural changes in isolated mitochondria from adult [9,21], fetal and neonatal rat liver [9]. The adenine nucleotide hypothesis to explain the rapid postnatal mitochondrial maturation was originally put forward by Pollak et al. [8,18,22]. In the newborn liver, the mitochondrial adenine nucleotide content increases three to four fold within a few hours after birth. Since ATP–ADP translocase operates as one-for-one exchange [23], the net accumulation of adenine nucleotides in mitochondria cannot be fully explained by this system. However, another carrier distinct from translocase, by being carboxyatractyloside insensitive, Mg^{2+}-dependent and ATP-specific, could be responsible for the enrichment in adenine nucleotides that takes place immediately after birth [1]. Although this carrier has not been isolated yet, its activity has been characterized [24] and it appears to be regulated by hormones, calcium, and other physiological stimuli [25].

The enrichment in adenine nucleotides promotes structural changes in the inner mitochondria membrane that result in a rapid increase in their osmotic activity and the enhancement of the 'de novo' protein synthesis involved in mitochondrial function [9,16], in particular the catalytic β-subunit of H^+-ATP synthase whose synthesis is controlled at the post-transcriptional level by increasing stability [15] and translational efficiency [26] of mitochondrial transcripts.

For a greater insight into such mitochondrial changes, flow cytometry has been applied to study isolated liver mitochondria during the perinatal period after exposure to Rh-123. These studies showed the occurrence of two mitochondrial populations distinguishable by their Rh-123-associated mean fluorescence intensity [27]. The relative percentages of these populations in the whole isolated mitochondria fraction changes during perinatal development ranging from 70/30 for the low (LFP) and high (HFP) fluorescence populations in the fetus to 90/10 in the newborn, and 95/5 in the adult (Fig. 1). Low-density Percoll gradient fractions were enriched in the HFP, supporting the notion that mitochondrial fluorescence fractions have different densities [28]. Similar changes in the percentages of these mitochondrial populations defined by their distinct Rh-123 fluorescence were observed in isolated mitochondria from pure hepatocytes during culture, which were not associated with cell cycle [29], suggesting that changes in the fluorescence-based mitochondrial populations are due to mitochondrial differentiation and correspond to a single cell type within the liver, i.e. the hepatocyte.

The postnatal increase in the LFP is prevented by inhibitors of nuclear DNA (nDNA)-encoded protein synthesis and to a lesser extent by those of mitochondrial protein synthesis, suggesting that the transition of HFP to LFP depends on protein synthesis [16]. Since hypothyroidism inhibits the transition of HFP to LFP together with the synthesis of β-subunit of H^+-ATP synthase, it may be speculated that the transition of HFP to LFP may involve the synthesis of β-subunit. It should be mentioned that the occurrence of two different mitochondrial populations distinguishable by their sensitivity to Rh-123 is not an exclusive feature of the liver since mitochondria from fetal or neonatal brains also proved to be heterogeneous when stained with Rh-123. Actually, two fluorescence populations similar to those found in the liver as judged by their 90° light scatter and Rh-123-associated green fluorescence parameters were observed in isolated mitochondria from rat brain (López-Mediavilla, C., Orfao, A. and Medina, J.M., unpublished). Consequently, it is reasonable to suggest that the occurrence of two mitochondrial populations with different sensitivity to Rh-123 is a universal feature of developing tissues.

Since the changes in respiratory parameters, in percentages of fluorescence populations and in the resistance of mitochon-

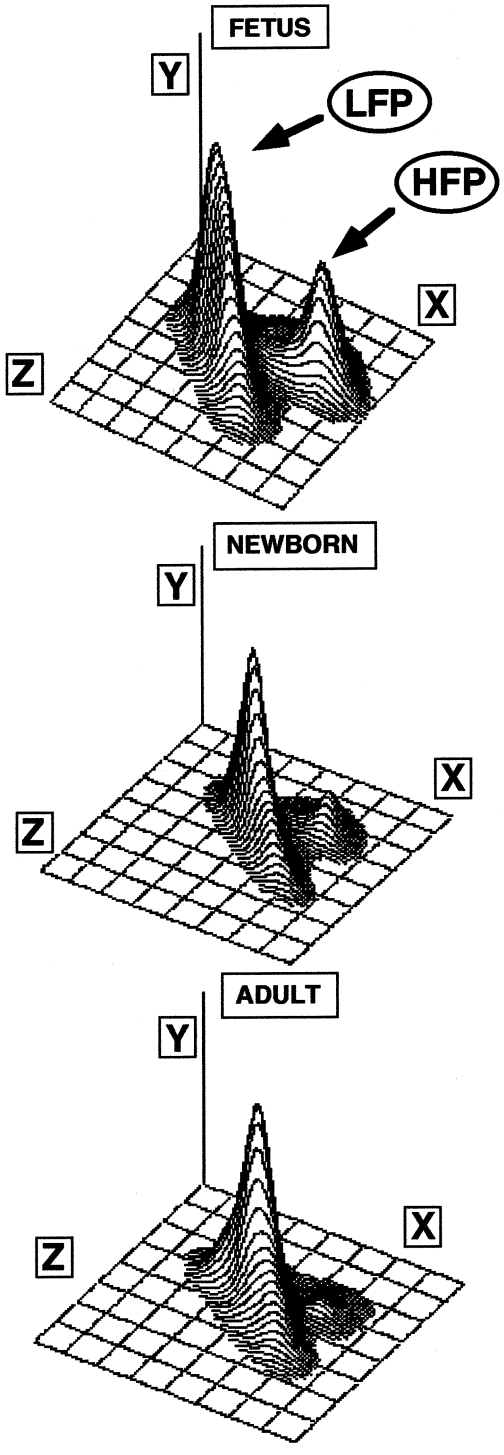

Fig. 1. Flow cytometric analysis of Rh-123-stained mitochondria from rat liver at different states of development. *x* axis: green fluorescence (log. scale); *y* axis: number of events; *z* axis: 90° angle light scatter ('side scatter'; linear scale). Two populations distinguished by the fluorescence emission intensities, i.e. LFP and HFP, were found. For more information see [28].

drial membranes to osmotic pressure observed during perinatal development are mimicked 'in vitro' by ATP [16,17], it is reasonable to suggest that adenine nucleotide accumulation is essential for the postnatal assembling of mitochondrial membranes and for the subsequent burst of mitochondrial function (Fig. 2). The relative distribution of the two mitochondrial populations changed 'in vitro' with the energy status of mitochondria at all the stages of development [28]. Nevertheless, these changes in the HFP/LFP ratio do not correlate with mitochondrial membrane potential but with the rate of ATP synthesis in every energy state of mitochondria [30]. In addition, the increase in cytochrome, respiratory complex and β-subunit of H^+-ATP synthase synthesis consolidates mitochondrial differentiation, thereby achieving a mature mitochondria with low rhodamine fluorescence and high resistance to osmotic pressure (Fig. 2). Accordingly, the observed differences in HFP/LFP ratio from newborn and adult rat could more likely reflect changes in the protein composition of the inner membrane with an increase in Rh-123 binding rather than differences in the mitochondrial membrane potential as previously suggested [30].

It has been suggested that the hormone status of the newborn probably modulates the rate of accumulation of adenine nucleotides either directly or indirectly through changes in the cytoplasmic ATP/ADP ratio [13,22,31]. Thus, immediately after birth, a sharp increase in plasma catecholamine concentrations occurs [32], which it has been suggested are responsible for resistance to postnatal hypoxia in the newborn [33]. The mechanism by which catecholamines are able to enhance the respiratory efficiency of newborns has been studied in the primary culture of rat hepatocytes [34]. Under these circumstances, the presence of adrenaline increased the mitochondrial RCR by some mechanism that was mimicked by dibutyryl-cAMP, suggesting that cAMP may be involved in the effect of adrenaline. Moreover, flow cytometric assessment of mitochondrial Ca^{2+} concentrations showed that dibutyryl-cAMP also increased free Ca^{2+} concentrations, suggesting that the effects of cAMP may be mediated by the increase in cytosolic Ca^{2+} level. These effects came about with the enrichment of mitochondria in adenine nucleotide which may be an intermediate step in the activation of mitochondrial respiratory function. Actually, adrenaline and dibutyryl-cAMP inhibited mitochondrial ATP/ADP translocase, a fact that may prevent the release of ATP from mitochondria during adenine nucleotide net accumulation, that takes place immediately after birth through the ATP-Mg^{2+}/Pi carrier [24].

3. Impairment of mitochondria differentiation by hypothyroidism

In adult liver, thyroid hormones regulate the coupling between respiratory chain and oxidative phosphorylation, resulting in the decrease of the mitochondrial membrane potential [35]. Although this effect might be explained by the increase in the synthesis of mitochondrial uncoupling proteins (UCPs) caused by thyroid hormones as shown in other tissues [36–39], it does not seem to be the case in the liver, where UCPs expression is restricted to non-parenchymal cells [40,41]. In addition, thyroid hormones regulate ATPase activity and the synthesis of H^+-ATP-synthase β-subunit during development [20]. Thus, experimental hypothyroidism halved ATPase activity and the amount of H^+-ATP-synthase β-subunit in the fetus and early newborn without affecting the synthesis of mitochondrial DNA (mDNA)-encoded H^+-ATP-synthase subunits [42]. Under the same experimental conditions, hypothyroidism inhibits the transition of the HFP to the LFP [43] and the increase in the resistance of mitochondrial membranes to osmotic pressure [17] occurring immediately after birth.

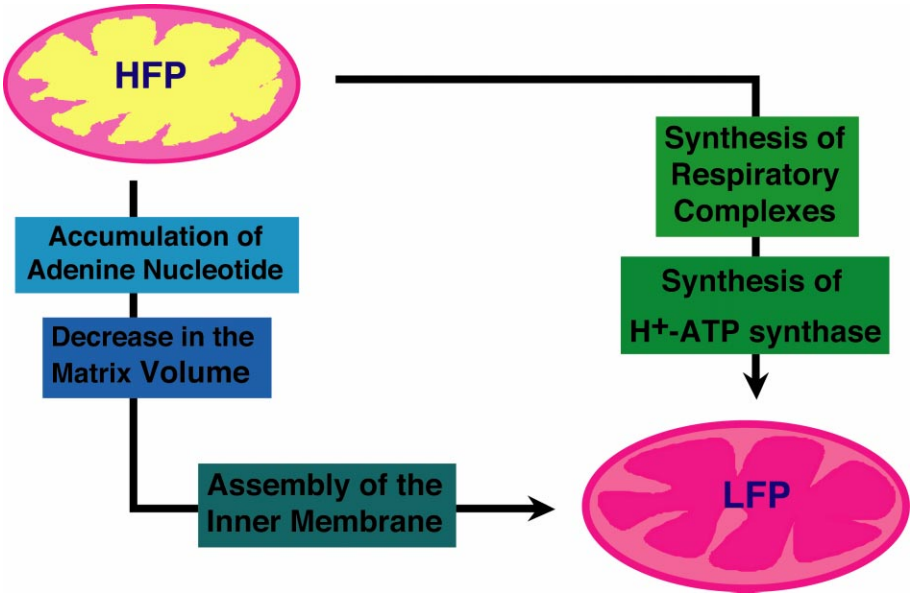

Fig. 2. Mitochondrial differentiation concerns the synergistic action of protein synthesis and adenine nucleotide accumulation. The coordinated induction of cytosolic and mitochondrial protein synthesis and the increase in intramitochondrial nucleotides in the HFP promotes a rapid increase in mitochondria enzymatic activities together with ultrastructural changes which result in a reduction of matrix volume and assembly of the inner mitochondrial membrane. This is the LFP able to support the osmotic activity of functional mature mitochondria.

Moreover, treatment of hypothyroid newborns with thyroid hormones counteracted the effects of hypothyroidism on fluorescence mitochondrial populations and membrane resistance, in accordance with the idea that thyroid hormones control mitochondrial differentiation (for review see [44]). The reversal effects of thyroid hormone treatment were avoided by the administration of inhibitors of nDNA-encoded protein synthesis but not by inhibitors of mDNA-encoded protein synthesis. Therefore, it might be speculated that hypothyroidism inhibits the synthesis of H^+-ATP synthase, preventing the HFP to the LFP transition and the appropriate assembling of mitochondrial membranes, altogether resulting in the impairment of postnatal mitochondrial differentiation.

4. Ethanol-induced changes in mitochondria populations

For a long time it has been shown that increased ethanol-intake exerts a toxic effect on liver cells and their organelles including mitochondria. Mitochondria exposed to ethanol show impaired functionality of the electron transport chain together with a decrease in the RCR and their cytochrome oxidase content and an impaired oxidative phosphorylation [45–47] associated at the microscopical level with enlargement and hyalin degeneration of mitochondria [48,49]. These abnormalities are translated into a decreased membrane potential of the major population of LFP mitochondria, as reflected by a lower Rh-123 uptake. Interestingly, a higher Rh-123-associated fluorescence intensity was observed for the less functional and minor HFP mitochondria, which could only be explained on the basis of an increased number of potential Rh-123 binding sites due to mitochondrial enlargement [50].

5. The role of mitochondria in aging

It is well known that mitochondria dysfunction plays a key role in normal cell aging and neurodegenerative diseases (for review, see [51–53]). Since mitochondria generates most of reactive oxygen species (ROS) [54], mDNA, proteins, lipids and the permeability transition pore complex (PTPC) are the most susceptible targets of ROS (for review see [55]). Most studies of aging-associated mitochondrial dysfunction are focused on the damage to the enzymes that participate in oxidative phosphorylation, and to the mDNA that controls the synthesis of key subunits on these enzymes [56–58]. Several studies have reported an age-related decrease in the levels of mitochondrial transcripts in some rat tissues [59] and in *Drosophila* [60]. In addition, aging increases the susceptibility to calcium-dependent permeability transition in isolated mitochondria from both brain and the liver [61]. In accordance with this, aging induces oxidative damage to the adenine nucleotide translocase [62], which is a critical component of the PTPC [63], a fact that may be the direct cause of the enhanced susceptibility of aged mitochondria to permeability.

The respiratory activity of isolated mitochondria decreases with age in liver [64], in skeletal muscle [65], and brain [66]. The decline in electron transport activity with age correlates with higher peroxide generation measured by flow cytometry in isolated mitochondria as well as a decrease in mitochondrial membrane potential measured in whole cells [67]. In addition, flow cytometric analysis with Rh-123 has shown that the effects of respiratory inhibitors or uncouplers are blunted in old rat liver mitochondria (López-Mediavilla C., Orfao, A. and Medina, J.M., unpublished), which may be accounted for by the well-documented inner mitochondria membrane changes related to free radical damage and/or DNA mutations described in old tissues (for review see [68]). It has also been reported that a respiratory chain deficiency occurs in cybrids (see below) containing mitochondria from older subjects [69]. In contrast, other cybrid experiments show that nDNA rather than mDNA mutations are responsible for the observed age-related reduction of the oxidative phosphorylation [70]

6. Cybrids and mDNA damage

Recent data [71,72] suggest that the mitochondrial and nuclear genotypes as well as their specific interactions play a role in the respiratory competence of the cell by impairing electron transport, formation of damaging free radicals and altered calcium handling. Accordingly, the different contribution of mDNA and nDNA to mitochondrial dysfunction has been studied in 'in vitro' cell culture of cybrids. Cybrid clones are cells obtained by fusion between human cell lines lacking mDNA (rho(0)) and enucleated cells from patients with an mDNA alteration. They provide valuable tools for investigation of mDNA defects excluding the influence of nDNA-encoded factors. Direct complementation between wild-type and mutant mDNA molecules was demonstrated in such cybrid cells [73,74]. In this context, the low mitochondrial membrane potential showed by rho(0) cells has been used to follow them with flow cytometry [75]. In addition, a flow cytometry technique has been developed to isolate single mitochondrial particles followed by polymerase chain reaction-based assays to determine the mDNA copy number and composition of individual particles [76]. The method may be applied to the study of copy numbers and distribution of the mDNA genome in different cell types.

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