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Regulation of mitochondrial oxidative phosphorylation through tight control of cytochrome *c* oxidase in health and disease – Implications for ischemia/reperfusion injury, inflammatory diseases, diabetes, and cancer

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A B S T R A C T

Mitochondria are essential to cellular function as they generate the majority of cellular ATP, mediated through oxidative phosphorylation, which couples proton pumping of the electron transport chain (ETC) to ATP production. The ETC generates an electrochemical gradient, known as the proton motive force, consisting of the mitochondrial membrane potential ($\Delta\Psi_m$, the major component in mammals) and ΔpH across the inner mitochondrial membrane. Both ATP production and reactive oxygen species (ROS) are linked to $\Delta\Psi_m$, and it has been shown that an imbalance in $\Delta\Psi_m$ beyond the physiological optimal intermediate range results in excessive ROS production. The reaction of cytochrome *c* oxidase (COX) of the ETC with its small electron donor cytochrome *c* (Cyt*c*) is the proposed rate-limiting step in mammals under physiological conditions. The rate at which this redox reaction occurs controls $\Delta\Psi_m$ and thus ATP and ROS production. Multiple mechanisms are in place that regulate this reaction to meet the cell's energy demand and respond to acute stress. COX and Cyt*c* have been shown to be regulated by all three main mechanisms, which we discuss in detail: allosteric regulation, tissue-specific isoforms, and post-translational modifications for which we provide a comprehensive catalog and discussion of their functional role with 55 and 50 identified phosphorylation and acetylation sites on COX, respectively. Disruption of these regulatory mechanisms has been found in several common human diseases, including stroke and myocardial infarction, inflammation including sepsis, and diabetes, where changes in COX or Cyt*c* phosphorylation lead to mitochondrial dysfunction contributing to disease pathophysiology. Identification and subsequent targeting of the underlying signaling pathways holds clear promise for future interventions to improve human health. An example intervention is the recently discovered noninvasive COX-inhibitory infrared light therapy that holds promise to transform the current standard of clinical care in disease conditions where COX regulation has gone awry.

1. Introduction

Approximately 90 % of ATP in the body is produced through oxidative phosphorylation (OxPhos) within mitochondria. The OxPhos machinery consists of two parts: the electron transport chain (ETC), which is composed of four multi-subunit protein complexes (complexes I-IV), combined with the final complex, ATP synthase (complex V), the enzyme that generates ATP from ADP and phosphate. The OxPhos complexes are localized in the inner mitochondrial membrane (IMM). The energy stored in food-derived molecules such as NADH and succinate is extracted through a series of steps where electrons are passed from complex to complex by the small electron carriers ubiquinone and cytochrome *c* (Cyt*c*). The movement of electrons is coupled to the pumping of protons from the matrix across the IMM into the intermembrane space (IMS). The transfer of protons across the membrane generates an electrochemical gradient referred to as the proton motive force, which consists of the mitochondrial membrane potential ($\Delta\Psi_m$) and a pH gradient across the IMM (ΔpH). In mammals, $\Delta\Psi_m$ is the primary contributor to the proton motive force [1,2] and is nowadays commonly analyzed through voltage-dependent probes such as

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tetramethylrhodamine ethyl ester (TMRE).

The proton gradient is utilized by ATP synthase, which couples proton flow down the concentration gradient back into the matrix to a rotational movement of the ring of c-subunits and the central stalk, forcing the formation of ATP from ADP and phosphate at the interface of the catalytic alpha and beta subunits in the mitochondrial matrix. Compared to just 2 molecules of ATP produced during glycolysis from 1 molecule of glucose, OxPhos generates more than 15 times as much, which makes it an efficient and evolutionarily optimized process.

Cytochrome *c* oxidase (COX) is the terminal oxidase in the ETC and catalyzes the final step by reducing oxygen to water using the electrons transferred by Cyt*c*, and it is the proposed rate-limiting step in higher organisms under physiological conditions [3–6]. The transfer of electrons from Cyt*c* to COX to oxygen is coupled to proton pumping. However, unlike complexes I and III, which pump 2 protons per electron, COX pumps only one proton per electron transferred, despite the fact that the reaction releases about twice as much free energy compared to that of complexes I and III [7]. The low H^+/e^- stoichiometry may allow the COX-catalyzed reaction to proceed quickly without releasing highly reactive ROS species that are generated during the conversion of O_2 to H_2O. The normal physiological $\Delta\Psi_m$ range in most mammalian cells is between 80 and 130 mV, allowing efficient ATP production [8] while minimizing the production of reactive oxygen species (ROS). High levels of ROS, which are generated at complexes I and III, have been correlated with increased $\Delta\Psi_m$ (>140 mV) due to an overall reduction of electron flux, resulting in the inhibition of the proton pumps and increased half-life of the semi-ubiquinone free radical intermediate [9], promoting the transfer of the unpaired electron to oxygen to generate superoxide. Basal levels of ROS production are required for some signaling pathways to function within and outside of mitochondria, and they are also important in regulating COX biogenesis and assembly [10,11]. COX contributes to $\Delta\Psi_m$ and ATP generation in the cell and does not directly generate ROS but, as the proposed rate-limiting step, controls $\Delta\Psi_m$ and thus indirectly controls ROS production at complexes I and III, which necessitates tight regulation of the essentially irreversible reaction catalyzed by the enzyme.

One monomer of COX is composed of 13 tightly bound subunits (I, II, III, IV, Va, Vb, VIa, VIb VIc, VIIa, VIIb, VIIc, VIII) where subunits I-III are mitochondrial encoded, and the remaining 10 are nuclear encoded. A less tightly bound 14th subunit exists, which was more recently discovered and will be discussed in a later section. Subunits I and II make up the catalytic core of the enzyme, are encoded by the mitochondrial DNA (mtDNA), and are surrounded by mitochondrially encoded structural subunit III and the other 11 nuclear encoded subunits. The COX complex is embedded within the lipid bilayer of the IMM. All of the subunits contain a hydrophobic transmembrane region except for subunits Va and Vb, which are bound to the matrix side of COX, and subunit VIb, which faces the IMS. Per COX monomer, there are two heme and two copper redox centers in subunits I and II: subunit I contains the heme *a* and Cu_B-heme a_3 redox centers and subunit II contains the Cu_A redox center with two copper ions. The Cu_A site is the electron acceptor site from Cyt*c* that then reduces the heme-*a* site. The electrons are then transferred to the Cu_B-heme a_3 site in subunit I, which is the catalytic oxygen binding site where the enzymatic reduction of dioxygen to water takes place. Known inhibitors of the Cu_B-heme a_3 oxygen binding site are carbon monoxide (CO), nitric oxide (NO), cyanide (CN^-), and azide (N_3^-), which compete with oxygen for binding, thus inhibiting COX.

Cyt*c* is a small electron carrier located in the mitochondrial IMS. It plays a critical role in the ETC by shuttling electrons from complex III (bc_1-complex) to COX. This electron transfer represents the final and the proposed rate-limiting step in the ETC, ultimately leading to the reduction of oxygen to water by COX. Given the importance of this reaction, it is not surprising that both Cyt*c* and COX are under stringent regulation. All three main regulatory mechanisms have been identified to act on COX and Cyt*c*, consisting of allosteric binding of ATP/ADP, post-translational modifications (PTMs), and expression of tissue-specific isoforms [12–14]. Interestingly, the presence of all three regulatory mechanisms above have not been reported for any of the other OxPhos complexes, highlighting the importance of fine-tuning the Cyt*c*-COX reaction and further supporting the concept that controlling this irreversible reaction is crucial in maintaining proper mitochondrial function and energy production. The tight regulation of the Cyt*c*-COX reaction is fine-tuned for meeting bioenergetic requirements of various tissues and cells under both normal and stress conditions. We and others have proposed that disruption or dysregulation of these mechanisms is associated with multiple diseases, such as inflammation [15], cancer [16–18], cardiovascular disease [19–22], diabetes [23,24] and ischemia/reperfusion injury [25–29]. In this review, we will discuss the main regulatory mechanisms of COX including allosteric regulation, tissue-specific isoforms, and provide a comprehensive catalog and discussion of post-translational modifications. These regulatory mechanisms work together to modulate oxygen consumption rate and ATP production, maintaining cellular energy homeostasis. This review will explore the causes and potential consequences of regulatory dysregulation of COX with the future goal of targeting COX in certain acute and chronic disease conditions as potential therapeutic interventions. Although the formation of supercomplexes is another essential aspect of COX regulation, it warrants a separate in-depth review and will only be touched upon here.

1.1. Allosteric regulation by ATP/ADP and other molecules

The most basic mechanism controlling ETC flux is traditionally referred to as respiratory control [30]. In isolated coupled mitochondria supplemented with substrates such as glutamate/malate, pyruvate/malate, or succinate there is a basal oxygen consumption rate (state 2 respiration), due to backflow of some protons from the IMS to the matrix through unspecific leak. When ADP and phosphate are added, ATP synthase utilizes the electrochemical gradient to produce ATP, leading to a drop of $\Delta\Psi_m$ and ΔpH. This causes a sharp increase in respiration (state 3 respiration), because the proton pumps do not have to pump against a high electrochemical gradient, which is the product of their reaction leading to product inhibition, i.e., respiratory control. When all the ADP is converted to ATP, high $\Delta\Psi_m$ and ΔpH levels build up again, inhibiting respiration (state 4 respiration, which is similar to state 2 respiration). State 3 respiration divided by state 4 respiration is called the respiratory control ratio, which serves as an indicator of mitochondrial coupling and integrity.

Later, Kadenbach and colleagues proposed a second mechanism of respiratory control that is independent of $\Delta\Psi_m$ and is based on direct inhibition of COX at high ATP/ADP ratios through allosteric binding of ATP (Fig. 1) [31]. They then called traditional respiratory control “first mechanism of respiratory control” and referred to allosteric regulation through ATP/ADP as the “second mechanism of respiratory control” [31]. Subunit IV of COX has a conserved ATP binding site on the matrix side modulating the allosteric inhibition at high ATP/ADP levels [32–34]. Additionally, Cyt*c* has also been shown to be regulated via ATP as an allosteric inhibitor [35]. When ADP levels are high, this causes the affinity of Cyt*c* to COX binding to increase fivefold [36–38]. In addition, it has been shown that COX can adjust the proton to electron stoichiometry. In cow heart COX in the presence of high ATP/ADP ratios, the H^+/e^- stoichiometry changes from 1.0 to 0.5 after exchanging the ADP with ATP on the matrix side [39].

There are additional small molecule COX allosteric regulators. The thyroid hormone T2 can turn off the allosteric ATP inhibition, leading to increased activation of the enzyme, likely by binding to the matrix-oriented subunit Va [40]. The fatty acid palmitate, but not other fatty acids, can decrease the H^+/e^- stoichiometry from 1.0 to 0.5 in cow liver, indicating lower proton pumping efficiency of COX by partial intrinsic uncoupling of proton pumping from electron transfer [41]. This effect, however, was not seen in heart COX, likely due to the differential expression of liver versus heart isoform of subunit VIa. Other examples

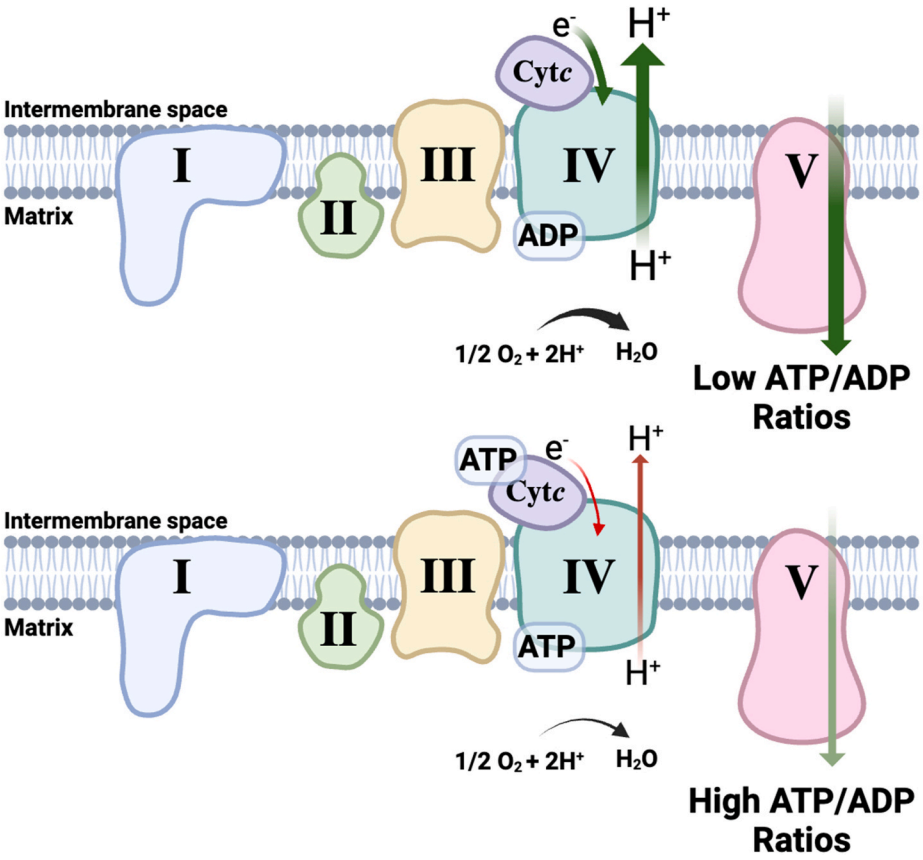

Fig. 1. ATP/ADP allosteric regulation of COX and Cyt*c* as an energetic sensor to regulate ATP production. Under low ATP/ADP ratios in the cell, ADP acts as an allosteric activator by binding to COX and the reaction between COX and Cyt*c* occurs at an increased rate resulting in more protons pumped into the intermembrane space (IMS). This generates an increased electrochemical gradient, and ATP synthase (Complex V) is able to generate more ATP. Under high ATP/ADP ratios, ATP acts as an allosteric inhibitor to both COX and Cyt*c* and decreases the rate at which Cyt*c* donates its electron to COX, resulting in fewer protons pumped into the IMS, thereby directly decreasing ATP production by complex V.

of regulatory molecules are nitric oxide, which acts as a competitive binder [42], protein-protein interaction with nitric oxide synthase [43,44], the androgen receptor [45], MNRR1 (also called CHCHD2) [46,47], the epidermal growth factor receptor (EGFR) [48], and the downstream protein of TGFβ signaling, Smad4 [49].

2. Tissue-specific isoforms

A subset of COX subunits has tissue-specific isoforms, further highlighting the importance of COX regulation being dependent on the cell type and energetic demand. Of the 14 COX subunits, six have isoforms, most of which are tissue-specific and are hypoxia-induced and/or developmentally regulated, as summarized in Table 1 and Fig. 2 and discussed in detail in the following sections. Generally, the majority of the isoform pairs historically termed 'liver-type' are expressed in most tissues except for heart and skeletal muscle where the 'heart-type' isoforms are expressed. The isoform regulated through oxygen can be classified as lung-type and is primarily present in the lung, carotid body, and placenta. Cyt*c* has also been identified to have a somatic and testes-specific isoform. The nuclear encoded subunits of COX in mammals have isoforms identified that modulate the activity and function of COX, and include subunits IV, VIa, VIb, VIIa, VIII, and NDUFA4. Originally, mammalian COX from bovine heart was crystallized as a homodimer and found to contain 13 subunits [50]. Later, a 14th subunit, NDUFA4, was identified by using the gentle detergent digitonin, a subunit originally thought to be a part of complex I [51]. These isoforms are all encoded by separate genes rather than alternative splicing, supporting their regulatory purposes by tighter control of gene expression specific to the corresponding cell type. The different isoforms provide slightly different structures, thereby fine-tuning the basal enzyme activity to compensate for mitochondrial capacity, allosteric regulation, tissue-specific cell signaling via PTMs, and COX dimerization or supercomplex formation. The isoforms can be broadly divided into two overlapping groups: hypoxia-induced and developmentally regulated.

2.1. Hypoxia-induced isoforms

Isoforms of subunit IV and NDUFA4 are differentially expressed depending on the oxygen tension in the tissue, and under hypoxic conditions, expression is upregulated. Subunit IV of COX has a ubiquitously expressed isoform that is present in all tissues and an alternative isoform expressed in specific tissues such as the lung. NDUFA4 is a ubiquitously expressed COX subunit, whereas its isoform exhibits more tissue-specific expression, being detected in specialized cell types, regulated under hypoxic conditions, and contributing to oxygen sensing.

2.1.1. Subunit IV isoforms

Subunit IV is the largest nuclear-encoded subunit of COX and is located next to the catalytic core with contact sites to both catalytic subunits I and II, allowing the isoform expressed to directly modulate the enzymatic activity. This subunit is also required for COX biogenesis and is part of the first assembly step by interacting with subunit I in mammals [52]. The yeast (*Saccharomyces cerevisiae)* COX subunit corresponding to mammalian subunit IV is subunit V (yeast nomenclature) and exists as two isoforms, Va and Vb. Under hypoxic conditions, there is a switch from Va to Vb, an isoform with a higher turnover and electron

Table 1
Six of the subunits of COX have isoforms that are differentially expressed depending on tissue type, developmental stage, and oxygen concentration. For subunits VIa, VIIa, and VIII, the isoform pairs can be classified as 'liver-type' expressed in most tissues, except skeletal muscle and heart, where the 'heart-type' isoforms are expressed. Subunit IV has a ubiquitous isoform that is expressed in all tissues, whereas the second isoform ('lung-type') is expressed in the lung, placenta, and carotid body. For subunits IV and NDUFA4, the alternative isoforms are regulated via oxygen concentration. Subunit VIb, which is exclusively localized to the IMS side on COX, contains a mostly ubiquitous ('somatic') isoform expressed in all tissues except for sperm cells and other testicular cell types.

Subunit	Class	Isoform	Tissue	Function
IV	Oxygen dependent	COX4i1 COX4i2	Ubiquitous Lung-type	Adaptation to oxygen concentration and tissue-specific energy requirements
NDUFA4	Oxygen dependent	NDUFA4	Ubiquitous?	Modifcation of supercomplexes?
		NDUFA4L2	Lung-type?	Adaptation to oxygen concentration
VIa	Developmental	COX6a1 COX6a2	Liver-type Heart-type	Modulation of proton pumping stoichiometry and thermogenesis
VIb	Developmental	COX6b1 COX6b2	Somatic Testes	Dimerization of COX and platform for tissue specific signaling/PTM?
VIIa	Developmental	COX7a1 COX7a2	Heart-type Liver-type	Adaptation to tissue-specific energy requirements
		COX7a3	Ubiquitous	Supercomplex assembly and stabilization
VIII	Developmental	COX8-1 COX8-2 COX8-3	Heart-type Liver-type Testes, pancreas, placenta	Adaptation to tissue-specific energy requirements

transfer rate, allowing efficient respiration despite low oxygen substrate levels [34,53]. A similar model was proposed for human COX subunit IV differential expression of the two isoforms, which is dependent on developmental stage and oxygen levels. Further highlighting the function of the isoform being regulated by oxygen tension, the isoform pair arose due to a gene duplication event about 320 million years ago at a time when atmospheric oxygen levels fluctuated and were higher compared to the current 21 %, possibly allowing adaptation to varying oxygen concentrations [34,54]. The two isoforms share 56 % sequence homology at the protein level but only 44 % nucleotide homology [34,55].

The principal isoform of subunit IV (COX4i1) is ubiquitously expressed and as mentioned earlier, can modulate COX activity through ATP/ADP allosteric regulation. The second isoform, COX4i2, is expressed in a tissue-specific manner and in response to oxygen concentration changes. Quantitative PCR in rat tissues showed that *Cox4i2* is primarily lung tissue-specific with comparable expression levels of *Cox4i1*, in addition to expression in the placenta [56]. It was also found to have low expression in heart (8 %) and brain (4 %) but no expression in liver or pancreatic tissue [21,34]. This isoform is also regulated developmentally, where *Cox4i2* is highly induced in the lung after birth [34]. The expression of the isoform is controlled by an oxygen responsive element (ORE) near the promoter, where under 4 % oxygen levels, there was maximal expression of *Cox4i2* with a three-fold induction compared to normoxic conditions [21]. This isoform has also been shown to be under the control of MNRR1 [57–59] and HIF-1 [60,61] under low oxygen conditions. Regulation of COX4i2 expression by oxygen appears to be unique to mammals, as in fish and reptiles, the transcript levels remain unchanged in response to varying oxygen concentrations [59].

COX enzyme purified from cow lung, which contains a mixture of both isoforms, was shown to have about two-fold increased basal activity compared to purified COX from cow liver, which only contains the ubiquitously expressed isoform [21]. Interestingly, unlike COX4i1, COX4i2 contains three cysteine residues where one is located in the transmembrane region and two on the matrix side near the proposed ATP binding site. The two cysteines on the matrix side are close enough to potentially form a disulfide bond, and the transmembrane cysteine residue may interact with other proteins to participate in redox reactions. We propose that the presence of these cysteine residues that are only present in COX4i2 but not in the ubiquitous isoform allows for redox signaling such as in response to hypoxic conditions. We earlier established a *Cox4i2* knockout mouse model and found a reduction in COX activity and decreased ATP levels. These knockout mice also display lung pathophysiology including reduced airway responsiveness due to a decreased ability for the airways to constrict [21]. High enzyme activity and ATP are required in response to low oxygen to allow the blood vessels to constrict and shunt blood flow to more efficient areas of the lung for oxygen uptake into the blood. Under acute hypoxic conditions, specialized cells of the lungs respond to optimize arterial oxygenation and blood flow by triggering hyperventilation and hypoxic pulmonary vasoconstriction (HPV) [62]. Although the exact mechanism of oxygen sensing is not well understood, mitochondrial hyperpolarization and thus increase of superoxide, which is then converted to hydrogen peroxide, causing cellular depolarization, is crucial to triggering HPV [63]. COX4i2 is essential to this response and is thought to be the main component of the primary oxygen sensor because the HPV response was abolished in *Cox4i2* knockout mice. Pulmonary arterial smooth muscle cells (PASMCs) isolated from *Cox4i2* knockout mice did not show an increase in intracellular calcium influx in response to hypoxia, a key downstream signal that triggers HPV. Additionally, unlike in wild-type mice, mitochondrial superoxide release was also absent.

2.1.2. *NDUFA4 isoforms*

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), a nuclear-encoded transmembrane protein initially thought to be a part of complex I, was recently found to be a structural component of COX and represents its 14th subunit [51,64]. Previous methods to purify and separate the different complexes of COX using stronger detergents were not able to preserve the bound NDUFA4 subunit: therefore, it was lost in the purification process and went undetected. Another important point to mention is that previous methods used to purify and separate COX subunits were almost always done using the homodimeric form. Balsa and colleagues were able to demonstrate that NDUFA4 was associated with COX by using the mild detergent digitonin, which solubilized the mitochondrial membrane to isolate intact COX while preserving protein-protein interactions [51]. Recently, Zong and coworkers provided structural evidence of the incorporation of NDUFA4 as part of the COX monomer at 3.3 Å resolution derived from human $I+III_2+IV_1$ supercomplex [65]. The authors noted that NDUFA4 lies at the dimeric interface of the COX homodimer, which would prevent dimerization.

Its role as a functional component of COX is strengthened by the identification of homozygous donor splice site mutations in NDUFA4 that resulted in protein loss of function and COX deficiency [66]. Additionally, NDUFA4 deletion was associated with COX deficiency in Leigh Syndrome [67]. The isoform of NDUFA4 known as NDUFA4L2 has mainly been studied in the context of complex I [68] but likely replaces NDUFA4 in a tissue-specific manner, somewhat similar to the COX4-i1/COX4i2 isoform pair in specific cell types of the lung including lung pericytes [69], the carotid body [70], and cerebral pericytes [71]. Regarding pathological conditions, both NDUFA4 and its isoform have

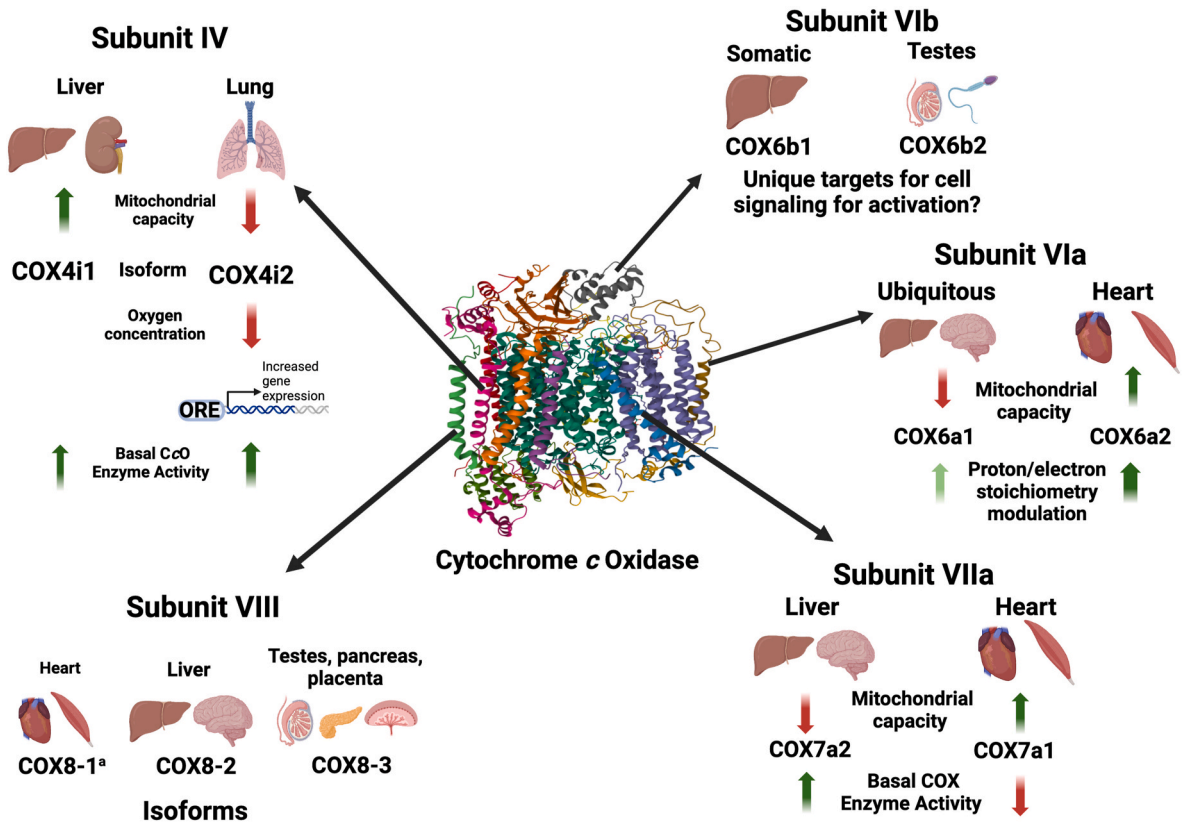

Fig. 2. COX subunits with isoforms including position on enzyme, tissue specificity and function. Subunit IV: pink, Subunit VIa: yellow, Subunit VIb: grey, Subunit VIIa: blue, Subunit VIII: light green. Crystal structure of bovine heart COX monomer (PDB DOI: https://doi.org/10.2210/pdb6JY3/pdb). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

been heavily studied in the context of cancer. In pancreatic adenocarcinoma, NDUFA4 was found to promote cell proliferation by increasing oxidative phosphorylation [72]. Overexpression in SW1990 cells significantly increased COX specific activity and ATP levels with slight increases to complex I and III activity. NDUFA4L2 transcriptional regulation through changes in oxygen concentrations is mediated by HIF-1 stabilization under hypoxic conditions, leading to NDUFA4L2 induction [68]. Interestingly, in HeLa cells, endogenous expression of the NDUFA4L2 isoform reduced oxygen consumption by 42 % under hypoxia, compared to when the expression was knocked down by 80 %, resulting in only a 27 % decreased oxygen consumption rate. This was proposed to be mediated through complex I inhibition. COX and Complex I specific activity were decreased by 38 % and 20 % under hypoxia, respectively. Silencing of the NDUFA4L2 isoform resulted in increased ROS and $\Delta\Psi_m$ and the same decrease in COX activity previously seen. However, the previously observed decrease in Complex I-specific activity was abolished. These findings suggest that this isoform may play a different role as a regulator of COX, such as potentially modulating supercomplex formation, and may have different functions depending on the cell type.

2.2. Developmentally induced isoforms

Subunit isoforms that are expressed differentially depending on the specific tissue type include VIa, VIb, VIIa, and VIII, in addition to the aforementioned oxygen-regulated isoforms. Subunits VIa, VIIa and VIII are expressed in most tissues as the so-called 'liver-type' (L), except for heart and skeletal muscle where the 'heart-type' (H) isoform is expressed instead. During fetal development, these subunits are expressed as the liver-type, and after birth, they are switched to the heart-type isoforms [73–75]. The mRNAs for the L-isoforms are expressed in all tissues, but the protein level is post-transcriptionally regulated by glutamate dehydrogenase (GDH) in a tissue-specific manner [76].

2.2.1. Subunit VIa isoforms

For complexes I and III of the ETC, the proton to electron stoichiometry remains constant, and for every electron transferred, two protons are pumped. Complex IV is unique in that it can adjust its stoichiometry under different conditions. Subunit VIa appears as an "S-shaped hook" connecting two COX monomers to stabilize the dimer and is thought to play a role in regulating the proton pumping efficiency. Usually, COX pumps one proton per electron transferred; however, it has been shown that COX purified from cow kidney, which expresses the liver-type isoform (COX6a1), had decreased proton to electron stoichiometry in the presence of the fatty acid palmitate [41]. COX purified from heart, which contains the heart isoform, did not show such an effect. The VIa heart isoform (COX6a2) was shown to bind to allosteric regulator ADP on the matrix side increasing enzyme activity and at high ATP/ADP ratios showed a 50 % decrease in proton to electron stoichiometry like the liver type isoform exposed to palmitate [77]. It was proposed that this effect may contribute to thermogenesis at high ATP/ADP ratios due to ATP-mediated intrinsic uncoupling. Isoform Cox6a2 knockout mice showed reduced COX activity and, interestingly, no changes in myocardial ATP levels under basal conditions, but they developed cardiomyopathy [19]. Incorporation of the liver isoform to compensate for loss of COX6a2 may explain the relatively mild phenotype in mice. Taken together, these findings highlight the importance of the heart isoform of subunit VIa on COX function in heart and skeletal muscle, tissues with high energetic demands.

2.2.2. Subunit VIb isoforms

Subunit VIb exists as a somatic (COX6b1) and testes (COX6b2)

isoform [78]. This subunit is located solely in the intermembrane space (IMS) side of COX and plays a role in connecting the two COX monomers by forming a teepee shape. If this subunit is removed from the enzyme, the enzyme still functions, but with a twofold increase in enzyme activity suggesting its importance in stabilizing dimerization, which itself regulates COX activity [79]. In rodents, COX6b2 is found only in the testes but in humans and bulls both isoforms are present. Interestingly, there is also a testes specific isoform of Cyt*c* present in most mammals; however, it is now a pseudogene in humans [80]. This suggests that there is coregulation of respiration at the level of COX and Cyt*c* in testes. This may be one way spermatozoa modulate COX activity to meet the unique energy demands through cell-specific signaling pathways where activation is only required upon movement. The presence of the different isoforms may also be a platform for cell type-specific signaling and PTMs.

2.2.3. Subunit VIIa isoforms

Subunit VIIa has three isoforms, with the liver-type isoform (COX7a2) and heart-type isoform (COX7a1) known since the mid-1980s [73,81]. Although the transcript levels of the liver isoform can be detected in muscle tissue, the expression of the protein is not present, implying that there is a posttranscriptional level of regulation for this gene as discussed above [76,82]. Whole body knockout of *Cox7a1* in mice leads to 32 % decreased COX specific activity and decreased respiratory control ratio, a measure of mitochondrial coupling [20,83]. When comparing the wild-type versus *Cox7a1* knockout mice, the wild-type mice were able to run 38 % longer than the knockouts when subjected to an incremental treadmill exercise test. Regarding anatomical effects of this knockout, there was a decrease in the capillary indices and differences in the fiber cross-sectional area and perimeter. These findings highlight the specific role this isoform plays in COX activity that contributes to muscle function and structure in addition to the vascular system that directly supplies these areas.

The third isoform of subunit VIIa was proposed to have a role in supercomplex formation. Supercomplexes are higher-order multi-complex structures that exist in various stoichiometries made up of the ETC complexes I, II, and IV. The gene that encodes this isoform, *COX7aR* (also known as *COX7a2L* or *SIG81*), was first identified through a silica-induced gene library [84]. This isoform is expressed in most tissues similar to the liver isoforms, with the highest expression in the kidney and liver. Due to recent studies on the function of this isoform that suggest it is crucial for supercomplex formation, it was renamed supercomplex assembly factor I (*SCAFI*) [85]. This was done by screening the respiratory complexes for proteins that are present in the supercomplexes only versus the free complexes. The study reported that the gene encoding SCAFI was 6 base pairs shorter, resulting in a 2 amino acid deletion in immortalized C57BL/6 mouse fibroblasts, which resulted in a defect of supercomplex formation [85]. However, truncated SCAFI in C57BL/6 mice showed no changes in biogenesis, had no respiratory defects, and normal levels of COX were incorporated into the supercomplexes; however, there was a difference in supercomplex subtypes [86,87]. In another study, the authors showed that SCAFI was required for the interaction between complex III dimer and COX and that different isoforms of subunit VIIa play a role in the different stoichiometries of the individual supercomplexes [88]. A separate study found that SCAFI binds mostly to free complex III and, to a lesser extent, to free COX and was required for complex III_2+complex IV supercomplex formation [89]. In human cells and postmitotic tissues, different COX7a isoforms, COX7a1, COX7a2, and SCAFI (COX7a2L), drive the formation of structurally distinct supercomplexes with varying bioenergetic properties [90,91]. In pig heart tissue, COX subunit VIIa facilitates the binding of complex IV to complex I and III in the I+III_2+IV supercomplex [92]. It is important to note, however, that the resolution of the crystal structure at 4 Å could not determine whether it was SCAFI or the heart isoform of subunit VIIa.

2.2.4. Subunit VIII isoforms

Subunit VIII is the smallest nuclear-encoded subunit, in which there are three known isoforms in rodents and two in humans. Again, the liver isoform of VIII (COX8-2) is expressed in most tissues [93,94]. In most mammals, including rodents, there is expression of the heart type isoform (COX8-1), which in catarrhines including humans has become a pseudogene [95]. Subunit VIII was found to have roles in supercomplex I+III_2+IV stabilization where it has direct contact with three subunits of complex I [92]. In a patient with Leigh-syndrome-like symptoms, there was an identified G to C transversion in the *COX8* gene that led to a nonfunctional protein and resulted in only 10 % COX activity in skeletal muscle and fibroblasts that could be restored by expressing the wild-type COX8 [96]. These findings highlight the importance of subunit VIII in COX function and stability. The third isoform of subunit VIII (COX8-3) has been identified in many mammalian species, including humans [97]. This isoform was detected at the highest levels in testes, pancreas, and placenta, but its functional role is poorly understood.

Six of the nuclear encoded subunits of COX have been found to express isoforms where functional studies have highlighted their role in modulating tissue specific regulation of enzyme activity. These subunits and their isoforms do not take part in the catalytic reaction but have, in some cases, well defined roles in the structure and functional regulation of COX. A proposed reason for the expression of these tissue-specific isoforms is due to the different mitochondrial capacities in a given tissue, such as in heart and skeletal muscle, which contain high amounts of mitochondria, versus other tissues, such as liver or brain, that do not have the capacity for such a high mitochondrial load and are therefore equipped with the liver isoforms that have higher basal activity [98]. Another example is the lung where there are even fewer mitochondria present, and therefore the lung-specific isoform allows higher basal activity with fewer mitochondria [99]. Another proposed purpose of these tissue-specific isoforms is to allow for tissue-specific cell signaling, resulting in PTMs and/or differential recognition for allosteric regulation.

3. Post-translational modifications

For over half a century, it has been known that protein PTMs are essential for mitochondrial metabolism and function with the well-characterized pyruvate dehydrogenase (PDH) regulation through phosphorylation [100,101]. PTMs are regulatory modifications that allow rapid and reversible functional adaptations induced and dependent on cell-signaling pathways. There have been identified PTMs on all the OxPhos complexes and Cyt*c*. The first phosphorylation on COX discovered and reported was on subunit IV-1, which was found using radioactive ATP labeling in mitochondria [102]. Since then, numerous phosphorylations and acetylations have been identified on various COX subunits. Despite the signaling pathways not being well defined, it is well established that PTMs have a regulatory function on the activity of COX. Current mass spectrometry (MS) screening tools have allowed researchers to discover and map many mitochondrial phosphorylation and acetylation sites. To date, over 100 mapped phosphorylations and acetylations have been identified across various COX subunits, summarized in Tables 2 and 3, respectively, most of which were identified by high-throughput MS experiments. However, these studies do not provide biological context but rather possible sites that can be modified. It is likely that a larger fraction of these PTMs, especially phosphorylations and acetylations, occur non-enzymatically because ATP and acetyl-CoA are reactive molecules that can react with amino acids such as serine, threonine, tyrosine, and lysine, respectively. It is therefore important to show that a given PTM is present on a larger portion of the protein pool to be biologically relevant. Arguably, it is unlikely for a PTM to be biologically relevant if less than a percent of the protein pool carries the modification. MS based methods have become very sensitive, allowing "background noise" PTMs to be easily detected. Therefore, it is essential that quantitative approaches including MS-based methods should be

Table 2
Identified phosphorylation sites of mammalian COX subunits for serine/threonine (S/T) and tyrosine (Y) residues. [a]Numbering is based on the mature peptide sequence on left and the right based on of the full-length sequence that includes the precursor. [b]Denotes phosphorylated amino acid identified is located in leader sequence and is not part of the mature protein. [c]Epitope sequence according to species identified. LTP: low-throughput, I/R: ischemia/reperfusion, HTP-MS: high-throughput mass spectrometry.

Subunit	Residue[a] Mature/Full Length	Species/Tissue	Phospho-epitope[c]	Reference	Method	Comment
COX1	S115	Rabbit heart	SLHLAGVSSILGAINF	132	LTP after I/R followed by MS	COX-inhibitory; PKA mediated
COX1	S116	Rabbit heart	SLHLAGVSSILGAINF			
COX1	Y304	Cow liver	MDVDTRAYFTSATMI	15, 130	LTP followed MS	COX-inhibitory; cAMP dependent/ inflammation signaling
COX2	S126	Cow heart	DSYMIPTSELKPGEL	140	HTP-MS	
COX2	Y218	Cow heart	LELVPLKYFEKWSAS	140	HTP-MS	
COX3	Y67	Human PC9	DVTRESTYQGHHTPP	168	HTP-MS	
COX4i1	S4/S26	Human Hues-9	VCVRAHESVVKSEDF	174	HTP-MS	
		Mouse liver	VCLRAHGSVVKSEDY	103, 139		
COX4i1	S8/S30	Human HeLa	AHESVVKSEDFSLPA	169, 170	HTP-MS	
		Rat brain and heart	AHGSVVKSEDYALPS	146		
COX4i1	S12/S34	Human liver	VVKSEDFSLPAYMDR	175	HTP-MS	
COX4i1	S15/S37	Rat brain and heart	SEDYALPSYVDRRDY	146	HTP-MS	
COX4i1	Y16/Y38	Human PC9	EDFSLPAYMDRRDHP	168	HTP-MS	
COX4i1	S34/S56	Mouse liver	VAHVTMLSASQKALK	103	HTP-MS	
		Multiple rat organs	VAHVKLLSASQKALK	146		
		Cow heart	VAHVKNLSASQKALK	137		
COX4i1	S36/S58	Mouse heart and cardiac fibers	HVTMLSASQKALKEK	32, 135	LTP followed MS	Promotes ATP allosteric inhibition; cAMP-PKA mediated
		Mouse heart, liver, brain, 3T3-L1 (adipocyte)	HVTMLSASQKALKEK	103, 139 144, 145, 147, 149,	HTP-MS	
		Cow heart	HVKNLSASQKALKEK	137		
		Multiple rat organs	HVKLLSASQKALKEK	146		
COX4i1	S50/S71	Multiple rat organs	EKEKADWSSLSRDEK	146	HTP-MS	
COX4i1	S51/S72	Human skeletal muscle	KEKASWSSLSMDEKV	113	HTP-MS	
		Mouse brain and kidney	KEKADWSSLSRDEKV	147, 172		
		Multiple rat organs	KEkADWSSLSRDEKV	146		
COX4i1	S53/S74	Human PC9	KASWSSLSMDEKVEL	168	HTP-MS	COX-inhibitory; PKA mediated
	T52	Rabbit heart	KAPWGSLTRDEKVEL	132	LTP after I/R followed by MS	
COX4i1	S67/S89	Human HeLa	YRIKFKESFAEMNRG	171	HTP-MS	
COX4i1	Y102/Y124	Human PC9	VIMWQKHYVYGPLPQ	168	HTP-MS	
COX4i1	S110/S132	Human HeLa	VYGPLPQSFDkEWVA	169	HTP-MS	
COX4i1	S135/S157	Mouse HL-1	ANPIQGFSAkWDYDk	146	HTP-MS	
		Rat intestine and kidney	VNPIQGFSAKWDYNK	144		
COX4i1	S136/S158	Human A498, HeLa	NPIQGLASKWDYEKN	171	HTP-MS	
COX4i2	S8[b]	Rat intestine and thymus	MFSRATRSLVMKTGG	146	HTP-MS	
COX5A	S1/S44	Cow heart	VQSLRCYSHGSHETD	140	HTP-MS	
COX5A	S4/S47	Cow heart	LRCYSHGSHETDEEF	137	MS	
COX5A	T7/T44	Mouse 3T3 fibroblasts	YSHGSHETDEEFDAR	177	HTP-MS	
COX5A	T35/T76	Human Hela	ELRKGINTLVTYDMV	171	HTP-MS	
		Cow heart	ELRKGMNTLVGYDLV	137		
COX5A	T38/T79	Human HeLa	KGINTLVTYDMVPEP	140	HTP-MS	
COX5A	Y39/Y80	Human PC9	GINTLVTYDMVPEPK	168	HTP-MS	
COX5A	T100/T141	Human liver	LNELGISTPEELGLD	175	HTP-MS	
COX5B	S2/S33	Cow heart	SVVRSMASGGGVPTD	140	HTP-MS	
COX5B	Y31/Y62	Human PC9	AKKGLDPYNVLAPKG	168	HTP-MS	COX-inhibitory; PKA mediated
COX5B	S40	Rabbit heart	MLPPKAASGTKEDPN	132	LTP after I/R followed by MS	
COX5B	S93/S124	Pig heart	GTHYKLVSHQLAH	114	HTP-MS	
COX6A	T11	Cow heart	AKGDHGGTGARTWRF	141	Crystal Structure	
COX6B1	T44	Human HeLa	HRCQKAMTAKGGDIS	178	HTP-MS	
COX6B1	S51	Human HeLa	TAKGGDISVCEWYQR	178	HTP-MS	
		Mouse brown fat, brain, heart, liver	TAKGGDVSVCEWYRR	103, 147, 149, 172		
COX6B1	S70	Mouse kidney, liver	LCPVSWVSAWDDRIA	103, 172	HTP-MS	
COX6B1	T80	Human HUES9	DEQRAEGTFPGKI	174	HTP-MS	
COX6B2	T63	Human HEK	CEYYFRVYHSLCPIS	180	HTP-MS	
COX6B2	S65	Human HEK	YYFRVYHSLCPISWV	180	HTP-MS	
COX6C	S2[b]	Mouse liver	____MSSGALLPK	144	HTP-MS	
		Rat heart and stomach	____MSSGALLPK	146		
COX6C	S3[b]	Mouse liver	___MSSGALLPKP	103, 144	HTP-MS	
COX6C	Y47/Y49	Human PC9	ADQRKKAYADFYRNY	168	HTP-MS	
COX6C	Y51/Y53	Human PC9	KKAYADFYRNYDVMK	168	HTP-MS	
COX6C	S71/S73	Human breast cancers	RKAGIFQSVS___	179	HTP-MS	
		Rat brain, intestine and kidney	RQAGVFQSAK___	146		
COX7A2	Y21/Y44	Human PC9	EDDEIPLYLkGGVAD	168	HTP-MS	
COX7A2	Y32/Y55	Human HUES9, PC9	GVADALLYRATMILT	168, 174	HTP-MS	

(*continued on next page*)

Table 2 (*continued*)

Subunit	Residue[a] Mature/Full Length	Species/Tissue	Phospho-epitope[c]	Reference	Method	Comment
COX7A3	S47[b]	Human PC9	ATPTKLTSDSTVYDY	168	HTP-MS	
COX7A3	Y52[b]	Human SK-N-BE, PC9, bone marrow	LTSDSTVYDYAGKNK	168, 173	HTP-MS	
COX7A3	Y32/Y87	Human HeLa	GLPDQMLYRTTMALT	171	HTP-MS	
COX7B	S2/S32	Mouse liver, 3T3-L1	SHQKRAPSFHDKYGN	103, 144, 145	HTP-MS	
COX7C	S5[b]	K562	__MLGQSIRRFTTS	181	HTP-MS	
COX7C	S1/S17	Human skeletal muscle	TTSVVRRSHYEEGPG	113	HTP-MS	
		Mouse 3T3-L1	TTSVVRRSHYEEGPG	139		
		Cow heart	TTSVVRRSHYEEGPG	140		
NDUFA4	S56/S66	Human Jurkat, K562	NDQYKFYSVNVDYSK	179, 181	HTP-MS	
		Mouse brown fat, BaF3, brain, heart, liver	NEQYKFYSVNVDYSk	103, 147, 172		
		Rat heart, intestine	NEQYKFYSVNVDYSK	146		
NDUFA4	Y61/Y71	Human PC9	FYSVNVDYSKLKKER	168	HTP-MS	
NDUFA4	S62/S72	Rat heart, intestine	YSVNVDYSKLKKEGP	146	HTP-MS	

used to demonstrate what portion of the protein carries the PTM.

PTMs are highly dynamic across various physiological and disease states, highlighting their importance [103–105]. MitoCarta suggests there are at least 25 known kinases that localized to the mitochondria. The mechanism in which many of these kinases are transported and folded into the mitochondria is not well understood [106]. As a better understood example, it was shown that the internalization of the membrane spanning receptor tyrosine kinases EGFR and ERBB2, which act on COX, is clathrin-mediated and it is assumed that clathrin coated vesicles then fuse with the mitochondrial membrane [17,48], exposing their kinase domain inside the mitochondria. For many of the soluble kinases that have been shown in the mitochondria, their mechanism of import is not known. One possibility is that some kinases are resident to the mitochondria and can be regulated through signal transduction pathways that are transmitted across the outer (for IMS targets) and inner (for matrix targets) mitochondrial membranes. Another possibility is that, upon activation, kinases from the cytosol are imported into the mitochondria, but it is unknown if this would require unfolding and traditional import through the TOM and TIM mitochondrial protein import machinery or an entirely different mechanism. Another point to strengthen the importance that PTMs play in mitochondria is the existence of resident protein phosphatases that are highly conserved across eukaryotes strengthened by their ancient origins [107]. MitoCarta suggests there are 12 mitochondrial candidate resident protein phosphatases that possess five distinct catalytic domains with specific residue preferences for substrate specificity and are also shown to be subcompartmentalized [106,108–110]. Analogous with the expression of tissue-specific COX isoforms, which can also provide a platform for cell signaling and PTMs, mitochondrial phosphatases are also suggested to be tissue-specific [111]. In a global mouse knockout of matrix localized mitochondrial phosphatase *Pptc7*, the animals displayed severe metabolic abnormalities and fully penetrant perinatal lethality within one day of birth [112]. There was also a change in mitochondrial content, highlighting the importance that PTM regulation has on proper mitochondrial health and development.

Phosphorylations on mitochondrial proteins as a means of regulation has been well known and it has been shown that ~91 % of these proteins have at least one phosphorylation site [106]. These findings highlight the importance and prevalence phosphorylation has across the mitochondrial proteome. Zhao and coworkers analyzed the phosphoproteome of resting human muscle which showed extensive phosphorylations on IMM proteins including the ETC complexes [113]. Despite the numerous sites mapped, it is important to consider the earlier point on whether these sites are biologically relevant or unspecific background signal. It is estimated that less than 5 % of the mapped sites have been investigated and characterized and even within these characterizations, many do not report aspects such as fraction of phosphorylated versus unphosphorylated protein [106]. Without further functional validation, it is difficult to know which sites are biologically relevant to the function and regulation of these proteins versus basal or unspecific phosphorylation [114]. Furthermore, it is possible that a subset of the detected PTM sites identified through high-throughput and even some low-throughput experiments could be due to protein phosphorylation in the cytosol before transport into the matrix and/or autophosphorylation [112,115]. Many phosphorylations are detected when enriching for phosphopeptides which suggests the mole fraction that is phosphorylated is low and may explain why some sites result in minimal or no enzymatic change. Despite not knowing how many of the PTMs possess a biological function, it cannot be disputed that at least a subset of these modifications are important to the homeostasis and regulation of mitochondrial function and bioenergetics. These high-throughput screens provide an important starting point and means of validation to begin characterizing the significance these PTMs have on COX and thus their biological relevance.

One method that allows quantitative monitoring of these modifications between samples is iTRAQ (multiplexed isobaric tagging technology for relative quantitation) labelling followed by MS [114]. This approach also allows identifying phosphorylation sites that are modulated by signaling pathways. This method labels the peptides with up to four to eight isobaric chemical tags in either a four-plex or eight-plex experiment. In pig heart mitochondria, for example, treated without or with (0.65 μM) calcium had 32 phosphorylation sites across 21 proteins with a 3.3-fold dephosphorylation rate at site S292 of PDH following calcium treatment. Corresponding PDH assays showed a 3.3-fold activation of activity as a result of dephosphorylation. These results confirmed previous work on the effect of calcium on PDH phosphorylation and activity as a proof-of-concept that this method can quantitively map and compare PTMs in a high-throughput way.

The different phosphorylation sites of COX exist in specific tissues and may have different effects on enzymatic function likely due to difference in stimuli and tissue-specific signaling. This can be further explained by the different kinases and a substrate specific phosphorylation site. The exact signaling pathway or kinase for many of the sites still remains not well understood. Only a few sites have been identified by low-throughput approaches. Protein kinase A (PKA) has been among the top signaling pathways studied in the context of COX modification and has been shown to interact directly with COX [116]. PKA has been shown to translocate into mitochondria and has thus been a kinase of interest for targeting COX subunits [117,118]. However, the cell-specific signaling of PKA activation that results in a PTM of COX is not well understood. Besides PKA, there have been other kinases that have been shown to translocate to mitochondria such as Src [119], Aurora kinases [120] and EGFR [48], suggesting a much more dynamic mode of operation than previously thought. Phosphorylation of a tyrosine residue on

Table 3
Identified acetylation sites of mammalian COX subunits for lysine (K) residues. [a]Numbering is based on the mature peptide sequence on left and the right based on of the full-length sequence which includes the precursor. [b]Mediates oxidative stress induced apoptosis. [c]Denotes acetylated amino acid identified is located in leader sequence and is not part of the mature protein. [d]Epitope sequence according to species identified. LTP: low-throughput, I/R: ischemia/reperfusion, HTP-MS: high-throughput mass spectrometry, H_2O_2: hydrogen peroxide.

Subunit	Residue[a]	Species/Tissue	Acetyl-epitope[d]	Reference	Method
	Mature/Full Length				
COX1	K13[b]	Human, 3T3 fibroblasts, rat neurons	WLFSTNHKDIGYLYL	152	LTP after I/R or H2O2
COX1	K264[b]	Human, 3T3 fibroblasts, rat neurons	IVTYYSGKKEPFGYM	152	
COX1	K319[b]	Human, 3T3 fibroblasts, rat neurons	IAIPtGVKVFSWLAT	152	
COX1	K481[b]	Human, 3T3 fibroblasts, rat neurons	EAFASKRKVLMVEEP	152	
COX3	K224	Rat kidney	VCLLRQLKFHFTSKH	150	HTP-MS
COX4i1	K7/K29	Mouse liver	RAHGSVVKSEDYAFP	155, 160	HTP-MS
		Multiple rat organs	RAHGSVVKSEDYALP	150	
COX4i1	K31/K31	Human A549, HeLa, MV4-11	LPEVAHVKHLSASQK	153, 154, 160	HTP-MS
		Multiple rat organs	LPDVAHVKLLSASQK	150	
COX4i1	K38/K60	Human A549, HeLa, Jurkat	KHLSASQKALKEKEK	153, 160	HTP-MS
		Mouse colon	TMLSASQKALKEKEk	156	
		Multiple rat organs	KLLSASQKALKEKEK	150	
COX4i1	K43/K65	Rat brown fat, brain, heart, kidney, stomach, thymus	SQKALKEKEKADWSS	150	HTP-MS
COX4i1	K45/K67	Human HeLa	KALKEKEKASWSSLS	160	HTP-MS
		Multiple rat organs	KALKEKEKADWSSLS	150	
COX4i1	K56/K78	Mouse liver, rat	SSLSRDEKVQLYRIQ	160	HTP-MS
		Multiple rat organs	SSLSRDEKVQLYRIQ	150	
COX4i1	K65/K87	Human A549, HeLa, Liver	ELYRIKFKESFAEMN	154, 157, 160	HTP-MS
COX4i1	K73/K95	Multiple rat organs	ESFAEMNKGTNEWKT	150	HTP-MS
COX4i1	K113/K135	Human A549	PLPQSFDKEWVAKQT	154	HTP-MS
COX4i1	K127/K149	Rat heart	TKRMLDMKVNPIQGF	150	HTP-MS
COX4i1	K137/K159	Mouse	PIQGFSAKWDYDKNE	160	HTP-MS
		Rat liver	PIQGFSAKWDYNKNE	150	
COX4i1	K142/K164	Human A549	ASKWDYEKNEWKK_	154	HTP-MS
		Mouse liver	SAKWDYDKNEWKK_	155, 160	
		Multiple rat organs	SAKWDYNKNEWKK_	150	
COX4i1	K146/K168	Rat kidney and stomach	DYNKNEWKK	150	HTP-MS
COX5A	K21/K62	Human A549, HeLa	RWVTYFNKPDIDAWE	154, 160	HTP-MS
		Mouse liver	RWVTYFNKPDIDAWE	155	
		Multiple rat organs	RWVTYFNKPDIDAWE	150	
COX5A	K31/K68	Mouse liver	IDAWELRKGMNTLVG	160	HTP-MS
		Rat heart	IDAWELRKGMNTLVG	150	
COX5A	K46/K87	Human HeLa	YDMVPEPKIIDAALR	160	HTP-MS
		Mouse colon and liver	YDLVPEPKIIDAALR	155, 156	
		Multiple rat organs	YDLVPEPKIIDAALR	150	
COX5A	K72/K109	Mouse colon and liver	VRILEVVKDKAGPHK	155, 156, 160	HTP-MS
COX5A	K108/K145	Mouse liver	PEELGLDKV____	155, 156, 158, 160	HTP-MS
		Rat white fat, brain, heart, kidney and liver	PEELGLDKV____	150	
COX5B	K26/K57	Mouse colon	EIMIAAQKGLDPYNM	156	HTP-MS
COX5B	K37/K67	Mouse colon	PYNMLPPKAASGTKE	155, 156	HTP-MS
		Rat brain, heart and stomach	PYNMLPPKAASGTKE	150	
COX5B	K43/K73	Mouse colon and liver	PKAASGTKEDPNLVP	155, 156, 160	HTP-MS
		Multiple rat organs	PKAASGTKEDPNLVP	150	
COX5B	K55/K86	Mouse colon and liver	LVPSISNKRIVGCIC	156, 158	HTP-MS
		Multiple rat organs	LVPSVSNKRIVGCIC	150	
COX5B	K76/K107	Human A549	VVWFWLHKGEAQRCP	154	HTP-MS
		Mouse	VIWFWLHKGESQRCP	155	
COX5B	K90/K121	Human A549	PRCGAHYKLVPQQLA	153	HTP-MS
		Mouse liver	PNCGTHYKLVPHQMA	158	
		Rat heart	PNCGTHYKLVPYQMV	150	
COX6C	K9	Mouse liver	SSGALLPKPQMRGLL	160	HTP-MS
		Multiple rat organs	SSGALLPKPQMRGLL	150	
COX6C	K18	Rat white fat, brain, heart and stomach	QMRGLLAKRLRVHIV	150	HTP-MS
COX6C	K48	Rat brown fat, heart and pancreas	GVAEPRKKAYADFYR	150	HTP-MS
COX6C	K60	Human A549	YRNYDVMKDFEEMRK	154	HTP-MS
	K61	Mouse liver	YRNYDSMKDFEEMRK	159, 160	
		Multiple rat organs	YRNYDSMKDFEEMRQ	150	
COX6C	K76	Mouse liver	AGIFQSAK_____	158	HTP-MS
COX7a1	K10/K31	Mouse	NRVAEKQKLFQADND	155	HTP-MS
COX7a2	K10/K33	Human HeLa	NKVPEKQKLFQEDDE	160	HTP-MS
		Mouse liver	NKVPEKQKLFQEDNG	155, 156	
		Multiple rat organs	NKVPEKQKLFQEDNG	150	
COX7a2	K23/K46	Rat heart	NGMPVHLKGGTSDAL	150	HTP-MS
COX7a3	K4[c]	Human A549	___MYYKFSGFTQK	154	HTP-MS
COX7a3	K10/K64	Mouse	NKVPELQKFFQKADG	155	HTP-MS
COX7a3	K14/K69	Human A549, MV4-11	ELQKFFQKADGVPVY	153	HTP-MS
COX7a3	K75	Rat brain, heart, muscle, stomach, thymus	PVGRVTPKEWRDQ_	150	HTP-MS
COX7C	K9/K25	Mouse colon, liver	HYEEGPGKNLPFSVE	156, 160	HTP-MS
		Multiple rat organs	HYEEGPGKNLPFSVE	150	
COX7C	K18/K34	Human A549	LPFSVENKWSLLAKM	154	HTP-MS

(*continued on next page*)

Table 3 (*continued*)

Subunit	Residue[a] Mature/Full Length	Species/Tissue	Acetyl-epitope[d]	Reference	Method
COX7C	K46/K62	Mouse liver	IVRHQLLKK____	158	HTP-MS
NDUFA4	K10[c]	Rat heart, kidney, stomach	RQILGQAKKHPSLIP	150	HTP-MS
NDUFA4	K45/K55	Human A549	NNPEPWNKLGPNDQy	154	HTP-MS
		Mouse liver	NNPEPWNKLGPNEQY	159, 160	
		Rat brain	NNPEPWNKLGPNEQY	150	
NDUFA4	K53/K64	Mouse liver	LGPNEQYKFYSVNVD	155, 160	HTP-MS
		Rat brain	LGPNEQYKFYSVNVD	150	
NDUFA4	K63/K73	Human A549	SVNVDYSKLKKERPD	154	HTP-MS
NDUFA4	K67/K74	Rat heart	VDYSKLKKEGPDF_	150	HTP-MS

COXII by activated c-Src resulted in increased COX activity in HEK cells [121]. In rat neonatal cardiac myocytes, PKCε was shown to translocate into mitochondria and interact with subunit IV, shown by ^{32}P labelling resulting in an overall increase of COX activity [122,123]. It is also possible that the known canonical kinases that exist in mitochondria such as PDH kinases (PDKs) have a wider substrate profile than originally thought. Proximity labeling studies show there are at least three PDK isoforms with non-PDH interactors [124] and that treatment of pig heart mitochondria with the pan-PDK inhibitor dichloroacetate and pyruvate decreased the phosphorylation of many unidentified mitochondrial proteins based on ^{32}P incorporation [125]. There are numerous kinases with well-defined substrates that reside in mitochondria such as adenylate kinases (AK2-4) for nucleotides, sphingosine kinase (SPHK2) for lipids, and phosphoenolcarboxykinase 2 (PCK2) for metabolites [106]. It is possible that other kinases such as the metabolic kinases could moonlight as a protein kinase and target COX subunits [126].

In purified cow heart COX incubated with PKA, cAMP and γ-^{32}ATP there was a phosphorylation on subunit Vb, either subunit II and/or III, and subunit I [127,128]. However, one should be cautious when interpreting these results as this was done in vitro with purified COX with auxiliary components missing, such as A-kinase anchoring proteins that are essential to the signaling cascade [129], and these results could therefore be unspecific. Follow up research on the role of cAMP dependent phosphorylation of COX using liver tissue and the phosphodiesterase inhibitor theophylline to increase cAMP levels showed that in the treated liver there was only a tyrosine phosphorylation of subunit I. MS mapped the phosphorylation site to tyrosine 304 (Y304) of COX subunit I (COX1) [130]. This phosphorylation reduced COX enzymatic activity, even in the presence of the allosteric activator ADP. Subunit I of COX is one of the catalytic subunits and the Y304 residue is located near to the oxygen binding site so its phosphorylation could lead to structural changes to alter enzymatic activity. Other molecules that increase cAMP levels such as the adenylyl cyclase activator forskolin and starvation hormone glucagon were also able to trigger the inhibitory effect on cAMP-dependent inhibition of COX. It is important to note that cAMP signaling to COX is tissue-specific. For example, in neuronal tissue following spinal cord injury, theophylline activated COX but had no effect in the heart [131]. Subsequently, the same residue was found to be phosphorylated in cow liver, simulating inflammatory conditions after treatment with the cytokine tumor necrosis factor alpha (TNFα), which also had strong inhibitory effect on the enzyme's activity [15]. Phosphorylation of Y304 resulted in pronounced sigmoidal kinetics and strong inhibition of oxygen consumption rate with a Hill coefficient close to 4. This result can be interpreted by dimerization of COX, assuming there is 1 productive and 1 non-productive Cyt*c* binding site per COX monomer. Although the initial signaling pathway may be different, both merge to potentially activate the same kinase to phosphorylate Y304 of subunit I, a residue which is conserved in eukaryotes and, based on crystal structure, is thought to be a part of an adenine nucleotide binding site and is also in contact with subunit VIa of the opposing monomer. Due to the hydrophobicity of subunit I, the protein sequence coverage by MS ranged from only 6–33 % and could result in other modifications being missed [15,130,132]. Recently, in a diabetic rat model, pY304 was found to be increased when rats were fed a high sucrose diet, leading to a drastic 80 % decrease in activity [24]. In this study, it was also determined that this phosphorylation promoted dimerization of the COX monomers, which helps to explain the previously found sigmoidal kinetics for Y304-phosphorylated COX.

Other groups have mapped cAMP-dependent phosphorylation sites of COX [32,133,134]. Acin-Perez and colleagues showed that S58 phosphorylation on subunit IV-1 promotes allosteric inhibition by ATP [32]. These studies demonstrate the regulation of cytosolic and matrix ATP/ADP regulation through interaction with subunit IV [33]. Interestingly, S58 phosphorylation on COX4i1 was reduced in hearts of septic mice, resulting in a decreased oxygen consumption rate [135]. In mouse cardiac fibers, it was shown that PKA regulates mitochondrial metabolism through phosphorylation of COX and other mitochondrial proteins [134]. The authors reported that cAMP is produced inside the mitochondrial matrix through the carbon dioxide/bicarbonate-regulated soluble adenylyl cyclase (sAC) in HEK cells. Furthermore, subunits I and IV were phosphorylated, increasing COX activity. In rabbit hearts and murine macrophage RAW 264.7 cells, PKA activity increased under hypoxic conditions (0.1 % oxygen for 10h) and was accompanied by hyperphosphorylation of COX subunits with a decrease in activity [133]. A follow-up study showed subunit I residues S115 and S116, subunit IV-1 T52, and subunit Vb S40 phosphorylation in rabbit heart tissue after ischemia/reperfusion and was verified by MS [132]. When treated with the kinase inhibitor H89, the phosphorylations were no longer present. It is unclear if these modifications are due to PKA signaling as H89 inhibits multiple kinases [136] and the identified sites do not have a traditional PKA consensus sequence.

There are additional phosphorylation sites of COX where the signaling pathways and functional studies are not known. In cow heart, subunits IV on Ser34 and Va on Ser4 and Thr35 are phosphorylated in vitro by PKA [137], with the same limitations as discussed above. In pig heart, subunit Vb S124 phosphorylation was identified [114]. In most other mammals, there is a proline residue rather than a serine in this position, located away from where the other subunits make contact and exposed to solvent, and therefore its functional significance may be minimal [138]. Subunit VIIc in human resting muscle was shown to be phosphorylated on S17 along with several other sites and was identified in separate high-throughput studies [113,139,140]. T11 phosphorylation on the heart isoform of subunit VIa in cow heart was suggested based on the crystal structure [141] but never reproduced, and S126 phosphorylation on subunit II was identified in three separate COX isolations of cow heart COX [142]. It is difficult to suggest any functional role these modifications have based on location alone. Subunit II is one of two catalytic subunits along with subunit I and accepts electrons donated from Cyt*c*. In cow heart, phosphorylation of Y218 on subunit II and S1 on subunit Va was observed in enzyme preparations with and without allosteric ATP-inhibition [140]. However, in the same samples, S2 on Vb was exclusively found in COX that showed allosteric ATP-inhibition. It was suggested that allosteric ATP-inhibition involves the binding sites for Cyt*c* in dimerized COX, and it is possible that this modification, which is located at the interface between the monomers,

promotes dimerization. Subunit IV has the most identified phosphorylation sites of COX and is uniquely located and in close contact with the two catalytic subunits I and II [50] and could therefore directly regulate enzyme activity. Subunit IV also has suggested ATP binding sites on both the matrix and IMS domains [143]. Finally, S58 phosphorylation on COX4i1 was identified in COX from cow heart tissue treated with fluoride, a non-specific S/T phosphatase inhibitor [137]. This modification showed a slight inhibitory effect when ATP was added but no sigmoidal kinetics were observed. Multiple high-throughput studies have also mapped phosphorylation on S58 on COX4i1 [103,137,139,140,144–151].

In addition, COX contains many acetylation sites mapped across the different subunits as summarized in Table 3, but very few functional studies exist [152]. The majority of these sites were mapped by high-throughput MS studies [150,153–160]. It is known that the OxPhos complexes are targeted by sirtuins (Sirts) that catalyze deacetylation reactions. Mitochondrial Sirt3 is well known as a regulator of oxidative stress [161,162]. It has been shown that Sirt3 can influence mitochondrial biology and metabolism through lysine deacetylations of various mitochondrial proteins including subunit 9 of complex I and flavoprotein of complex II [163–165]. Subunits I and II of COX are vulnerable to ROS due to their heme groups and copper centers which can result in mitochondrial dysfunction and apoptosis [166,167]. Based on the importance of subunit I for COX activity, it was questioned as to how acetylations can affect the enzyme after oxidative stress. Using 2, 2′-azoibis(2-aminopropane) dihydrochloride (AAPH)/hydrogen peroxide and cerebral ischemia/reperfusion to induce oxidative stress, the authors found that subunit I was deacetylated by Sirt3 as shown by immunoprecipitation, which inhibited oxidative stress-mediated apoptosis [152]. The authors then mapped the acetylation sites of subunit I in NIH/3T3 cells by mutating individually each of the 10 lysine residues and replaced with arginine, followed by an acetylation assay. The authors identified sites K13, K264, K319, and K481 as potential targets. However, it is unclear if the mutated proteins expressed exogenously in the cytosol and not in the mitochondrial matrix are incorporated into the COX holoenzyme, thus further studies are needed to confirm these results.

Overall, the vast majority of PTMs identified for COX (Table 2) have been found by high-throughput studies, usually with phosphopeptide enrichment and, as noted, very few of these sites have been functionally studied [103,113,114,139,145,168–181]. It is thus difficult to distinguish between PTMs that are biologically important and those that represent unspecific background noise. Another aspect is the number of PTMs missed and not identified either due to experimental difficulties such as analyzing hydrophobic proteins, inappropriate purification methods that do not persevere PTMs, and low molar fractions. From those PTMs studied in more detail, it cannot be disputed that PTMs can decisively regulate protein function. Clearly, more work is needed to better understand how specific PTMs and their combinations regulate oxidative phosphorylation and specifically COX activity under basal conditions and how they change in disease states.

4. Dysregulation of phosphorylations on COX and their connection to disease

The ETC carries out a crucial biological process for life, and regulatory mechanisms are in place to maximize energy production to meet tissue demand while maintaining low ROS. However, these regulatory mechanisms are compromised under various stress conditions and can result in several outcomes. As an example, further discussed below, respiration can completely stall during ischemia in the absence of oxygen, followed by uncontrolled respiration when blood flow is restored to the tissue, $\Delta\Psi_m$ hyperpolarization, and excessive ROS production, triggering cell death cascades. We propose that under stress conditions there is hyper-dephosphorylation of COX and Cytc, increasing ETC flux. In addition, loss of allosteric inhibition by ATP further drives $\Delta\Psi_m$ hyperpolarization and ROS production [182]. Such loss of regulation is cause for many disease states. The degree of dysregulation may be highest in acute conditions such as ischemia/reperfusion injury and sepsis, whereas less dysfunction would be expected in more chronic conditions such as Noonan syndrome, diabetes, and cancer. Below we discuss the role of selected disease-implicated COX phosphorylations that have been functionally studied.

4.1. Noonan syndrome

Noonan syndrome presents an example of the importance of regulating PTMs on mitochondrial function. Mutations in the gene *PTPN11*, which encodes the tyrosine phosphatase SHP-2, accounts for over half of the identified cases of Noonan syndrome [183,184]. It is an autosomal dominant disorder that presents with congenital heart defects, dysmorphic facial features, chest deformity, and cognitive deficits. SHP-2 was the first identified mitochondrial localized tyrosine phosphatase that exists in the IMS and is mostly associated with the Ras/mitogen-activated signaling pathway [185]. The mutated forms of SHP-2 in these patients have increased basal phosphatase activity [186], and in both patient and mouse cell lines, there was an increase in COX activity [22]. There is a suggested compensatory mechanism due to a decrease in the overall protein levels for both COX and Cytc. Alongside these results, there was an increase in basal ROS and a decrease in ATP levels. Currently it is not known if SHP-2 can dephosphorylate COX subunits or Cytc directly, but it is clear that a change in phosphorylation levels of mitochondrial proteins can alter activity and function as discussed earlier.

4.2. Ischemia/reperfusion

During ischemia as seen in ischemic stroke, myocardial infarction, and cardiac arrest patients, there is a lack of blood flow and therefore oxygen bioavailability to the affected organ. This alone can cause damage and death of cells through necrosis. However, the blockage can be medically removed, triggering excessive additional damage during reperfusion when blood flow is restored [187–193]. During reperfusion, the ETC is the primary generator of mitochondrial ROS due to hyperpolarization of the $\Delta\Psi_m$, which inhibits proton pumping from all complexes and causes an increased half-life of the ubisemiquinone free radical intermediate, which can then transfer the free electron to oxygen to generate superoxide [9,194]. We propose that COX and Cytc are primary regulators of mitochondrial ROS production by controlling electron flux in the ETC. During ischemia, there is massive loss of inhibitory phosphorylations of mitochondrial proteins including both Cytc and COX in an attempt by the cell to boost energy production. This attempt is futile given the absence of oxygen as the terminal electron acceptor. However, the ETC is now primed for hyperactivity and when oxygen is returned to the tissue there is uncontrolled respiration, leading to a pathological increase in $\Delta\Psi_m$ and ROS, and eventually triggering the release of Cytc and apoptosis [184,193,195].

Calcium is a known signaling molecule for mitochondrial activation [196] and is a critical player in activating cellular stress signaling. Under stress conditions such as ischemia, there is an increase in intracellular calcium concentrations [197] causing mitochondria to sequester calcium [198]. In pig heart mitochondria, excessive calcium leads to dephosphorylation of most mitochondrial proteins, including the OxPhos complexes [199]. Several studies showed when mitochondria sequester calcium, there is an increase in mitochondrial respiration and COX activity with loss of ATP allosteric regulation [127,197,200]. Mitochondria treated with calcium have an increase in state 4 respiration [201] that can be explained by dephosphorylation of the enzyme. Additionally, calcium is a known activator of protein phosphatases and therefore would result in dephosphorylation of most mitochondrial proteins [196,199]. Consistent with this concept are our previous findings that phosphorylation of COX on Y304 [15,24,130] and

phosphorylation of T28 [202], T58 [203], S47 [27], Y48 [204], and Y97 [135] of Cyt*c* decrease respiration whereas dephosphorylation increases respiration, which may partially explain the effect seen during stress conditions mediated by elevated calcium, causing hyperactive ETC complexes, $\Delta\Psi_m$, and ROS.

Protein kinase C (PKC) has been shown to act in mitochondria, and PKCβ has been found associated with the ETC in the brain after global brain ischemia [205]. Interestingly, PKCβ was found to translocate to mitochondria in neurons that were resistant to cell death whereas in regions that die after ischemia there was no PKCβ translocation. Compounds that activate PKCε and δ were shown to provide protection in the heart [206] and brain [207] after ischemia/reperfusion, preserving the phosphorylation state of mitochondrial proteins and providing regulation and protection during stress. Another role PKCε can play in ischemia/reperfusion injury is phosphorylating the calcium-sensing receptor (CaSR) on T888, reducing intracellular calcium release from the endoplasmic reticulum, preventing hyper-dephosphorylation [208]. In rat neonatal cardiac myocytes after activation of PKC with diacylglycerol or 4β-PMA, subunit IV was phosphorylated, and COX activity was increased [122,123]. A follow up study suggested PKCε is a candidate kinase as it co-immunoprecipitated with COX [209].

4.3. Inflammation

Acute inflammation has been shown to be associated with metabolic changes and suppression of mitochondrial respiration. Sepsis, or severe systemic inflammation, is the leading cause of mortality in intensive care units with more than 210,000 deaths in the US annually [210]. Despite patients having increased lactate levels [211], systemic oxygen delivery during sepsis does not improve outcomes, suggesting that oxygen consumption rather than delivery is impaired, a condition known as cytopathic hypoxia. A study of 96 septic patients showed a positive correlation between survival and COX activity and amount [212]. In cow and mouse livers treated with TNFα, there was a substantial decrease in COX activity within 5 min after treatment [15]. Phosphorylation on Y304 of subunit I was identified to be the result of inflammatory signaling that correlated with decreased enzymatic activity. TNFα treatment of mouse liver and cells also showed a decrease of $\Delta\Psi_m$ and cellular ATP levels. In cases of localized inflammation, such as a small wound, it would make sense to shut down cellular processes such as OxPhos to cut off essential metabolites to the isolated area to fight off the infection because some pathogens exploit the host's metabolites. However, if inflammation persists and becomes systemic, this can result in a system-wide shut down of the major organs, leading to multiple organ dysfunction syndrome (MODS) and death [142].

4.4. Diabetes

Diabetes pathology and mitochondria dysfunction have long been connected, but the molecular mechanisms underlying this dysfunction are not well understood [213–217]. Using the Cohen diabetic rat model, generated by selective multi-generation inbreeding to select for genetic factors that make rats resistant or sensitive to diabetes, the sensitive animals quickly develop diabetes when fed a high sucrose diet. Analyzing liver mitochondria, we recently found an unexpectedly high 80 % reduction in COX-specific activity in both the resistant and sensitive strains when fed a high sucrose diet [24]. The sensitive strain fed a regular diet had already a 40 % reduction in OCR. These results suggest that both genetic predisposition to diabetes and diet play a role in mitochondrial dysfunction, contributing to disease pathogenesis. Additionally, using a custom pY304-specific antibody and blue native polyacrylamide gel electrophoresis (BN-PAGE), Y304 phosphorylation was only identified in the dimeric form of COX, suggesting that it promotes the homodimerization of the enzyme.

4.5. Cancer

Mitochondrial alterations and dysfunction are known to be implicated in cancer. During carcinogenesis, there are changes in not only metabolism but most solid tumors have shown a 25–60 % reduction in mitochondrial mass [218]. Cancer cells are also known to shift their aerobic metabolism to glycolysis even in the presence of oxygen, which is known as aerobic glycolysis, called the Warburg effect [219]. It was later proposed that many of the established cancer related signaling pathways in fact reprogram catabolic cellular metabolism found in normal cells to anabolic metabolism seen in cancer cells. Epidermal growth factor receptor (EGFR) has been widely studied in many types of cancer including breast, colon, and lung and was the first receptor tyrosine kinase shown to directly affect COX. In breast cancer cell lines stimulated with EGF, EGFR translocated to mitochondria, where it physically interacts with subunit II [48]. Only activated EGFR phosphorylated on Y845 was shown to interact with SRC kinase, which was also required for EGFR-mediated COX phosphorylation [16]. The specific tyrosine residue on COX subunit II was not mapped, but this modification was associated with 60 % decreased COX activity. A similar mechanism was identified for another cancer related receptor tyrosine kinase, ERBB2, which also translocates to mitochondria and interacts with COX [17]. When cell signaling pathways go unchecked, this can lead to a dysregulation of PTMs on COX resulting in aberrant activity, and an emerging strategy for some anticancer drugs is to target metabolic reprogramming [220]. Inflammation is also a known promoter of cancer and is associated with metabolism changes [221,222]. Patients with ulcerative colitis have a 5–7 fold increase risk of colorectal cancer and this risk is reduced by 80 % with cyclooxygenase-2 inhibitors, a type of non-steroidal anti-inflammatory drug (NSAID) [223]. The proposed explanation for the correlation between inflammation and cancer is a two-step model: reactive oxygen and nitrogen species (RONS) produced by inflammatory cells cause mutations in neighboring cells that are further amplified by RONS production in pre-malignant cells. The second step is tumor promotion accompanied by immune cell cytokine production that can activate other signaling pathways that promote tumorigenesis. ROS are prevalent in both inflammation and cancer and are tied to mitochondria. Therefore, it makes sense that mitochondria play a key role in cancer. Another aspect that ties mitochondria to cancer is that mitochondrial ROS production can serve as an important signal for cell proliferation [148].

5. Restoring COX regulation as a therapeutic target

As discussed, the interaction between COX and Cyt*c* is the proposed rate-limiting step in the ETC and therefore requires tight regulation. There are many mechanisms in place to regulate COX that are unique to just this complex, further highlighting its importance in maintaining mitochondrial homeostasis. Oxidative phosphorylation dysfunction is involved in many diseases [152] and therefore is a logical therapeutic target. It has been known that COX is the primary photo-acceptor of near infrared light (IRL) of wavelengths between 700 and 1000 nm due to the two copper centers that are crucial for its enzymatic function and is the most abundant copper containing enzyme in the body [224,225]. The binuclear Cu_A and mononuclear Cu_B sites broadly absorb IRL as seen in the COX absorption spectrum [226]. The exact mechanism of IRL modulation of COX activity is not well understood. We propose that inhibitory IRL wavelengths are absorbed by the Cu_A center, leading to increased vibrational movement, therefore impairing the optimal binding of Cyt*c* with COX, slowing down electron transfer. IRL has been shown to be beneficial in several cases including wound healing and it was presumed that it exclusively activates mitochondrial respiration [227–229]. Recently, however, the discovery of two specific IRL wavelengths (750 and 950 nm) that inhibit COX opened up novel and potentially transformative treatment options for conditions in which hyperactive mitochondria are present. COX-inhibitory IRL resulted in

reduced $\Delta\Psi_m$, decreased superoxide production, and suppression of cell death in HT22 cells when subjected to glutamate toxicity and oxygen-glucose deprivation [193,230]. The non-invasive use of these inhibitory wavelengths was profoundly neuroprotective in rat and pig global brain ischemia/reperfusion and stroke models, limiting ischemia/reperfusion injury and neuronal cell death by attenuating mitochondrial hyperactivity and ROS production [25,28,29,231]. Thus, the inhibitory IRL wavelengths tone down COX activity and, when oxygen is returned during reperfusion, COX functions more closely within physiological levels, similarly to when PTMs are present (Fig. 3).

The benefit of the IRL treatment is that it is non-invasive and, in contrast to pharmacologic drugs, does not rely on delivery through the blood and thus has an immediate effect, an especially notable point during blockage of blood flow during ischemic stroke. Furthermore, instead of drugs that act systemically, the light is applied locally where needed, another benefit of this approach. It should be noted that specific IRL wavelengths may not penetrate deeply enough to some of the internal organs of the human body to deliver therapeutic doses. With the correct application design, however, we have shown for COX-inhibitory wavelengths of 750 nm and 950 nm to achieve tissue penetration up to 4 cm into the human brain without the need of active cooling to deliver therapeutic doses of IRL in the double-digit $\mu W/cm^2$ range [25,230,232]. There are also major research efforts on the use of drugs to influence mitochondrial function and bioenergetics. Protamine sulfate is an example, which is clinically used as an antidote for heparin during heart surgery. It has an inhibitory effect on the ETC at lower concentrations and interferes with Cytc binding to COX [233].

Due to the inability of drug administration during ischemia, therapeutics that do not solely rely on blood flow are crucial, such as gasotransmitters nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S), which have been choice molecules of study for treatment of I/R injury [234]. Nitric oxide (NO) administration has long been studied in the context of I/R injury for pulmonary dysfunction and lung transplants [235–237]. NO administration either prior to ischemia or after provided protection against myocardial I/R injury [238]. Additionally, both endogenous and exogenous NO were able to attenuate I/R injury in the liver such as in the case of liver transplants [239–241]. It has also been found that NO production can mediate protective preconditioning effects [242]. One caveat is that NO has a short half-life in vivo. Therefore, the use of NO inducers and donors are now being explored such as nitrite [243,244]. Similar to the inhibitory effect of NO on COX activity, another example used for treatment is CO and has been used to limit anesthesia induced neurotoxicity and oxidative stress [245–247]. Hydrogen sulfide (H_2S) was found to bind the copper center of COX and inhibit activity at high concentrations, originally labelling this molecule as toxic [248]. H_2S was identified as the third gaseous signaling molecule after CO and NO and has also been studied in the context of I/R injury [249–251].

There is still a lot to understand about the regulation of COX in different tissues and conditions, but we expect that more research focused on this important aspect will open the door to many therapeutics to mitigate and treat diseases related to mitochondrial dysfunction.

6. Conclusion and perspectives

PTMs can decisively control the activity of COX and other OxPhos components including Cytc. They can also enable or disable other regulatory mechanism such as allosteric regulation through ATP/ADP binding [128]. When COX is phosphorylated, ATP interaction is increased, thus giving high flux-control coefficients for mitochondrial respiration and during times of stress, cellular signals dephosphorylate the enzyme and the ATP/ADP control is abolished [182,252]. Isolated mitochondria, show higher rates of respiration than in intact cells or tissues, which may in part be explained by the loss of auxiliary signaling pathways that control and target the ETC.

It has been shown that ATP production becomes saturated in *Escherichia coli* at a $\Delta\Psi_m$ of $\geq$130 mV [8] ;therefore, having further increased $\Delta\Psi_m$ levels is not necessary for the production of ATP. In contrast, it is now accepted that ROS production exponentially correlates with $\Delta\Psi_m$ [9,253]. Although some conditions require $\Delta\Psi_m$ hyperpolarization and ROS, such as when cells commit to apoptosis, under most physiological conditions the relationship between $\Delta\Psi_m$ and ROS

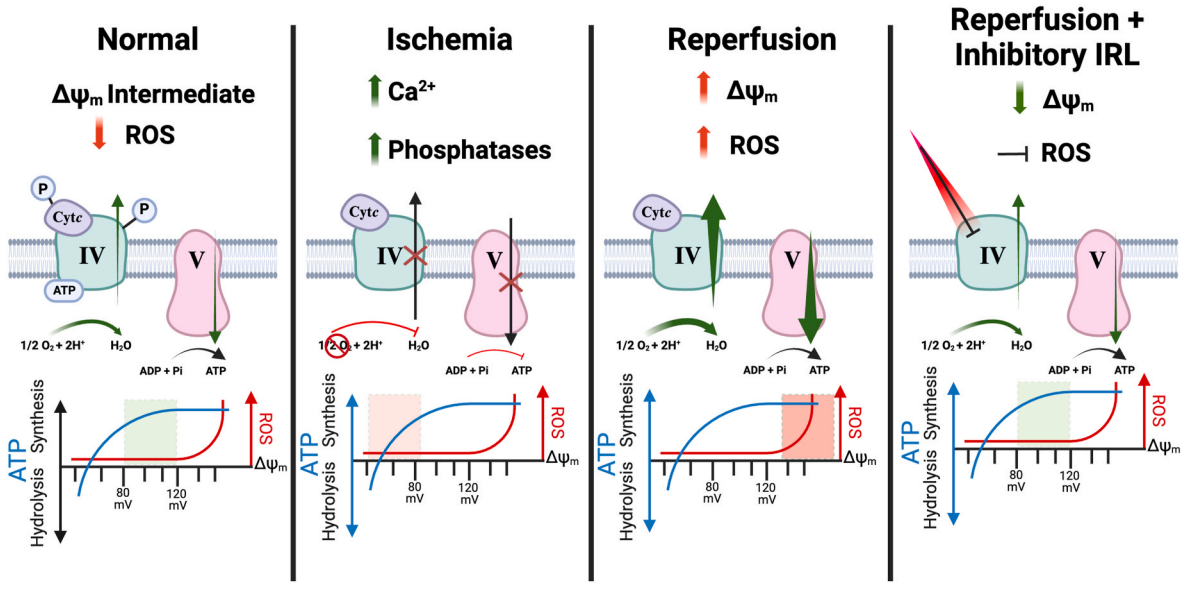

Fig. 3. Molecular changes of COX and Cytc during ischemia and reperfusion and the application of inhibitory infrared light to prevent $\Delta\Psi_m$ hyperpolarization and increased ROS as a therapeutic intervention. Under physiological conditions, $\Delta\Psi_m$ is maintained in the optimal intermediate range (80–120 mV), allowing efficient ATP production and minimal ROS (left). $\Delta\Psi_m$ is primarily regulated by PTMs such as phosphorylations on COX and Cytc to maintain this optimal intermediate range. During ischemia, increased calcium influx into mitochondria activates stress pathways including phosphatases, resulting in dephosphorylation of mitochondrial proteins. This includes Cytc and COX, priming them for hyperactivity. During reperfusion, oxygen, the substrate for COX returns resulting in hyperpolarization of $\Delta\Psi_m$ and an exponential increase in ROS. COX inhibitory infrared light applied during reperfusion counteracts mitochondrial hyperactivity, limits ROS production, and is neuroprotective.

necessitates fine-tuned regulatory mechanisms to control $\Delta\Psi_m$ in order to limit ROS. This is achieved through a combination of all three known primary mechanism, PTMs, allosteric regulation, and tissue specific isoforms. In the OxPhos system the combination of all three mechanisms has only been reported for COX and Cyt*c*, further supporting the concept that they are the main valve for ETC flux and thus represent the rate limiting step of the ETC. This puts COX and Cyt*c* in the spotlight as therapeutic targets for many diseases with bioenergetic dysfunction and/or increased ROS production, including the most common diseases such as ischemia/reperfusion injury, cardiovascular diseases, neurodegeneration, inflammatory diseases, diabetes, and cancer.

CRediT authorship contribution statement

Lucynda Pham: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Tasnim Arroum:** Writing – review & editing, Visualization. **Junmei Wan:** Writing – review & editing. **Lauren Pavelich:** Writing – review & editing. **Jamie Bell:** Writing – review & editing. **Paul T. Morse:** Writing – review & editing. **Icksoo Lee:** Writing – review & editing. **Lawrence I. Grossman:** Writing – review & editing. **Thomas H. Sanderson:** Writing – review & editing. **Moh H. Malek:** Writing – review & editing. **Maik Hüttemann:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Conceptualization.

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Declaration of competing interest

Drs. Maik Hüttemann and Thomas Sanderson are co-founders of Mitovation, Inc., that develops IRL therapy for I/R injury applications. All other authors declare no potential conflict of interest.

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Data availability

No data was used for the research described in the article.

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