Biochimica et Biophysica Acta 1797 (2010) 1171–1177

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Hypoxia and mitochondrial oxidative metabolism

Giancarlo Solaini *, Alessandra Baracca, Giorgio Lenaz, Gianluca Sgarbi

Department of Biochemistry "G. Moruzzi", University of Bologna, Bologna, Italy

ARTICLE INFO

Article history:
Received 11 December 2009
Received in revised form 2 February 2010
Accepted 7 February 2010
Available online 11 February 2010

Keywords:
Mitochondrion
Oxidative phosphorylation
Hypoxia
ROS
Autophagy
F_1F_0 ATPase
IF_1

ABSTRACT

It is now clear that mitochondrial defects are associated with a large variety of clinical phenotypes. This is the result of the mitochondria's central role in energy production, reactive oxygen species homeostasis, and cell death. These processes are interdependent and may occur under various stressing conditions, among which low oxygen levels (hypoxia) are certainly prominent. Cells exposed to hypoxia respond acutely with endogenous metabolites and proteins promptly regulating metabolic pathways, but if low oxygen levels are prolonged, cells activate adapting mechanisms, the master switch being the hypoxia-inducible factor 1 (HIF-1). Activation of this factor is strictly bound to the mitochondrial function, which in turn is related with the oxygen level. Therefore in hypoxia, mitochondria act as $[O_2]$ sensors, convey signals to HIF-1directly or indirectly, and contribute to the cell redox potential, ion homeostasis, and energy production. Although over the last two decades cellular responses to low oxygen tension have been studied extensively, mechanisms underlying these functions are still indefinite. Here we review current knowledge of the mitochondrial role in hypoxia, focusing mainly on their role in cellular energy and reactive oxygen species homeostasis in relation with HIF-1 stabilization. In addition, we address the involvement of HIF-1 and the inhibitor protein of F_1F_0 ATPase in the hypoxia-induced mitochondrial autophagy.

1. Introduction

Over the last two decades a defective mitochondrial function associated with hypoxia has been invoked in many diverse complex disorders, such as type 2 diabetes [1,2], Alzheimer's disease [3,4], cardiac ischemia/reperfusion injury [5,6], tissue inflammation [7], and cancer [8–12].

The $[O_2]$ in air-saturated aqueous buffer at 37 °C is approx. 200 μM [13]; however, mitochondria *in vivo* are exposed to a considerably lower $[O_2]$ that varies with tissue and physiological state. Under physiological conditions, most human resting cells experience some 5% oxygen tension, however the $[O_2]$ gradient occurring between the extracellular environment and mitochondria, where oxygen is consumed by cytochrome *c* oxidase, results in a significantly lower $[O_2]$ exposition of mitochondria. Below this oxygen level, most mammalian tissues are exposed to hypoxic conditions [14]. These may arise in normal development, or as a consequence of pathophysiological conditions where there is a reduced oxygen supply due to a respiratory insufficiency or to a defective vasculature. Such conditions include inflammatory diseases, diabetes, ischemic disorders (cerebral or cardiovascular), and solid tumors. Mitochondria consume the greatest amount (some 85–90%) of oxygen in cells to allow oxidative phosphorylation (OXPHOS), which is the primary metabolic pathway for ATP production. Therefore hypoxia will hamper this metabolic pathway, and if the oxygen level is very low, insufficient ATP availability might result in cell death [15].

When cells are exposed to an atmosphere with reduced oxygen concentration, cells readily "respond" by inducing adaptive reactions for their survival through the AMP-activated protein kinase (AMPK) pathway (see for a recent review [16]) which *inter alia* increases glycolysis driven by enhanced catalytic efficiency of some enzymes, including phosphofructokinase-1 and pyruvate kinase (of note, this oxidative flux is thermodynamically allowed due to both reduced phosphorylation potential $[ATP]/([ADP][P_i])$ and the physiological redox state of the cell). However, this is particularly efficient only in the short term, therefore cells respond to prolonged hypoxia also by stimulation of hypoxia-inducible factors (HIFs: HIF-1 being the mostly studied), which are heterodimeric transcription factors composed of α and β subunits, first described by Semenza and Wang [17]. These HIFs in the presence of hypoxic oxygen levels are activated through a complex mechanism in which the oxygen tension is critical (see below). Afterwards HIFs bind to hypoxia-responsive elements, activating the transcription of more than two hundred genes that allow cells to adapt to the hypoxic environment [18,19].

Abbreviations: HIF-1, hypoxia-inducible factor 1; HIF, hypoxia-inducible factor; IF_1, ATP synthase natural inhibitor protein; F_1F_0 ATPase, ATP synthase; COX, cytochrome *c* oxidase; $\Delta\Psi_m$, electrical membrane potential of mitochondria; mtDNA, mitochondrial DNA; AMPK, AMP-activated protein kinase; PHD, prolyl hydroxylase; PDK1, pyruvate dehydrogenase kinase 1; BNIP3, Bcl-2/adenovirus E1B 19 kDa interacting protein 3; IEX-1, immediate early response gene X-1; NOS, nitric oxide synthase

* Corresponding author. Department of Biochemistry "G. Moruzzi", University of Bologna, Via Irnerio 48, 40126 Bologna, Italy. Tel.: +39 051 2091215; fax: +39 051 2091224.

E-mail address: giancarlo.solaini@unibo.it (G. Solaini).

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doi:10.1016/j.bbabio.2010.02.011

Several excellent reviews appeared in the last few years describing the array of changes induced by oxygen deficiency in both isolated cells and animal tissues. In *in vivo* models, a coordinated regulation of tissue perfusion through vasoactive molecules such as nitric oxide and the action of carotid bodies rapidly respond to changes in oxygen demand [20–24]. Within isolated cells, hypoxia induces significant metabolic changes due to both variation of metabolites level and activation/inhibition of enzymes and transporters; the most important intracellular effects induced by different pathways are expertly described elsewhere (for recent reviews, see [25–27]). It is reasonable to suppose that the type of cells and both the severity and duration of hypoxia may determine which pathways are activated/depressed and their timing of onset [3,6,10,12,23,28]. These pathways will eventually lead to preferential translation of key proteins required for adaptation and survival to hypoxic stress. Although in the past two decades, the discovery of HIF-1 by Gregg Semenza et al. provided a molecular platform to investigate the mechanism underlying responses to oxygen deprivation, the molecular and cellular biology of hypoxia has still to be completely elucidated. This review summarizes recent experimental data concerned with mitochondrial structure and function adaptation to hypoxia and evaluates it in light of the main structural and functional parameters defining the mitochondrial bioenergetics. Since mitochondria contain an inhibitor protein, IF_1, whose action on the F_1F_0 ATPase has been considered for decades of critical importance in hypoxia/ischemia, particular notice will be dedicated to analyze molecular aspects of IF_1 regulation of the enzyme and its possible role in the metabolic changes induced by low oxygen levels in cells.

2. Mechanism(s) of HIF-1 activation

HIF-1 consists of an oxygen-sensitive HIF-1α subunit that heterodimerizes with the HIF-1β subunit to bind DNA. In high O_2 tension, HIF-1α is oxidized (hydroxylated) by prolyl hydroxylases (PHDs) using α-ketoglutarate derived from the tricarboxylic acid (TCA) cycle. The hydroxylated HIF-1α subunit interacts with the von Hippel–Lindau protein, a critical member of an E3 ubiquitin ligase complex that polyubiquitylates HIF. This is then catabolized by proteasomes, such that HIF-1α is continuously synthesized and degraded under normoxic conditions [18]. Under hypoxia, HIF-1α hydroxylation does not occur, thereby stabilizing HIF-1 (Fig. 1). The active HIF-1 complex in turn binds to a core hypoxia response element in a wide array of genes involved in a diversity of biological processes, and directly transactivates glycolytic enzyme genes [29]. Notably, O_2 concentration, multiple mitochondrial products, including the TCA cycle intermediates and reactive oxygen species, can coordinate PHD activity, HIF stabilization, hence the cellular responses to O_2 depletion [30,31]. Incidentally, impaired TCA cycle flux, particularly if it is caused by succinate dehydrogenase dysfunction, results in decreased or loss of energy production from both the electron-transport chain and the Krebs cycle, and also in overproduction of free radicals [32]. This leads to severe early-onset neurodegeneration or, as it occurs in individuals carrying mutations in the non-catalytic subunits of the same enzyme, to tumors such as phaeochromocytoma and paraganglioma. However, impairment of the TCA cycle may be relevant also for the metabolic changes occurring in mitochondria exposed to hypoxia, since accumulation of succinate has been reported to inhibit PHDs [33]. It has to be noticed that some authors believe reactive oxygen species (ROS) to be essential to activate HIF-1 [34], but others challenge this idea [35], therefore the role of mitochondrial ROS in the regulation of HIF-1 under hypoxia is still controversial [36]. Moreover, the contribution of functional mitochondria to HIF-1 regulation has also been questioned by others [37–39].

3. Effects of reduced oxygen level on energy metabolism in cells

Oxygen is a major determinant of cell metabolism and gene expression, and as cellular O_2 levels decrease, either during isolated hypoxia or ischemia-associated hypoxia, metabolism and gene expression profiles in the cells are significantly altered. Low oxygen reduces OXPHOS and Krebs cycle rates, and participates in the generation of nitric oxide (NO), which also contributes to decrease respiration rate [23,40]. However, oxygen is also central in the generation of reactive oxygen species, which can participate in cell signaling processes or can induce irreversible cellular damage and death [41].

As specified above, cells adapt to oxygen reduction by inducing active HIF, whose major effect on cells energy homeostasis is the inactivation of anabolism, activation of anaerobic glycolysis, and inhibition of the mitochondrial aerobic metabolism: the TCA cycle, and OXPHOS. Since OXPHOS supplies the majority of ATP required for cellular processes, low oxygen tension will severely reduce cell energy availability. This occurs through several mechanisms: first, reduced oxygen tension decreases the respiration rate, due first to nonsaturating substrate for cytochrome *c* oxidase (COX), secondarily, to allosteric modulation of COX [42]. As a consequence, the phosphorylation potential decreases, with enhancement of the glycolysis rate primarily due to allosteric increase of phosphofructokinase activity; glycolysis however is poorly efficient and produces lactate in proportion of 0.5 mol/mol ATP, which eventually drops cellular pH if cells are not well perfused, as it occurs under defective vasculature or ischemic conditions [6]. Besides this "spontaneous" (thermodynamically-driven) shift from aerobic to anaerobic metabolism which is mediated by the kinetic changes of most enzymes, the HIF-1 factor activates transcription of genes encoding glucose transporters and glycolytic enzymes to further increase flux of reducing equivalents from glucose to lactate [43,44]. Second, HIF-1 coordinates two different actions on the mitochondrial phase of glucose oxidation: it activates transcription of the *PDK1* gene encoding a kinase that phosphorylates and inactivates pyruvate dehydrogenase, thereby shunting away pyruvate from the mitochondria by preventing its oxidative decarboxylation to acetyl-CoA [45,46]. Moreover, HIF-1 induces a switch in the composition of cytochrome *c* oxidase from COX4-1 to COX4-2 isoform, which enhances the specific activity of the enzyme. As a result, both respiration rate and ATP level of hypoxic cells carrying the COX4-2 isoform of cytochrome *c* oxidase were found significantly increased with respect to the same cells carrying the COX4-1 isoform [47]. Incidentally, HIF-1 can also increase the expression of carbonic anhydrase 9, which catalyses the reversible hydration of CO_2 to HCO_3^- and H^+, therefore contributing to pH regulation.

4. Effect of reduced oxygen level on mitochondria

4.1. Effects of hypoxia on mitochondrial structure and dynamics

Mitochondria form a highly dynamic tubular network, the morphology of which is regulated by frequent fission and fusion events. The fusion/fission machineries are modulated in response to changes in the metabolic conditions of the cell, therefore one should expect that hypoxia affect mitochondrial dynamics. Oxygen availability to cells decreases glucose oxidation, whereas oxygen shortage consumes glucose faster in an attempt to produce ATP via the less efficient anaerobic glycolysis to lactate (Pasteur effect). Under these conditions, mitochondria are not fueled with substrates (acetyl-CoA and O_2), inducing major changes of structure, function, and dynamics (for a recent review see [48]). Concerning structure and dynamics, one of the first correlates that emerge is that impairment of mitochondrial fusion leads to mitochondrial depolarization, loss of mtDNA that may be accompanied by altered respiration rate, and

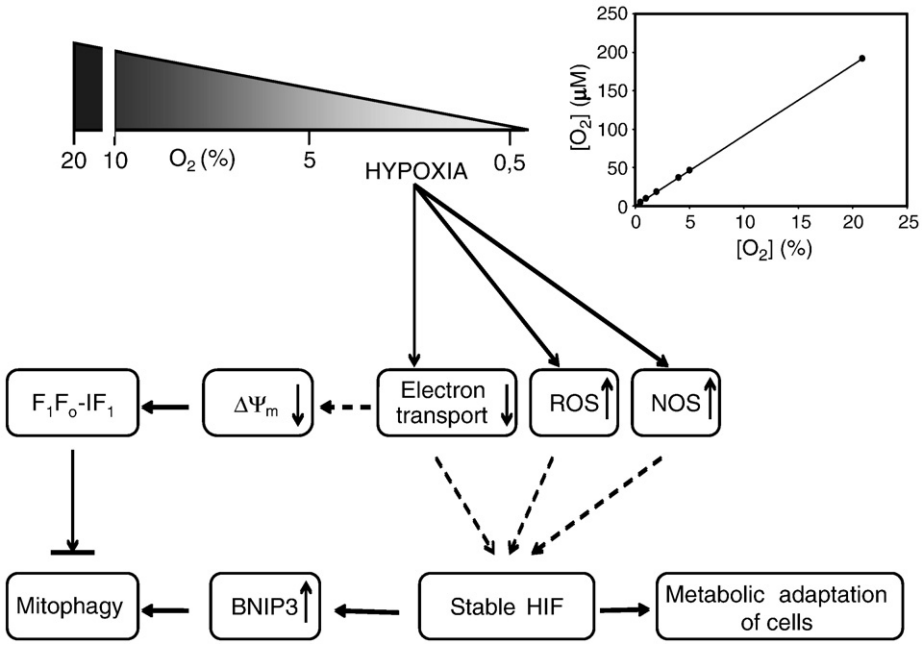

Fig. 1. Major mitochondrial changes in hypoxia. Hypoxia could decrease electron-transport rate determining $\Delta\psi_m$ reduction, increased ROS generation, and enhanced NO synthase. One (or more) of these factors likely contributes to HIF stabilization, that in turn induces metabolic adaptation of both hypoxic cells and mitophagy. The decreased $\Delta\psi_m$ could also induce an active binding of IF_1, which might change mitochondrial morphology and/or dynamics, and inhibit mitophagy. Solid lines indicate well established hypoxic changes in cells, whilst dotted lines indicate changes not yet stated. *Inset*, relationships between extracellular O_2 concentration and oxygen tension.

impaired distribution of the mitochondria within cells [49–51]. Indeed, exposure of cortical neurons to moderate hypoxic conditions for several hours, significantly altered mitochondrial morphology, decreased mitochondrial size and reduced mitochondrial mean velocity. Since these effects were either prevented by exposing the neurons to inhibitors of nitric oxide synthase or mimicked by NO donors in normoxia, the involvement of an NO-mediated pathway was suggested [52]. Mitochondrial motility was also found inhibited and controlled locally by the [ADP]/[ATP] ratio [53]. Interestingly, the author used an original approach in which mitochondria were visualized using tetramethylrhodamineethylester and their movements were followed by applying single-particle tracking.

Of notice in this chapter is that enzymes controlling mitochondrial morphology regulators provide a platform through which cellular signals are transduced within the cell in order to affect mitochondrial function [54]. Accordingly, one might expect that besides other mitochondrial factors [30,55] playing roles in HIF stabilization, also mitochondrial morphology might reasonably be associated with HIF stabilization. In order to better define the mechanisms involved in the morphology changes of mitochondria and in their dynamics when cells experience hypoxic conditions, these pioneering studies should be corroborated by and extended to observations on other types of cells focusing also on single proteins involved in both mitochondrial fusion/fission and motion.

4.2. Effects of hypoxia on the respiratory chain complexes

O_2 is the terminal acceptor of electrons from cytochrome *c* oxidase (Complex IV), which has a very high affinity for it, being the oxygen concentration for half-maximal respiratory rate at pH 7.4 approximately 0.7 μM [56]. Measurements of mitochondrial oxidative phosphorylation indicated that it is not dependent on oxygen concentration up to at least 20 μM at pH 7.0 and the oxygen dependence becomes markedly greater as the pH is more alkaline [56]. Similarly, Moncada et al. [57] found that the rate of O_2 consumption remained constant until [O_2] fell below 15 μM. Accordingly, most reports in the literature consider hypoxic conditions occurring in cells at 5–0.5% O_2, a range corresponding to 46–4.6 μM O_2 in the cells culture medium (see Fig. 1 inset). Since between the extracellular environment and mitochondria an oxygen pressure gradient is established [58], the O_2 concentration experienced by Complex IV falls in the range affecting its kinetics, as reported above.

Under these conditions, a number of changes on the OXPHOS machinery components, mostly mediated by HIF-1 have been found. Thus, Semenza et al. [59] and others thereafter [46] reported that activation of HIF-1α induces pyruvate dehydrogenase kinase, which inhibits pyruvate dehydrogenase, suggesting that respiration is decreased by substrate limitation. Besides, other HIF-1 dependent mechanisms capable to affect respiration rate have been reported. First, the subunit composition of COX is altered in hypoxic cells by increased degradation of the COX4-1 subunit, which optimizes COX activity under aerobic conditions, and increased expression of the COX4-2 subunit, which optimizes COX activity under hypoxic conditions [29]. On the other hand, direct assay of respiration rate in cells exposed to hypoxia resulted in a significant reduction of respiration [60]. According with the evidence of Zhang et al., the respiration rate decrease has to be ascribed to mitochondrial autophagy, due to HIF-1-mediated expression of BNIP3. This interpretation is in line with preliminary results obtained in our laboratory where the assay of the citrate synthase activity of cells exposed to different oxygen tensions was performed. Fig. 2 shows the citrate synthase activity, which is taken as an index of the mitochondrial mass [11], with respect to oxygen tension: [O_2] and mitochondrial mass are directly linked.

However, the observations of Semenza et al. must be seen in relation with data reported by Moncada et al. [57] and confirmed by others [61] in which it is clearly shown that when cells (various cell lines) experience hypoxic conditions, nitric oxide synthases (NOSs) are activated, therefore NO is released. As already mentioned above, NO is a strong competitor of O_2 for cytochrome *c* oxidase, whose apparent K_m results increased, hence reduction of mitochondrial cytochromes and all the other redox centres of the respiratory chain occurs. In addition, very recent data indicate a potential de-activation of Complex I when oxygen is lacking, as it occurs in prolonged hypoxia [62]. According to Hagen et al. [63] the NO-dependent inhibition of

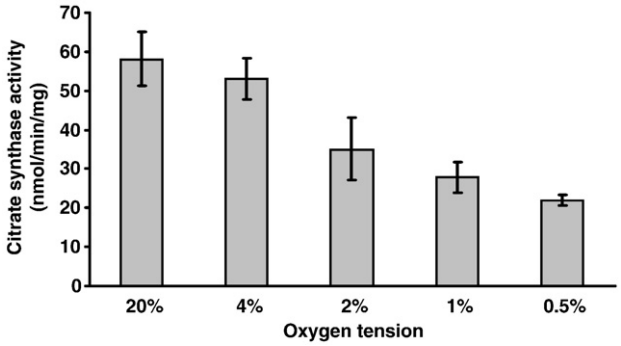

Fig. 2. Citrate synthase activity. Human primary fibroblasts, obtained from skin biopsies of 5 healthy donors, were seeded at a density of 8,000 cells/cm^2 in high glucose Dulbecco's Modified Eagle Medium, DMEM (25 mM glucose, 110 mg/l pyruvate, and 4 mM glutamine) supplemented with 15% Foetal Bovine Serum (FBS). 18 h later, cell culture dishes were washed once with Hank's Balanced Salt Solution (HBSS) and the medium was replaced with DMEM containing 5 mM glucose, 110 mg/l pyruvate, and 4 mM glutamine supplemented with 15% FBS. Cell culture dishes were then placed into an INVIVO2 humidified hypoxia workstation (Ruskinn Technologies, Bridgend, UK) for 72 h changing the medium at 48 h, and oxygen partial pressure (tension) conditions were: 20%, 4%, 2%, 1% and 0.5%. Cells were subsequently collected within the workstation with trypsin-EDTA (0.25%), washed with PBS and resuspended in a buffer containing 10 mM Tris/HCl, 0.1 M KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM EDTA, and 2 mM $MgCl_2$ pH 7.4 (all the solutions were preconditioned to the appropriate oxygen tension condition). The citrate synthase activity was assayed essentially by incubating 40 μg of cells with 0.02% Triton X-100, and monitoring the reaction by measuring spectrophotometrically the rate of free coenzyme A released, as described in [90]. Enzymatic activity was expressed as nmol/min/mg of protein. Three independent experiments were carried out and assays were performed in either duplicate or triplicate.

cytochrome *c* oxidase should allow "saved" O_2 to redistribute within the cell to be used by other enzymes, including PHDs which inactivate HIF. Therefore, unless NO inhibition of cytochrome *c* oxidase occurs only when [O_2] is very low, inhibition of mitochondrial oxygen consumption creates the paradox of a situation in which the cell may fail to register hypoxia. It has been tempted to solve this paradox, but to date only hypotheses have been proposed [23,26]. Interestingly, recent observations on yeast cells exposed to hypoxia revealed abnormal protein carbonylation and protein tyrosine nitration that were ascribed to increased mitochondrially generated superoxide radicals and NO, two species typically produced at low oxygen levels, that combine to form $ONOO^-$ [64]. Based on these studies a possible explanation has been proposed for the above paradox.

Finally, it has to be noticed that the mitochondrial respiratory deficiency observed in cardiomyocytes of dogs in which experimental heart failure had been induced lies in the supermolecular assembly rather than in the individual components of the electron-transport chain [65]. This observation is particularly intriguing since loss of respirasomes is thought to facilitate ROS generation in mitochondria [66], therefore supercomplexes disassembly might explain the paradox of reduced [O_2] and the enhanced ROS found in hypoxic cells. Specifically, hypoxia could reduce mitochondrial fusion by impairing mitochondrial membrane potential, which in turn could induce supercomplexes disassembly, increasing ROS production [11].

4.3. Complex III and ROS production

It has been estimated that, under normoxic physiological conditions, 1–2% of electron flow through the mitochondrial respiratory chain gives rise to ROS [67,68]. It is now recognized that the major sites of ROS production are within Complexes I and III, being prevalent the contribution of Complex I [69] (Fig. 3). It might be expected that hypoxia would decrease ROS production, due to the low level of O_2 and to the diminished mitochondrial respiration [6,46], but ROS level is paradoxically increased. Indeed, about a decade ago, Chandel et al. [70] provided good evidence that mitochondrial reactive oxygen species trigger hypoxia-induced transcription, and a few years later the same group [71] showed that ROS generated at Complex III of the mitochondrial respiratory chain stabilize HIF-1α during hypoxia (Figs. 1 and 3). Although others have proposed mechanisms indicating a key role of mitochondria in HIF-1α regulation during hypoxia (for reviews see [64,72]), the contribution of mitochondria to HIF-1 regulation has been questioned by others [35–37]. Results of Gong and Agani [35] for instance show that inhibition of electron-transport Complexes I, III, and IV, as well as inhibition of mitochondrial F_0F_1 ATPase, prevents HIF-1α expression and that mitochondrial reactive oxygen species are not involved in HIF-1α regulation during hypoxia. Concurrently, Tuttle et al. [73], by means of a non invasive, spectroscopic approach, could find no evidence to suggest that ROS, produced by mitochondria, are needed to stabilize HIF-1α under moderate hypoxia. The same authors found the levels of HIF-1α comparable in both normal and ρ^0 cells (i.e. cells lacking mitochondrial DNA). On the contrary, experiments carried out on genetic models consisting of either cells lacking cytochrome *c* or ρ^0 cells both could evidence the essential role of mitochondrial respiration to stabilize HIF-1α [74]. Thus, cytochrome *c* null cells, being incapable to respire, exposed to moderate hypoxia (1.5% O_2) prevented oxidation of ubiquinol and generation of the ubisemiquinone radical, thus eliminating superoxide formation at Complex III [71]. Concurrently, ρ^0 cells lacking electron transport, exposed 4 h to moderate hypoxia failed to stabilize HIF-1α, suggesting the essential role of the respiratory chain for the cellular sensing of low O_2 levels. In addition, recent evidence obtained on genetic manipulated cells (i.e. cytochrome *b* deficient cybrids) showed increased ROS levels and stabilized HIF-1α protein during hypoxia [75]. Moreover, RNA interference of the Complex III subunit Rieske iron sulfur protein in the cytochrome *b* deficient cells, abolished ROS generation at the Qo site of Complex III, preventing HIF-1α stabilization. These observations, substantiated by experiments with MitoQ, an efficient mitochondria-targeted antioxidant, strongly support the involvement of mitochondrial ROS in regulating HIF-1α. Nonetheless, collectively, the available data do not allow to definitely state the precise role of mitochondrial ROS in regulating HIF-1α, but the pathway stabilizing HIF-1α appears undoubtedly mitochondria-dependent [30].

4.4. Hypoxia and ATP synthase

The F_1F_0 ATPase (ATP synthase) is the enzyme responsible of catalysing ADP phosphorylation as the last step of OXPHOS. It is a rotary motor using the proton motive force across the mitochondrial inner membrane to drive the synthesis of ATP [76]. It is a reversible enzyme with ATP synthesis or hydrolysis taking place in the F_1 sector at the matrix side of the membrane, chemical catalysis being coupled to H^+ transport through the transmembrane F_0 sector.

Under normoxia the enzyme synthesizes ATP, but when mitochondria experience hypoxic conditions the mitochondrial membrane potential ($\Delta\psi_m$) decreases below its endogenous steady-state level (some 140 mV, negative inside the matrix [77]) and the F_1F_0 ATPase may work in the reversal mode: it hydrolyses ATP (produced by anaerobic glycolysis) and uses the energy released to pump protons from the mitochondrial matrix to the intermembrane space, concurring with the adenine nucleotide translocator (i.e. in hypoxia it exchanges cytosolic ATP^{4-} for matrix ADP^{3-}) to maintain the physiological $\Delta\psi_m$ (Fig. 3). Since under conditions of limited oxygen availability the decline in cytoplasmic high energy phosphates is mainly due to hydrolysis by the ATP synthase working in reverse [6,78], the enzyme must be strictly regulated in order to avoid ATP dissipation. This is achieved by a natural protein, the $H^+/\Delta\psi_m$-dependent IF_1, that binds to the catalytic F_1 sector at low pH and low $\Delta\psi_m$ (such as it occurs in hypoxia/ischemia) [79]. IF_1 binding to the ATP synthase results in a rapid and reversible inhibition of the enzyme [80], which could reach about 50% of maximal activity (for recent reviews see [6,81]).

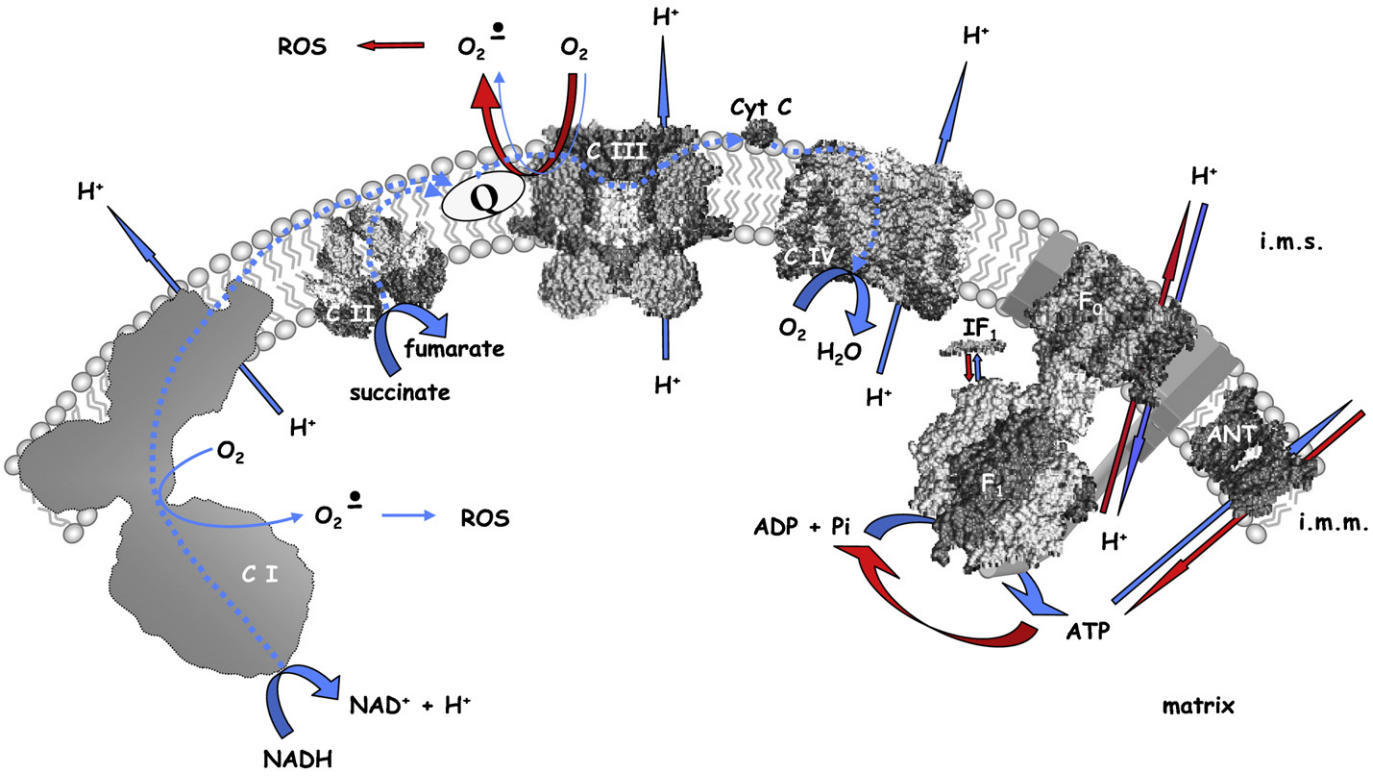

Fig. 3. Overview of mitochondrial electron and proton flux in hypoxia. Electrons released from reduced cofactors (NADH and $FADH_2$) under normoxia flow through the redox centres of the respiratory chain (r.c.) to molecular oxygen (blue dotted line), to which a proton flux from the mitochondrial matrix to the intermembrane space is coupled (blue arrows). Protons then flow back to the matrix through the F_0 sector of the ATP synthase complex, driving ATP synthesis. ATP is carried to the cell cytosol by the adenine nucleotide translocator (blue arrows). Under moderate to severe hypoxia, electrons escape the r.c. redox centres and reduce molecular oxygen to the superoxide anion radical before reaching the cytochrome *c* (red arrow). Under these conditions, to maintain an appropriate $\Delta\psi_m$, ATP produced by cytosolic glycolysis enters the mitochondria where it is hydrolyzed by the F_1F_0 ATPase with extrusion of protons from the mitochondrial matrix (red arrows).

Besides this widely studied effect, IF_1 appears to be associated with ROS production and mitochondrial autophagy (mitophagy). This is a mechanism involving the catabolic degradation of macromolecules and organelles via the lysosomal pathway that contributes to housekeeping and regenerate metabolites. Autophagic degradation is involved in the regulation of the ageing process and in several human diseases, such as myocardial ischemia/reperfusion [82], Alzheimer's Disease, Huntington diseases, and inflammatory diseases (for recent reviews see [83,84], and, as mentioned above, it promotes cell survival by reducing ROS and mtDNA damage under hypoxic conditions.

Campanella et al. [81] reported that, in HeLa cells under normoxic conditions, basal autophagic activity varies in relation to the expression levels of IF_1. Accordingly, cells overexpressing IF_1 result in ROS production similar to controls, conversely cells in which IF_1 expression is suppressed show an enhanced ROS production. In parallel, the latter cells show activation of the mitophagy pathway (Fig. 1), therefore suggesting that variations in IF_1 expression level may play a significant role in defining two particularly important parameters in the context of the current review: rates of ROS generation and mitophagy. Thus, the hypoxia-induced enhanced expression level of IF_1 [81] should be associated with a decrease of both ROS production and autophagy, which is in apparent conflict with the hypoxia-induced ROS increase and with the HIF-1-dependent mitochondrial autophagy shown by Zhang et al. [60] as an adaptive metabolic response to hypoxia. However, in the experiments of Zhang et al. the cells were exposed to hypoxia for 48 h, whereas the F_1F_0-ATPase inhibitor exerts a prompt action on the enzyme and to our knowledge, it has never been reported whether its action persists during prolonged hypoxic expositions. Pertinent with this problem is the very recent observation that IEX-1 (immediate early response gene X-1), a stress-inducible gene that suppresses production of ROS and protects cells from apoptosis [85], targets the mitochondrial F_1F_0-ATPase inhibitor for degradation, reducing ROS by decreasing $\Delta\psi_m$. It has to be noticed that the experiments described were carried out under normal oxygen availability, but it does not seem reasonable to rule out IEX-1 from playing a role under stress conditions as those induced by hypoxia in cells, therefore this issue might deserve an investigation also at low oxygen levels.

In conclusion, data are still emerging regarding the regulation of mitochondrial function by the F_1F_0 ATPase within hypoxic responses in different cellular and physiological contexts. Given the broad pathophysiological role of hypoxic cellular modulation, an understanding of the subtle tuning among different effectors of the ATP synthase is desirable to eventually target future therapeutics most effectively. Our laboratory is actually involved in carrying out investigations to clarify this context.

5. Conclusions and perspectives

The mitochondria are important cellular platforms that both propagate and initiate intracellular signals that lead to overall cellular and metabolic responses. During the last decades, a significant amount of relevant data has been obtained on the identification of mechanisms of cellular adaptation to hypoxia. In hypoxic cells there is an enhanced transcription and synthesis of several glycolytic pathway enzymes/transporters and reduction of synthesis of proteins involved in mitochondrial catabolism. Although well defined kinetic parameters of reactions in hypoxia are lacking, it is usually assumed that these transcriptional changes lead to metabolic flux modification. The required biochemical experimentation has been scarcely addressed until now and only in few of the molecular and cellular biology studies the transporter and enzyme kinetic parameters and flux rate have been determined, leaving some uncertainties.

Central to mitochondrial function and ROS generation is an electrochemical proton gradient across the mitochondrial inner membrane that is established by the proton pumping activity of the respiratory chain, and that is strictly linked to the F_1F_0-ATPase function. Evaluation of the mitochondrial membrane potential in hypoxia has only been studied using semiquantitative methods based on measurements of the fluorescence intensity of probes taken up by cells experiencing normal or hypoxic conditions. However, this approach is intrinsically incorrect due to the different capability that molecular oxygen has to quench fluorescence [86,87] and to the uncertain concentration the probe attains within mitochondria, whose mass may be reduced by a half in hypoxia [60]. In addition, the uncertainty about measurement of mitochondrial superoxide radical and H_2O_2 formation *in vivo* [88] hampers studies on the role of mitochondrial ROS in hypoxic oxidative damage, redox signaling, and HIF-1 stabilization.

The duration and severity of hypoxic stress differentially activate the responses discussed throughout and lead to substantial phenotypic variations amongst tissues and cell models, which are not consistently and definitely known. Certainly, understanding whether a hierarchy among hypoxia response mechanisms exists and which are the precise timing and conditions of each mechanism to activate, will improve our knowledge of the biochemical mechanisms underlying hypoxia in cells, which eventually may contribute to define therapeutic targets in hypoxia-associated diseases. To this aim it might be worth investigating the hypoxia-induced structural organization of both the respiratory chain enzymes in supramolecular complexes and the assembly of the ATP synthase to form oligomers affecting ROS production [65] and inner mitochondrial membrane structure [89], respectively. An investigation in due course in our laboratory suggests a significant influence of hypoxic conditions to induce reduction of mitochondrial mass and respiratory chain complexes, and morphology changes (manuscript in preparation).

Future work will continue to explore hypoxia-induced effects and help to position mitochondrial function, dynamics and signaling within multiple cellular pathways, including those involved in many diverse complex disorders, such as tumors, ischemic injury, complications of diabetes, and hypoxia-associated neurodegeneration.

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