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Mitochondrial biogenesis as a cellular signaling framework

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Abstract

The identification, more than 50 years ago, of mitochondria as the site of oxidative energy metabolism has prompted studies that have unraveled the complexity of the numerous biosynthetic and degradative reactions, fundamental to cell function, carried out by these organelles. These activities depend on a distinctive mitochondrial structure, with different enzymes and reactions localized in discrete membranes and aqueous compartments. The characteristic mitochondrial structural organization is the product of both synthesis of macromolecules within the mitochondria and the import of proteins and lipids synthesized outside the organelle. Synthesis and import of mitochondrial components are required for mitochondrial proliferation, but rather than producing new organelles, these processes may facilitate the growth of pre-existing mitochondria. Recent evidence indicates that these events are regulated in a complex way by several agonists and environmental conditions, through activation of specific transcription factors and signaling pathways. Some of these are now being elucidated. Generation of nitric oxide (NO) appears to be a novel player in this scenario, possibly acting as a unifying molecular switch to trigger the whole mitochondriogenic process.

Keywords: Mitochondrial biogenesis; Cellular signaling; Energy metabolism

> It is a good thing for the entire enterprise that mitochondria and chloroplasts have remained small, conservative, and stable, since these two organelles are, in a fundamental sense, the most important living things on earth. Between them they produce oxygen and arrange for its use. In effect, they run the place.
>
> Lewis Thomas
>
> ''The Lives of a Cell—Notes of a Biology Watcher'' (1975)

1. Introduction

Mitochondria first captured the attention of cell physiologists some 50 years ago. The role of these organelles as the producers of most of the energy in animal cells was soon discovered. The steps involved in such process, i.e. the passing of electrons along the series of respiratory enzyme complexes located in the inner mitochondrial membrane, and the ensuing build up of a transmembrane electrochemical proton gradient, enabling adenosine 5′-triphosphate (ATP) synthase, to synthesize the energy carrier ATP are now characterized [1].

Recent evidence, suggests that this important bioenergetic process occurs in organelles that are not static. Mitochondria have indeed been shown to be in constant movement within the cells, with several fusion/fission events taking place. This high degree of plasticity is accompanied by variations of mitochondrial size, number and mass, in complex processes triggered by a variety of physiological stimuli and differentiation states, involving approximately 1000 genes and producing about 20% of cellular proteins. Such complexity underlies the existence of a complex network connecting many different regulatory pathways coordinated and regulated tightly [2–4].

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Among the factors involved in the regulation and coordination of mitochondrial gene expression identified thus far, Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) and PGC-1β appear to play a crucial role. In brown adipocytes and myocytes PGC-1α enforces the expression of nuclear respiratory factors (NRF-1 and NRF-2), which are transcription factors that trigger the expression of genes coding for both nuclear subunits of the respiratory chain and proteins involved in mitochondrial DNA transcription and replication.

Recently, we have provided evidence that elevated levels of nitric oxide (NO) stimulate mitochondrial biogenesis in a number of cells, including brown adipocytes, via a soluble guanylate-cyclase-dependent signaling pathway that activates PGC-1α [5]. These results raise possibilities for a role of NO in modulating mitochondrial content in response to physiological stimuli such as cold exposure or exercise. Whether this signaling cascade represents a widespread mechanism by which mammalian tissues regulate mitochondrial content, and how it might integrate with other pathways that control PGC-1α expression, remain, however, unsolved issues.

In this article, after a brief historical overview, we will discuss the implications of this biogenetic process in the framework of mitochondrial biology, as well as its possible involvement in the pathophysiological mechanisms underlying diseases, such as metabolic syndrome.

2. The developing concept of mitochondria as cellular organelles: a historical overview

2.1. Mitochondria as cytoplasmic structures

In the years 1850–1890, many cytologists observed granular elements and inclusions in the cytoplasm of different cells, some of which undoubtedly artifacts, to which they assigned many names and functions. Perhaps Kölliker deserves particular mention, since he was among the first to describe characteristically arranged granules in the sarcoplasm of striated muscle and to study them systematically over a period of years beginning about 1850 (see [6]). These granules, which were later to be called *sarcosomes* by Retzius in 1890, were at first thought to be present only in muscle, but today we recognize the sarcosomes as the mitochondria of muscle cells. Kölliker should also be credited with the first separation of mitochondria from cell structure. He also showed them to possess a membrane.

In the 1890s, Flemming recognized characteristic filamentous structures in the cytoplasm of many cells. These structures, which were named *fila*, were undoubtedly mitochondria. It was not only until 1890, however, that Altmann developed a greatly superior stain that was relatively specific for the granules and made possible their systematic intracellular observation. Altmann wrote a somewhat visionary and highly speculative book, entitled *Elementarorganismen*, in which he suggested that these structures were autonomous, elemental living units, which he called *bioblasts*; he postulated them to be the ultimate ''elementary living particles'' of cellular life. He suggested that these bioblasts are similar to bacteria, and capable of living independently or in colonies in the cytoplasm of host cell.

It was Benda in the last 1800s that first coined the name *mitochondrion*, derived from the Greek *mitos*, a thread, and *chondros*, a grain. However, this term was not immediately accepted; many other terms were applied to the granules by various investigators: blepharoblasts, chondriokonts, chondriomites, chondrioplasts, chondriosomes, chondriospheres, fila, interstitial bodies, mitogel, parabasal bodies, plasmasomes, spheroplasts, and vermicules, among others.

Lewis and Lewis, from 1914 onwards, painstakingly studied the behavior of individual mitochondria in living cultured cells and found them to undergo changes in their shape, size, and location [7]. These observations suggested that mitochondria play a dynamic role and possess plasticity of structure; they were forerunners of modern exploration of mitochondria in living cells with phase-contrast optics and time-lapse microscopy. These and other observations indicated that the number, size, and location of the mitochondria in the cell were often expressions of the cell's nutritional and metabolic state.

2.2. Bioenergetics and biochemistry of mitochondria

The first explorations on animal and cell respiration, with the work by Liebig, Berzelius, Batelli and Stern, Schardinger and Thunberg, were followed in the period 1928–1933 by the classical and powerful work of Warburg on the effect of light of different wavelengths on the relief of the inhibition of cellular respiration by carbon monoxide. Furthermore, following Szent-Györgyi's discovery of the catalytic effect of the four-carbon dicarboxylic acids on respiration, Krebs, on the basis of simple but penetrating experiments on the respiration of muscle suspensions, postulated the citric acid cycle as the primary cellular mechanism for the oxidation of carbohydrate [8]. ATP was discovered in 1931 by Lohmann, but its real significance in cellular energy transformation was not appreciated until the demonstration of Warburg in 1937–1938 of the formation of ATP coupled to enzymatic oxidation of glyceraldehyde phophate and the work of Meyerhof on the formation of ATP from phosphopyruvate.

In 1940, at the Rockefeller Institute, Claude began his systematic investigations on the structure, behavior, and chemical composition of ''large granules'' (mitochondria) and ''small granules'' (microsomes) isolated by differential centrifugation of liver homogenates [9]. The full significance of the role of the mitochondria in respiration was revealed by the studies of the multienzyme system of liver that catalyzed oxidation of fatty acids conducted by Leloir and Munoz, Lehninger and Kennedy, Schneider and Potter

in the period 1943–1947, which demonstrated a functional individuality of mitochondria.

2.3. Mitochondrial structure

Further evidence for individuality of mitochondrial structure and function also came from new developments in the field of electron microscopy. In 1952 and 1953, Sjöstrand and Palade independently published the first high-resolution images of mitochondria in thin-sectioned tissues and described the major features of mitochondrial structure. They both recognized that mitochondria contained more than one membrane system, but their interpretations of their micrographs led to different models. Sjöstrand's early model contained a double limiting membrane with internal membrane-bounded compartments forming septs that divide the matrix into many compartments [10]. Palade also observed the internal membrane compartments that he christened *cristae mitochondriales*, but in his model they were buffles rather than septa, which projected into the matrix with broad paths around them [11]. Palade's model evolved into the one currently depicted in textbooks in which "the inner mitochondrial membrane is one continuous closed surface with a complex morphology, and the cristae formed from folds similar to the bellows of an accordion. This model, sometimes called the baffle model, shows the cristae with broad openings to the intermembrane space on one side of the mitochondrion and protruding across the matrix nearly to the other side" [12].

Application of electron microscopic (EM) tomography in mid-1990s to mitochondria *in situ* in several different tissues provided striking evidence that the standard structural model was incorrect. The 3D images clearly showed that the cristae are not baffles with wide openings into the intermembrane space but that they display a pleomorphic, extensively tubular nature [13–15]. This feature would have significant functional implications, in particular, that internal metabolite gradients might regulate rates of ATP production under some conditions.

2.4. Mitochondrial biogenesis

The word biogenesis has been used to describe both the formation of mitochondria during the life cycle of a cell [3,16] and the phylogenesis and ontogenesis of mitochondria [17,18]. A comprehension of the evolutionary origin is a prerequisite for understanding any biological structure or process. We, therefore, address the interested readers to thoughtful reviews on this topic [19–23].

Here we would only remark that an endosymbiotic origin of the mitochondrion [24,25], which was postulated over a century ago [26], is the hypothesis that draws much of its contemporary support from the discovery of a unique genome in this organelle, a relic of the mitochondrion's evolutionary past. Studies of mitochondrial DNA (mtDNA) and its expression have amply affirmed the eubacterial roots of this genome [27]; mitochondrial gene sequences have enabled researchers to trace the evolutionary antecedents of mitochondria to a single ancestor related to the division of the Proteobacteria [28]. Members of the rickettsial subdivision of the α-Proteobacteria, a group of obligate intracellular parasites that includes genera such as Rickettsia, Anaplasma, and Ehrlichia, are considered to be among the closest known eubacterial relatives of mitochondria [29]. This evolutionary origin might condition the mitochondrial biogenesis processes.

The theories on the biogenesis of mitochondria that have been proposed may be classified into three categories: (i) *de novo* synthesis of mitochondria from submicroscopic precursors present in the cytoplasm; (ii) formation from other membranous structures of the cell; (iii) growth and division of pre-existing mitochondria.

The bulk of the experimental evidence on the biogenesis of mitochondria favors the concept that pre-existing mitochondria may grow and divide, even if the other two possibilities may not be ruled out conclusively. Mitochondria divide during mitosis, providing daughter cells with a normal complement of mitochondria. Mitochondrial mass increases from the onset of S-phase through M-phase [30]. There are, however, instances in which mitochondrial divisions are not tied to the cell cycle. For example, muscle mitochondria will proliferate during myogenesis, but also following exercise [31,32]. Mitochondrial division can be induced by a wide range of substances, including benzodiazepine, inhibitors of oxidative phosphorylation, phorbol esters and calcium fluxes [33–36]. In vertebrates, the number of mitochondria, or rather the volume of mitochondrial mass per cell, are further controlled by thyroid hormones, such as T3, which broadly influence metabolic rates in vertebrates and may specifically induce mitochondrial division [37]. In addition, exposure of mammals to low-temperature environment for prolonged periods of time induces a marked increase in mitochondrial mass in brown adipocytes, originating an important control mechanism to maintain body energy balance and core temperature [38].

Mitochondria display an amazing plasticity of form and distribution. Although their internal structural organization is highly conserved, the external shape of mitochondria is variable. As already seen, in addition to the classic bean-shaped organelles observed in electron micrographs, mitochondria are frequently found as extended reticular networks [39]. These networks are extremely dynamic in growing cells, with tubular sections dividing in half, branching, and fusing to create a fluid tubular web [40]. Even in cells with a seemingly "stable" network of mitochondrial tubules, there are frequent and continual cycles of mitochondrial fusion and fission, opposing processes that exist in equilibrium and serve to maintain the overall architecture of these organelles [33,41]. Recently the fusion and fission apparatus have been analyzed deeply and the involved protein complexes characterized (see [42,43]).

Moreover, it has been suggested that the dynamic nature of mitochondrial protects these organelles by ensuring that regional losses of membrane potential, caused perhaps by local depletion of metabolic substrates or mtDNA, are always transient. In particular, mitochondrial fusion enables intermitochondrial cooperation by allowing exchange of both membrane and matrix components and therefore may help to restore local depletions and maintain mitochondrial function [44]. This suggests that the biogenesis of functional mitochondria derives from a mitochondrial fusion process, implying the notion that mitochondrial biogenesis is a shift of the equilibrium between the fission and fusion processes towards fusion.

3. Regulation of mitochondrial functions

3.1. Regulation of energy metabolism

Aerobic tissues obtain most of their energy from mitochondrial oxidative phosphorylation (OXPHOS) (Fig. 1). OXPHOS is tightly regulated, not only by allosteric and covalent regulation of catalytic properties of several OXPHOS complexes, mediated by ATP/free ADP ratios, but also by numerous fine controls [45–51]. The phosphocreatine shuttle can influence the ability of adenylates to mediate changes in OXPHOS [46,47]. Changes in NADH production can arise through regulation of Ca^{2+}-sensitive mitochondrial enzymes [52] and metabolic fuel availability [47]. Modulation of OXPHOS activity may also result from regulation of oxygen binding to complex IV, an action brought about by the gaseous messenger NO (see below).

3.2. Membrane leakiness, reactive oxygen intermediates (ROI) production, and thermogenesis

The observation that mitochondria consume small amounts of oxygen in the complete absence of ADP is usually attributed to proton leak [53]. Protons crossing the mitochondrial membrane dissipate Δp, inducing a very low level of respiration to replenish the proton gradient. Although membranes in general are relatively impermeable to protons, even pure phospholipid preparations show some permeability to protons [54].

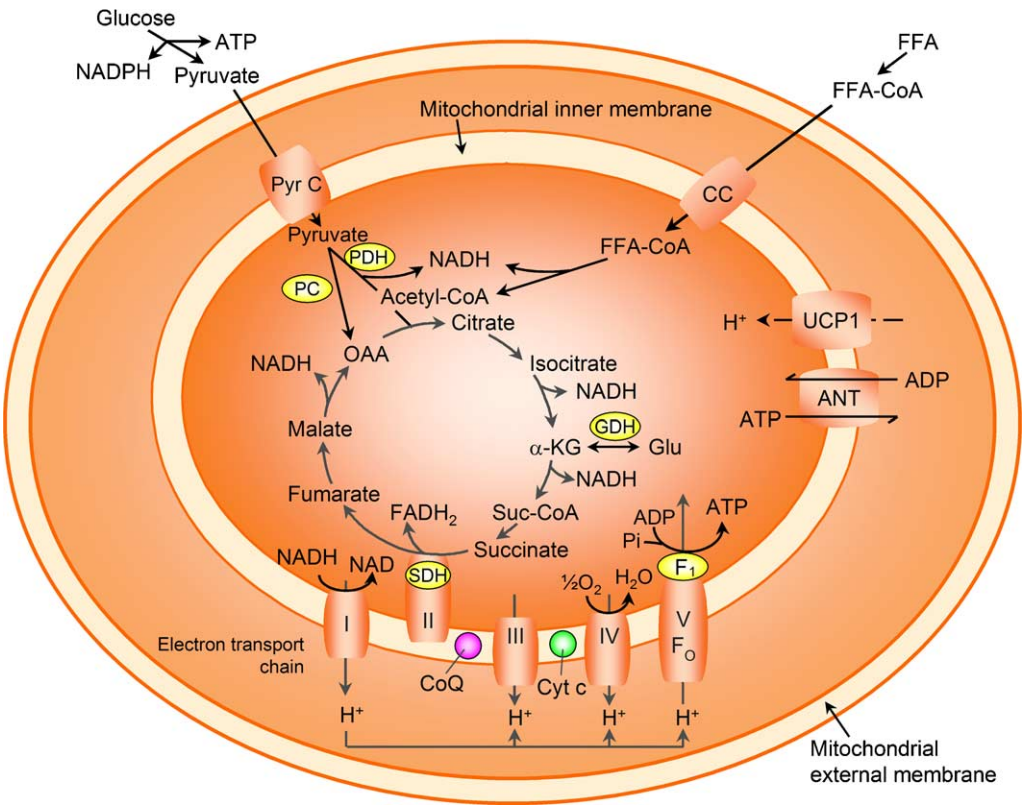

Fig. 1. The tricarboxylic acid (TCA) cycle and respiratory chain in a mitochondrion. Substrate oxidation in the TCA cycle activates the respiratory chain, leading to the generation of ATP, which is subsequently translocated to the cytosol. The respiratory enzyme complexes involved in OXPHOS are complex I [NADH: ubiquinone oxidoreductase], which includes a flavin mononucleotide and six Fe–S centers (designated with a cube); complex II [succinate: ubiquinone oxidoreductase], which includes a flavin-adenine dinucleotide, three Fe–S centers, and a cytochrome *b*; complex III [ubiquinol: cytochrome *c* oxidoreductase], which includes cytochrome *b*, cytochrome *c*1, and the Rieske Fe–S center; complex IV [cytochrome *c* oxidase], which includes cytochromes $a^{+}a3$, CuA, and CuB; and complex V [H^{+}-translocating ATP synthase]. Pyruvate from glucose enters the mitochondria via pyruvate dehydrogenase (PDH), generating acetyl-CoA, which enters the TCA cycle by combining with oxaloacetate (OAA). *cis*-Aconitase converts citrate to isocitrate and contains an 4Fe–4S center. Lactate dehydrogenase (LDH) converts excess pyruvate plus NADH to lactate. Abbreviations: ANT, adenine nucleotide translocator; GDH, glutamate dehydrogenase; Glu, glutamate; KG, ketoglutarate; PC, pyruvate carboxylase; Suc-CoA, succinyl-CoA; SDH, succinate dehydrogenase; UCP1, uncoupling protein 1.

Mitochondrial proton leak regulates energy metabolism in several ways. First, it prevents mitochondrial membranes from undergoing electrical collapse associated with excessive field strength. Second, because the production of ROI is greatest at the highest Δp, the leak may also serve to reduce oxidative stress [55]. At rest, leak respiration may be a significant component of resting metabolic rate and heat production [56,57].

Heat is released as a result of unavoidable inefficiencies in the process of electron transport. In any given system, mitochondrial heat production should increase in relation to respiration rate. In order to reach comparable levels of ATP synthesis, systems with higher inherent leakiness will require higher respiration rates and produce more heat as a by-product.

3.3. Uncoupling proteins and uncoupled mitochondrial respiration

Uncoupling proteins (UCPs) are expressed by all eukaryotic organisms and appear to increase proton conductance [58]. In particular, the brown adipose tissue uses UCP1 to generate heat [59–61]. It had long been thought that UCP1 acted as a proton channel [60], but recently Garlid *et al.* [59] proposed an alternative model whereby increases in proton conductance arise through futile cycling of fatty acids between membrane layers. Indeed, data from experiments with UCP1 in reconstituted systems tend to indicate that fatty acids (and ubiquinone [62,63]) are essential for UCP1 function [64,65], i.e. they may function as cofactors. There is still some degree of controversy, however, since in some experiments addition of fatty acids (or ubiquinone) to isolated brown fat mitochondria was found not required in order to observe high uncoupling. This may, of course, be explained as being due to the presence of sufficient levels of fatty acids in these preparations (although even high amounts of fatty acid-free albumin cannot inhibit the high proton permeability [66–68]). However, the question of the mechanism of regulation of the activity of the UCP1 remains a central area of discussion [63,67,69].

The functions of other isoforms of UCP (UCP2 and UCP3) are not yet clear, although they have been proposed to increase proton conductance [70], based on the fact that they are fatty acid-transporters *in vitro* [71]. Recent studies, however, show that these isoforms may not have a central role in thermogenesis, but rather function to prevent excessive Δp and participate in regulation of glucose and lipid metabolism [70,72,73].

The degree of uncoupling and the leakiness of the system render the mitochondrial electron transport system an important source of ROI, specifically of superoxide [74]. The risk of losing electrons depends upon the redox state of the carriers. ROI production is highest when the electron transport system is reduced, which occurs at low respiratory rates [55]. Mitochondria also possess vigorous antioxidant defenses, including antioxidant enzymes (Mn^{2+} superoxide dismutase, glutathione peroxidase) and free radical scavengers (e.g. cytochrome *c*, thioredoxin, tocopherol) [75]. Thus, the net release of mitochondrial ROI depends upon the factors that influence both production and scavenging.

3.4. Mitochondrial reticulum

The organization of mitochondria into a reticulum is thought to be beneficial for tissues highly dependent on aerobic metabolism, such as muscle [76]. If the reticulum operates as an electrical cable, electrogenic events in one location could rapidly be conducted throughout the cellular network, although the increased electrical conductance could imply a high vulnerability to depolarizing events [77]. The mitochondrial network might also facilitate oxygen diffusion, particularly in muscles devoid of myoglobin [78]. In addition, such dynamic reticulum, constantly undergoing fission and fusion, might help reducing the regional accumulation of defective mitochondrial proteins and DNA, delaying the onset of mitochondriopathies. The mobility in the mitochondrial network ensures also the selective removal of excesses in defective proteins/mtDNA, which are then targeted to the quality control apparatus as part of the normal process of mitochondrial turnover [79].

Many important regulators of mitochondrial fission/fusion processes have been identified. Mitofusins (Mfn) are the vertebrate homologues of Drosophila fuzzy onion, a GTPase located in the outer mitochondrial membrane that mediates mitochondrial fusion. Mfn1 is expressed in most tissues, but Mfn2 is expressed primarily in striated muscle [80]. In comparison, dynamin-related proteins (Drp), which are distributed throughout the cell, are required for mitochondrial fission [81]. Thus, the balance of the relative activities of Mfn and Drp appears to be the major determinant of the continuous remodeling of the mitochondrial reticulum [79].

4. Transcriptional control of mitochondrial biogenesis

Cellular control over adaptive changes in mitochondrial content demands a capacity to sense the need for additional mitochondrial energy production, followed by triggering of signaling pathways that culminate in an increased and coordinated expression of respiratory genes.

Many conditions that lead to changes in bioenergetics result in mitochondrial proliferation. Although most attention focuses on the control of respiratory gene expression, it is important to recognize that other processes (e.g. mRNA stability, the post-translational modification, import, folding, assembly) contribute to the mitochondrial proliferative response [82]. Despite advances in understanding the roles of transcription factors/nuclear receptors (NRFs/PPARs)

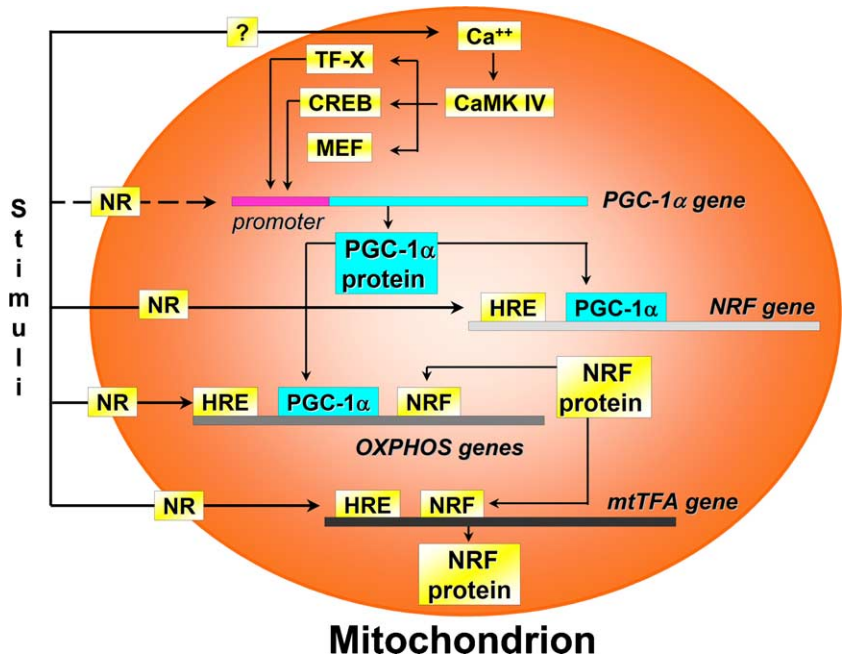

Fig. 2. Coordination of transcription of mitochondrial and nuclear genes encoding subunits of OXPHOS by different extracellular stimuli, such as hormone. In the nucleus, the hormone–receptor complex can interact with the hormone response elements (HREs) of OXPHOS genes, to directly activate them, and also with the HREs of transcription factor genes (NRF, PGC-1α), to induce transcription factors which exert a positive effect on the OXPHOS genes. By way of nongenomic modulation of intracellular Ca^{2+} concentration and activation of CaMK IV, the master regulator of mitogenesis, PGC-1α, is induced, which can directly or indirectly stimulate the transcription of OXPHOS genes and the mitochondrial transcription factor A (mtTFA) gene. The effect of extracellular stimuli, such as hormones, on mitochondrial OXPHOS can be direct, by interaction of the hormone–receptor complex with mitochondrial HREs, or indirect, via induction of nuclear-encoded mitochondrial transcription factors. Abbreviation: NR, nuclear receptor.

and coactivators (PGC-1α) in transcriptional control of respiratory genes, relatively little is known of the pathways by which these proteins are regulated, in particular about the possible involvement of metabolic signals.

Several lines of evidence offer support for links between energy metabolism and transcription factor levels or DNA binding activity (Fig. 2). Many studies have shown increases in nuclear respiratory factor-1 (NRF-1) in response to contractile activity in skeletal muscle [83]. Uncoupling of HeLa cells, through expression of UCP1, triggers expression of NRF-1 and NRF-1-sensitive genes [84] and subsequent increase in the activity of δ-aminolevulinate synthase, a rate-limiting enzyme for the synthesis of hem, which is regarded as an early marker of mitochondrial biogenesis [84]. The metabolic disturbance produced by the chronic treatment of animals with β-guanidinopropionic acid, which reduces cellular phosphocreatine and ATP, led to an increased NRF-1 binding to DNA with the subsequent increases of mitochondrial proteins and mitochondrial volume [85]. This treatment causes also an increased activity of AMP-activated protein kinase (AMPK). Of importance, an increased AMPK activity is responsible for the enhanced expression of definite mitochondrial enzymes in animals treated with 5-aminoimidazole-4-carboxamide-ribofuranoside (AICAR) for 4 weeks [86]. In addition to direct sensitivity to AMP levels, AMPK levels are regulated at the transcriptional levels, and AMPK activity is regulated by AMPK-kinase (AMPKK) [87]. In skeletal muscle, the activation of AMP-activated protein kinase is associated with increases of NRF-1 binding, cytochrome *c* levels and muscle mitochondrial density, which suggests that a decrease in the cellular ATP/AMP ratio may trigger mitochondrial biogenesis [85]. Finally, AMPK signaling has been implicated in mediating responses to changed metabolic conditions in other signaling pathways [87]. Thus, AMPK appears to act as a central trigger of mitochondrial biogenesis in response to metabolic changes. This is further supported by the fact that this enzyme may also trigger activation of the endothelial NOS (see below) [88].

Alterations in ROI production accompany physiological and pharmacological effectors of respiratory gene expression, including exercise [89]. Both AP-1 and NFκB are thought to respond at many levels to ROI and oxidative stress, however, few direct links between the activity of AP-1 or NFκB and expression of either respiratory genes or their transcription regulators have been established.

There are many clear links between Ca^{2+} and respiratory gene expression mediated via Ca^{2+}-dependent regulatory enzymes, including the calcium/calmodulin-dependent protein kinase IV (CaMK IV) and the protein phosphatase calcineurin A (CnA). Recently, transgenic mice were developed that selectively express in skeletal muscle a constitutively active form of CaMK IV [90]. Skeletal muscle of these mice demonstrated pronounced mitochondrial biogenesis, as indicated by morphological (i.e. increased

subsarcolemmal mitochondrial content), biochemical (i.e. increased nuclear and mitochondrial gene products), functional (i.e. improved fatigue resistance), and molecular (i.e. augmented transcription of PGC-1α) indices [90]. More recently, the control of PGC-1α expression by CaMK IV and CnA in muscle cells has been investigated, and it has been reported that the PGC-1α promoter is subject to positive regulation by these key calcium-signaling factors [91]. In addition, PGC-1α has been demonstrated to regulate its own promoter through interactions with components of the calcium-signaling pathway, resulting in an autoregulatory loop that potentially can provide a certain stability to the expression of genes characteristic of type 1 muscle fibers [91].

The interpretations of data regarding Ca^{2+} are that imbalances between cellular ATP demand and mitochondrial ATP supply, leading to alterations in Ca^{2+} homeostasis, can trigger the induction of signal transduction pathways leading to the phosphorylation or dephosphorylation of transcription and/or stability factors.

5. Mitochondria and brown adipose tissue

The brown adipose tissue (BAT) is a specialised tissue, which in small mammals and newborns is responsible for the nonshivering thermogenesis (NST), the main mechanism for thermoregulatory heat production [92]. Isolated brown fat mitochondria are innately ''uncoupled'', i.e. show a spontaneous high rate of oxygen consumption [92]. In addition, brown fat cell mitochondria represent an extreme as they do not appear to contain tubular cristae but display only very large crista lamellae, orientated parallel to one another and perpendicular to the longer axis of the mitochondrial cross section [93]. Crista junctions, defined as the tubular membranes of relatively uniform diameter that connect a crista membrane with the inner boundary membrane, are similar to those visualized in neuronal mitochondria [93,94]. Their inner mitochondrial membrane contains UCP1 (Fig. 1).

During cold acclimation, the sympathetic innervation triggers the recruitment of BAT by hyperplasia, which involves the proliferation and differentiation of precursor cells, and by hypertrophy of mature brown adipocytes [92]. The acute stimulation of NST and the recruitment of increased NST capacity in response to cold exposure are primarily mediated by the sympathetic neuronal connection between the brain and BAT [95]. In cold-exposed animals, sympathetic nerve fibers, which densely innervate BAT, increase noradrenaline release in the direct vicinity of brown adipocytes. Noradrenaline released by sympathetic nerve fibers in BAT binds with different affinities to multiple α- and β-adrenergic receptor (AR) isoforms expressed in brown adipocytes. Brown fat hyperplasia is stimulated through β_1-AR [96–98], whereas the lipolytic and thermogenic action is primarily mediated by β_3-AR [99,100]. β-AR signaling is coupled to various signaling pathways, among these particularly relevant appear the activation of adenylyl cyclase and the generation of NO. The effects of NO will be discussed in a specific section later on (see below). The adenylyl cyclase-mediated rise of intracellular cAMP level stimulates lipolysis and uncoupled respiration in brown adipocytes. Increased cAMP levels release the catalytic subunits of protein kinase A, which phosphorylate multiple targets including hormone-sensitive lipase [101] and perilipin [100–102]. The fatty acids thus produced might activate UCP1 (see above).

The role of this uncoupling protein in mediating BAT thermogenic effects have been well characterized in animal models in which UCP1 was either knocked-out or knocked-in. Brown fat mitochondria from UCP1-ablated mice show a low rate of spontaneous respiration [103,104]. In addition, they possess a high membrane potential which is not influenced by GDP, a known inhibitor of uncoupled respiration of BAT, that increases the mitochondrial membrane potential of wild-type brown adipocytes [105]. In transgenic mice overexpressing UCP1, there is an increased mitochondrial protein content [106] associated with elevated COX IV mRNA levels and increased cytochrome content also in white adipocytes. Ultrastructural appearance and morphometric analysis of mitochondria was consistent with stimulation of mitochondrial biogenesis in this tissue.

Taken together, these findings suggest that high expression levels of UCP1, as such, may activate mitochondrial biogenesis in brown adipocytes through the proposed energy-sensing mechanism.

The data summarized above suggest the involvement of transcriptional as well as translational control mechanisms in the mitochondrial biogenesis occurring in brown fat during cold acclimation. Translational control appears to be of major importance in mature brown adipocytes which undergo hypertrophy in response to cold exposure, whereas transcriptional regulation is most likely to be crucial for hyperplasia, when brown adipocytes are recruited from preadipocytes.

6. NO as a regulator of mitochondrial functions

NO is synthesized from L-arginine and O_2 by NO synthase (NOS) (EC 1.14.13.39) in almost all mammalian cells [107,108]. Three distinct isoforms of NOS have been identified, two of which, namely the endothelial (eNOS) and neuronal (nNOS) isoforms are regulated by second messengers, whereas one is inducible by cytokines and bacterial products (iNOS). However, it is now clear that all three NOS isoforms can be induced by different, appropriate stimuli through transcriptional and post-transcriptional mechanisms and can be constitutively expressed in some tissues or cells [107,108].

NO may act on mitochondria at several levels. Because of its vasodilating properties, it regulates blood flow to

tissues, and thus indirectly it supplies of respiratory substrates to mitochondria, as well as the redistribution of the heat generated by respiring mitochondria. In addition, NO directly regulates oxygen binding to, and release from, hemoglobin [109], and thus the supply of oxygen to mitochondria.

NO regulates mitochondrial function also by direct binding to cytochrome *c* oxidase. This binding has the following three important characteristics: it is always reversible, it occurs in competition with oxygen, and results in inhibition of enzyme activity [110–112]. These characteristics result in an NO-dependent negative modulation of OXPHOS particularly at the low oxygen concentrations, as those usually found in tissues [113]. For this, the NO/cytochrome *c* oxidase system has been proposed to act as the acute oxygen sensing system in the cells [114]. The recent finding that mitochondria have some form of NO synthase (often referred to as mitochondrial NOS, mtNOS) associated with them is consistent with NO regulating mitochondrial functions directly [115,116].

7. NO generation by eNOS: the unifying link in mitochondrial biogenesis?

The hypothesis of an involvement of NO in the regulation of BAT functions came from observations by our group and others that NO generation is triggered by most, if not all stimuli initiating the BAT differentiation programme. In particular, cold exposure triggers eNOS and iNOS [117,118] and PGC-1α expression [119], through activation of β_3-adrenergic receptors and increases in intracellular cAMP and Ca^{2+}, all of which stimulate NO production in brown adipocytes [117].

Recently, both *in vivo* and *in vitro* evidence has indicated that NO, either exogenous or generated by eNOS, plays a significant role in brown adipocytes [118,120–122]. Systemic inhibition of NO synthase (NOS) with N^G-monomethyl-L-arginine causes defective adaptive thermogenesis in rodents [123,124]. Interestingly, mitochondrial conversion of 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide (MTT) to formazan, a method usually used for the indirect measurement of cell proliferation on the basis of mitochondrial dehydrogenase activity on several cell types [125], was reduced only by 30–40% in spite of the complete inhibition of brown fat cell proliferation after chronic treatment with NO donors [120]. These results suggest that NO, while inhibiting cell proliferation, may affect mitochondrial mass in brown fat cells.

These studies indeed suggested that NO might be playing an obligatory role in mitochondrial biogenesis, at least in BAT. We decided to investigate this issue by studying the mitochondrial biogenesis in primary cultures of mouse brown adipocyte precursors. We found that treatment with NO donors increased the mtDNA content in a way which was sensitive to NO removal by the NO scavenger oxyhemoglobin, indicating that it was mediated by NO generation [5].

We also found that the NO-stimulated mitochondriogenesis occurred through activation of PGC-1α—the principal regulator of mitochondrial biogenesis in BAT, and cardiac and skeletal muscle as previously seen. Using a cyclic GMP analogue and a guanylate-cyclase inhibitor, we showed that the mitochomdrial biogenesis depends on cGMP. Similar types of experiments gave consistent results also in mouse white fat 3T3-L1 and human monocytic U937 cell lines, revealing that the NO-dependent mitochondrial biogenesis was not restricted to brown adipocytes and their differentiation processes.

To investigate the role of endogenous NO, we stably transfected HeLa cells with eNOS, i.e. the only NOS isoform that is expressed constitutively in brown adipocytes and found that its induction was sufficient to initiate mitochondrial biogenesis in the transfectant cells.

The obligatory role of eNOS in mitochondrial biogenesis was proved by using $eNOS^{-/-}$ mice. Histological analysis indicated that $eNOS^{-/-}$ BAT was functionally inactive, and indeed exposure of these animals to cold did not result in the well known mitochondriogenetic process which is commonly observed in wild-type animals. In addition, deletion of eNOS was enough to reduce the number of mitochondria even in tissues that have a basal level of neuronal, and possibly iNOS expression, such as the brain, liver, and heart.

eNOS can be activated through increases in cytosolic Ca^{2+} concentrations and phosphorylation by various protein kinases, including AMPK (see previously), explaining the ability of the various mitochondriogenetic stimuli to generate NO. This, together with the observation that NO generation by eNOS is sufficient to induce mitochondrial biogenesis strongly suggest that NO is the common mediator presiding over this process, no matter which is the initial triggering stimulus.

The importance of NO as a mitochondriogenetic stimulus has also broad implications in term of pathology. In $eNOS^{-/-}$ mice, oxygen consumption rates—an indicator of metabolic rate—were decreased, indicating that BAT-dependent thermogenesis is impaired. In genetic models of obesity, defective energy expenditure is involved in increased food intake and body-weight gain; 8-week-old $eNOS^{-/-}$ mice showed similar food consumption but weighed more than wild-type mice. So, the increased body weight of $eNOS^{-/-}$ mice could be accounted for by higher feed efficiency (i.e. weight gain/food intake) caused by defective energy expenditure.

The features shown by $eNOS^{-/-}$ mice—reduced mitochondrial mass and energy expenditure, weight gain, insulin resistance, and hypertension—are all typical of the so-called metabolic syndrome. Millions of people are estimated to have metabolic syndrome, placing them at an increased risk of developing diabetes and cardiovascular disease. If the results reported above are applicable to

humans, then we might have clues for the prevention or treatment of this condition.

8. Oxidative metabolism in humans with metabolic syndrome

In 1988, Reaven [126] proposed the existence of a metabolic syndrome (sometimes referred to as syndrome X) in which atherogenic risk factors combine with underlying insulin resistance. Others have developed this concept and several key features of this syndrome are now recognised including hyperinsulinemia, abnormal glucose metabolism (i.e. impaired glucose tolerance or diabetes), hypertension, dyslipidemia (low high-density lipoprotein (HDL) cholesterol, high triglycerides), obesity (especially visceral), hyperuricemia, microalbuminuria, and hypercoagulability (with elevated levels of fibrinogen and plasminogen activator inhibitor-1).

The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (ATP III) draws attention to the importance of the metabolic syndrome and provides a working definition of it for the first time [127]. Participants displaying three or more of the following criteria were defined as having the metabolic syndrome: (1) abdominal obesity: waist circumference >102 cm in men and >88 cm in women; (2) hypertriglyceridemia: 150 mg/dL (1.69 mmol/L); (3) HDL cholesterol: <40 mg/dL (1.04 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women; (4) high blood pressure: 130/85 mm Hg; (5) high fasting glucose: 110 mg/dL (6.1 mmol/L). In view of this definition, is not surprising the recently reported 3-fold excess of coronary heart disease and stroke in subjects with the metabolic syndrome phenotype [128]. It has been estimated that 75% of individuals with type 2 diabetes mellitus (DM) meet the diagnostic criteria for this syndrome, and there is increasing recognition that central obesity is a key etiological factor in the development of the underlying insulin resistance [128].

A recent report suggested that the prevalence of the metabolic syndrome increases with age, affecting more than 40% of those older than 60 years [129]. Based on age-adjusted estimates, about a quarter of the population in the western world has a metabolic syndrome, with about 47 million affected in the 13 Crown Colonies in the New World [129].

By using high-density oligonucleotide arrays to identify genes differentially expressed in skeletal muscle from nondiabetic subjects or insulin-resistant subjects at high risk for diabetes (''prediabetes'') on the basis of family history of DM and Mexican–American ethnicity or type 2 diabetic subjects, Patti *et al.* [130] have recently demonstrated that prediabetic and diabetic muscle is characterized by decreased expression of oxidative phosphorylation genes, many of which are regulated by NRF-dependent transcription. Furthermore, expression of PGC-1α and PGC-1β is significantly reduced in both prediabetic and diabetic subjects. Taken together, these data would indicate that decreased PGC-1 expression may be responsible for decreased expression of NRF-dependent metabolic and mitochondrial genes and may contribute to the metabolic disturbances characteristic of insulin resistance, DM and, perhaps, obesity.

In both obesity and common forms of type 2 DM, glucose oxidation and storage are reduced, in parallel with reduced activity of the tricarboxylic acid cycle, β-oxidation, and electron transport enzymes, especially complex I [131,132] with reductions in mitochondrial area and number [132–134]. A potential role for dysregulation of oxidative metabolism gene expression in DM may be inferred from other studies. In streptozotocin-induced DM mice [135], expression of oxidative phosphorylation genes is decreased. Similarly, expression of multiple energy metabolism genes is altered in poorly controlled type 2 DM humans [136]. In both studies, some differences were partially normalized by insulin, suggesting that differential regulation in DM may partly reflect secondary changes, perhaps due to decreases in NRF-1 expression or transcriptional activity. However, although less pronounced, alterations in oxidative phosphorylation genes were also observed in insulin-resistant nondiabetics. Accordingly, expression of two electron chain subunits (NADH dehydrogenase 1 and ATP5C1) is reduced in insulin-resistant nondiabetic Pima Indians [137], and ATP synthase subunit F expression is reduced in the insulin-resistant normoglycemic *ob*/*ob* mouse [138]. Mootha *et al.* [139] found similar reductions in expression of oxidative phosphorylation genes in Caucasians with impaired glucose tolerance and type 2 DM.

Both primary sequence alterations and environmental risk factors for DM may contribute to decreased PGC-1α and PGC-1β expression and/or function and thus NRF-dependent transcription. On the basis of our results on the NO-induced mitochondrial biogenesis and on the evidence that eNOS$^{-/-}$ mice show features reminescent of metabolic syndrome [5], we suggest that *eNOS* primary sequence polymorphism may also play a significant role in the pathogenesis of metabolic syndrome.

9. NO and mitochondrial biogenesis: signaling system sensors of metabolic state of the cell

NO share the ability of ROI to activate a series of signaling pathways. ROI, with reactive nitrogen intermediates (RNI), are sets of related molecules with individually distinct chemical and biological properties. ROI refers to all oxidation and excitation states of O_2 that arise in physiological environments, including superoxide ($O_2^{\bullet -}$), singlet oxygen ($1O_2^*$), ozone (O_3), hydrogen peroxide (H_2O_2), hypohalites, and hydroxyl radical ($OH^{\bullet}$). RNI refers to

all oxidation states and reactive adducts of nitrogenous NOS products, that arise in physiological settings, including nitroxyl (NO^-), nitrosonium (NO^+), higher oxides of nitrogen, S-nitrosothiols (RSNOs), peroxynitrite ($OONO^-$), and dinitrosyl iron complexes. None among ROI have identical biological properties, nor do any of the RNI. Their distinctive properties arise from differences in such features as reactivity, half-life, and lipid solubility.

More and more frequent is documentation of the participation of ROIs and RNIs in intracellular signaling [140]. Nonetheless, it has been difficult to understand how their involvement meets the requirement of signaling for specificity, and hence, to accept that their role could be physiologic.

The frame of reference of the present discussion is that ROI and RNI are routinely produced throughout the aerobic biome. Evolution has capitalized on their properties to put them to work as signaling molecules. From this perspective, the molecules usually referred to as antioxidant and antinitrosative defenses spend most of their time acting as integral parts of homeostatic signaling systems. As with any aspect of physiology, production of ROI and RNI can become excessive to the point that it is maladaptive, if not for the producing cell, at least for a target cell. In those circumstances, the systems that catabolize ROI and RNI, or reverse their effects, act as defense mechanisms.

The list of signaling molecules known to be regulated by ROI and/or RNI has expanded far beyond the original examples—soluble guanylyl cyclase for RNI [141–143] and NFκB [144] and activation protein-1 [145] for ROI—to include ion channels and transporters, G protein-coupled receptors, small GTPases, phosphatases, kinases, proteases, metabolic enzymes, cytoskeletal elements, translation regulators, cell-cycle control factors, transcription factors, histone (de)acetylases, and DNA methylases (see [140]). Through these reactions, ROI and RNI help regulate the development of plasmodia [146], flies [147], frogs [148], and mice [149]; cells' motility, matrix [150], interconnections [151], secretion, respiration, metabolism, gene expression, replicative cycle, and apoptosis; and clocks controling circadian rhythms and senescence (see [140]).

ROI and RNI can act both locally and distantly to tune responses to agonists, a role for which they are suited not only because of their small size and diffusibility but also because of their chemical reactivity. Thus, a major homeostatic role of ROI and RNI may be to link the behavioral and differentiative commitments of a cell to its metabolic budget.

ROI and RNI may be of particular importance as alert signals reporting about changes in the metabolic status in a cell, because of their origin from both oxygen and NAD(P)H. ROI and RNI can also regulate metabolic status. NO physiological regulation of the mitochondrial electron transport chain may lead to generation of ROI in most mammalian cells [152]. In this line, demonstration that NO can induce mitochondrial biogenesis in a variety of cells [5] is of relevance. Indeed, NO might represent a sensor of

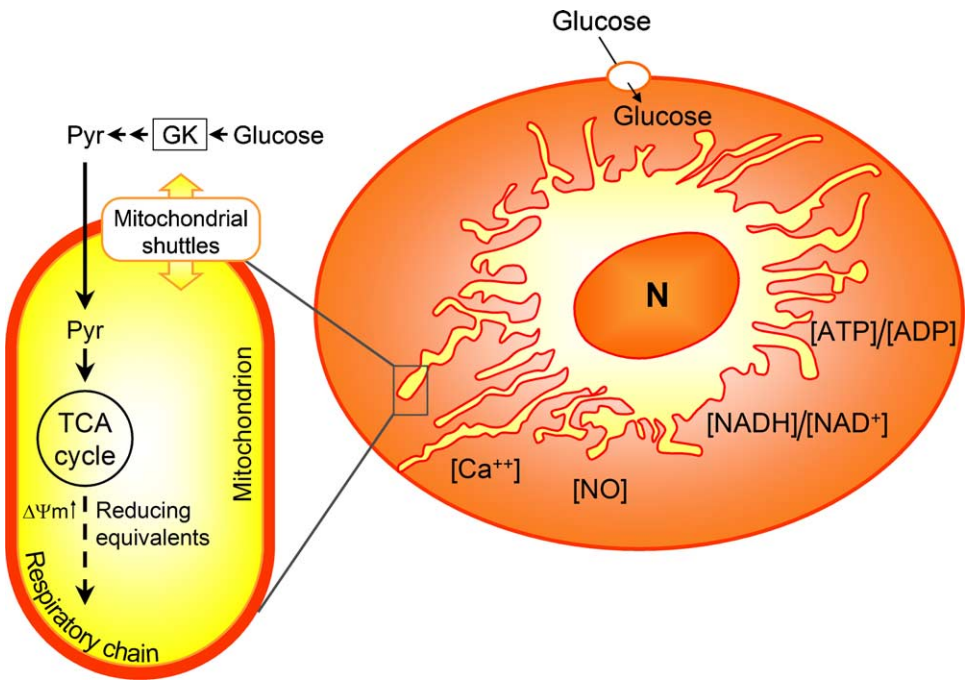

Fig. 3. Mitochondrial reticulum as a framework for cell signaling. Cells might monitor their own metabolic state (and activity) through the intracellular concentration of NO (and possibly other mediators like this). They may dynamically shape their behavior according to fluctuations in NO levels and consequent changes in mitochondrial networks that allow very rapid transport of information through the cell volume. This could imply a direct control of locally and temporally distant second messengers in the cell volume, including intracellular Ca^{2+} levels, and of metabolic markers, including intracellular ATP/ADP and NADH/NAD^+ ratios and glucose concentrations.

metabolic availabilities of cells and it might regulate cell metabolism through molecular control of mitochondrial biogenesis.

Furthermore, the NO-induced mitochondrial biogenesis, i.e. the dynamics of the mitochondrial reticulum [44], would allow a fine-tune control of cell biology and metabolism as a whole, despite continuos genetic and environmental perturbations (Fig. 3). In at least some cases, there is reason to believe that this robustness (or cell stability) is a consequence of the organizational control of mitochondrial networks rather than being genetically encoded. This would allow facing continuous metabolic variations. If ATP or metabolites turn over frequently, how does the cell maintain such a stable state? An interesting possibility is that cells may monitor their own metabolic state (and activity) through the intracellular concentration of NO (and possibly other mediators like this). They may dynamically shape their behavior according to fluctuations in NO levels and consequent changes in mitochondrial networks that allow very rapid transport of information through the cell volume. This form of robustness would be maintained by mitochondrial networks that are dynamic rather than organizationally stable. This idea is to be highlighted by experimentation, but data reported in this review seem to suggest it is likely.

10. Conclusions

Mitochondria are important dynamic organelles for cell survival and functions. Mitochondrial dynamics and biogenesis may be involved in cell metabolism control and signaling transduction. Moreover, mitochondrial biogenesis requires the choreographed expression of diverse transcription activators, including PGC-1α and NRF-1. Recently, this mitochondrial biogenesis program has been suggested to be involved in the pathogenesis of obesity, insulin resistance and type 2 DM. We have shown that NO acts as a key messenger to activate the mitochondrial biogenesis program in diverse cell types, and that this is obtained through cGMP-dependent and -independent pathways. The question whether eNOS polymorphism may be relevant in the pathogenesis of metabolic syndrome remains to be evaluated.

Many other open questions will be important to elucidate, such as the precise mechanism(s) by which NO/cGMP activates PGC-1α and/or NRF-1 to trigger mitochondrial biogenesis, the functional relevance of the NO-induced mitochondrial biogenesis, i.e. oxygen consumption and cell respiration, as well as the exact nature of the NO effects on mitochondrial dynamic properties. This picture may be even more complex: dietary factors (i.e. proteins formed by certain aminoacids, carbohydrates and lipids, or minerals and vitamins) that are able to modulate NO production in different tissues specifically acting on the diverse NOS isoforms [153], may be at work to modulate mitochondrial properties and thus become useful in disease prevention or treatment.

Future research on this fascinating organelle and its properties will give us interesting insights in the evolution of life on the earth and in the cell life. Thus, we can conclude with L. Thomas:

> [Mitochondria] feel like strangers, but (...) the same creatures, precisely the same, are out there in the cells of sea gulls, and whales, and dune grass, (...). Through them, I am connected; I have close relatives, once removed, all over the place. This is a new kind of information, for me, and I regret somewhat that I cannot be in closer touch with my mitochondria.
>
> "The Lives of a Cell—Notes of a Biology Watcher" (1975)

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