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Review

Cytochrome *c* oxidase deficiency: Patients and animal models

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ABSTRACT

Cytochrome *c* oxidase (COX) deficiencies are one of the most common defects of the respiratory chain found in mitochondrial diseases. COX is a multimeric inner mitochondrial membrane enzyme formed by subunits encoded by both the nuclear and the mitochondrial genome. COX biosynthesis requires numerous assembly factors that do not form part of the final complex but participate in prosthetic group synthesis and metal delivery in addition to membrane insertion and maturation of COX subunits. Human diseases associated with COX deficiency including encephalomyopathies, Leigh syndrome, hypertrophic cardiomyopathies, and fatal lactic acidosis are caused by mutations in COX subunits or assembly factors. In the last decade, numerous animal models have been created to understand the pathophysiology of COX deficiencies and the function of assembly factors. These animal models, ranging from invertebrates to mammals, in most cases mimic the pathological features of the human diseases.

1. Introduction

Mitochondria, ubiquitous organelles of eukaryotic cells, are the main generators of cellular ATP and carry several important metabolic reactions such as the tricarboxylic acid cycle (TCA), lipid β-oxidation and oxidative phosphorylation (OXPHOS). Mitochondria also play an active role in survival and death signaling and cellular calcium homeostasis. Defects in mitochondrial function are associated with numerous neurodegenerative diseases (Parkinson's, Alzheimer's and Huntington's disease) and in particular with mitochondrial diseases. Mitochondrial diseases are characterized by defects in the oxidative phosphorylation (OXPHOS) system with an incidence of 1 in 5000 [1]. They are heterogeneous in nature, affecting multiple organs particularly those with higher energetic demands such as muscle and brain. Mitochondria have their own genome which encodes for about 1% of the total mitochondrial proteins, therefore, mitochondrial biogenesis occurs as a result of a highly coordinated action between the nuclear and the mitochondrial genome that requires the import of several proteins into the mitochondria that are encoded by the nuclear genome (reviewed in [2]). For this reason, mitochondrial diseases are caused by mutations in mitochondrial genes, in nuclear genes encoding for subunits of the respiratory chain complexes or in genes coding for proteins involved in the nuclear–mitochondrial communication (reviewed in [3]). This review focuses on defects in cytochrome *c* oxidase (COX) or complex IV, one of the most common defects of the OXPHOS system.

2. Oxidative phosphorylation

The mitochondrial metabolic pathway converting substrates generated from glycolysis and β-oxidation into energy in the form of ATP is known as oxidative phosphorylation (OXPHOS). The OXPHOS system is composed of 5 multi-enzyme complexes (complexes I through V) and two electron carriers, a quinone (coenzyme Q) and a small heme containing protein (cytochrome *c*) that are located in the inner mitochondrial membrane. These respiratory complexes are formed by subunits encoded by both the mitochondria and the nuclear genome with the exception of complex II which is entirely encoded by the nuclear DNA. In this energy generating pathway, the reducing equivalents NADH and FADH2 formed during TCA cycle and β-oxidation enter into the electron transport chain (complexes I through IV of the OXPHOS system) at the level of complex I (NADH: ubiquinone oxidoreductase) or at the level of complex II (succinate dehydrogenase) respectively. Electrons are subsequently transferred to the non-protein electron carrier coenzyme Q and then to complex III (ubiquinol:cytochrome *c* oxidoreductase or bc1 complex). The second electron carrier, cytochrome *c*, acts as a bridge for the transfer of electrons between complexes III and IV (cytochrome *c* oxidase). Once the electrons have reached complex IV they are transferred to molecular oxygen to form water. During the transfer of electrons through the electron transport chain (ETC, complexes I–IV), protons are translocated simultaneously from the matrix to the mitochondrial intermembrane space by complexes I, III and IV. This proton translocation creates an electrochemical gradient that is utilized by complex V (ATPase synthase) to generate ATP with the concomitant translocation of protons back into the matrix. In the last few years, studies on the respiratory complexes have revealed that they are assembled in large structures or supercomplexes to form a functional

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"respirasome" [4]. It has been postulated that the organization into "respirasomes" allows for a more efficient substrate channeling, including electron transfer and proton translocation, thus minimizing the formation of free radicals by reducing the direct transfer of electrons to oxygen [5]. Nevertheless, the electron transport chain is one of the major pathways leading to the production of reactive oxygen species (ROS) in the cell.

3. Cytochrome *c* oxidase

3.1. Structure and function

Cytochrome *c* oxidase (COX) is the terminal component of the electron transport chain and catalyzes the transfer of electrons from cytochrome *c* to oxygen. The mammalian enzyme is a 200 kDa complex composed of 13 subunits encoded by both the mitochondrial (COX1, COX2 and COX3 subunits) and the nuclear genome (COX4, COX5a, COX5b, COX6a, COX6b, COX6c, COX7a, COX7b, COX7c and COX8 subunits) [6,7]. The large catalytic subunits are encoded by the mitochondrial DNA (mtDNA) and constitute the core of the enzyme. The small peripheral structural subunits surrounding the catalytic core are encoded by the nuclear DNA.

COX is active as a dimer and requires several prosthetic groups for catalytic function: 2 hemes (*a* and a_3), two copper centers (Cu_A and Cu_B), zinc and magnesium. Tsukihara and collaborators resolved the crystal structure of the metal sites and the whole complex with the 13 subunits of the bovine heart cytochrome oxidase at 2.8 Å resolution. Both heme *a* and the binuclear center a_3-Cu_B are located in COX1 (subunit 1) whereas the binuclear Cu_A center is located in COX2 (subunit 2). A Zn^{2+} ion is in COX5b on the matrix side of the complex and a Mg^{2+} ion is in proximity to the a_3-Cu_A site between COX1 and COX2. Additionally, complex IV can also bind calcium and sodium ions [7,8].

During catalysis, the electrons from reduced cytochrome *c* are transferred consecutively to the Cu_A site, to heme *a*, and then to oxygen in the heme a_3-Cu_B center producing water. This transfer of electrons is coupled to the translocation of 2 protons from the matrix into the intermembrane space in each cycle [9–11].

The catalytic function related to the redox chemistry is carried out by both COX1 and COX2 subunits whereas the proton translocation activity appears to be associated to the COX3 subunit [9]. The function of the peripheral structural subunits in the eukaryotic enzyme is still not completely understood, since the catalysis is carried out by the mitochondrial encoded subunits. The bacterial complex functions with only the 3 catalytic subunits. The nuclear-encoded subunits appear to play a role in the regulation and stability of the fully assembled complex [12,13].

3.2. Regulation of enzyme activity

As a rate limiting enzyme in the OXPHOS system, COX activity is subjected to regulation by a gamut of molecules to meet the energetic demands of the cell including hormones, membrane lipids and second messengers. For example: thyroid hormone regulates COX activity by binding to COX5a [14] and oxidized cardiolipin and nitric oxide inhibit COX activity [15,16]. Moreover, COX activity is also regulated by allosteric inhibition/activation by ATP/ADP and by phosphorylation of different subunits by kinases like c-SRC, cAMP dependent protein kinase, PKCε and PKCδ [17–23].

Another level of regulation of COX activity appears to be mandated by the existence of some tissue-specific, species-specific isoforms or by the protein content of some of the small subunits in a tissue/cell type. In humans, isoforms for subunits COX4, COX6a, COX6b, COX7a and COX8 have been described. COX6a and COX7a have liver (ubiquitous) and heart specific isoforms; COX4 has a lung specific isoform [24] and COX6b a testis specific isoform. In the case of species-specific isoforms, isozymes for COX8 in bovine, dog and rat are not found in human, sheep and rabbit [18,25–27]. In yeast (*Saccharomyces cerevisiae*), there are two Cox5 isoforms (homolog of human COX4) whose expression is dependent on the environmental oxygen [28]. During normoxia, the Cox5a isoform is expressed whereas in low oxygen the expression switches to the Cox5b isoform. This switch confers higher enzymatic efficiency during hypoxia [29]. Similarly, this homeostatic mechanism and higher enzymatic efficiency is also observed in mammalian cells. In response to hypoxia, an isozyme switch of COX4-1 for COX4-2 occurs and is mediated by Hif-1 [30]. In the case of mammalian COX5b, no isoforms have been reported albeit this subunit can modulate COX activity by varying its content in relation to the whole complex [31]. Moreover, COX5b is required for assembly and stability of complex IV [13].

3.3. Enzyme biogenesis

Although great advances have been made on the understanding of the mechanism of COX biogenesis in the last 10 years, this process is still not completely unraveled. What is known about this complicated mechanism has been elucidated in its majority either from systematic studies in yeast, using mutant strains defective in COX assembly or from studies in cell lines derived from patients with COX deficiencies. Studies in yeast revealed that the COX assembly process requires more than 30 auxiliary proteins, encoded in the nuclear genome, that do not form part of the final assembled complex. Recent findings, not described in this review, imply that not all the COX assembly factors have been already discovered even in yeast [32]. These auxiliary proteins or assembly factors play an important role in several aspects related to heme *a* biosynthesis [33]; copper delivery and homeostasis [34]; transport of nuclear-encoded subunits across the mitochondrial membrane; and translation, membrane insertion and maturation of mitochondrial encoded subunits [35]. The function of some of this newly identified assembly factors, many with unknown mammalian homologues to date, are described in more details in recent reviews [36–38].

Among the accessory proteins that participate in COX assembly with clinical implications in humans are COX10, COX15, Surf1, LRPPRC, SCO1 and SCO2. The functions of COX10 and COX15 are related to heme *a* biosynthesis. Heme *a* is a unique molecule exclusive of COX that is found in two chemical forms, the *a* which is a low-spin heme and the a_3 which is a high-spin heme. The first step of heme *a* synthesis starts with the conversion of heme *b* into heme *o* which requires the farnesylation of the vinyl group present in carbon 2. This step is catalyzed by COX10, a farnesyl transferase [39]. Then heme *o* is converted to heme *a* by COX15 and a monooxygenase, yet to be identified in humans [40]. These enzymes hydroxylate the methyl group in carbon 8 which is subsequently oxidized into a formyl group [41–43].

The function of Surf1, in spite of numerous studies remains elusive. However, Surf1 is known to play a role in the early assembly steps of COX. In the protobacteria *Rhodobacter sphaeroides* Surf1 assists in the insertion of heme a_3 into COX1 and in the maturation of the heme a_3-Cu_B center [44]. The Surf1 yeast homologue, Shy1, has been shown to genetically interact with the COX1 translation machinery. These include mss51 and COX14, proteins required for COX1 translation in yeast [45]. Additionally, Shy1 remains associated with COX subassembly intermediates that are able to interact with complex III to form supramolecular arrangements [46]. Interestingly, a recent report on the analysis of tissues from patients with Surf1 deficiency suggests that this protein has an additional role in copper homeostasis [47].

SCO1 and SCO2 participate in copper delivery to COX, cellular copper homeostasis and redox signaling [48–50]. These two copper chaperones albeit sharing high similarity, do not have overlapping functions but rather act in combination [51]. The copper delivery to the two metal centers in COX (Cu_A located in COX2 and Cu_B located in COX1) is carried out by several proteins. COX17 another copper chaperon delivers the

metal to COX11 or to SCO1 and SCO2 proteins. In turn, COX11 participates in the formation of the Cu_B center whereas SCO1 and SCO2 participate in the formation of the binuclear Cu_A center (reviewed in [52]). Additional roles for SCO1 and SCO2 have been recently proposed [47,53]. Stiburek and colleagues showed that SCO1 interacts with the fully assembled complex in human muscle mitochondria, and that in SCO1 patients, the mutant SCO1 was associated with subassembly intermediates containing COX2. These results suggest a SCO1 is involved in a different posttranslational step in the maturation of COX2 other than or additional to copper delivery [47]. The incorporation of copper to COX2 was thought to occur while the subunit was embedded in the membrane but prior to its incorporation into the complex. Leary et al. found that although both SCO proteins are required for Cu_A site metal loading, SCO2 alone is required for COX2 synthesis and additionally acts as a thiol reductase of SCO1 [53].

3.3.1. Assembly intermediates

The assembly of COX has been characterized in numerous cell lines or tissues from patients with defects in the assembly proteins using blue native gel electrophoresis [54]. This technique permitted the separation of respiratory complexes in their active native conformation, allowing the detection of subassembly intermediates and of supramolecular interactions [55]. Using information provided by this technique, Nijtmans and collaborators proposed a sequential assembly process that starts with COX1 and the addition of its respective prosthetic groups (heme *a* and Cu_B center). This constitutes the first subassembly intermediate or S1. Then COX4 is added to form the S2 intermediate to which COX2, COX3, COX5a, COX5b, COX6b, COX6c, COX7a, COX7c and COX8 subunits are subsequently added to form the S3 intermediate. Lastly, COX6a and COX7b join S3 to form the final intermediate S4. This last intermediate constitutes the monomeric form of COX that subsequently dimerizes to form the active complex [56,57]. Further analysis of different tissues from patients with Surf1 and SCO2 mutations prompted Stiburek et al. to refine Nijtmans' model. In this analysis, the authors confirmed that COX4 and COX5a are in a preexisting subcomplex [58] previous to their addition to the S1 intermediate. The addition of COX4/COX5a subcomplex occurs after the incorporation of prosthetic groups in COX1 but previous to the addition of COX2 [59].

The assembly of COX as well as other respiratory complexes has been recently reviewed by Fernandez-Vizarra and colleagues [60].

4. Mitochondria and cytochrome *c* oxidase in neurons

In addition to mitochondrial diseases, mitochondrial malfunction and defects in respiratory complexes have been associated with many neurodegenerative diseases and aging. This section highlights the importance of this organelle not only in metabolism but also in neuronal plasticity. Mitochondria have a wide distribution in neurons. They are present in the cell body and in functional domains with high energetic requirements during neuronal activity such as dendrites, nodes of Ranvier, synapses and axonal growth cones (reviewed in [61]). The wide distribution of these organelles in neurons is achieved by mitochondrial transport through actin and microtubules tracks. Mitochondria are indispensable to provide the energy necessary for synaptic transmission and play an important role on neurotransmitters release and uptake, calcium buffering, vesicle cycling in presynaptic terminals, repolarization after depolarizing stimulation, and growth cone formation. Recent studies have showed that mitochondrial biogenesis (including mtDNA replication and fusion and fission events) occurs in distal axons independently of the cell body [62].

Neurons have compartmentalized protein synthesis. Although the majority of the protein synthesis occurs in the cell body, there are functional regions distal from the soma (axons, dendrites and synaptic terminals) that require fast protein synthesis [63,64]. This compartmentalization plays a crucial role on synaptic plasticity required for neuronal activity. Kaplan's group [64] found that those mRNAs that are transported for local protein synthesis encode a wide variety of proteins of diverse functions i.e. cytoskeleton proteins, translation machinery proteins (ribosomal and translation factors), molecular motors proteins and metabolic enzymes [64]. Additionally, a high abundance of mRNAs (about 25%) encoding mitochondrial proteins and chaperons has been found in the axon terminal of the squid giant axon [65]. The authors showed that protein synthesis in the axon terminal was necessary for mitochondrial function and axonal maintenance. In a series of elegant experiments, Hillefors and colleagues [66] showed that inhibition of axonal protein synthesis resulted in axonal retraction, altered mitochondrial membrane potential and impaired restoration of axonal ATP levels following membrane depolarization. In contrast, when mitochondrial protein synthesis was blocked by chloramphenicol treatment, none of the previous effects were observed [66].

COX activity has been proposed to be a good indicator of metabolic capacity required for neuronal function in neurons. Wong-Riley's group [67,68] had extensively studied the transcriptional regulation of the mitochondrial and nuclear genome in COX biosynthesis during neuronal activity. They have shown that neuronal stimulation upregulated mRNAs for all the COX subunits after 5 h whereas the blocking of neuronal activity with tetrodotoxin produced a downregulation of COX transcripts in a time differential manner in primary rat visual cortical neurons. Mitochondrial encoded COX mRNAs were downregulated two times faster (2 days) than the nuclear-encoded COX mRNAs (4 days). These changes in mRNA levels also were reflected at the protein and at the enzyme activity levels. During neuronal activity, membrane repolarization after an excitatory stimulus requires higher energy expenditure in neurons. The investigators propose that the availability of mitochondrial subunits upon neuronal stimulation play a central role in regulating cytochrome *c* oxidase levels and activity in neurons [67].

Dhar and collaborators [68] proposed that the tight coupling between the energetic metabolism and the neuronal activity, regulated at the level of transcription, is reached by having a common transcription factor [68]. The authors showed that both AMPA glutamate receptor subunits and COX subunits are regulated by the same transcription factor, nuclear respiratory factor 1 (NRF-1). Moreover, knocking down NRF-1 prevented the upregulation of GluR2 (AMPA glutamate receptor subunit that regulates the calcium permeability of the receptor) and COX subunits during synaptic stimulation [69].

Recently, an additional level of regulation of COX biogenesis in neurons has been identified. This novel mechanism involves the presence of microRNAs in axons and dendrites that can repress translation of specific mRNAs or target them for cleavage. Like mRNAs, microRNAs have been shown to be also located in different neuronal compartments [70]. Aschrafi et al. discovered that COX4 mRNA levels were regulated in axonal terminals by a specific microRNA (MiR-338) therefore controlling the local mitochondrial respiration and ATP levels [71].

5. Cytochrome *c* oxidase deficiency in patients

COX deficiencies in humans comprehend a wide variety of disorders and the pathological features and genotype have been extensively reviewed [72–77]. This section summarizes some of the features of the COX deficiencies, includes some new findings and discusses some of the pathological phenotypes of mutations in COX subunits and assembly factors with high clinical relevance. Table 1 shows a summary of the genes associated with COX deficiencies and the different clinical presentations.

Genotypically, isolated COX deficiencies could arise from mutations in any of the 3 subunits encoded in the mtDNA, from any of the 10 structural subunits encoded in the nucleus or from mutations in any of the assembly factors. Phenotypically, all these possibilities create a wide variation on the clinical symptoms that could affect

Table 1
Gene defects associated with cytochrome *c* oxidase deficiencies in humans.

Mutated gene	Function	Clinical features	Reference
mtDNA-encoded subunits			
MTCOX1	Catalytic core	Encephalopathy	[137]
		Sideroblastic anemia	[81]
		Myoglobinuria	[138]
		Motor neuron disease	[139]
		MELAS-like syndrome	[140]
MTCOX2	Catalytic core	Myopathy	[141]
		Encephalopathy	[80]
		Multi systemic disease (bilateral cataracts, sensorineural hearing loss, myopathy, ataxia) and metabolic acidosis	[142]
MTCOX3	Catalytic core	MELAS	[143]
		Encephalopathy	[82]
		Leigh-like syndrome	[144]
		Exercise intolerance and rhabdomyolysis	[142]
Nuclear-encoded subunits			
COX6b1	Structural subunit	Encephalopathy	[84]
Assembly factors			
Surf1	Unknown Assists in early CIV assembly	Leigh syndrome	[85,86]
COX10	Heme *a* biosynthesis	Leukodystrophy	[97]
		Tubolopathy	[88]
		Hypertrophic cardiomyopathy	[98]
		Leigh syndrome	
		Leigh-like syndrome	
COX15	Heme *a* biosynthesis	Cardiomyopathy	[99]
		Leigh syndrome	[100,101]
SCO1	Copper delivery and homeostasis	Hepatopathy	[96]
		Metabolic acidosis	[47]
		Hypertrophic cardiomyopathy	
		Encephalopathy	
SCO2	Copper delivery and homeostasis	Hypertrophic cardiomyopathy	[94]
		Encephalopathy	[95]
LRPPRC	COX1 and COX3 mRNA stability	French–Canadian Leigh syndrome	[91]
			[92]
TACO1	COX1 translational activator	Slowly progressive Leigh syndrome	[104]
FASTKD2	unknown	Encephalopathy	[102]

MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes.

single or multiple organs. Moreover, defects in the mtDNA such as deletions, duplications or mutations in any of the tRNA genes can also affect COX function along with defects in complexes I, III and V. This is the case of diseases such as progressive external ophthalmoplegia and Kearns–Sayre syndrome [78]. One of the pathogenic characteristic of mtDNA deletions or tRNA point mutations is the presence of a mosaic pattern of COX positive and COX negative fibers in muscle biopsies as well as the presence of ragged-red fibers (RRF) which are due to the presence of an increased number of mitochondria that stain red with Gomorri Trichrome [79]. This increase in mitochondrial mass or mitochondrial biogenesis is believed to be the result of a compensatory mechanism for respiratory defects.

5.1. Mutations in COX subunits

Few COX deficiencies involve mutations in the mitochondrial encoded subunits [80–83]. The clinical presentations of such mutations encompass myopathy, sideroblastic anemia, amyotrophic lateral sclerosis (ALS)-like syndrome, encephalomyopathy and MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [3,74]. The muscle of patients with mutations in the mtDNA-encoded COX subunits showed large amounts of COX negative fibers without RRF [73]. These mtDNA mutations could affect a single tissue (myopathies) or multiple systems (MELAS and encephalomyopaties) and the severity of the COX deficiency could vary in different tissues. The difference in severity and tissue specificity remains elusive although heteroplasmic load of the mtDNA mutation (coexistence of wild type and mutant mtDNA) and threshold effect of a particular mutation (amount of mutant mtDNA required to cause a defect, usually very high) appear to be the main culprits. In terms of defects derived from nuclear mutations, the differences in tissue specificity could be explained by differences in the levels of certain subunits or assembly factors, presence of specific isozymes, specific regulatory mechanisms (phosphorylation and allosteric regulation) or by differential availability of cofactors.

Mutations in the nuclear-encoded subunits were not found for many years in patients with COX deficiency leading to the idea that these mutations were incompatible with live. Recently, Massa and colleagues [84] described two patients (siblings from cousin parents in third degree) suffering from severe infantile encephalopathy with COX deficiency. Both patients were homozygous for a mutation in the COX6b1 gene [84]. Because this was the first report of a mutation in a nuclear-encoded COX subunit, the pathogenicity of the mutation was functionally validated in yeast and indeed it caused COX deficiency. Further analysis of these patient tissues showed that even though the mutant COX6b1 protein was synthesized, it failed to be incorporated into the holoenzyme, therefore causing the enzymatic defect [84].

5.2. Mutations in COX assembly factors

The majority of the genetic defects related to COX deficiencies have been found in nuclear genes coding for auxiliary proteins. The first identified COX deficiency with a nuclear origin was Leigh syndrome (LS), a fatal subacute neurodegeneration with an early onset affecting the whole body [85,86]. LS patients die between 6 months to 12 years of age. The typical pathology of Leigh syndrome includes the presence of bilateral symmetric necrotic lesions in subcortical areas of the brain (brain stem, basal ganglia and thalamus) that develop into ophthalmoparesis, nystagmus, ataxia, dystonia and optic atrophy.

These spongiform necrotic lesions consist of neuronal loss, demyelination and vascular proliferation [87]. The residual levels of COX activity observed in LS patients range from 10 to 25% of control values with no apparent correlations in terms of severity and tissue specificity. The gene responsible for LS in cases associated with COX deficiency was the assembly factor Surf1. However, not all the mutations in this gene produced Leigh syndrome, some Surf1 patients did not develop the characteristic neurodegenerative lesions. In some cases, the pathology of Leigh syndrome has been associated with mutations in the COX10 gene or with mutations in the mtDNA [88,89]. Moreover, LS can result from defects in other genes altering energetic metabolism such as pyruvate dehydrogenase, NADH:ubiquinone oxidoreductase (complex I) or ATP synthase (complex V) [90].

A less severe form of LS is the French–Canadian Leigh syndrome (FCLS, exclusive of the Charlevoix and Saguenay-Lac-St. Jean region of Quebec). Clinical presentations of this form include mild regression of psychomotor skills and a fatal lactic acidosis with death occurring between 3 and 10 years of age. In this disorder, the tissues mostly affected are brain (basal ganglia) and liver [91]. Mootha et al. identified LRPPRC (leucine-rich pentatricopeptide repeat cassette) as the gene responsible for this disease [92]. LRPPRC is a homologue of the yeast P309, another COX assembly factor, which is required for the expression of COX1 by assisting in the stability and translation of COX1 mRNA. Analysis of LRPPRC mRNA levels in different tissues from patients with FCLS revealed that those tissues that were mostly affected (higher COX deficiency) had the lowest levels of expression [93]. Confirming the role of LRPPRC as an mRNA stabilizing protein, studies in FCLS patients showed a severe decrease on the levels of COX1 and COX3 mRNAs (23 and 30% of control levels respectively) [93].

Another COX deficiency characterized by hypertrophic cardiomyopathy with encephalopathy also with an early onset is due to mutations in the copper related assembly factor SCO2. Although the COX deficiency was more severe in cardiac and skeletal muscle, autopsy analysis of the brain revealed spongiform neurodegeneration in midbrain, pons and medulla accompanied by gliosis and microglia proliferation in the thalamus, but lacked the typical LS lesions [94,95]. Mutations in the other copper chaperone, SCO1 have been associated with completely different phenotypes. Valnot and collaborators [96] reported two neonate cases, the first one with severe metabolic acidosis and with hepatic failure consisting in microvesicular steatosis (lipid accumulation) and enlarged liver. The patient died at 2 months of age. A severe COX deficiency in liver and muscle (less than 1% of control values) was determined in postmortem tissues. The second case was a sibling born in early pregnancy that also presented with metabolic acidosis and neuronal involvement and died at 5 days of age. In both cases, the defect mapped to the SCO1 gene. The patients were compound heterozygous [96]. A third case of SCO1 defect was just recently reported [47]. This last patient presented with an early onset of hypertrophic cardiomyopathy accompanied by encephalopathy and hepatomegalia and died at 6 months of age. Ultrastructural analysis of the cardiac tissue showed accumulation of abnormal mitochondria of varying sizes, containing hemidense deposits. The liver had microvesicular steatosis and the muscle lacked ragged-red fibers [47].

Defects in the heme *a* biosynthetic pathway lead to different clinical presentation also with fatal outcome early on life (months to 2 years of age). The first COX10 mutation reported by Valnot et al. was found in a pediatric patient suffering from ataxia, muscle weakness, hypotonia and pyramidal syndrome, leukodystrophy and proximal tubolopathy accompanied by elevated lactate in both cerebrospinal fluid and blood [97]. Other patients with mutations in COX10 presented with anemia, sensorineural deafness and hypertrophic cardiomyopathy [88]; with anemia and Leigh syndrome [88]; and with Leigh-like syndrome [98]. The levels of COX activity in COX10 patients ranged from 5 to 25% of control, depending on the tissues analyzed. Also few patients with COX15 defects have been documented. The first patient studied by Antonicka et al. had lactic acidosis, hypotonia and seizures and after developing a massive biventricular cardiomyopathy died before the first month of age [99]. The residual COX activity in this patient ranged from 7 to 25% of control values on the tissues analyzed. Another COX15 pediatric patient presented a completely different clinical phenotype. At 7 months of age this second patient had hypotonia, nystagmus, motor regression, retinopathy and microcephaly, increased lactate levels in blood, and cerebrospinal fluid and at 1 year of age had developed the typical Leigh syndrome lesions. The patient died before the fourth year of age. COX activity in primary cultured fibroblast was undetectable [100]. The third COX15 patient was also diagnosed with Leigh syndrome but with a very slow progression of the disease and only showing skeletal muscle and brain involvements. At the time of the report the patient was 16 years old. Initially lactate levels were elevated but progressively went down to normal range while other features remained unchanged. The COX deficiency in muscle and fibroblast (42 and 22% of control values respectively) was not as severe as in the case of the other two COX15 patients perhaps accounting for the slow progressive clinical phenotype [101].

A report of two patients with an early onset of mitochondrial encephalopathy presenting asymmetrical brain atrophy, convulsions, hemiplegia and psychomotor developmental delay associated with low levels of cytochrome *c* oxidase activity in skeletal muscle showed that the defect mapped to the FASTKD2 (fas activated serine–threonine kinase domain 2) gene [102]. Although the function of FASTKD2 is not known, this protein appears to be involved in apoptosis. Preliminary results showed no abnormalities in COX assembly in the patient's muscle as assessed by blue native gels [102]. Further investigations are required to determine if the ablation of the FASTKD2 directly affects COX activity or if this effect is a consequence of a more generalized dysfunction.

The assembly factors described above along with mtDNA genes constitute candidate genes screened to diagnose the causes of COX deficiency in patients. Recently, another yeast assembly gene with a human homolog that has been proposed as a screening candidate for COX deficiencies is COX18, which is required for the insertion of COX2 into the inner mitochondrial membrane. Unfortunately, mutational screening of COX18 gene in a large cohort of patients with an unknown cause of COX deficiency and where mutations in other candidate genes were already ruled out, failed to reveal any sequence abnormality for COX18 suggesting that mutations in this gene could be incompatible with life or not as frequent [103].

As mentioned earlier, not all the steps of COX biogenesis and assembly factors discovered in yeast have been identified in humans. This is the case of factors related to mtDNA transcription and translation. There are fundamental differences between yeast and humans such as the presence of introns in the mtDNA and the presence of 5′ UTRs in mitochondrial mRNAs in yeast. Additionally, yeast mitochondrial mRNAs require translational activators that recognize their 5′ UTRs and promote translation. These differences suggest different mechanisms of mitochondrial mRNA translation and perhaps different type of factors to accommodate the lack of mRNA regulatory elements found in humans. A new human COX assembly factor was identified by Shoubridge's group while studying a COX deficient patient with Leigh syndrome with a slow progression of the disease [104]. The authors found that in this particular patient the COX defect was due to a mutation in the CCDC44 gene that impaired COX1 protein synthesis. This gene encodes a protein, conserved in bacteria, that functions as a translational activator of COX1, and therefore was renamed TACO1 after its function [104]. This recent finding perhaps opens the door to the study and search of new COX assembly factors yet to be discovered in humans.

6. Animal models of cytochrome *c* oxidase deficiency

This section describes the different animal models for isolated COX deficiencies created by deletion or knock down of COX subunits and

assembly factors. The creation of animal models is of great importance in the mitochondrial field to the understanding of the pathophysiological mechanisms of mitochondrial diseases and the development of better therapeutic approaches. Fig. 1 summarizes the existing animal models, the different clinical phenotypes and the genes responsible for COX deficiencies observed in humans. Information of animal models that include additional defects of the oxidative phosphorylation system can be found in recent reviews by Torraco et al. [105] and Vempati et al. [106]. Animal models with defects in the mtDNA can be found in reviews by Kahn et al. [107] and Tyynismaa and Suomalainen [108].

6.1. Animal models with defects in COX subunits

In mice, all fetal tissues express the COX6a liver isoform (COX6a-L). As mice develop, the liver isoform is gradually replaced only in heart and muscle by the heart isoform (COX6a-H). In the cardiac tissue, this isoform switch is completed when mice are 28 days old [109]. To get insights into the function of the heart isozyme, Radford et al. [110] created a knockout (KO) mouse. The COX6a-H null mouse developed a subtle myocardial diastolic dysfunction with no apparent ultrastructural abnormalities. The disruption of COX6aH affected neither fertility nor life span. The KO mice had a COX deficiency of about 23% compared to control animals in cardiac tissue and the levels of the fully assembled complex were significantly decreased. The authors concluded that the COX6a-H subunit is required for proper assembly and stability of the complex [110].

Liu and colleagues found the *levy* mutant when screening temperature sensitive *Drosophila melanogaster* mutants. The *levy* flies suffered from reversible paralysis in response to increased temperature (38 °C). For young flies (2 days old) the paresis occurred within 5 min of exposure to increased temperature and the movement recovery occurred within 30 min. In contrast, older flies (1 month old) paralyzed faster, within 30 s and were slower in recovering movement (3 h). The *levy* mutation was mapped to the COX6a gene and caused a progressive COX deficiency with premature death. The life span of the mutant flies was about half of the wild type. Other phenotypes included motor dysfunction as well as a spongiform type of neurodegeneration in the brain and optic lobes. This is the first *Drosophila* model with a mutation in a COX structural gene that rather than lethality caused a mitochondrial encephalomyopathy [111].

A knock down (KD) of the structural subunit COX5a was created by Baden et al. in *Danio rerio* (zebrafish) [112]. The zebrafish constitutes a convenient animal model to study events during early development because of the transparency of the embryonic and larval stages and their extra uterine development. Disruption of translation of the COX5a mRNA was achieved using antisense morpholino oligonucleotides allowing the study of dosage dependence phenotype. The KD fish had about 50% COX deficiency when compared with control animals that led to a series of developmental defects (small rostral–caudal axis, impair gut development, microphtalmia, unabsorbed yolk sac and abnormal cephalic shape). All these pathological features resulted in early death (about 7 days after fertilization). In addition, the COX5a KD fish had cardiac pathology although no apoptotic cells were observed in this tissue. Neurological abnormalities were also observed in this COX deficient fish. Neurodegeneration was observed in brain and neuronal tube in conjunction with peripheral neuropathy were secondary motoneurons were severely impaired [112].

Recently, Suthammarak et al. knocked down subunits COX4 and COX5a by RNAi in *Caenorhabditis elegans* [113]. These two subunits are involved in the allosteric regulation of COX. The KD worms showed developmental delay (taking an extra day to reach adulthood),

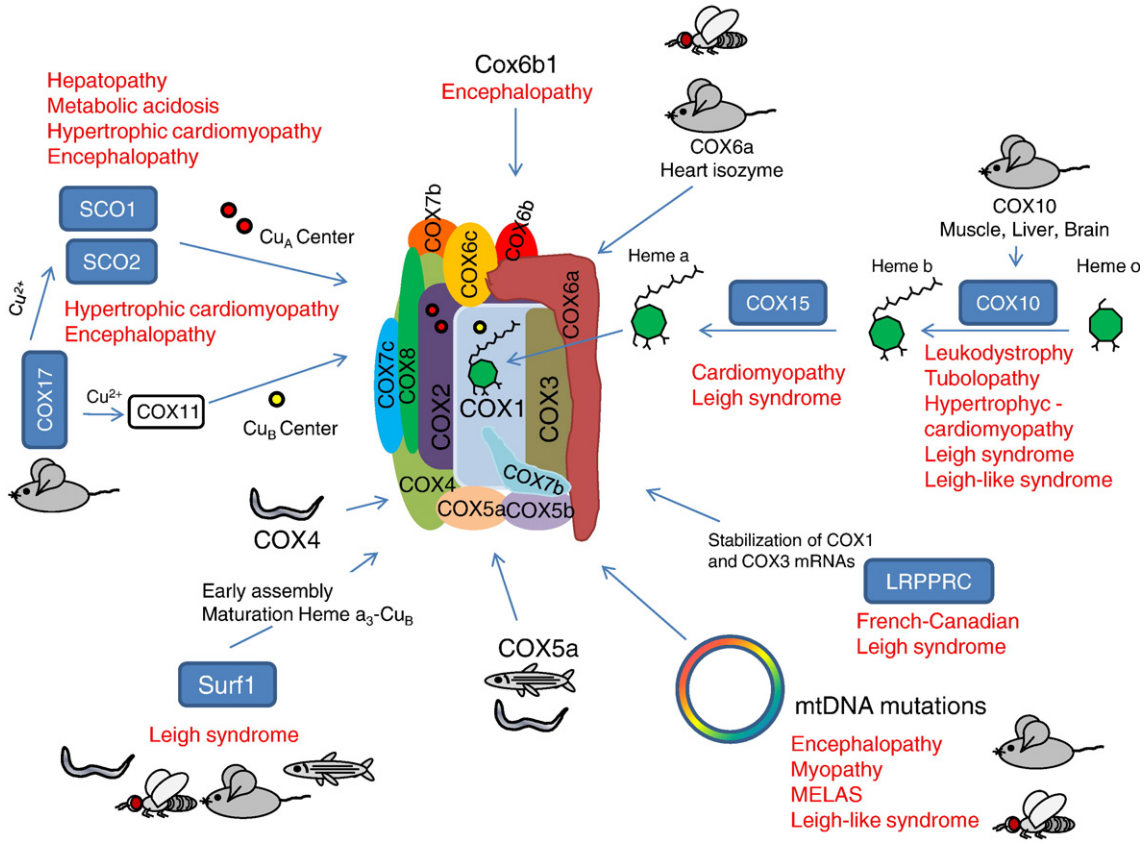

Fig. 1. Gene defects associated with cytochrome *c* oxidase deficiencies in humans and existing animal models. Clinical pathologies associated with mutations in respective COX subunits or assembly factors associated with COX deficiencies are labeled in red. The different animal models created for a corresponding protein or mitochondrial DNA are shown in the figure: mouse, zebrafish, *Drosophila*, and *C. elegans*.

reduced number of eggs/worm (about half of the controls), and reduced life span (12–14 days compared to 17 days for control worms). The COX deficiency obtained in the nematodes after knocking down either subunit was less than 50% of control values. Moreover, the absence of these subunits affected also complex I activity although the protein levels of the fully assembled complex I were not altered. Additionally, the formation of supercomplexes containing COX was also affected [113].

Using a mitochondrial targeted endonuclease to the germ line, Xu and colleagues created a *Drosophila* model carrying a homoplasmic mutation in the mitochondrial COX1 gene [114]. The mutant flies displayed growth retardation, with progressive neurodegeneration and myopathy with locomotor impairment resulting in a severe reduction in their life span [114].

6.2. Animal models with defects in COX assembly factors

6.2.1. Surf1

To study Leigh syndrome, the group of Zeviani has generated two KO mice for the COX assembly factor Surf1. The first model was obtained by disrupting the gene by targeted insertion of a neomycin cassette and replacement of exons 5 to 7. This genetic disruption caused embryonic lethality in the majority of the KO mice obtained. Those KO mice that survived presented a wide variation on their life span with the males being more affected and the majority dying within the first two months of age. The severity of the COX deficiency varied among the tissues being higher in muscle and liver and lower in heart and brain. Surf1 null mice had decreased muscle strength and deficit in motor skills. Ultrastructural analysis of muscle tissue showed accumulation of enlarged abnormal mitochondria in subsarcolemmal area. Surprisingly, the Surf1 null mouse did not show any apparent neurological abnormalities. Cortex, hippocampus and thalamic regions were comparable to control animals. Morphological features of neurons in common oculomotor and red nuclei (regions frequently affected in Leigh syndrome) appeared normal in KO mice [115]. To create the second animal model the authors only disrupted the last portion of the Surf1 gene (exon 7). The COX deficiency in the second model was comparable to the first one but no embryonic lethality was obtained. Moreover and unexpectedly, these animals had a prolonged life span (about 5 months longer than control mice) but again this new model failed to show any neurodegenerative pathology. The reasons for increased longevity were unexplained although the same phenotype was observed in CNS specific Surf1 knockdown in *Drosophila*. The constitutive Surf1 knockout caused embryonic lethality in the fruit fly [116]. To produce a neurological phenotype and test for the sensitivity of COX deficient neurons to toxic stimulus, Surf1 KO mice were treated with kainic acid (glutamate agonists that induces Ca^{++} excitotoxicity). Kainic acid exposure produces epilepsy due to the stimulation of AMPA glutamate receptors that leads to Na^{+} influx, membrane depolarization and consequently a massive Ca^{++} influx through NMDA receptors. This cascade alters cellular homeostasis and induces neuronal death [117]. There was no difference in the frequency, duration and severity of the seizures or mortality rate between the Surf1 KO and control animals treated with kainic acid. Surprisingly, in the surviving animals treated with kainic acid no signs of neuronal degeneration were observed in the Surf1 KO mice when compared to the surviving controls. Moreover, calcium induced apoptosis was absent in KO mice whereas it was prominent in control mice. Apparently, this apoptosis resistance was due to a reduced increase of the intracellular and mitochondrial calcium levels in the Surf1 deficient neurons [118].

A zebrafish model for Surf1 was created by knocking down gene expression with morpholino oligonucleotides by Baden et al. The authors obtained the same phenotypical abnormalities described above for the COX5a KD by knocking down Surf1 in fish (small rostral–caudal axis, abnormal head shape, small eyes, underdeveloped intestinal track and unabsorbed yolk sac) that resulted in early death [112].

As mentioned earlier, ubiquitous knockdown of Surf1 in *Drosophila* caused impaired larval development and lethality whereas the neuron specific KD bypassed this lethality. The neuron specific Surf1 KD flies reached adulthood and did not present evident signs of neurodegeneration albeit they had abnormal photo-behavior responses [116]. Recently, Fernandez-Ayala et al. tested in the *Drosophila* Surf1 KD model a new strategy to bypass the COX defect that consisted in expressing the alternative oxidase enzyme (AOX) from *Ciona intestinalis* to transfer electrons directly from coenzyme Q to oxygen. The tunicate enzyme, not found in vertebrates, was able to circumvent the lethality of the fruit fly Surf1 KD [119].

6.2.2. COX17

Deletion of the copper chaperone COX17 in mouse by Takahashi et al. showed that this protein is indispensable not only for COX activity but also for embryonic development and the KO mice died between E8.5 and E10 [120]. These results indicate that functionally relevant mutations in the COX17 gene are incompatible with life and to date mutations in this gene have not been found in humans.

6.2.3. COX10

Conditional ablation of COX10 by the Cre-loxP system has allowed us to create tissue-specific models of COX deficiency in muscle, liver and brain. To ablate this gene, loxP sites were introduced flanking exon 6, which encodes part of the active site of the enzyme. Disruption of the floxed gene using the Cre recombinase driven by the myosin light chain promoter allowed the creation of a myopathy mouse model [121]. The myopathy mouse had a progressive severe COX deficiency already manifested at 1 month of age (13% of control values of COX activity in muscle), although no signs of myopathy were observed. Muscles were able to contract at forces similar to control animals and myopathyc signs were observed at approximately 3 months of age. The COX deficiency progressed with age, showing a mosaic pattern mimicking mitochondrial myopaties observed in patients and ended with an early death. The COX10 KO females were more affected than the males, having shorter life span and pronounced exercise intolerance [121].

In search for mitochondrial disease treatments, we induced mitochondrial biogenesis, a natural compensatory mechanism observed in many mitochondrial diseases, in the COX10 KO myopathy model. The stimulation of mitochondrial biogenesis by induction of PGC-1α (peroxisome proliferator activator receptor γ co-activator-1α) was achieved by either genetic manipulation (PGC-1α overexpression in skeletal muscle), bezafibrate treatment (PPAR panagonist) or exercise training and showed that increased mitochondrial mass in all cases ameliorated the onset of myopathy and extended the life span of the COX10 KO mice [122,123].

A mitochondrial hepatopathy mouse model was created by ablating the floxed COX10 gene with Cre recombinase driven by the rat albumin promoter [124]. In these KO mice, despite the severe COX deficiency and liver pathology (increased transaminases, lipid accumulation and decrease glycogen storage), hepatocytes that escaped COX10 deletion had proliferated and restored complete liver function and COX deficiency at a later age. The animals had a complete reversion of all pathological phenotypes resulting on a normal life span. In contrast, the progeny of the cross of two KO mice, those animals homozygous for both the floxed allele and the Cre recombinase, presented a more severe hepatopathy due to the higher expression of the recombinase. The pathology included a higher number of COX deficient hepatocytes, hepatomegaly and microvesicular steatosis. These mice died at around 2 months of age [124].

We also created two models of CNS specific COX deficient mice. In the first model, the ablation of the floxed COX10 gene was driven by the synapsin-1 promoter which is expressed early during embryonic development by embryonic day 12.5 (E12.5). In the second model, the

COX10 deletion was achieved using the recombinase under the regulation of the CamKIIα promoter which produces a postnatal expression of Cre mainly in the hippocampus and cortex. The first model will be referred as the infantile and the second as the adult neuron specific COX10 KO mice. The infantile COX10 KO mice did not show any gross brain abnormalities and the COX deficiency in brain homogenates was very mild (about 80% of control values) although they died at 10 days of age (Diaz and Moraes, unpublished results). In spite of the mild COX deficiency observed and lack of gross brain abnormalities, further characterization of this animal model is required. This will establish if neurogenesis and cell differentiation of specific neuronal populations is impaired by the lack of mitochondrial respiration. Neurons are generated from neuronal progenitor cells before birth whereas the majority of glia cells (astrocytes and oligodendrocytes) are generated after birth (during the first month of age) in the mammalian CNS. During mouse brain development neurogenesis starts at E12 to E15 and ends at birth [125,126]. This period of neurogenesis coincides with the expression of Cre recombinase in our infantile COX10 KO mice. In addition, the COX defect may affect apoptosis and correct balance of neuronal populations required during brain development.

The adult COX10 neuron specific deficient mouse was phenotypically undistinguishable from control littermates until they reached 3.5 to 4 months of age when behavioral abnormalities became evident. They presented with alternated hypo- and hyperactive episodes, increased appetite, hypersensitivity to environmental noise and aggressive behavior. Eventually as disease progressed, the animals became more irresponsive. The behavioral phenotype was accompanied by neuronal degeneration that started after 4 months of age and resulted in a dramatic cortical atrophy by 8 months of age, followed by death although some mice reached and passed 1 year of age [127]. Initial characterization of this model showed a progressive COX deficiency in cortex and hippocampus homogenates in the KO mice starting at 1 month of age. Gross inspection of brains at 4 months of age did not show any apparent abnormalities or altered steady-state levels of Tuj1, a neuronal marker [127]. Further examination of the brain pathology in the COX10 KO mice revealed early molecular neurodegeneration signs including the presence of tunel positive cells and reactive astrocytes (increased GFAP expression) starting at 4 months of age (Diaz and Moraes, unpublished results). In the adult COX10 KO model, neurons seem to survive for about 2 months from the onset of the COX deficiency until first signs of neurodegeneration appeared. This same prolonged neuronal survival in the absence of oxidative phosphorylation was previously observed in a conditional KO for the mitochondrial transcription factor TFAM where all respiratory complexes were impaired [128]. These results suggest that neurons are capable of metabolically adapting (at least temporarily) to the lack of mitochondrial respiration. They possibly accomplish this by upregulating other energy generating pathways such as glycolysis.

Numerous neurodegenerative disorders have been associated with mitochondrial dysfunction, accumulation of mtDNA mutations and oxidative damage. Particularly, in Alzheimer's disease (AD), numerous investigators reported a reduction of COX activity [129] and increase in oxidative damage in brain from AD patients. In addition, recent reports described mtDNA mutations including point mutations in COX encoded subunits as well as mutations in regulatory regions of the mtDNA in AD patients [130–133]. In an effort to expand our understanding of the implications of COX deficiency in AD, we crossed the adult COX10 KO mouse with a previously established AD transgenic mouse resulting in a COX deficient AD mouse (COXd/AD mouse) [127]. The AD mouse model used to accomplish this study expresses mutant forms of both APP and presenilin that leads to the formation of amyloid plaques, however this mouse does not developed cognitive abnormalities [134]. Unexpectedly, the COXd/AD mice had a reduced number of amyloid plaques concomitant with a reduction in Aβ42 levels, reduction of β-secretase activity and reduction in oxidative damage when compared with the AD mice at 4 months of age. In the AD transgenic model, damages caused by oxidative stress have been associated to the pathology and accumulation of amyloid plaques. Moreover, treatment of the AD mice with antioxidants such as vitamin E decreased accumulation of amyloid plaques and reduction of the antioxidant mechanisms such as SOD2 increased the accumulation of plaques [135,136]. Extensive analysis measuring ROS production, protein carbonylation or nucleic acid oxidation failed to reveal any oxidative damage induced by COX deficiency, on the contrary, all the values obtained for the COXd/AD mice were significantly lower than the ones for the AD mice. Therefore, COX deficiency does not promote the oxidative stress observed in AD but rather is a consequence of the accumulation of Aβ in neurons [127].

7. Concluding remarks

In the last 10 years the understanding of COX biogenesis and its regulation have taken a great forward leap albeit many unanswered questions remain. The creation of numerous animal models that mimic the pathological conditions seen in humans are paving the way for the development of new and alternative treatments for mitochondrial diseases.

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