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Review

Respiratory chain complex I, a main regulatory target of the cAMP/PKA pathway is defective in different human diseases

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This paper is dedicated to the memory of Antonio Xavier, an inspired, innovative, rigorous scientist and a charming person, whose friendship was always a privilege and a real pleasure.

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ABSTRACT

In mammals, complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial respiratory chain has 31 supernumerary subunits in addition to the 14 conserved from prokaryotes to humans. Multiplicity of structural protein components, as well as of biogenesis factors, makes complex I a sensible pace-maker of mitochondrial respiration. The work reviewed here shows that the cAMP/PKA pathway regulates the biogenesis, assembly and catalytic activity of complex I and mitochondrial oxygen superoxide production. The structural, functional and regulatory complexity of complex I, renders it particularly vulnerable to genetic and sporadic pathological factors. Complex I dysfunction has, indeed, been found, to be associated with several human diseases. Knowledge of the pathogenetic mechanisms of these diseases can help to develop new therapeutic strategies.

1. Introduction

Complex I (NADH-ubiquinone oxidoreductase, E.C. 1.6.5.3) is the first enzyme of the mitochondrial respiratory chain, being the entry port in the chain of the reducing equivalents of NADH, produced by mitochondrial dehydrogenases of respiratory substrates [1–4]. The enzyme catalyzes the transfer of electrons from NADH to ubiquinone in the inner mitochondrial membrane and conserves the free energy, so made available, as a transmembrane electrochemical proton gradient ($\Delta\mu H^+$) according to reaction (1).

$$NADH + UQ + 5H^+_{in} \leftrightarrow NAD^+ + UQH_2 + 4H^+_{out} \quad (1)$$

The complex thus contributes by 40% to the generation of the respiratory chain $\Delta\mu H^+$, which is utilized to make ATP from ADP in the mitochondrial process of oxidative phosphorylation (OXPHOS) [4,5]. In human cells the ATP produced by OXPHOS covers, under physiological conditions, more than 80% of the ATP that cells need. This requirement is particularly stringent in organs like, for example, the human brain, which even though it only represents 2% of the body weight, utilizes around 20% of all the oxygen we consume [6].

Mammalian complex I, with a molecular mass of $\approx 1 \times 10^6$ Da, is the largest enzyme of the mitochondrial OXPHOS system. The complex contains multiple redox centers: one FMN, nine iron–sulfur (FeS)-centers and two protein bound semiquinones [1–4,7,8]. A series of these provides the sequential, stepwise transfer of electrons from NADH to the ubiquinone of the pool, coupled to proton

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pumping [4,7–9]. During this process direct electron leakage to oxygen may, however, occur at different redox sites in the enzyme [4,10–12].

In humans and mammals mitochondrial complex I has, in addition to the 14 subunits of the catalytic core, conserved from prokaryotes to humans, at least 31 supernumerary subunits [4,13]. Seven subunits of the complex are encoded by the mitochondrial DNA, 38 by nuclear genes located in different chromosomes [1,4,12,13]. What is the hidden code of these numerous supernumerary subunits? Some of them appear to be involved in the assembly of the complex [4,14–17], its regulation [4,18] and ancillary functions [4,19,20]. The GRIM-19 (B16.6) subunit, for example, belongs to the group of genes associated with retinoid–interferon induced mortality [19]. Moreover, a plethora of post-translational modifications of complex I subunits have been detected. In particular, various subunits of the complex, NDUFS4 [21,22], NDUFB11 [23], NDUFA1 [23], NDUFA10 [24], NDUFA7 (B14.5a) [25] have been found to be phosphorylated. Other post-translational modifications have also been observed [26]. In addition, several proteins have been identified which assure the proper assembly and stability of the complex [27–29].

Complex I is a regulable pacemaker of the mitochondrial respiratory function [15,30–32], is a major site of cellular oxygen superoxide production [10–12,33], is involved in apoptosis [34,35] and age-related functional decline [36–38]. Various hormones, neurotransmitters and cell growth factors regulate, through the activation of cellular signal transduction networks, the expression and functional activity of complex I [30,39–42].

Genetic dysfunctions of complex I represent, on the other hand, the largest of primary mitochondrial diseases [43–46] and dysfunctions of the complex have been found in sporadic and familial forms of Parkinson [47], cancer [48,49] and other disorders [46,50,51].

Understanding the mechanisms of the biogenesis, energy transduction, reactive oxygen species (ROS) production and regulation of complex I is crucial to comprehend the pathophysiological role of the complex in human diseases and to develop of therapeutical strategies.

The present paper reviews features of the regulation by the cAMP/PKA pathway of the biogenesis, functional capacity, ROS interaction in complex I and the pathophysiological implication of dysfunctions of the complex.

2. Complex I subunits are phosphorylated by the cAMP-dependent protein kinase

Various mitochondrial proteins have been found in the phosphorylated form [52–54]. In the nineties our group discovered that among the phosphorylated proteins of the inner mitochondrial membrane, proteins of 18 and 6–10 kDa are structural subunits of complex I. We showed that phosphorylation of these subunits is catalyzed by the cAMP-dependent protein kinase (PKA) [55,56]. Phosphorylation of complex I subunits can, in fact, be mediated by the PKA present in both the cytosol [57] and mitochondria [58].

Edman sequencing of the electrophoretic protein band of ≈18 kDa of the purified bovine heart complex I showed the presence in this band of two subunits of the complex, namely the GDHG and the AQDQ subunits (NDUFS4 gene) [21,22]. The position of the latter corresponded to the band labeled by ^{32}P in the presence of PKA and [γ-^{32}P]ATP [21]. Chen et al. [23] confirmed PKA-dependent phosphorylation of protein(s) in bovine heart mitochondria, and showed, in the 18 kDa ^{32}P-labeled protein band of complex I, an additional serine phosphorylated, previously undetected [59], subunit (ESSS subunit) [23]. Two-dimensional IEF/SDS–PAGE electrophoresis of purified bovine heart complex I, ^{32}P labeling and immunoblotting with an antibody specific for the serine-173 phosphorylated C-terminus of the NDUFS4 protein, which is conserved in animal species [22], showed that this site is phosphorylated by PKA. It was also shown that precursor and mature human NDUFS4 protein produced by "in vitro" heterologous expression, were both ^{32}P-labeled when incubated with [γ-^{32}P]ATP and PKA [22]. Thin layer chromatography amino-acid mapping of radioactive proteins showed the presence of phosphothreonine and phosphoserine both in the precursor and mature form of the NDUFS4 protein [22]. S173A replacement in the conserved phosphorylation site resulted in disappearance of phosphoserine from the mature form [22]. It should be mentioned that mass spectrometry of the trypsin digest of the heterologous human NDUFS4 protein, phosphorylated by PKA, identified peptide ions of the protein with 82% coverage of the sequence, but the in silico predicted carboxy terminal peptide ions of the last C-terminal 4–7 residues were, however, not detected [22] see also [23]. It is worth noting that this can be due to general problems in detection of this and other phosphoproteins, like the low dynamic state of phosphorylation of the protein, loss of the C-terminus in the preparation/treatment of the mass-spectrometer sample and the small size (217.63 m/z) of the terminal peptide ion generated by tryptic proteolysis (see also [60]).

3. The cAMP/PKA pathway regulates complex I activity and ROS balance in cell cultures

In mammalian cells there are subcellular pools of cAMP and PKA [57,58,61–63]. In response to activation, by extracellular effectors, the plasma membrane adenylyl cyclase (tmAC) produces and releases cAMP in the cytosol, where its level is determined by the opposite activities of the cyclase and phosphodiesterase(s) (Fig. 1) [57,62].

Cytosolic cAMP cannot apparently move into the internal space of subcellular organelles. There is, however, in the mitochondrial matrix, and other organelles, a soluble bicarbonate-activable adenylyl cyclase (sAC), which produces cAMP inside the organelles (Fig. 1) [64–66].

All the members of the complex, involved in reversible cAMP-dependent protein phosphorylation, are associated with the outer [70] and the inner mitochondrial membrane [58,69,71]. The complex consists of A-kinase anchor protein(s) (AKAPs) [58,67,68], catalytic and regulatory subunits of PKA, phosphodiesterase(s) and serine phosphatase(s) [58,67–71] (Fig. 1).

A strong depression of the NADH-ubiquinone oxidoreductase activity of complex I, increase in the mitochondrial level of oxygen superoxide and in the cellular level of hydrogen superoxide have been observed in cells brought in the G0 phase by serum deprivation [40,72], or impaired by genetic disorder [73,74] as well as in K-ras oncogenic transformated cells [75]. It has to be recalled that complex I, besides being the major mitochondrial producer of ROS [4,10–12], is by itself particularly vulnerable to oxidative stress [10,76]. The question remains open whether, under the conditions described, it is a dysfunction in complex I that causes ROS overproduction, or if it is the latter to cause inhibitory, oxidative damage of the complex. The two processes can, in fact, set up a detrimental cycle.

In all the cases described, addition to the cells of a permeant derivative of cAMP, or activation of tmAC by cholera toxin [40] or the β-adrenoceptor agonist isoproterenol [42], reversed the inhibition of complex I activity and the accumulation of ROS, without having, however, any effect on ROS scavengers [40,42,72]. The rescue effect exerted by cAMP production in response to activation of tmAC, shows that, under these conditions at least, it is the

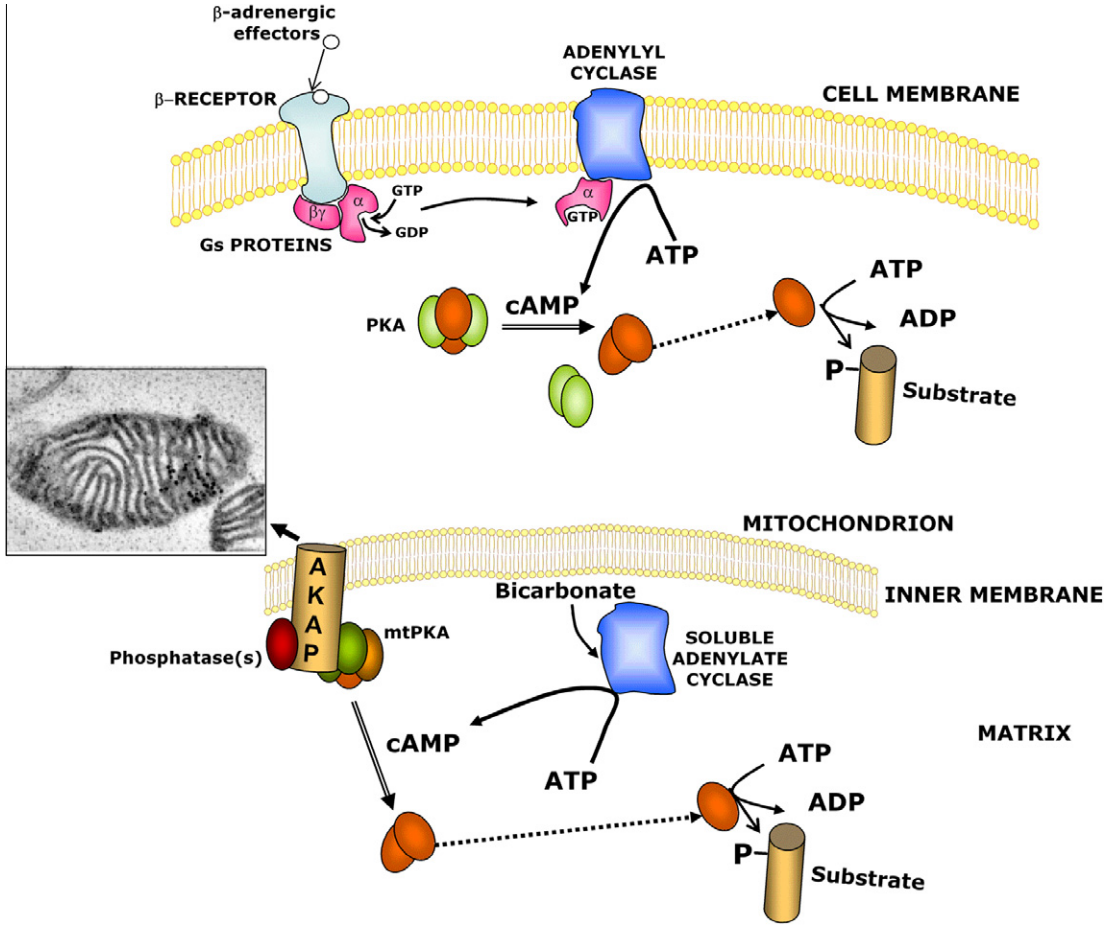


Fig. 1. Subcellular compartmentalization of the cAMP/PKA system. cAMP is produced by the plasma membrane adenylyl cyclase, in response to extracellular signals [40,42,57] and by the bicarbonate-activated adenylyl cyclase localized in mitochondria and other subcellular structures [64–66]. Different subcellular pools of cAMP [63] can thus be generated, with activation of PKA and other cAMP effectors. PKA, consisting of two cAMP-binding regulatory subunits (in green) and two catalytic subunits (in red), dissociates upon binding of cAMP to the regulatory subunits (R-PKA). Specific AKAP proteins, associated with various subcellular membranes, bind R-PKA and in this way tether the PKA tetramer to membranes together with specific protein phosphatases [58,62,67,68]. These complexes accomplish phosphorylation/dephosphorylation of proteins bound or close to the complexes [67–69]. PKA has been found to be associated with both the outer [70] and the inner membrane/matrix fraction of mammalian mitochondria [58] where it can phosphorylate various mitochondrial proteins. In the left inset a transmission electron microscopy picture of mitochondria isolated from rat heart exposed to the AKAP121 antibody, conjugated with colloidal-gold secondary antibody is shown. Numerous gold particles of AKAP protein appear to be associated with the mitochondrial cristae (from [58]).

elevation of cytosolic cAMP concentration to promote the activity of complex I and to lower the ROS level, although also mitochondrial cAMP can provide an additional contribution.

Immunodetection with specific antibodies showed that in cell cultures cAMP promoted, under the conditions in which it activated complex I and reversed ROS accumulation, phosphorylation of serine [15,40,69] in the RVS site in the carboxy terminus of the NDUFS4 protein [22]. Evidence that PKA-mediated phosphorylation of the NDUFS4 subunit is, indeed, responsible for the cAMP activation of complex I, was clearly provided by the finding that cAMP-dependent serine phosphorylation of complex I subunit(s) and complex I activation were missing in patient's fibroblasts with an NDUFS4 mutation, which caused disappearance of the protein product of the gene from the cells [15,18]. It has also been found that addition to cell cultures of phosphatase inhibitors also promotes complex I activity [42].

The mechanism by which PKA dependent phosphorylation of the NDUFS4 subunit, and possibly others, can result in activation of complex I and lowering of ROS level is presented in the last part of Section 4. In any case, the activation of complex I by cAMP/PKA system shows that the functional capacity of the complex can respond, in the cell, to a series of regulatory factors, which include ligands of plasma membrane receptors of the cAMP cascade, networks of cell signal transduction pathways, as well as metabolic processes which contribute to cellular bicarbonate homeostasis (see Fig. 1).

4. The cAMP/PKA system regulates the expression and the assembly of complex I

In addition to the regulatory effects due to post-translational protein phosphorylation, the cAMP/PKA system regulates also the biogenesis of the mitochondrial OXPHOS system [41,77,78]. A central role in this regulatory function is played by the cAMP/Ca^{2+} response element (CRE) binding protein (CREB) [79]. CREB binds to the DNA consensus sequence TGACGTCA in the promoters of target nuclear genes [79]. The transcriptional activity of CREB is induced by serine phosphorylation in its conserved kinase inducible domain by PKA, Ca^{2+}/calmodulin protein kinases or other kinases [80,81].

A role of CREB in mitochondrial biogenesis was highlighted by the discovery that the CREB transcriptional complex activates the expression of the master gene PGC-1α (the transcriptional coactivator PPARc coactivator 1α) [93,94]. PGC-1α is also activated by

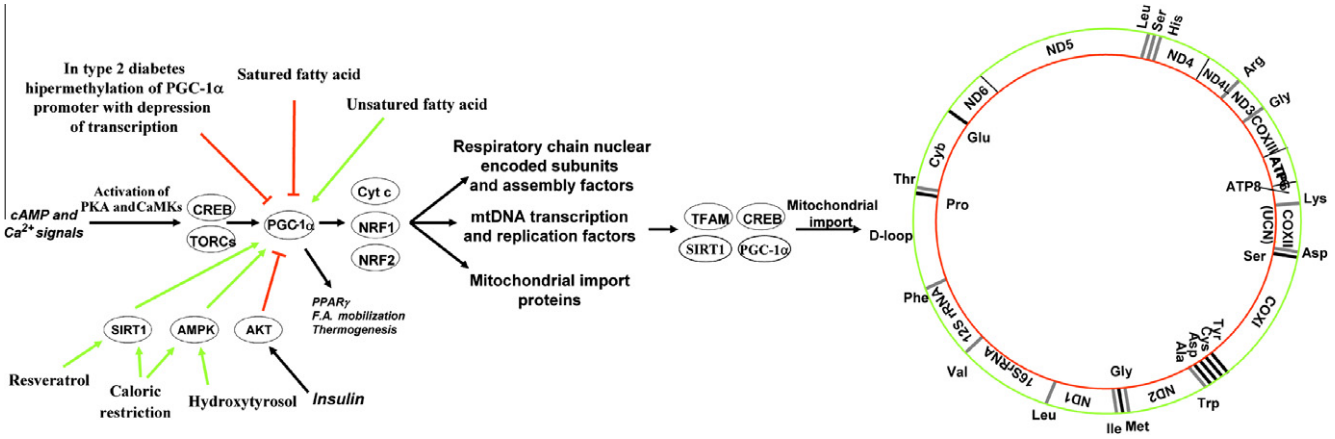


Fig. 2. Schematic representation of PKA and CREB mediated pathways, which control mitochondrial biogenesis. Activation of protein kinases by cAMP and/or Ca2+ results in phosphorylation of CREB and TORCs proteins. Phosphorylated CREB and TORCs proteins bind to the CREB complex on gene promoters and exert their transcriptional coactivator action [82]. CREB and TORC promote the transcription of PGC-1α and cytochrome c genes. PGC-1α is also activated by post-translational modifications, like deacetylation by SIRT1 [83], phosphorylation by AMPK [84] and inhibited by phosphorylation by the insulin activated AKT [85]. The expression of PGC-1α can be regulated at transcriptional level by methylation of the promoter, as found in type II diabetes in which hypermethylation of the promoter inhibits transcription [86]. The diet also influences the expression of PGC-1α. Caloric restriction, and thus the AMP/ATP and NAD^+/NADH ratios, regulate the activities of AMPK and SIRT1 respectively [87,88]. A diet rich in polyunsaturated fatty acids apparently promotes, one rich in saturated fatty acids down-regulates PGC-1α expression [89]. Several compounds present in the diet like hydroxytyrosol of olive oil [90], and resveratrol of red wine [91], regulate the expression of PGC-1α. PGC-1α, in combination with other factors, activates the expression of the nuclear respiratory transcription factors, NRF1 and NRF2. NRF1 and NRF2 activate the expression of the mitochondrial transcription and replication factors (TFAM, TFB1M, TFB2M and MRP RNA) and nuclear genes coding for structural proteins and assembly factors of OXPHOS complexes, mitochondrial import, ion channel and shuttle proteins. The CREB protein, after mitochondrial import, promotes the expression of mtDNA [78]. Recently PGC-1α and SIRT1 have been found also in mitochondria, where they physically interact with mTFA [92].

post-translational modification, such as deacetylation by the NAD-dependent deacetylase sirtuin-1 (SIRT1) [83], whose expression is promoted by fasting [88]. Compounds present in the diet like hydroxytyrosol of olive oil [90], and resveratrol of red wine [91], regulate the expression of PGC-1α, through the activation of AMP kinase, and/or SIRT1 (Fig. 2).

Induced expression of PGC-1α activates a transcriptional regulatory cascade, which amplifies the impact of CREB-mediated signal transduction on mitochondrial biogenesis [94]. PGC-1α promotes the expression and activity of the nuclear respiratory transcription factors 1 and 2 (NRF1 and NRF2) [95]. NRF1 and NRF2 control the expression of nuclear genes coding for structural proteins of mitochondrial respiratory chain and the F_oF_1-ATP synthase, enzymes of heme biosynthesis and proteins involved in mitochondrial import of nuclear encoded subunits of OXPHOS complexes and complex assembly (Fig. 2) [96].

NRF1 and NRF2 also promote replication and transcription of the mitochondrial genome (see Fig. 2) [96]. The transcriptional activity of both the heavy and light mtDNA strands is controlled by the mitochondrial transcriptional factor A (TFAM). This interacts with the mitochondrial RNA polymerase [97] together with two additional transcription factors (mitochondrial transcription factor TFB1M and TFB2M) and a fraction of PGC-1α found in mitochondria [92]. In this way, a transcriptional cascade (Fig. 2) confers to mammalian cells the capacity to upregulate in a concerted process the expression of nuclear and mitochondrial genes encoding subunits of OXPHOS complexes.

It has been discovered that, in addition to the nucleus, CREB is also localized in the inner mitochondrial compartment [98]. In neuronal cell cultures CREB protein was found to bind to the mtDNA D-loop [99]. In these cells, the overexpression of mito-tagged CREB increased the transcript levels of the ND2, ND4, and ND5 mitochondrial genes of complex I. We have found [78] that "in vitro" expressed [^{35}S] methionine-labeled CREB is imported in isolated mitochondria by the membrane potential-dependent mechanism [100]. Both mitochondrial surface binding and import into the inner mitochondrial compartment of radioactive CREB was inhibited by an antibody against Tom20 (outer membrane translocator subunit 20) [78]. CREB protein, once imported in mitochondria, produced a strong stimulation of the synthesis of subunits of OXPHOS complexes encoded by mitochondrial genes, an effect that required CREB phosphorylation by PKA [78]. The stimulatory effect was particularly potent in the case of ND1, ND6, and cox III/ATP6 proteins, whose synthesis was two- to three-fold enhanced by the combination of CREB and cAMP [78].

The assembly of a mature, functional complex in the inner mitochondrial membrane is achieved in a multi-step process which, in addition to the quantitatively coordinated expression of the 38 nuclear-encoded and the seven mitochondrial-encoded subunits, involves: (i) chaperon-assisted folding changes of the newly synthesized proteins; (ii) mitochondrial import of the nuclear-encoded precursor proteins through the outer membrane (TOM) and inner membrane (TIM) translocator systems; (iii) proteolytic maturation of the precursor proteins; (iv) stepwise incorporation in the complex, in which one copy of each subunit has to be incorporated in the proper near-neighbor functional position [27–29,46]. In addition, the synthesis and assembly of the protein subunits has to be coordinated with the production and the correct binding at the catalytic centers of FMN and non-heme iron–sulfur centers [101].

It has been found that, in addition to de novo synthesis of an entire complex from all the individual subunits, an exchange of individual subunits, in particular those more superficially located in the complex, with newly synthesized subunits can also take place [102].

Work in our laboratory showed that the $\Delta\psi$ dependent import in isolated mitochondria of [^{35}S]-methionine labeled NDUFS4 precursor protein, produced "in vitro" by expression of the full-length NDUFS4 cDNA, is promoted when it is phosphorylated by added PKA and inhibited by phosphatase (Fig. 3) [103].

The import/maturation in mitochondria of the NDUFS4 protein, completed in the same time-span in which cAMP stimulated complex I activity in cell cultures, was completely suppressed by site-specific substitution of serine 173 with alanine in the C-terminal RVS phosphorylation site. These observations, showing that the functional capacity of complex I depends on phosphorylation and

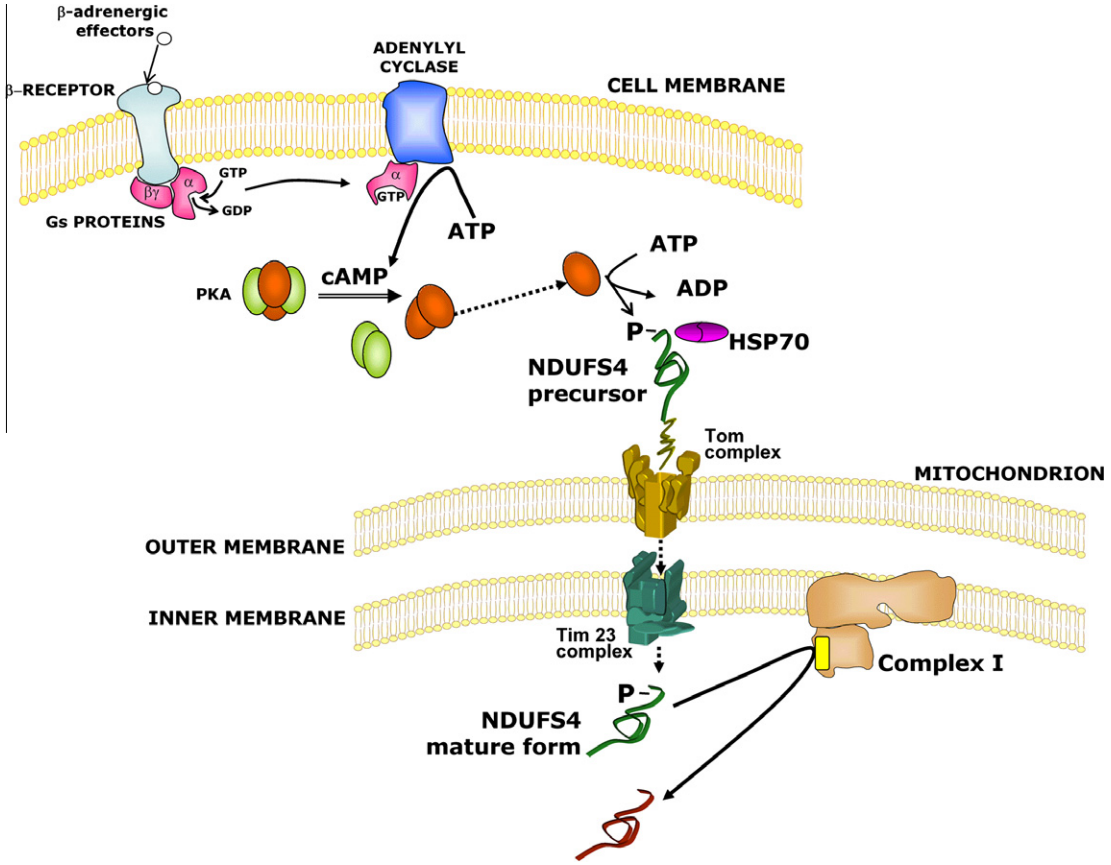


Fig. 3. Scheme describing the impact of the cAMP/PKA system on the mitochondrial import of the NDUFS4 subunit and its assembly in complex I. Elevation of the cytosolic level of cAMP by stimulation of plasma membrane adenylyl cyclase results in PKA-dependent phosphorylation of the NDUFS4 C-terminus. This promotes the binding of the NDUFS4 precursor to the cytosolic heat shock protein 70 (Hsp70). Phosphorylated NDUFS4 precursor is imported into mitochondria where it undergoes to cleavage of the presequence [103]. Newly imported NDUFS4 protein (in green) is assembled in complex I during de novo synthesis of the complex or in exchange with the pre-existing (damaged, in red) subunit in the mature complex.

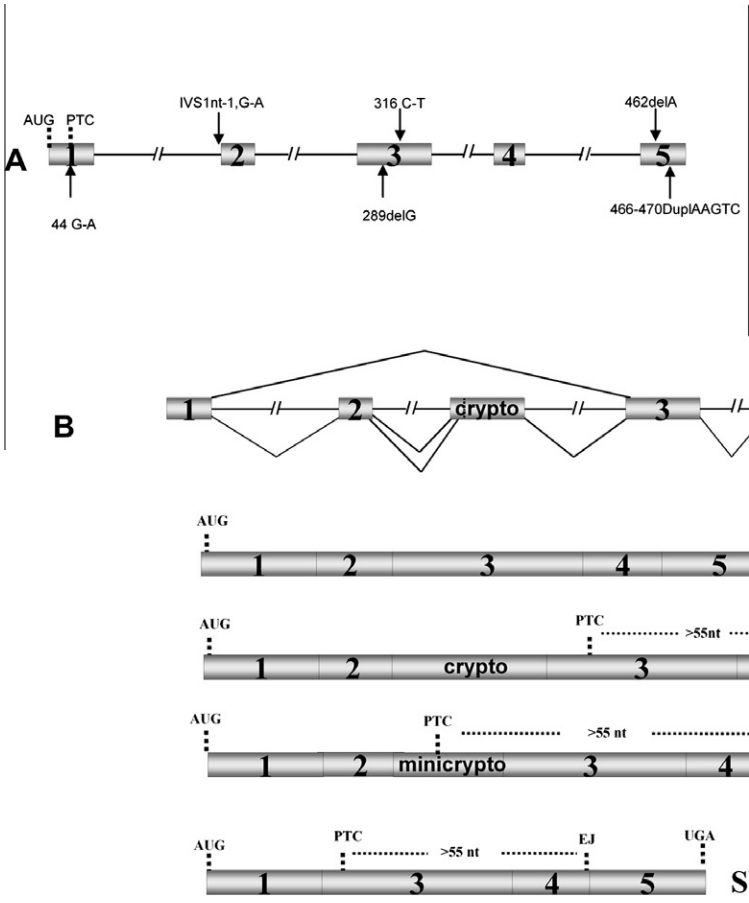
import of newly synthesized NDUFS4 protein, support the proposed mechanism of exchange of subunits in the assembled complex with newly imported ones to maintain complex I homeostasis (Fig. 3) [102]. This process can, in fact, play a proof-checking role for subunits, superficially exposed to the matrix space where they can be oxidatively-damaged by ROS, with depression of the catalytic activity of the complex. Substitution of the oxidatively damaged subunits with newly synthesized ones can play a role in the functional rescue of complex I. Oxidation of complex I subunits, accompanied by reduced functional capacity of the complex, have been observed in aging [37,38]. Results from the author's laboratory has shown that oxidation of complex I subunits, by the addition to fibroblast cell cultures of ROS generating tert-butyl hydroperoxide, depressed the functional capacity of complex I. Activation of the cAMP/PKA system promoted the import of newly synthesized NDUFS4 subunit into mitochondria and restored its dynamic assembly (unpublished observations).

5. Genetic dysfunctions of complex I are associated with different human diseases

Isolated complex I deficiency is the most frequent case of primary mitochondrial defects [43–46]. Complex I pathological mutations have been initially found in mitochondrial-DNA encoded subunits [104]. The first fatal mutation in a nuclear gene of complex I, detected in a newborn [105], was a base duplication in exon 5 of the NDUFS4 gene encoding the 18 kDa AQDQ subunit of the complex, previously found to be phosphorylated by PKA [21]. Later, other mutations in the NDUFS4 gene were detected in different encephalopathic patients [46]. In all these cases the NDUFS4 mutations resulted in: (i) disappearance of the protein from the patient's cells; (ii) suppression of the last step in complex I assembly; (iii) disappearance of cAMP dependent serine phosphorylation of complex I subunits and of the stimulatory effect of cAMP on mitochondrial respiration; (iv) stabilization of alternative splice products of the gene [46]. In the NDUFS4 mutations, which resulted in complete suppression of the NADH-ubiquinone oxidoreductase activity of complex I, no ROS accumulation was observed [73].

A pathological C1564A mutation was also detected in the nuclear NDUFS1 gene coding for the 75 kDa iron–sulfur protein subunit of complex I [73]. This is the largest conserved subunit of the complex and contains the N1b, N4, N5 Fe–S clusters of the electron transfer pathway in the enzyme [7–9].

In the fibroblasts of the patient harboring the C1564A mutation (Q522K substitution in the protein), the mitochondrial content of the normally assembled complex decreased as compared to normal cells and an additional lower molecular mass of the complex appeared [73]. The mutation resulted also in a marked, but not complete, suppression of the NADH-ubiquinone oxidoreductase activity. In the patient's fibroblasts large accumulation of H_2O_2 and intramitochondrial O_2^- was detected. The Q522K mutation in the NDUFS1 subunit alters, apparently, the redox function of the Fe–S center(s) bound to this protein, with inhibition of the normal catalytic activity of the complex and increased production of oxygen superoxide. The enhanced production of ROS can in turn cause further oxidative damage in a "vicious cycle" leading to



Mutations	Disease associated	Ref.
44G-A	Leigh-like syndrome	[108]
IVS1nt-1, G-A	Leigh syndrome	[109]
289DelG	Leigh-like syndrome	[110]
316C-T	Leigh-like syndrome	[110]
466-470DuplAAGTC	Leigh syndrome	[105]
exons 3,5 deletion	Not specified	[111]
462DelA	Leigh syndrome	[112]

Fig. 4. (Panel A) Structure of the *NDUFS4* human gene of complex I with location of pathological mutations found in neurological patients with complex I deficiency. In the Table five exonic mutations, one intronic mutation shown in the structure of the gene and a sixth mutation with deletion of a region including exons 3 and 5, with the associated diseases are listed. For details see text and the references in the table. (Panel B), alternative splicing products of the NDUFS4 gene detected in human fibroblasts. The scheme shows the mechanism of production of the alternative splicing products normally produced by the NDUFS4 gene but up-regulated in the exonic 44G–A mutation as well as in the intronic IVS1nt-1, G–A mutation. For details see text and refs. [105,108–112].

amplification of the biochemical damage and disease progression. The depression of the activity of complex I was counteracted by the addition to the fibroblast culture of dibutyryl-cAMP, which also prevented the accumulation of oxygen free radicals [73]. Selected antioxidants and β-agonists could offer a rational therapeutical strategy in patients carrying mutations in *NDUFS1* or in other complex I genes, which result in a dysfunction centered on excess of ROS production and oxidative stress.

A more recent example of how a dysfunction in the cAMP/PKA system can result in a detrimental impairment of the catalytic activity of complex I and overproduction of ROS was provided by a study of the mitochondrial bioenergetic function in human skin fibroblasts of Down Syndromes patients [74]. Down Syndrome is a multifactorial disorder caused by trisomy of human chromosome 21, resulting in mental retardation, premature aging and neurodegeneration. A strong decrease in the cellular level of cAMP and depression of PKA activity were found in the patient's fibroblasts [74]. The serine phosphorylation of the NDUFS4 subunit and the catalytic activity of complex I were markedly reduced and the level of mitochondrial oxygen superoxide and cellular H_2O_2 increased in the patient's fibroblasts. All these changes were reversed by the addition to the fibroblasts of dibutyryl-cAMP. These observations, besides contributing to understand the pathogenetic mechanism of the Down Syndrome, may help to develop therapeutical approaches based on the use of β-adrenoceptor agonists which, as previously reported, rescue complex I deficiency and decrease ROS levels in human cell cultures.

It can also be mentioned that work in progress along other lines in the author's laboratory, in collaboration with the Department of Biotechnology and Biosciences, University of Milano-Bicocca, showed that K-ras oncogenic expression in "in vitro" transformed cells and in human tumors was associated with a dysregulation of the cAMP/PKA pathway. This appeared to result in fragmentation of mitochondria and depression of complex I activity. These defects also were all rescued by promoting formation of cAMP with forskolin (unpublished observations).

6. Pathological mutations of the NDUFS4 subunit of complex I stabilize alternative splice transcripts of the gene

A study of the NDUFS4 gene transcription in patients with pathological mutations of the gene revealed new features of surveillance mechanisms regulating the levels of alternative transcripts [106,107]. The five exons of the gene result in a canonical mRNA coding for the 175 residues precursor protein of the 18 kDa (AQDQ) subunit of complex I [22] (Fig. 4).

In a patient with a 44G → A, nonsense mutation, in the first exon caused, as expected, disappearance of the mature protein. This mutation which introduced a premature termination codon (PTC) in the proximity of the canonical AUG start codon, rather than eliciting nonsense mediated decay (NMD) degradation of the canonical mRNA, upregulated three PTC containing alternative transcripts (splice variants 1, 2 and 3) (Fig. 4) [106,107]. The splice variants 1 and 2 (SV1 and SV2) result from insertion between

exons 2 and 3 of a crypto exon which uses two alternative acceptor sites (Fig. 4). The splice variant 3 (SV3) derives from exon 2 skipping. These three alternative transcripts are also produced in normal cells, but their level is kept low by different RNA surveillance mechanisms. SV1 and SV2 were found to be degraded in the cytosol by NMD, the level of SV3 was down-regulated directly in the nucleus by a process different from NMD, which affected the production of the transcript rather than its stability [107]. In the patient fibroblasts the 44G → A nonsense mutation inactivated NMD degradation of SV1 and SV2 and nuclear down regulation of SV3 (Fig. 4). In a neurological patient with a homozygous splice acceptor site mutation in intron 1 (IVS1nt-1, G → A) of the *NDUFS4* gene (Fig. 4) only a mRNA transcript, in which exon 2 was skipped, was detected [111]. Amplification of this transcript and sequencing showed that it corresponded to the PTC containing SV3 isoform detected in the patient with 44 G → A nonsense exonic mutation in the *NDUFS4* gene (Fig. 4) [107]. The accumulation of the aberrant alternative transcripts, caused by exonic or intronic mutation in the *NDUFS4* gene, able to escape the mRNA surveillance mechanisms, can represent another deleterious event contributing to the pathogenetic mechanism of the NDUFS4 mutations in neurological patients. In vitro translation experiment showed, in fact, that the aberrant alternative transcripts were able to produce potential toxic proteins [107].

7. Selective degradation of the NDUFS3 subunit of complex I. An involvement of granzyme A mediated NDUFS3 cleavage in type I diabetes?

Type I diabetes is an autoimmune disease in which, killer lymphocytes (natural killer and cytotoxic T lymphocytes) destroy the insulin producing β-cells of the pancreas [113]. The destruction of β-cells by lymphocytes involves: formation of immune synapse through lymphocyte-binding to class I hystocompatibility complex proteins on the surface of β-cells [113] and perforin mediated injection into β-cells of members of granzyme serine proteases, where they initiate a cascade of events leading to cell death [113]. A mechanism of cell death induced by granzyme B has been proposed that involves caspase and Bid-dependent apoptosis [113].

Recent work has provided evidence showing that killer lymphocytes can induce a caspase independent cell death by delivery of granzyme A into mitochondria of the target cells [114]. Granzyme A once entered into the inner mitochondrial compartment digests, selectively, the NDUFS3 subunit of complex I [114]. Degradation of this subunit results in inhibition of the normal catalytic activity of the complex and promotion of its capacity to produce ROS. Elevation of the cellular ROS level is proposed to trigger a series of downstream events, which include translocation of the endoplasmic reticulum-associated SET complex into the nucleus, where its cleavage by granzyme A induces the release of proteins, responsible for DNA fragmentation and ultimately cell death [114].

Mitochondrial proteomic analysis, carried out in our laboratory, has revealed a selective decrease of the content of the NDUFS3 subunit of complex I in brain mitochondria of rats in which insulin-deficient type I diabetes was induced by streptozotocin treatment. The decreased content of the NDUFS3 subunit, which was essentially due to post-transcriptional events, resulted in a severe impairment of the enzymatic activity of complex I (unpublished observations).

How insulin deficiency, hyperglycemia and/or other secondary alterations do result in the selective degradation of the NDUFS3 subunit of complex I in the brain of diabetic rats remains to be clarified. It is possible that the streptozoticin-induced diabetes results in activation of granzyme A or of different endogenous protease(s) in neuronal mitochondria (see also Ref. [114]). Is it possible that selective NDUFS3 degradation by granzyme A is critically involved in a mechanism of destruction of pancreatic β-cells in human diabetes? (see also Ref. [115]).

8. Conclusions

The work presented provides an appraisal of the regulation by the cAMP/PKA pathway of the expression, assembly and catalytic activity of complex I of the mitochondrial respiratory chain in humans and mammals. The control of complex I functional capacity and of ROS production/level by the cAMP/PKA pathway has far-reaching implications in human pathophysiology. Complex I is a pace-maker of the overall activity of mitochondrial oxidative phosphorylation. In organs like brain, heart, kidney, skeletal muscle, and others, which undergo changes from rest to various degrees of activity, the ATP production by OXPHOS has to respond to the continually changing energy demand. Complex I with its abundance of structural and ancillary proteins offers the prerequisites to respond to a variety of regulatory stimuli. Regulation of complex I by the cAMP/PKA pathway, which is at the cross-road of signal transduction networks, amplifies the signals to which mitochondrial respiratory metabolism can respond. The same complexity of the regulatory network of complex I makes it particularly vulnerable to gene mutations, as well as to sporadic, endogenous and exogenous factors. The deleterious consequence of this, is, in fact, underlined by the discoveries of the different human diseases in which complex I dysfunction was, so far, found to play a determinant role. The possibility emerges, from what reported, of new therapeutical strategies for diseases in which combined dysregulation of the cAMP/PKA pathway and of complex I are involved.

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