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Cytochrome c Oxidase Dysfunction in Oxidative Stress

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Abstract

Cytochrome c Oxidase (CcO) is the terminal oxidase of the mitochondrial electron transport chain. This bigenomic enzyme in mammals contains 13 subunits, of which, three catalytic subunits are encoded by the mitochondrial genes. The remaining ten subunits with suspected roles in the regulation, and/or, assembly are coded by the nuclear genome. The enzyme contains two heme groups (heme a and a3) and two Cu^{2+} centers (Cu^{2+} A and Cu^{2+} B) as catalytic centers and handles more than 90% of molecular O_2 respired by the mammalian cells and tissues. CcO is a highly regulated enzyme which is believed to be the pace setter for mitochondrial oxidative metabolism and ATP synthesis. The structure and function of the enzyme is affected in a wide variety of diseases including cancer, neurodegenerative diseases, myocardial ischemia/reperfusion, bone and skeletal diseases and diabetes. Despite handling a high O_2 load the role of CcO in the production of reactive oxygen species still remains a subject of debate. However, a volume of evidence suggests that CcO dysfunction is invariably associated with increased mitochondrial reactive oxygen species production and cellular toxicity. In this article we review literature on mechanisms of multimodal regulation of CcO activity by a wide spectrum of physiological and pathological factors. We also review an array of literature on the direct or indirect roles of CcO in reactive oxygen species production.

Keywords

Cytochrome C Oxidase; mitochondrial dysfunction; oxidative stress; alcohol toxicity; drug effects; myocardial ischemia; regulation of enzyme activity; phosphorylation; Protein kinse A; cAMP; respirasome assembly; mtDNA mutations

Introduction

Under normal physiological conditions, mitochondrial oxidative phosphorylation accounts for more than 90% of the cellular ATP production in most cells and tissues. Mitochondria are also involved in the maintenance of Ca^{2+} homeostasis, in addition to carrying out critical reaction steps of steroid hormone metabolism, pyrimidine synthesis, elimination of ammonia through urea cycle, and programmed cell death. In addition, they are the major cellular source for reactive oxygen species (ROS) [23,75]. Almost all functions of mitochondria are either directly or indirectly linked to the working of oxidative phosphorylation machinery

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and energy coupling. Most part of this machinery is in the inner mitochondrial membrane and comprises the four electron transfer chain complexes (complex I, II, III and IV), ATP synthase (complex V) and ubiquinone, and cytochrome c as electron carriers. Complex IV or CcO is the terminal enzyme of the electron transport chain, which catalyses the final step of electron transfer from reduced cytochrome c to oxygen to make H_2O. CcO is also one of the three proton pumps along with complexes I and III that generate the proton gradient across the inner mitochondrial membrane, which powers the ATP synthesis. This review focuses on function and the multilayered regulation of cytochrome c oxidase (CcO) and explores the mechanisms by which CcO defects play a role in mitochondrial dysfunction and oxidative stress.

Highly oxidative tissues and cells such as the heart, brain and kidney nephrons produce generally higher levels of ROS compared to less oxidative tissues due to higher rates of electron transfer chain activities and highly polarized transmembrane potential. Mechanisms of ROS production by highly polarized mitochondria with high $\Delta\varphi m$ have been discussed elegantly in a recent review by Murphy [99]. It should however be recognized that high transmembrane potential is not the only cause of ROS production since mitochondria with defective, dysfunctional electron transport complexes, or imbalance in the levels of different complexes and electron donors also produce higher rates of ROS. The literature on this aspect of mitochondrial biology and oxidative stress is highlighted in this review.

CcO is a bigenomic complex with subunits coded by both mitochondrial and nuclear DNA. A coordinated expression of these subunits and their assembly in the right stoichiometry provides cells different modes of regulation of enzyme content in mitochondria. Of the thirteen subunits of the mammalian CcO, the mitochondrial genome encodes subunits I, II and III, which form the catalytic core of the enzyme [31]. During a catalytic cycle, the copper center CuA associated with subunit II, accepts electrons from reduced cytochrome C and transfers to the heme a in subunit I. From heme a, electrons are transferred to the bimetallic center heme a3/CuB, also in subunit I, to reduce molecular oxygen to water; the function of subunit III is not clearly understood [31]. The nuclear genome codes for the remaining ten subunits of CcO, which are synthesized in cytosol and imported into mitochondria. While some of the nuclear subunits provide structural stability to the complex, in general, they are thought to be involved in the regulation of enzyme activity [31]. The importance of CcO is underlined by the fact that organisms have evolved different levels of regulation of its activity. Under normal physiological conditions, CcO acts as the rate limiting step of respiratory chain and its activity is an indicator of the oxidative capacity of the cells.

Regulation of Cytochrome c Oxidase Function

Subunit Isoforms

Organisms from different phylogenetic order contain CcO with variable number of subunits ranging from 4-13 in different species; 4 in bacteria, 7 in slime-molds, 12 in *Yeast* and 13 in vertebrates [88]. The three largest and evolutionarily conserved subunits I, II and III carry the heme and Cu^{+2} redox centers and form the catalytic core of the enzyme. In eukaryotes, these subunits are encoded by the mitochondrial genome and the remaining are coded by nuclear genes. Many of the nuclear subunits exist as multiple isoforms that are constitutively expressed in a tissue specific manner or are induced under certain physiological conditions. In vertebrates, subunits VIa, VIIa and VIII are expressed as L and H isologs [6,87,158]. The L isologs are expressed ubiquitously in all tissues, while the H isologs are expressed exclusively in the skeletal and cardiac muscle [87,130,132]. It has been suggested that subunits VIaH and L isologs may contribute to the tissue specific differences in ADP/ATP binding to Cytochrome oxidase [71]. As explained in a later section, binding of ADP/ATP is

an important mechanism for allosteric regulation of CcO activity which directly relates to the bioenergetic status of the cell.

Regulation by subunit switching

In Yeast, COX 5 is expressed as 5a and 5b isologs. Subunit 5a is expressed under aerobic conditions and is replaced by 5b under low oxygen levels [28]. The main difference between the yeast enzymes containing Va and Vb subunits is the rate of electron transfer to the binuclear center and thus the catalytic function [155]. The mammalian homolog of this subunit, designated as IV, is expressed as two isoforms, IVi1 and IVi2 [72]. Unlike the yeast homolog, regulation of mammalian subunit IV appears to be more complex. This stems from the fact that isoform IVi2 is both inducible and differentially expressed in mammalian tissues. Huttemann and coworkers, showed high levels of constitutively expressed IVi2 in lung and trachea, with undetectable levels in other tissues like heart, brain, muscle and liver [72]. Mouse models with CcO IVi2 gene knock out show decreased CcO activity and ATP levels in lung and develop lung pathology that deteriorates with age [73]. Promoter analysis showed presence of a 24bp proximal promoter region called oxygen response element (ORE), which is essential for constitutive expression of IVi2 in human lung cells [74]. Semenza's lab on the other hand, showed that in both liver and lung, hypoxic environment causes induction of isoform IVi2 that switches subunit composition of CcO from IVi1 to IVi2 [59]. Further, they showed two hypoxia response elements (HRE) in the 5′ flanking region and intron 1 of Cox4I2 gene. Both hypoxia (1% O_2) and over expression of Hif 2 in HeLa cells and 293T cells strongly induced the transcription activity of Cox4I2 promoter luciferase reporter constructs showing the presence of a Hif inducible mechanism of IVi2 up regulation [59]. These studies show that altered subunit composition by switching isoforms is an important mechanism by which the catalytic function of CcO complex is regulated.

Tissue distribution

Vijayasarathy et al. reported that CcO activity varies in heart, brain, kidney and liver and in different heart compartments depending on the oxidative capacity and workload of the tissue [150]. Immunoblots and mRNA analysis showed a variation in CcO subunit content in these tissues as well as in different cardiac compartments. Tissues with low energy demand such as liver exhibited lower levels of CcO subunits IV and Vb and CcO activity compared to high oxygen consuming tissues such as the brain and kidney [150]. Interestingly, subunit IVi1 expression in rat testis was similar to heart although CcO enzyme activity is among the lowest in testis [152]. Similarly among the cardiac compartments, left ventricle and right atrium contain nearly twice the amount of Vb and VIa compared to left atrium and right ventricle. Liver CcO exhibited a high Km for cytochrome C and low turnover number (TN) while heart had the highest TN with low Km. Kidney and brain showed intermediate kinetic values [150].

Substrate availability and allosteric regulation

A host of small molecules have been shown to interact with CcO subunits, affecting the kinetic properties of the enzyme.

Both ATP and ADP bind to CcO. Using kinetic analysis, seven ATP and ten ADP binding sites have been determined in bovine CcO [102]. Subunits, IV and VIa have been identified as ATP binding subunits [17,102]. Titration of ATP versus ADP showed that while all of the ATP binding sites can also be occupied by ADP, three of the ADP binding sites are exclusive. This suggested that rather than the concentration of ATP, the ratio of ATP to ADP determines the allosteric status of the enzyme. A higher ADP content increases enzyme activity while higher steady state ATP results in allosteric inhibition [102]. Based on ATP/ADP mediated regulation of CcO activity Kadenbach and co workers proposed a

second mechanism of respiratory control termed RC2 to explain the variable relationships between respiration and Δp (proton gradient) [10]. They showed that ATP/ADP mediated allosteric regulation of CcO activity in turn regulates the rate of respiration, independent of Δp [10,76]. More recently, using isolated cow and rat heart mitochondria these investigators showed that high matrix ATP/ADP ratios keep Δφm at low level possibly through allosteric modulation of CcO activity to reduce ROS production [76,117,118].

Although allosteric modulation provides a mechanism to regulate enzyme activity in response to ATP levels, cells can bypass the ATP/ADP dependent allosteric regulation to meet their changing energy demands. At least three mechanisms have been described by which allosteric inhibition of CcO by ATP is reversed. One such mechanism is by assembling CcO with subunit IVi2, an isoform that is not subject to allosteric inhibition by ATP [59,69]. Hypoxia in cortical astrocytes and neurons induces expression of subunit IVi2 [69]. In astrocytes, ATP levels even under hypoxia are sufficient to negatively regulate CcO activity [69]. Subunit IVi2 expression under hypoxia in these cells therefore prevents any further inhibition of CcO by ATP when cells are under bioenergetic crisis. Similarly, in HeLa and 293T cells, hypoxia causes an isoform switch from subunit IV i1 to IV i2 [59]. CcO complexes containing IVi2 have also been shown to be catalytically more efficient with higher turnover number (TN) and lower Km for cytochrome C [69,137]. Similarly in yeast, at low oxygen concentrations substitution of COX5b, the yeast homolog of mammalian IVi2 for COX5a resulted in higher turnover number of cytochrome c oxidase, which is an adaptive advantage under hypoxia [4,155]. The second mechanism of relieving ATP mediated regulation of CcO activity is by binding thyroid hormone 3,5 diiodothyronine (T2). T2 has been shown to directly bind to subunit Va and abrogate ATP mediated allosteric inhibition of CcO [9]. This effect represents a forced activation of CcO activity in response to hormonal stimulation and is in accordance with the effect of T2 on energy metabolism [9]. Phosphorylation and dephosphorylation of specific subunits are yet another mechanism to either abrogate or activate ATP dependent allosteric inhibition and is discussed in detail in the next section.

Apart from ATP/ADP, other small molecules have been shown to interact with Cytochrome oxidase, although their physiological significance has not been as extensively studied. CcO subunit Vb interacts through its amino terminal end with RI , the regulatory subunit of cAMP dependent protein kinase (PKA) [159]. In CHO cells, binding of RI is sensitive to cAMP level and has been shown to regulate CcO activity. In these cells, under normal conditions, binding of RI is required for basal CcO activity. It is speculated that conditions leading to increased cAMP levels might dissociate RI from Vb leading to inhibition of CcO activity [159].

Four different gases, Nitric oxide (NO), Carbon monoxide (CO), Hydrogen Sulfide (H_2S) and Hydrogen Cyanide bind to CcO and invariably inhibit the enzyme activity. Their interaction with CcO and its physiological significance have been reviewed in detail by Cooper and Brown [48]. NO has been established as an important second messenger, which is involved in diverse physiological and pathological functions. Although soluble guanylate cyclase is one of its most prominent targets, NO interacts with metal centers of many proteins. Reversible inhibition of CcO by NO was demonstrated nearly two decades ago [25,43,131]. Since then, physiological significance of this interaction and its role in cellular pathologies have been extensively investigated. Since O_2 and NO compete for the same binding site in CcO, an important question has been whether endogenously generated NO can reach concentrations that are inhibitory to CcO under physiological oxygen levels. Several lines of experimental evidence indeed suggest that this is the case. In endothelial cells under basal conditions, NOS inhibitor N monomethyl l arginine, dramatically increased oxygen consumption [44]. Similarly NOS inhibitors increased oxygen consumption in whole

body and specific tissues of conscious dogs [134,144]. Conversely, treatment with bradykinin and ATP, which increase endogenous NO by Ca^{2+} dependent NOS, decreased respiration [44]. An interesting physiological role for NO CcO signaling has been put forward by Darley-Usmar's group. According to their model, inhibition of CcO by NO controls oxygen gradients in tissues by regulating consumption of O_2 in actively respiring mitochondria [135,136]. Changes in local oxygen levels, when CcO is inhibited by NO, have been shown by multiple studies [64,89,142]. In addition, actively respiring mitochondria (State 3) have been shown to be more susceptible to NO inhibition than those undergoing state 4 respiration [19,24]. These findings have led to the suggestion that NO increases the availability of oxygen by minimizing its consumption by active CcO, thus preventing hypoxia in deeper regions of tissue [19,24].

CO is generated endogenously by degradation of Heme by Heme Oxygenase. Zuckerbraun et. al, showed that anti inflammatory effects of CO in macrophages is a result of direct inhibition of CcO [165]. However, this study used exogenously added CO and did not provide insight on physiological conditions that generate high CO in cells. In case of both NO and CO, since CcO inhibition is by competing with O_2, the effects are likely to be more significant under pathological conditions of low oxygen tension such as hypoxia or when CcO complex is altered leading to lower affinity for oxygen. H_2S is produced endogenously by both enzymatic and non enzymatic reactions and has been shown to affect vasodilation, neurotransmission and both proinflammatory and anti inflammatory pathways [48]. Hydrogen sulfide non competively inhibits CcO activity with Ki of 0.2 μM [113]. The resulting decrease in ATP level was thought to activate K_{ATP} channels and mediate many of the biological effects of H2S [48]. However, a more recent estimate of H2S concentration in tissue was found to be about 15 nM [60]. This suggests that physiological effects of H2S are likely to be due to its direct action on ion channels, transcription factors and other cellular proteins as reviewed in [85].

Covalent modification

Phosphorylation at eighteen different sites of CcO complex has been reported thus far under physiological and pathological conditions [67]. The kinases involved and the precise physiological role of phosphorylation are still not completely understood. Depending on the residues and subunits involved, the effect of phosphorylation has been shown to both activate and inhibit enzyme activity. T11 of subunit VIa, S34 and S58 of subunit IV, and S4 and T35 of subunit Va have been shown to be phosphorylated under normal physiological conditions [2,68]. Phosphorylation at S58 and its function was reported only recently by Manfredi's lab [2]. Previous work by their group showed that metabolically generated CO_2 increases mitochondrial cAMP by activating a CO_2 bicarbonate regulated soluble adenylyl cyclase [3]. This in turn activates cAMP dependent protein kinase (PKA) in the mitochondrial matrix and phosphorylates CcO subunits I and IV [3]. Subunit IV was shown to be phosphorylated at S58, one of the ATP binding sites involved in allosteric regulation of CcO activity [2]. By computational modeling and induced fit, the authors propose that phosphorylation of S58 reduces ATP binding to the complex and abolishes ATP dependent inhibition of CcO activity. The proposed model allows regulation of CcO activity in accordance with the metabolic flux of the cell. The phosphorylation site on subunit I by this signaling and its role in CcO activation is yet to be determined.

Earlier studies showed that, in vitro phosphorylation of CcO in presence of cAMP and PKA resulted in phosphorylation of only subunits II and Vb [18]. Intriguingly, under these conditions phosphorylation resulted in inhibition of CcO activity, possibly by increasing ATP dependent allosteric inhibition [18]. cAMP dependent phosphorylation of CcO, however appears to be more complex than just regulation by metabolic flux. Lee et al. showed that in presence of theophylline, Tyrosine 304 of CcO subunit I is phosphorylated in

a cAMP dependent manner [81]. In this case phosphorylation resulted in a decrease of enzyme activity and increased affinity for cytochrome c binding. However, phosphorylation by theophylline action differs in that Tyr 304 of CcO subunit I faces the intermembrane space, and thus, likely to be different from the site phosphorylated by mitochondrial matrix PKA. In addition, effect of cAMP is indirect and probably through activation of a tyrosine kinase [81]. Glucagon, a hormone produced during starvation to conserve energy also promotes subunit I phosphorylation at Y304 and CcO inhibition [81]. Prabu et al, have shown that subunits I, IVi1 and Vb are phosphorylated under hypoxia and ischemia/reperfusion stress in a cAMP and PKA dependent manner [115]. Although the phosphorylation sites on subunits IVi1 and Vb characterized in this study are not canonical PKA sites, results using H89 and specific inhibitor PKI, strongly suggest the role of PKA [115]. The nature of these phosphorylations and their effect on CcO activity will be discussed in a later section. In osteoclasts, phosphorylation of CcO is used to meet the high energy demand of their bone resorption activity. c-Src, a non-receptor tyrosine kinase was shown to phosphorylate subunit II [97]. Although not experimentally determined, phosphorylation site prediction suggests that Tyr 113 is the likely residue involved. Phosphorylation increases CcO activity and is positively correlated with increasing c-Src activity [97]. Apart from phosphorylation, the only other covalent modification of CcO reported is acetylation. Several mitochondrial proteins have been shown to be acetylated, and significance of some of them has been demonstrated only by activity of deacetylases. A recent high resolution mass spectrometry has identified N6 acetylation of subunit Vb at K121 and of subunit IVi1 at K53 and K60 [41]. However, the physiological significance of these modifications still remains to be established.

Biogenesis and organization in supercomplexes

CcO assembly is a concerted process and involves a sequential addition of subunits to form the final complex. More than 30 assembly factors have been identified so far [56,57]. They are involved at different stages of assembly like biosynthesis of heme, import and incorporation of nuclear subunits to the membrane, insertion of copper and regulation of assembly itself [56,57]. Strikingly, a majority of inherited CcO deficiencies are associated with defects in CcO assembly [51,53,111]. CcO assembly starts with a seed subunit, identified as Cox1 in mammals and is pulsated with formation of stable assembly intermediates as additional subunits are incorporated to the growing complex [57]. In the yeast *S. cerevesiae*, synthesis of Cox1p itself has been shown to be regulated by the availability of other assembly factors shy1p, Mss51 and Cox14p [15,16,112].

Fully assembled CcO has been shown to exist along with other electron transfer chain complexes, specifically complex I and III in larger assemblies called super complexes [1]. It has been proposed that super complexes increase the efficiency of OXPHOS by substrate channeling and reducing the distances of electron transfer in between centers [1,125,153]. They also prevent leakage of electrons and their premature oxidation to form reactive oxygen radicals. Interestingly, the composition of super complexes is variable resulting in a pool of free OXPHOS complexes and those present as supercomplexes [1,125,153]. The individual complexes are in a dynamic state of association with supercomplexes depending on the metabolic state of the cell. A single functional unit of supercomplex consisting of I III2 IV4 is called respirasome [128]. Experimental evidence suggests that these repirasomes are arranged in rows to form supramolecular structures called respiratory strings. Through its existence in a supercomplex, CcO (complex IV) has been shown to be an essential component for the assembly of functional complex I in fibroblasts [52]. Similarly assembly and stability of CcO itself has been shown to require the presence of cytochrome C and ATP synthase in mitochondria [121]. The multimodal regulation of CcO by allosteric and

hormonal activators, subunit phosphorylation and organization in respirosome complexes has been summarized in Figure 1.

Cytochrome Oxidase Dysfunction and Oxidative Stress

As a critical component of the electron transport chain, CcO activity affects all aspects of mitochondrial function. While a robust CcO activity setting up steeper $\Delta\varphi m$ is expected to promote higher rates of mitochondrial superoxide production, dysfunctional CcO also promotes oxidative stress in many diseases. A diverse array of pathological conditions cause altered cytochrome oxidase function in cells and tissues. In addition to diseases, age related loss of CcO activity has been reported in many studies [108,138]. Except in cases of genetic defects, it is commonly seen that mitochondrial dysfunction is a cumulative effect of failure of more than one complex of the electron transport chain. Some of the common mechanisms of CcO dysfunction include assembly defects, covalent modifications and loss of subunits, disassembly of supercomplex organization and direct inhibition of enzyme activity. The impact of these events include energy crisis due to lower ATP production, lactic acidosis and increased formation of ROS in mitochondria. Although there is a volume of literature on CcO activity in many diseases, detailed investigation on the mechanism of CcO dysfunction and its consequence in the disease process are not completely understood. In this section, we review mechanisms of CcO dysfunction with reference to some of the conditions and assess their impact on mitochondrial function leading to oxidative stress.

Nuclear and mitochondrial mutations which cause defective assembly

Genetic defects resulting in either specific loss of mitochondrial subunits or defects in complex assembly are perhaps the only conditions leading to exclusive loss of CcO activity. They are ideal for understanding the role of CcO in mitochondrial dysfunction. MtDNA mutations that involve large scale rearrangements causing deletion or insertions and point mutations have been reported in all three mitochondrially encoded subunits of CcO [77,91,92]. They are responsible for mild to severe depletion of the subunits resulting in varying levels of loss of CcO activity. At least five different mutations have been identified for CoxI including nonsense mutations, microdeletion and heteroplasmic point mutations [14,27,47,62]. Mutations of CoxII include missense and initiation codon mutations [42,116]. CoxIII mutations include nonsense mutations, frame shift and micro deletions [66,77,143]. However, a majority of the mutations leading to CcO deficiency are those occurring in nuclear encoded assembly factors. The most common form is associated with Leigh disease, with mutations in SurfI, a protein involved in CcO assembly [164]. Other assembly factor mutations are found in LRPPRC and Taco1 [94,156]. Mutations in Copper chaperones Sco1 and Sco2, and factors involved in heme biosynthesis, Cox10 and Cox15 have been shown to cause varied CcO deficiencies with different clinical manifestations [7,45,147,148]. More recently, a single mutation in subunit COX VIb1 has been reported as the first ever structural subunit mutation of CcO [93]. Phenotypically these mutations are responsible for hypertrophic cardiomyopathy, encephalomyopathy, sideroblastic anemia, amyotrophic lateral sclerosis like syndrome, Leigh's syndrome and MELAS. The severity and type of symptom are dependent on the mutation load and the organ affected. Energy crisis leading to lactic acidosis appears to be an underlying cause of most of the symptoms of genetic CcO deficiency.

Alterations in nuclear subunit levels

The prototypic cytochrome oxidase complex consists of three catalytic core subunits, I, II and III, which are sufficient to carry out the catalytic cycle of the enzyme. The additional subunits coded by nuclear genome found in higher eukaryotes, are used for regulating enzyme activity and to provide structural stability to the complex. Not surprisingly, many

pathological conditions involving CcO dysfunction are due to alterations in the expression and regulation of some of these nuclear subunits.

Hypoxia and ischemia cause a time dependent damage to the electron transport chain in mitochondria. While short duration of hypoxia affects activities of complexes I and III [55,83,120], prolonged hypoxia (0.2 to 2% O_2 for 8 12h) and global or focal ischemia of hearts have been shown to significantly decrease CcO activity [82,114,115,146,151]. Prabu et. al, showed in RAW 264.7 macrophages, 12h of hypoxia results in more than 50% decrease in CcO activity. Analysis of immunoprecipitated holoenzyme complex showed that under both hypoxia and rabbit heart ischemia/reperfusion the levels of subunits I, IVi1 and Vb were reduced [115]. Interestingly, using phospho specific antibodies, these same subunits were shown to be phosphorylated at serine and threonine residues under hypoxia. Mitochondrial PKA activity was increased under these conditions and inhibition of PKA activity by either H89 (50nM) or specific inhibitor, myristoylated peptide inhibitor (MPI, 40 nM) prevented both phosphorylation and loss of the subunits [115]. Although at higher concentrations, H89 is known to inhibit other kinases such as certain MAP kinases and AMPK, at the concentration of 50 100 nM used in these studies, it is more specific for PKA [100]. By mass spectrometry, the PKA responsive phosphorylations were shown to be at S115 and S116 of subunit I, S40 of subunit Vb and T52 of subunit IVi1 [54]. It is likely that phosphorylation of these subunits under hypoxia is a trigger for their degradation. Notably, mass spectrometric analysis of rabbit heart enzyme did not show any other phosphorylation thought to occur under physiological conditions [54]. It is possible that the phosphorylation dependent regulation of CcO under low oxygen tension is quite different from the regulation under normoxic conditions and involves different kinases, phosophorylation sites and perhaps different phosphatases.

Fukuda et. al., reported the mechanism of degradation of subunit IVi1 under hypoxia [59]. Exposing HeLa cells to 1% O_2 or hypoxia of lung and liver of mice induces the expression of Lon protease in a Hypoxia inducible factor (HIF) dependent manner [59]. Lon is a stress responsive mitochondrial protease that degrades oxidatively damaged proteins [20,21,103]. Fukuda et. al showed that under hypoxia, Lon degrades subunit IVi1 of CcO and that under same conditions, there is an upregulation of subunit IVi2, as an adaptive response to hypoxia [59]. Specific, although modest decrease in subunits I and IV of CcO has also been reported in cardiac mitochondria of transgenic rat model of heart failure [124]. Loss of these subunits resulted in about 50% decrease in TMPD/ascorbate stimulated respiration in mitochondria [124]. Similarly, a rat model of cardiac hypertrophy exhibited loss of subunit Vb of CcO [80]. Differential loss of CcO subunits due to ischemia reperfusion has also been reported in rat heart. Prolonged ischemia/reperfusion of rat heart resulted in about 70% decrease in subunit I and more than 50% in subunit Va [162]. However, there was no significant difference in levels of subunit IV and Vb in these hearts. The differences in the subunits involved in all these studies could arise from species specific variation in sequences that might alter their susceptibility to oxidative damage.

Loss of subunit IV and Vb has also been reported in ethanol toxicity [12,36]. Chronic alcohol consumption has long been shown to decrease CcO protein content and activity [8,141]. A consistent observation is that about 50 to 70% decrease in the activity and heme aa3 content occurs under ethanol treatment in rodents [141]. Cunningham and others have demonstrated that ethanol consumption decreases synthesis of the 13 mitochondrially encoded proteins that are components of respiratory complexes I, III, and IV and the ATP synthase [110]. It is proposed that the inhibition of mitochondrial protein synthesis following chronic ethanol exposure is due to defects in mtDNA and a decline in the number of functional mitochondrial ribosomes resulting from chronic ethanol exposure [110]. However, this does not completely explain the observed decreases in nuclear encoded

subunits of CcO. Recent work from our lab has shown that in both COS and HepG2 cells, ethanol treatment results in lower levels of subunits IV and Vb of CcO and accompanying decrease in enzyme activity [12]. Loss of CcO activity was exacerbated in cells expressing higher levels of mitochondrial targeted P450 2E1, suggesting its role in CcO dysfunction [12]. Excessive ROS produced during ethanol toxicity has an important role in oxidatively damaging subunits of CcO. Chen et. al., have shown 4 hydroxy nonenal (HNE), a toxic lipid peroxidation product produced when rats are exposed to ethanol, forms aldehyde adducts mainly with subunit IVi1 of CcO [35,36]. In addition to HNE, ethanol treatment generates malondialdehyde (MDA), another lipid peroxidation product. MDA also forms adduct with CcO and in vitro treatment showed greater inhibition of enzyme activity by MDA compared to HNE [34]. The more potent effect of MDA could be due to the fact that it forms adducts with both subunits IVi1 and Va/b of CcO [34]. Proteins modified by HNE and other lipid peroxidation derived aldehydes are actively degraded by proteases and probably explains the loss of subunits IVi1 and Vb in ethanol toxicity.

Loss of supercomplex organization

The supercomplex arrangement of the electron transport chain is a fairly recent observation. Supercomplexes are proposed to increase the efficiency of electron transfer and minimize electron leakage that could generate partially reduced oxygen species. The nature of interaction between complexes and the dynamic equilibrium between free forms of the complex and supercomplex forms are not completely understood. However, in a growing number studies effect of pathological conditions on supercomplex assembly is being reported. CcO is found in two major forms of mitochondrial supercomplexes, I/III2/IV1 4 and III2/IV1 2 [126,129]. Pathologies that result in decreased protein levels of CcO eventually affect stoichiometry and assembly of these supercomplexes. Heart failure in dogs induced by coronary microembolism resulted in the loss of Complex IV containing supercomplexes of the electron transport chain [61,119]. Similarly, in RAW 264.7 macrophages knockdown of either subunit CcO Vb or CcO IV resulted in significant decrease in cytochrome oxidase containing supercomplexes [61]. Liver mitochondria from ethanol treated rat also showed a lower level of supercomplexes concomitant with the loss of CcO protein [12]. CcO has been shown to be necessary for maintaining stability of complex I in the supercomplexes. In mouse fibroblast cell lines reduced expression of subunit IVi1 or nonsense mutation in subunit I, not only resulted in lower CcO content, but also caused significant reduction in complex I [86]. Similarly, mouse fibroblasts from COX10 knockout mice, which have defective CcO assembly, also exhibited reduced complex I levels [52]. However, RAW 264.7 macrophages with more than 90% reduced subunit Vb levels and in mouse cell lines with reduced expression of IV, Va, VIa, VIIa and VIIb, there was no significant reduction in complex I content [58,61]. Similarly, muscle mitochondria from patients with SurfI mutation had defective CcO assembly with no effect on complex I level [127]. This apparent discrepancy can be explained by a recent finding that structural intermediates of CcO, which usually accumulate during CcO assembly defects, are involved in the initial stabilization of complex I during supercomplex formation [98].

Drug induced inhibition of enzyme activity

Direct inhibition of CcO activity occurs by inhibitors interfering with the catalytic cycle or by binding at distal allosteric sites. Nitric oxide, which has been shown to have both physiological and pathological effects, inhibits CcO activity by competing with oxygen for the binuclear center. Inhibition by NO is shown to be reversible. However, under oxidative stress, NO reacts with superoxide radical to form peroxynitrite. *In vitro* experiments show that peroxynitrite interacts irreversibly with CcO subunits inhibiting its activity [133]. Carbon monoxide and Hydrogen sulphide are other gaseous molecules that inhibit CcO activity. Although enzyme systems in the cell are capable for producing CO (Heme

oxygenase) and H_2S (Cystathionine gamma lyase and Cystathionine –beta synthase), the cellular concentrations are unlikely to affect CcO activity. Poisoning by inhalation of these gases is a more common reason for high cellular concentration and inhibition of CcO activity. Many externally administered drugs and xenobiotics have also been shown to inhibit CcO activity leading to mitochondrial stress. Mechanisms of action of most of these have not been elucidated in great detail. Anthracyclines like daunomycin (DAU) and doxorubicin (DOX), are antitumor drugs that cause cardiomyopathy as a side effect during long term treatment [33,107]. Cell culture and animal studies showed DAU directly inhibits CcO activity in a dose dependent manner. Further, DAU toxicity could be reversed by treatment with hemin, suggesting that DAU may act by binding to heme groups in CcO [107]. Interestingly, DOX treatment resulted in both CcO inhibition and lower levels of CcO subunits. DOX effects were reversed by treatment with MitoQ, a mitochondria targeted antioxidant [33]. Anthracyclines probably act by more than one mechanism to affect CcO expression and activity. CcO activity is significantly inhibited during inflammatory condition like sepsis [84]. Tyr 304 phosphorylation of subunit I induced by the inflammatory cytokine TNF is thought to be the mechanism of inhibition of CcO [122]. The pathophysiological factors that induce mitochondrial dysfunction have been summarized in Figure 2.

Role of dysfunctional CcO in oxidative stress

Energy crisis and acidosis

As one of the three proton pumps that contribute to mitochondrial membrane potential and as the enzyme that completes electron transport chain by transferring electrons to oxygen, CcO dysfunction has a direct effect on cellular ATP level. Knockdown of CcO subunits that cause loss of activity also resulted in lower membrane potential and reduced ATP generation [58,61]. Cells compensate for decreased mitochondrial ATP production through oxidative metabolism by up regulating less efficient glycolytic pathway. However, a consequence of this adaptation is increased acidification due to lactate accumulation. In Leigh's disease CcO assembly defect leads to more than 80% reduction in enzyme activity [63,65,101]. Fibroblasts generated from patient samples were found to generate only 15 20% of the ATP compared to control cells [65]. A compensatory increase in glycolysis is thought to cause severe lactic acidosis and pyruvate depletion affecting aerobic regions of the patient's brain [63].

ROS production

Mitochondria are the principal source of cellular reactive oxygen species (ROS). Mechanisms of ROS production by mitochondrial respiratory chain complexes have been reviewed extensively [30]. ROS, particularly superoxide anions are formed invariably as by products of electron transport chain and other redox reactions in mitochondria through one electron reduction of molecular oxygen (O_2). Superoxide is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs), present both within the mitochondria and in the cytosol. Depending on their type and rate of production, ROS have both physiological role and pathological effects in the context of mitochondrial as well as whole cell function. Excessive production of ROS and the associated cytotoxic effects are generally called oxidative stress. Peroxidation of membrane lipids, direct oxidation of amino acids and oxidative cleavage of peptide bonds in proteins and DNA damage are some of the hallmarks of oxidative stress and are responsible for many of the disease symptoms. Although several redox reactions take place in mitochondria, only a few of them have been shown to generate detectable oxygen free radicals [5]. While Complexes I and III are the major sites of ROS formation [23,29,30,38,83,145], recent reports show that complex II can readily generate superoxide radicals in the absence of electron acceptors [105,163]. In

addition, ketoglutarate dehydrogenase, electron transfer flavoprotein:CoenzymeQ oxidoreductase, mono amine oxidase, and few other mitochondrial enzymes have been shown to produce ROS [30,75,139,140].

One of the major challenges in the field of free radical biology has been to determine which of the respiratory sites generate pathological levels of ROS. It is widely accepted that mitochondria with robust respiration coupled electron transport chain activity and high $\Delta\varphi m$ produce higher levels of $O_2^{\cdot -}$ [75,79,99]. A recent review nicely highlights possible mechanisms by which complex I, II and III contribute to ROS production both as part of forward and reveres electron flow [99].

The contribution of CcO, the terminal enzyme of the electron transport chain to ROS production and oxidative stress remains controversial. It is important to note that, in sequential systems that carry out a series of redox reactions such as the electron transport chain, ROS production from a particular site can be influenced by dysfunction of other complexes or redox centers in the series. Irrespective of the state of other complexes, most cases of CcO deficiency induce mitochondrial ROS production. The direct and indirect roles of CcO in ROS production have been investigated by a number of groups over the years.

A notable characteristic of hypoxic stress is the massive burst of reactive oxygen species produced in mitochondria. Decreased flux through CcO due to low oxygen availability results in accumulation of reduced intermediates upstream in the ETC. Loss of electrons from reduced intermediates of the electron transport chain is considered one of the mechanisms of ROS formation [49,50]. Contribution of CcO dysfunction to ROS production under ischemia/reperfusion has been shown in an earlier study from our group [115]. In this study, CcO containing vesicles of asolectin and cardiolipin were prepared from sub mitochondrial particles from control and ischemic rabbit hearts. When reduced cytochrome c was added as the electron donor, CcO vesicles prepared from ischemic heart generated nearly 2 fold higher reactive oxygen species compared to control heart [115]. Since global stress conditions such as ischemia and hypoxia affect more than one complex, a more exclusive role of CcO in increasing mitochondrial ROS was shown in a stable CcO knock down cell line. Stable expression of sh-RNA to CcO Vb mRNA produced cells containing only 10% CcO activity compared to control wild type macrophages. In these cells, even under normoxic conditions, isolated mitochondria generated much higher ROS compared to control cells [61]. The specificity and mitochondrial origin of this ROS was verified by electron paramagnetic resonance spectroscopy using mito DEPMPO, a mitochondria targeted spin trap [61]. Similarly, HeLa cells expressing sh-RNA to subunit IV 1 exhibited increased H_2O_2 level compared to control cells [59]. Another mechanism of CcO dysfunction that leads to higher ROS formation during hypoxia and ischemia is possibly by the loss of cardiolipin [37]. CcO is associated with cardiolipin in the inner membrane and this interaction is essential for maximal CcO activity. ROS produced during hypoxia/ischemia depletes cardiolipin by peroxidation [109]. This decreases cytochrome C level resulting in higher ROS formation by complex I. As mentioned above, paradoxically, a more robust CcO activity has also been shown to increase ROS production [137]. In astrocytes, hypoxic and toxin mediated induction of subunit IV i2 is thought to play a role in elevated mitochondrial peroxide production [69,137]. Knockdown of IV i2 by siRNA in these cells resulted in decreased mitochondrial peroxide formation in addition to decreasing CcO activity [96,137]. This observation is however opposite to an earlier report in which knockdown of subunit IV i2 under hypoxia increased H_2O_2 production [59]. The difference could be due to the cell types used and the stress condition used to induce subunit IV i2. CcO dysfunction can also contribute to higher ROS formation by the loss of supercomplex structures. Heart failure in dogs induced by coronary microembolism resulted in a loss of Complex IV containing supercomplexes of the electron transport chain [61,119]. Similarly,

in both CcO Vb and CcO IV knockdown RAW 264.7 macrophages, there was a significant decrease in cytochrome oxidase containing supercomplexes [61]. As discussed earlier in this review, an important function of these supercomplexes is thought to reduce electron leakage to minimize ROS formation, which seems to be affected under conditions when cytochrome oxidase levels are reduced. Chronic ethanol treatment augments CcO subunit degradation, which is one of the factors leading to high levels of mitochondrial $O_2^{\cdot -}$ and H_2O_2, resulting in oxidative damage. Ethanol treatment also increases NO production in hepatocytes which has been shown to affect mitochondrial functions [13,149,160]. Inhibition of CcO by NO itself has been shown to increase ROS formation from other complexes of the electron transport chain [22]. In addition, NO reacts spontaneously with superoxide anion in the mitochondria to form peroxinitrite, a highly reactive nitrogen radical [70]. Peroxinitrite covalently modifies mitochondrial proteins by nitrosation of tyrosine and cysteine residues and damages Fe S centers causing futher oxidative damage to the mitochondrial compartment [104]. The interaction of CcO and NO pathway to increase mitochondrial ROS production under ethanol and hypoxic stress is summarized in figure 3.

All of these mechanisms describe indirect contribution of CcO to ROS formation in mitochondria. So far, there is no evidence for CcO acting as a direct source of ROS, although mechanistic studies on the catalysis by CcO have shown formation of reactive oxygen intermediates at the metal centers of the enzyme [95]. Additionally, Electron paramagnetic resonance (EPR) spin trap studies suggest the formation of protein derived radicals on mammalian CcO [39,40,90], though other studies suggest that amino acid radicals may be part of the proton loading and release mechanism [161]. While many redox centers can leak reactive intermediates, the rapid kinetics of CcO ensures full reduction without releasing the reactive species into the medium. The rapid kinetics of electron transfer to oxygen prevents formation of reactive oxygen species and release into medium. Interestingly, however, in cells subjected to hypoxia or loss of some select CcO subunits, there is a marked accumulation of subcomplexes [61]. It would be interesting to see if such partially assembled complexes exhibit kinetics that is different from fully assembled CcO. Using functional models of CcO active site, Collman et al, have shown the importance of correct spatial arrangement of elements of active site [46]. In their model, masking of a phenol, representing Tyr244 of the bovine CcO active site resulted in much greater leakage of partially reduced oxygen species (PROS). Recently Khalimonchuk et al. showed in yeast that CcO subunit I haem a is a pro oxidant intermediate formed during the assembly of cytochrome oxidase [78]. However, evidence for the direct contribution of CcO to ROS production is still lacking.

Role of CcO in the formation of Nitric Oxide has been shown under hypoxic conditions. Castello et al, have shown that isolated yeast CcO produces NO in presence of Nitrite under oxygen limiting conditions [32]. Nitrite reductase activity of CcO has also been shown by earlier studies [26,106,123]. Further, under hypoxia, NO generated by CcO has been shown to form peroxynitrite by reacting with superoxide and modify mitochondrial proteins by nitration. HEK293 cells that do not possess nitric oxide synthase were shown to generate mitochondrial NO under hypoxia, which was prevented by CcO inhibitor, sodium azide [11]. NO generated in these cells was shown to stabilize Hif . Both NO inhibitors and CcO inhibitors prevented stabilization of Hif under hypoxia in these cells proving the role of CcO generated NO in hypoxia signaling [11].

Calcium homeostasis and apoptosis

Mitochondria have an important function in modulating cellular calcium homeostasis. They act as calcium stores by taking up calcium in an energy dependent manner. Calcium enters mitochondria through a uniporter in the inner mitochondrial membrane and is driven by

membrane potential. Physiological levels of calcium are required for stimulating mitochondrial metabolism. Any impairment of mitochondrial function leading to loss of membrane potential and decreased ATP levels reduces the activity of the calcium pump and mitochondrial uptake. Given the important role of CcO in ATP synthesis it seems logical to expect aberrant calcium homeostasis in CcO deficient cells. Surprisingly there has been only a single report of abnormal calcium homeostasis in fibroblasts of a CcO deficient patient [154]. Leigh syndrome is caused by mutations in Surf I leading to impaired CcO assembly. The authors report a greatly impaired influx of calcium into mitochondria of patient fibroblasts, even in presence of Thapsigargin that activates mitochondrial store operated calcium entry by blocking ER calcium entry [154]. Mitochondria play an important role in programmed cell death. They are the sites of initiation of the intrinsic pathway of apoptosis. Myocardial infarction (MI) resulting from cardiac ischemia reperfusion is characterized by tissue damage due to apoptosis. Stress by MI leads to specific up regulation of COX III [157]. A recent study has established a connection between COX III over expression and apoptosis. Over expression of COX III in HL 1 myocytes resulted in decreased expression of COX I and lower Cytochrome oxidase activity. Interestingly the cells became susceptible to oxidative stress and had increased cell death due to apoptosis [157].

Summary and conclusions

Cytochrome oxidase is a major regulatory enzyme of the electron transport chain. Its importance is shown by multiple levels of regulation of its activity and content in the mitochondria. Disruption of any of these regulatory cues can result in a pathological condition. Defective cytochrome oxidase activity either directly or indirectly affects all aspects of mitochondrial function and consequently an array of diseases are associated with reduced contents or altered activity of CcO. While it is expected to see a severe impairment of aerobic ATP generation due to CcO deficiency, there is also evidence of CcO affecting calcium homeostasis. Further, there is considerable literature to show the role of defective CcO in increased mitochondrial ROS, though the precise mechanism of its contribution remains unclear.

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Abbreviations

OXPHOS	Oxidative phosphorylation system
ROS	Reactive oxygen species
sh-RNA	Short hairpin RNA
ER	Endoplasmic reticulum
cAMP	Cyclic adenosine mono phosphate
$\Delta\psi m$	Mitochondrial transmembrane potential

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Highlights

- Mammalian Cytochrome oxidase (CcO) is a bigenomic enzyme with 13 subunits
- The enzyme is regulated by subunit phosphorylation and allosteric effectors
- CcO function is affected by hypoxia/ischemia, drugs and by alcohol
- CcO function is also compromised in many diseases
- CcO dysfunction often leads to increased production of reactive oxygen species

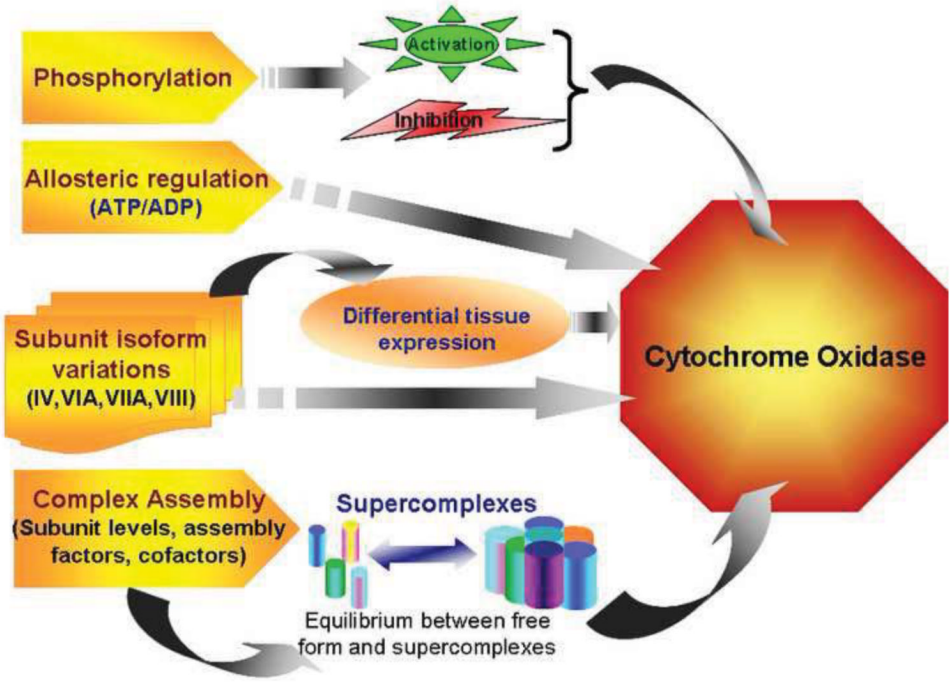

Figure 1.

Regulation of Cytochrome C Oxidase. Cells control CcO function by regulating both protein content and enzyme activity. Co ordinated expression of subunits, their assembly into complexes and supercomplexes provide a global control over protein levels of the enzyme. Differential cell and tissue specific expression of subunit isoforms and their relative abundance are used to change enzyme composition and alter kinetic properties of CcO activity in accordance with oxidative capacity of the tissues. Rapid adaptation to cellular bioenergetic requirements is brought about by binding small molecules like ATP/ADP, di-iodo thyronine, NO and by phosphorylation of specific subunits that either increase or decrease CcO activity. The multimodal regulation of CcO activity is summarized in this figure.

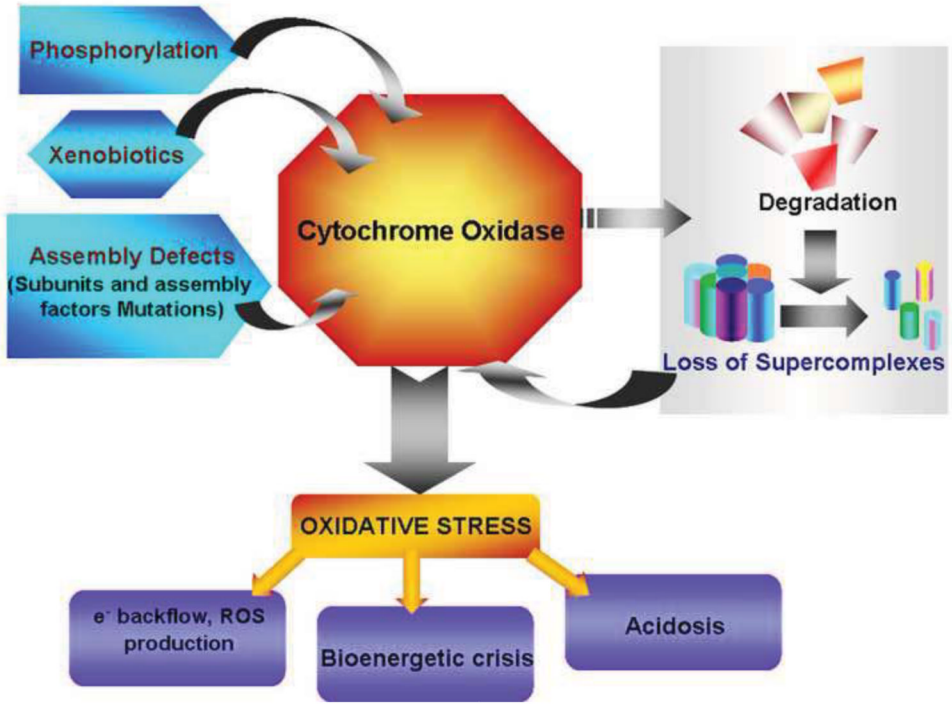

Figure 2.

Factors which cause dysfunction of Cytochrome C Oxidase. Multiple factors affect CcO function leading to mitochondrial stress and dysfunction. Genetic mutations of subunits and assembly factors are responsible for significant decrease in fully assembled complexes. Environmental changes such as hypoxia induce phosphorylation which either increase the activity or decrease activity by targeting the subunits for degradation. Many xenobiotics act by directly binding to active site heme and inhibit enzyme activity. Dysfunctional CcO contributes greatly to oxidative stress by increasing ROS production, ATP depletion and lactic acidosis.

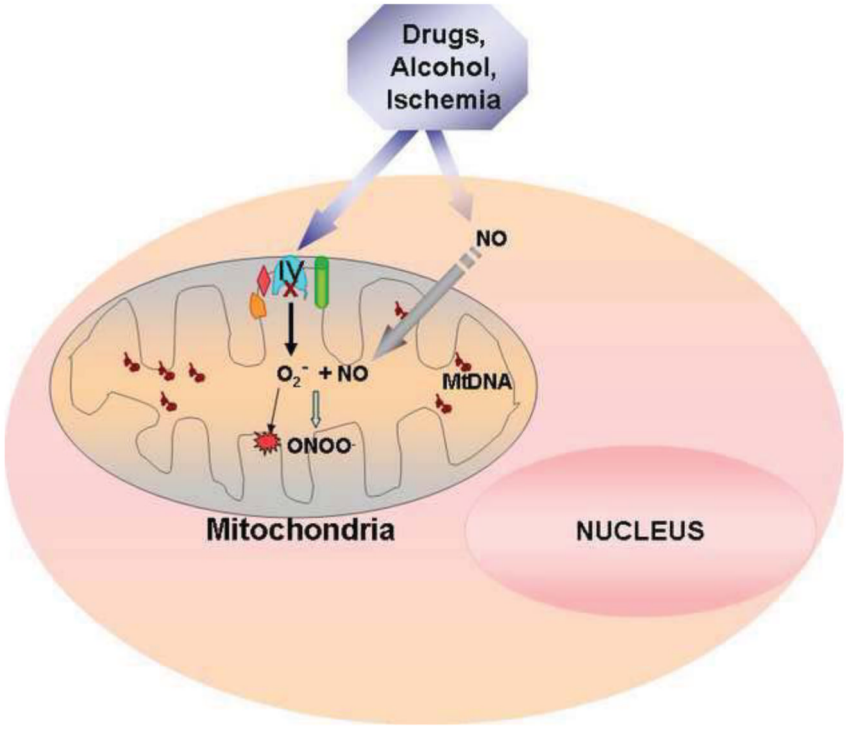

Figure 3.

Mechanism of ethanol, ischemia and drug induced mitochondrial ROS production. Results show that ethanol, myocardial ischemia and drugs such as doxorubicin cause CcO dysfunction and induce ROS production. In the case of ethanol induced toxicity, increased mitochondrial superoxide anion reacts with NO to form the highly reactive peroxynitrite which covalently modifies proteins and Fe S centers.