



## Review

# Regulation of mitochondrial respiration and apoptosis through cell signaling: Cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation<sup>☆</sup>

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## ABSTRACT

Cytochrome c (Cyt c) and cytochrome c oxidase (COX) catalyze the terminal reaction of the mitochondrial electron transport chain (ETC), the reduction of oxygen to water. This irreversible step is highly regulated, as indicated by the presence of tissue-specific and developmentally expressed isoforms, allosteric regulation, and reversible phosphorylations, which are found in both Cyt c and COX. The crucial role of the ETC in health and disease is obvious since it, together with ATP synthase, provides the vast majority of cellular energy, which drives all cellular processes. However, under conditions of stress, the ETC generates reactive oxygen species (ROS), which cause cell damage and trigger death processes. We here discuss current knowledge of the regulation of Cyt c and COX with a focus on cell signaling pathways, including cAMP/protein kinase A and tyrosine kinase signaling. Based on the crystal structures we highlight all identified phosphorylation sites on Cyt c and COX, and we present a new phosphorylation site, Ser126 on COX subunit II. We conclude with a model that links cell signaling with the phosphorylation state of Cyt c and COX. This in turn regulates their enzymatic activities, the mitochondrial membrane potential, and the production of ATP and ROS. Our model is discussed through two distinct human pathologies, acute inflammation as seen in sepsis, where phosphorylation leads to strong COX inhibition followed by energy depletion, and ischemia/reperfusion injury, where hyperactive ETC complexes generate pathologically high mitochondrial membrane potentials, leading to excessive ROS production. Although operating at opposite poles of the ETC activity spectrum, both conditions can lead to cell death through energy deprivation or ROS-triggered apoptosis. This article is part of a Special Issue entitled: "Respiratory Oxidases".

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## 1. Introduction

In multicellular organisms communication between cells and within subcellular compartments is essential to coordinate diverse cellular and overall organ functions. The communication system in higher organisms utilizes a battery of mechanisms such as hormonal regulation through the bloodstream, electrical conduction to transmit signals in neurons, and small molecules or ions inside the cell such as

cAMP and calcium. Cell signaling pathways streamline communication within and among cells. Central roles are played by receptors, kinases, phosphatases, and scaffolding proteins that together generate a specific signal that eventually leads to the chemical modification, i.e., phosphorylation or dephosphorylation of downstream target proteins. A key cellular unit, the mitochondrial oxidative phosphorylation (OxPhos) machinery, is currently being explored for evidence of regulation by cell signaling pathways.

The primary role of OxPhos is the provision of energy, which drives all cellular processes. An average human synthesizes 65 kg of ATP from ADP and phosphate per day [1], highlighting the importance of this process for organismal health. A secondary role is the participation of OxPhos in apoptosis, a concerted sequence of events including changes in OxPhos activity, mitochondrial membrane potential

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( $\Delta\Psi_m$ ) hyperpolarization, reactive oxygen species (ROS) production, release of cytochrome *c*, formation of the apoptosome, and execution of apoptosis via caspase activation. Given the dual role of OxPhos in cellular life and death decisions, tight regulation is warranted.

OxPhos generates more than 15 times the amount of ATP compared to anaerobic glycolysis. OxPhos consists of the electron transport chain (ETC) and ATP synthase. The ETC consists of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), the non-protein electron carrier ubiquinone, *bc*<sub>1</sub>-complex (complex III), cytochrome *c* (Cyt<sub>c</sub>), and cytochrome *c* oxidase (COX; complex IV). Electrons enter the ETC primarily via complex I from NADH. Complex II links OxPhos with the citric acid cycle and feeds electrons derived from succinate directly into the ubiquinone/ubiquinol pool. Except for complex II, which does not function as a proton pump, the other ETC complexes pump protons from the matrix into the intermembrane space, generating the mitochondrial membrane potential ( $\Delta\Psi_m$ ).  $\Delta\Psi_m$  is utilized by ATP synthase (complex V) to synthesize ATP from ADP and phosphate, driven by the backflow of protons from the mitochondrial intermembrane space to the matrix. In contrast to many unicellular organisms including yeast, which can grow and survive in the absence of oxygen, animals fully depend on aerobic energy metabolism, with few exceptions restricted to parts of the organism, such as the fetus during the first half of pregnancy [2], and cancer, where the exception is known as the Warburg effect [3,4].

## 2. Regulation of mitochondrial OxPhos

In the following sections we review the multifaceted regulation of mitochondrial respiration through respiratory control, tissue-specific isoforms, allosteric regulation, and post-translational modifications, which will be the basis for the subsequent discussion of the role of OxPhos in selected human diseases.

### 2.1. The traditional view of the regulation of mitochondrial OxPhos: respiratory control

Depending on the activity of the cell, cellular energy demand varies widely and energy production thus has to be adjusted accordingly. The traditional principle by which OxPhos is controlled is called respiratory control, first described by Lardy and Wellman [5] as stimulation of mitochondrial respiration by a phosphate acceptor (e.g., ADP), and later extended by Chance and Williams [6] as an inhibition of respiration during transition from state 3 (high rate) to state 4 (low rate), when ADP is converted to ATP.

When cells utilize energy ATP is converted into ADP, which is converted back into ATP by ATP synthase using the proton gradient. This reduction in  $\Delta\Psi_m$  allows the proton pumps to resume electron transfer and to pump protons across the inner membrane. When cells are at rest and the vast majority of ADP has been converted into ATP, ATP synthesis slows, and  $\Delta\Psi_m$  builds up, which inhibits the proton pumps and thus mitochondrial respiration. When working with isolated mitochondria two extreme states are often reported to characterize mitochondrial function: state 3 respiration after the addition of ADP and phosphate in the presence of complex I or II substrates, which gives maximal respiration rates, and state 4, when all ADP has been converted into ATP, which gives low respiration rates (state 1 is baseline respiration of mitochondria in the absence of substrates; state 2 is defined as respiration after the addition of ETC substrates such as succinate but in the absence of exogenous ADP). Analysis of respiratory states of isolated mitochondria is useful to determine the number of ATP molecules produced per oxygen consumed, which is referred to as the P/O ratio; the ratio of state 3/state 4 respiration serves as a measure of mitochondrial “tightness” or coupling and is referred to as the respiratory control ratio.

Respiratory control is the most basic level of regulation and is defined by substrate concentrations. For example, substrates for

complexes I and II, and ADP and phosphate for ATP synthase, and the reaction intermediates and products, such as  $\Delta\Psi_m$  generated by the proton pumps and ATP generated by ATP synthase, all affect the respiration rate. However, additional regulatory mechanisms exist, as recently established for mammalian COX and Cyt<sub>c</sub>, and these will be discussed.

### 2.2. Tissue-specific isoforms of cytochrome *c* oxidase and cytochrome *c*

In contrast to OxPhos complexes I, II, III, and V, for which no tissue specific isoforms have been reported, both Cyt<sub>c</sub> and COX occur as distinct tissue-specific isozymes in mammals. This suggests that the terminal step of the ETC, i.e., the electron transfer from Cyt<sub>c</sub> to O<sub>2</sub> via COX, has to be functionally adapted to tissue-specific energy needs.

#### 2.2.1. Cytochrome *c*

Mammalian Cyt<sub>c</sub> is a one-electron carrier that shuttles electrons from *bc*<sub>1</sub> complex to COX. It contains a covalently attached heme group and 104 amino acids (Fig. 1, top left). Cyt<sub>c</sub> is highly positively charged with a pI of 9.6 and is located in the mitochondrial intermembrane space, where it is associated with negatively charged phospholipids of the inner membrane, in particular cardiolipin.

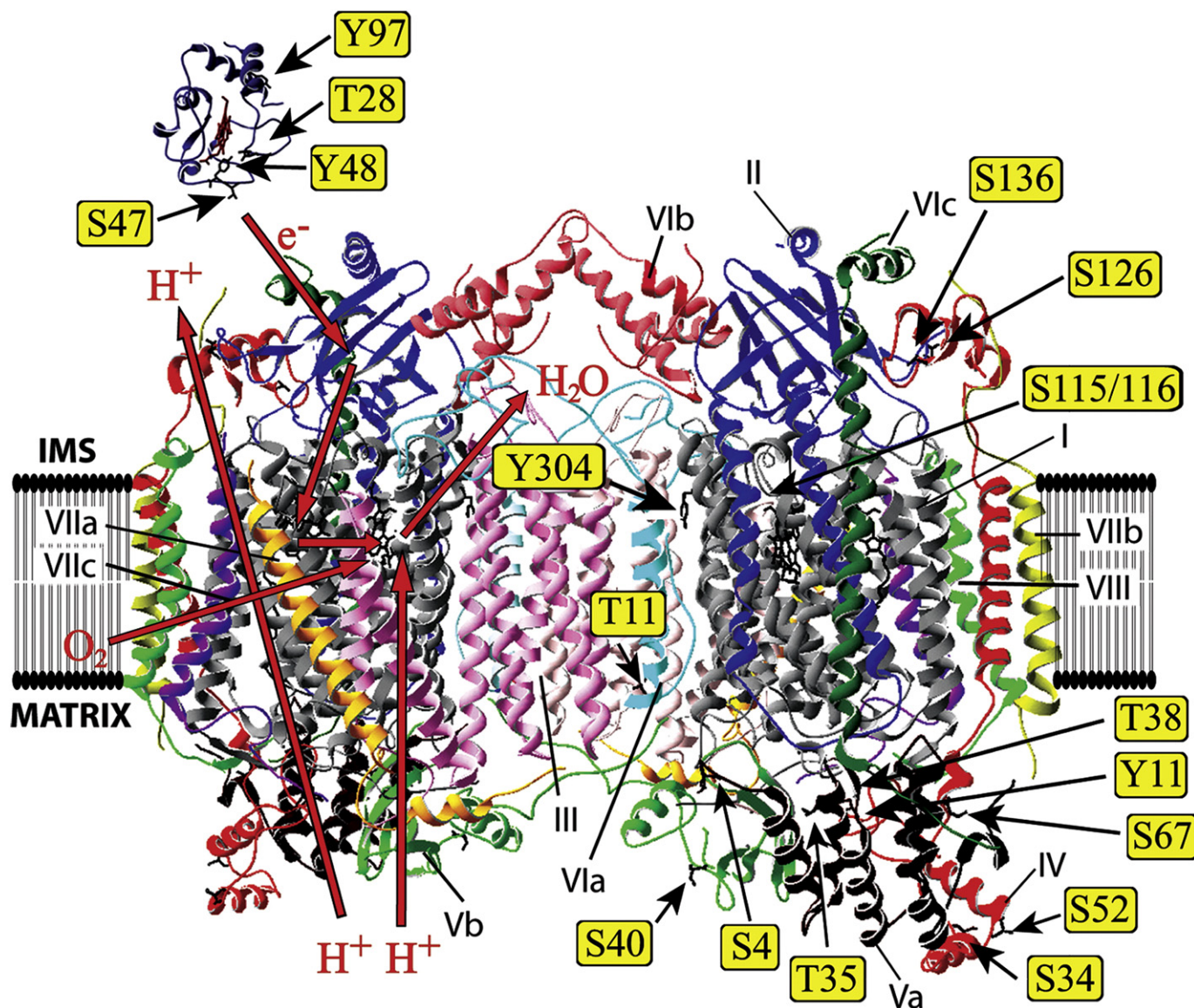
In rodents, Cyt<sub>c</sub> occurs as a testes-specific isoform (Cyt<sub>c</sub>-T), which is expressed in the germinal epithelial cells [7], and a somatic isoform (Cyt<sub>c</sub>), which is expressed in all other tissues. In humans there is only a single functional Cyt<sub>c</sub> gene because the Cyt<sub>c</sub> testis isoform gene was lost during primate evolution. The former Cyt<sub>c</sub>-T in humans is now a non-transcribed pseudogene [8,9].

In mice the two Cyt<sub>c</sub> isoforms share 86% sequence identity but their enzymatic functions are clearly distinct as discussed elsewhere [10]. For example, Cyt<sub>c</sub>-T shows a threefold increased activity to reduce hydrogen peroxide compared with the somatic isoform, but also shows an approximately fourfold increased ability to induce apoptosis [11]. The authors proposed that these functional changes may prevent radical damage of sperm and at the same time serve as a selective agent by initiating apoptosis in damaged sperm. In addition to its role in respiration, as a ROS scavenger under healthy conditions, and as an initiator of apoptosis, Cyt<sub>c</sub> participates in several other activities including Erv1-Mia40-coupled protein import, cardiolipin oxidation during apoptosis, and p66<sup>shc</sup>-coupled ROS production (reviewed in [10]), where it would be interesting to assess possible differences between the Cyt<sub>c</sub> isoform pair.

#### 2.2.2. Cytochrome *c* oxidase

COX is the terminal enzyme of the ETC. It accepts electrons from reduced Cyt<sub>c</sub> and transfers them to molecular oxygen, which is reduced to water. This reaction is coupled to the transfer of protons from the matrix to the intermembrane space by COX. In addition to these ‘pumped’ protons COX takes up ‘chemical’ protons, which are needed when oxygen is reduced to water. Both pumped and chemical protons contribute to  $\Delta\Psi_m$ . Cow heart COX has been crystallized as a dimer [12]. Per monomer, mammalian COX is composed of two heme and two copper redox centers located in catalytic subunits I and II. COX contains 13 subunits, three of which are encoded by the mitochondrial DNA and ten by nuclear DNA (Fig. 1).

For six of the smaller, nuclear-encoded subunits tissue-specific isoforms have been identified. All isoforms are encoded by separate genes and are not generated through differential splicing. Three liver- and heart-type isoform pairs of subunits VIa, VIIa, and VIII exist, which have long been known (reviewed in [13]). Liver-type COX is expressed in most organs such as liver, brain, and kidney, whereas the heart-type isozyme is expressed in heart and skeletal muscle. Heart-type COX is expressed in tissues with a high aerobic capacity and a large number of mitochondria, whereas other tissues including the brain cannot afford such a high mitochondrial load due to their specialized functions. Since these tissues still fully depend on aerobic



**Fig. 1.** Mapped phosphorylation sites on cytochrome *c* and cytochrome *c* oxidase. Crystal structure data of horse heart cytochrome *c* [119] and cow heart COX [12] were used and processed with the program Swiss-PDBViewer 3.7. Identified phosphorylated amino acids in mammals are shown in sticks. See Table 1 for a detailed description of the sites including phospho-epitopes and references. Note that Thr52 in rabbit corresponds to Ser52 in cow COX subunit IV.

energy production they are equipped with a more active enzyme, the liver-type. In addition, we recently identified three more COX subunit isoforms. These are a lung-specific isoform of COX subunit IV (COX4-2), a testis-specific isoform of subunit VIb, and a third isoform of subunit VIII [9,14,15].

Functional data including enzyme kinetics have so far been reported only for the better-studied liver, heart, and lung isoforms showing that enzymatic activity follows the order heart-type < liver-type < lung-type [16,17]. The reason for lung COX being the highest activity enzyme is not clear. It was proposed that the expression of COX4-2 in lung, a highly oxygenated tissue, might drain electrons from the ETC leaving fewer substrate electrons for the formation of ROS [17,18].

### 2.3. Allosteric regulation of cytochrome *c* and cytochrome *c* oxidase

Another regulatory mechanism found in key metabolic enzymes is allosteric regulation, which allows fine-tuning of enzymatic activity based on the concentrations of reaction substrates, intermediates, and products. A common mechanism found in COX and Cytc is that they

bind adenine nucleotides. At physiological concentrations, ATP binds to Cytc, which changes the binding of Cytc to COX, leading to an inhibition of the reaction between Cytc and COX, and the elimination of the low  $K_m$  phase of the otherwise biphasic kinetics with COX [19].

For COX, the largest nuclear-encoded subunit, subunit IV, has been shown to bind ATP at the matrix side [20,21]. ATP binding leads to an allosteric inhibition of enzyme activity at high intra-mitochondrial ATP/ADP ratios, adjusting energy production to demand. It was then proposed that COX subunit IV is a pivotal regulator for COX activity in higher organisms in contrast to bacteria that lack this subunit [22,23].

COX subunit Va, which localizes to the matrix (Fig. 1), was shown to bind the thyroid hormone T2. Upon binding, the allosteric ATP inhibition is released, allowing a high turnover even at high ATP/ADP ratios [24].

The muscle specific isoform of subunit VIa was suggested to decrease the pumped proton-to-electron stoichiometry at high ATP/ADP ratios, in contrast to the VIa liver-type-containing isozyme, which pumps protons at a high ratio independently of the ATP/ADP ratio [25]. However, liver-type COX shows a reduced proton/electron stoichiometry after addition of palmitate [26].



In addition to allosteric regulation COX is inhibited by nitric oxide (NO), which competes with O<sub>2</sub> for binding at the binuclear heme a<sub>3</sub>–Cu<sub>B</sub> reaction center [27]. Altered NO concentrations are found in a variety of human diseases including amyotrophic lateral sclerosis, cancer [28], arthritis [29], sepsis and septic shock [30], trauma [31], neurodegenerative diseases [32], and obesity and diabetes [33]. Elevated levels of NO result in COX inhibition and overall reduced ETC flux, leading to decreased energy levels, which might at least in part explain some aspects of NO-related pathologies.

#### 2.4. Regulation of cytochrome c and cytochrome c oxidase by phosphorylation

Research analyzing other compartments of the cell including the plasma membrane, cytosol, and nucleus has long established the central role of cell signaling in the regulation and functioning of the living cell. However, mitochondria have only recently been recognized as targets of cell signaling pathways, and are now rousing from a century-long hibernation. Phosphorylation sites have been identified in all OxPhos complexes, but the signaling pathways involved including kinases and phosphatases remain unknown or uncertain in most cases [34]. Phosphorylation of Cytc and COX has been most intensively studied, both structurally and functionally, and those phosphorylations will be discussed in detail, including a new phosphorylation site in COX subunit II that we present here. In Cytc and COX, 4 and 14 phosphorylation sites have been mapped, respectively, mainly through mass spectrometry (MS) (Fig. 1 and Table 1). MS methodology has become increasingly sensitive and can be operated in a high throughput manner. More and more such data sets are available now, including a study on human skeletal muscle showing phosphorylation of Cytc Thr28 and Ser47 [35], and a study on human HeLa cells that indicated phosphorylation of COX subunit IV-1 Ser67 and Ser136 COX subunit Va Thr35 and Thr38 [36] (see Fig. 1 and Table 1). Such information is important but two points clearly need to be addressed: 1) What are the functional consequences of individual phosphorylations, and 2) How much of the protein is phosphorylated at a given condition, i.e., what are the dynamics of these phosphorylations. Below we summarize the known phosphorylations on Cytc and COX and focus our discussion on phosphorylations and signaling pathways that affect the function of Cytc and COX.

##### 2.4.1. Phosphorylation of cytochrome c

After Cytc had been studied for more than a century, we discovered that it is the target of cell signaling pathways. We isolated Cytc from cow heart tissue under conditions that preserve the physiological phosphorylation state and found, by MS, that it was phosphorylated on Tyr97 (Fig. 1) [37]. The phosphorylated form showed functional differences, including a shift of the heme iron–methionine 80 absorption band from 695 to 687 nm. In addition, phosphorylated Cytc showed enhanced sigmoidal kinetics and inhibition in the reaction with COX by shifting the K<sub>m</sub> of COX for Cytc. Half maximal turnover was observed at a Cytc substrate concentration of 5.5 μM compared to 2.5 μM for unphosphorylated Cytc.

We later analyzed Cytc phosphorylation in cow liver tissue and, to our surprise, found that it was phosphorylated on a different residue, Tyr48 [38]. There were no spectral changes, and enzyme kinetics with isolated COX was distinct compared to Tyr97-phosphorylated heart Cytc. Tyr48-phosphorylated Cytc produced a hyperbolic response, similar to unphosphorylated Cytc. At maximal turnover, COX activity was more than 50% reduced with Tyr48-phosphorylated Cytc. The finding of partial (but not full) respiratory inhibition with *in vivo* Tyr97- and Tyr48-phosphorylated Cytc in heart and liver supports our model that, under healthy conditions, ETC activity does not operate at full capacity, undoubtedly to prevent ΔΨ<sub>m</sub> hyperpolarization. This prevents the production of significant amounts of ROS, which are generated at high ΔΨ<sub>m</sub> levels, a concept we discuss in Section 3.

In a follow-up study we generated and purified phosphomimetic Cytc by replacing Cytc Tyr48 with Glu, which mimics the negative charge of the phosphate group. Similar effects compared to *in vivo* Tyr48-phosphorylated Cytc were observed in the reaction with COX, suggesting that it is a good model for phosphorylated Cytc. Tyr48Glu Cytc showed reduced binding affinity to cardiolipin, a mitochondria-specific lipid with which it is normally associated. Strikingly, the ability of Cytc to trigger down-stream caspase activation was completely abolished in the Tyr48Glu mutant suggesting that Cytc phosphorylation may function as a switch for the regulation of apoptosis. This finding may have a broader impact for the molecular mechanism underlying the ability of cancers to evade apoptosis. Although the kinases that mediate Cytc phosphorylation are unknown, the fact alone that Cytc can be tyrosine phosphorylated might suggest a link to cancer because receptor and non-receptor tyrosine kinase signaling often causes cancer and it may target Cytc.

**Table 1**  
Identified phospho-epitopes in mammalian cytochrome c and cytochrome c oxidase<sup>1</sup>.

| Enzyme | Species & tissue      | Phosphorylated amino acid <sup>2</sup> | Phospho-epitope <sup>3</sup> | Reference  | Method  | Kinase prediction <sup>4</sup>  |
|--------|-----------------------|--|------------------------------|------------|---|---------------------------------|
| Cytc   | Human skeletal muscle | Thr28                                  | EKGKKHKITGPNLHGL             | [37]       | HT-MS <sup>5</sup>                                | AMP kinase, low confidence      |
| Cytc   | Human skeletal muscle | Ser47                                  | TGQAPGYSYTAANKN              | [37]       | HT-MS   | no known motif                  |
| Cytc   | Cow liver             | Tyr48                                  | GQAPGFSYTDANKKN              | [40]       | MS  | no known motif                  |
| Cytc   | Cow heart             | Tyr97                                  | EREDLIAYLKKATNE              | [39]       | MS  | no known motif                  |
| COX    | Cow liver             | Tyr304, SU I                           | MDVDTRAYFTSATMI              | [48]       | MS, cAMP-dependent                                | no known motif                  |
| COX    | Cow liver             | Tyr304, SU I                           | MDVDTRAYFTSATMI              | [93]       | Phospho-epitope-specific antibody; TNFα-dependent | no known motif                  |
| COX    | Rabbit heart          | Ser115 and Ser116, SU I                | SLHLAGVSSILGAINF             | [51]       | MS; after ischemia/reperfusion                    | no known motif                  |
| COX    | Cow heart             | Ser126, SU II                          | DSYMIPTSELKPGEL              | This study | MS  | no known motif                  |
| COX    | Cow liver             | Tyr11, SU IV-1                         | SVVKSEYALPSYVD               | [39]       | MS  | Abl kinase, low confidence      |
| COX    | Cow heart             | Ser34, SU IV-1                         | VAHVKNLSASQKALK              | [56]       | MS  | CAMK2G, low confidence          |
| COX    | HeLa cells            | Ser67, SU IV-1                         | YRIKFESFAEMNRG               | [38]       | HT-MS   | PKCε                            |
| COX    | Rabbit heart          | Thr52, SU IV-1                         | KAPWGSLTRDEKVEL              | [51]       | MS; after ischemia/reperfusion                    | DNA PK, low confidence          |
| COX    | HeLa cells            | Ser136, SU IV-1                        | NPIQGLASKWDYEKN              | [38]       | HT-MS   | no known motif                  |
| COX    | Cow heart             | Ser4, SU Va                            | SHGSHETDEEF                  | [56]       | MS  | no known motif                  |
| COX    | Cow heart             | Thr35, SU Va                           | ELRKGMENTLVGYDLV             | [56]       | MS  | PKCε, low confidence            |
| COX    | HeLa cells            | Thr35 and Thr38, SU Va                 | LRKGINTLVTDYDMVPE            | [38]       | HT-MS   | PKCε and DNA PK, low confidence |
| COX    | Rabbit heart          | Ser40, SU Vb                           | MLPPKAASTCTKEDPN             | [51]       | MS; after ischemia/reperfusion                    | no known motif                  |
| COX    | Cow heart             | Thr11, SU VIa                          | AKGDHGGTGTARTWRF             | [57]       | Crystal structure                                 | no known motif                  |

<sup>1</sup> Identified phosphorylation sites are presented in an ascending amino acid and subunit (SU) order.

<sup>2</sup> Numbering according to mature peptide based on cow.

<sup>3</sup> Sequence is based on the species where the phosphorylation site was identified.

<sup>4</sup> Based on the Scansite Motif Scan web tool ([http://scansite.mit.edu/motifscan\\_seq.phtml](http://scansite.mit.edu/motifscan_seq.phtml)). The predictions are purely speculative and have to be viewed with extreme caution.

<sup>5</sup> High throughput mass spectrometry sequencing.

Two more phosphorylation sites were recently mapped on Cyt<sub>c</sub> from human skeletal muscle tissue by high throughput phosphoproteomic MS analysis. Thr28 and Ser47 were identified on Cyt<sub>c</sub> [35], suggesting that Cyt<sub>c</sub> in this tissue is targeted for phosphorylation by a signaling pathway that is distinct from heart and liver. The functions of these phosphorylations remain unknown as does the extent of Cyt<sub>c</sub> phosphorylation.

Interestingly, all four identified phosphorylation sites are located on the right side of Cyt<sub>c</sub> in the conventional view (Fig. 1). It remains to be seen how phosphorylation of Cyt<sub>c</sub> affects its other functions, including ROS scavenging under healthy conditions [39,40] and ROS generation under stressed conditions through the p66<sup>shc</sup> pathway [41].

#### 2.4.2. Phosphorylation of cytochrome c oxidase

In 1997, the first paper was published suggesting that COX is phosphorylated on subunit IV by an endogenous kinase after incubation of mitochondria with [ $\gamma$ -<sup>32</sup>P]ATP [42]. After that the cAMP-dependent signaling pathway was studied by several groups in more detail and therefore deserves an in-depth discussion. The localization of PKA has been proposed by various investigators to occur in all mitochondrial compartments, including the outer side of the outer mitochondrial membrane, the intermembrane space, and the matrix, but it still remains a controversial issue depending on the tissue or cell-type and methodology (reviewed in [34]).

In initial work, the Kadenbach group incubated isolated cow heart COX with PKA, cAMP, and [ $\gamma$ -<sup>32</sup>P]ATP *in vitro* [43]. After autoradiography they obtained a signal for subunit Vb and a faint signal for subunits II and/or III. In a follow-up study, using the same approach they showed that subunit I was also phosphorylated [44]. It must be emphasized that data obtained with an *in vitro* approach should be viewed with great caution, because auxiliary components, such as scaffolding proteins that mediate specificity, are absent. For example, PKA signaling specificity is mediated through A-kinase anchoring proteins (AKAPs) that provide a platform for the docking of kinases and other proteins near membranes and their targets [45]. Therefore, an *in vitro* approach should only be considered when similar results are obtained *in vivo*. Based on our experience (e.g., comparing phosphorylation patterns of COX after incubation with several kinases *in vitro*, which did not match results obtained *in vivo* after stimulation or inhibition of the corresponding signaling pathway) we strongly caution the reader against pursuing an *in vitro* approach.

Our laboratory later revisited cAMP-dependent phosphorylation of COX *in vivo* in liver tissue, where cAMP serves as a starvation signal, triggered by the starvation hormone glucagon. We purified COX after treatment with the phosphodiesterase inhibitor theophylline, which generates high cAMP levels. During the purification of mitochondria and subsequently of COX, we included activated vanadate and fluoride, two unspecific tyrosine and serine/threonine phosphatase inhibitors, respectively. The isolated enzyme was tyrosine phosphorylated on subunit I, whereas the untreated control enzyme did not show a signal. The site of phosphorylation was identified by MS as COX subunit I tyrosine 304. Tyr304 is located adjacent to the oxygen binding center on COX, an ideal site for enzyme regulation (Fig. 1). Indeed, Tyr304-phosphorylated COX was fully or strongly inhibited up to 10  $\mu$ M Cyt<sub>c</sub> substrate concentrations, even in the presence of allosteric activator ADP [46]. Control experiments using the physiological hormone glucagon, the strongest physiological signal for increasing intracellular cAMP levels in liver, or the adenylyl cyclase activator forskolin, both of which increase intracellular cAMP, showed a similar inhibitory effect. Notably, Tyr304 phosphorylation cannot be directly mediated by Ser/Thr-specific PKA, and it was proposed that a downstream tyrosine kinase phosphorylates COX [46].

It should be noted that there are distinct tissue-specific responses to cAMP and probably other signaling pathways as well. For example, in neuronal tissue theophylline treatment leads to COX activation [47]. In heart, theophylline showed no effect, and other heart-specific

cAMP inducers such as 3-isobutyl-1-methylxanthine (IBMX) did not show significant effects (unpublished).

One study proposed that cAMP/PKA signaling leads to phosphorylation of COX in heart after ischemia. There, COX subunits I, IV, and Vb were found to be phosphorylated after ischemia in rabbit hearts and these phosphorylations were abolished in the presence of alleged PKA inhibitor H89 [48]. The authors later identified the phosphorylated amino acids by MS to be Ser115 and Ser116 of subunit I, Thr52 of subunit IV, and Ser40 of subunit Vb [49]. These sites (Fig. 1 and Table 1) are not PKA consensus sites, and it thus seems unlikely that PKA signaling is directly involved. Kinase inhibitors often target a group of kinases, and H89 inhibits at least five other kinases [50], one or more of which may be involved in the phosphorylations observed after ischemia.

The Manfredi group has recently added another interesting facet to cAMP-dependent signaling by identifying a matrix-localized carbon dioxide/bicarbonate-regulated adenylyl cyclase [51]. Via sensing of CO<sub>2</sub> generated by the citric acid cycle, this enzyme links nutrient availability to OxPhos activity. COX is a target of this signaling, and the authors showed that subunits I and IV are targets of a matrix-localized PKA. The phosphorylation sites need to be mapped to provide additional evidence of direct PKA involvement. The effect of matrix adenylyl cyclase and PKA-dependent signaling is activation of COX [51], and such activation was shown to improve mitochondrial function in COX-deficient cells [52]. Additional support for matrix localized PKA signaling to COX was reported earlier. The R1 $\alpha$  regulatory subunit of PKA can bind to matrix-localized subunit Vb of COX [53]. It is noteworthy that the observed activation of COX through matrix-localized adenylyl cyclase and PKA results in the opposite effect compared to cAMP-dependent *in vivo* Tyr304 phosphorylation. This phosphorylation occurs on the intermembrane space side, and *in vitro* phosphorylation of COX with commercially available PKA, which can presumably occur on the matrix and intermembrane space sides, also leads to COX inhibition [43]. Mapping of the phosphorylation sites in these studies would shed light on this controversial point.

Additional phosphorylation sites have been mapped on COX, but the signaling pathways involved and their functional consequences are unknown. Those are Tyr11 of subunit IV-1 in isolated cow liver [37], Ser67 and Ser136 of subunit IV-1, Thr35 and Thr38 of subunit Va in human HeLa cells by high-throughput MS [36], Ser34 of subunit IV-1, and Ser4 and Thr35 of subunit Va in cow heart [54], and Thr11 of subunit VIa heart isoform, which was identified in the cow heart crystal structure [55]. The presence of phosphorylation sites in tissue-specific isoforms suggests that one of their functions is the provision of a platform for cell signaling that can mediate tissue-specific regulation. This is accompanied by tissue-specific expression of signaling molecules, such as mitochondrial tyrosine phosphatase PTP 1B, which is only found in brain but is absent in muscle, heart, and liver tissue [56].

We here present another new phosphorylation, which we identified in cow heart COX (Fig. 2). This site, Ser126 on catalytic subunit II, was unambiguously mapped by MS in three independent COX isolations, in which the enzyme was isolated from heart tissue that was immediately frozen on dry-ice, kept on ice for 20 min, and kept ischemic for 45 min at 37 °C prior to enzyme isolation. The phosphorylation site is located on the intermembrane space side (Fig. 1). It is adjacent to but not part of the Cyt<sub>c</sub>-COX docking site based on computer modeling [57]. In that model the nearest amino acid of Cyt<sub>c</sub>, Lys27, is located within 11 Å of Ser126. Ser126 is located at the interface of subunits II and IV, with His109 of subunit IV being the closest amino acid (within 5 Å). Therefore, this phosphorylation might be involved in enabling allosteric regulation of COX via ATP and ADP that bind to subunit IV, which regulation is lost when COX is fully dephosphorylated [13].

One concern regarding the above studies is that there is little overlap of phosphorylation sites identified by different labs. Likely explanations are that different purification protocols were used and

that treatments differed, including buffer compositions and the use of phosphatase inhibitors. In addition, MS methodology is problematic with hydrophobic peptides. For COX as a membrane protein this makes it difficult to identify hydrophobic phospho-epitopes. For example, phospho-Tyr304 identification of hydrophobic subunit I required significant optimization of the sample preparation [46].

There is another series of studies suggesting that COX is a target of signaling pathways, but in these cases the phosphorylation sites have not been identified. Non-receptor tyrosine kinase Src has been shown to localize to the mitochondrial intermembrane space [58], and it phosphorylates COX subunit II in osteoblasts [59]. This phosphorylation leads to COX activation and appears to be important for the bone-resorbing activity of these cells, which might be altered in rheumatoid arthritis [60]. Another COX regulator is protein kinase C $\epsilon$  (PKC $\epsilon$ ). In rat neonatal cardiac myocytes treatment with diacylglycerol or 4 $\beta$ -PMA, which are PKC activators, resulted in the phosphorylation of an 18 kDa protein *in vitro* [61,62]. It was shown that PKC $\epsilon$  co-immunoprecipitated with COX and that the radiolabeled 18 kDa band contained subunit IV. Activation of PKC $\epsilon$  resulted in about two- to fourfold increased COX activity. Src kinase is also phosphorylated and activated by PKC $\epsilon$  [63,64]; however, no tyrosine phosphorylation of COX subunit II was observed after PKC $\epsilon$  stimulation [62]. This might be explained with tissue specific differences in cell signaling including presence or absence of additional required components such as scaffolding proteins. PKC $\epsilon$  is implicated in ischemia/reperfusion injury, a topic that we will revisit in the next section.

Mitochondrial metabolism shows major alterations in cancer. Most solid tumors show a reduction in mitochondrial mass of 25–60% compared to healthy tissue [65]. Cancer cells rely less on respiration and more on glycolysis, which is known as the Warburg effect [3,4]. The molecular changes that mediate this metabolic switching are now emerging. Often, cancers show increased receptor tyrosine kinase signaling, which promotes proliferation and is anti-apoptotic. One such receptor is epidermal growth factor receptor (EGFR), which is implicated in numerous cancers including colon, lung, breast, cervical, and head and neck cancers. Recent research has changed the picture of how signaling from the plasma membrane is transmitted to the mitochondria, which now appears to be much more dynamic than thought previously. Notably, EGFR, upon stimulation with EGF, translocates to the mitochondria where it directly interacts with COX subunit II, as shown by phage display screening and co-immunoprecipitation experiments [66]. EGFR binding to COX is dependent on the activation state of the receptor and requires Tyr845 phosphorylation, which is mediated by Src kinase. EGFR in combination with Src leads to an increase in COX subunit II phosphorylation *in vitro* as was shown after incubation with [ $\gamma$ -<sup>32</sup>P] ATP followed by autoradiography [67]. However, the phosphorylation sites still have to be identified. Importantly, the effect of EGFR/Src signaling on COX activity is distinct from Src signaling alone discussed above because it leads to 60% inhibition after cells were treated with EGF for 20 min. This finding may explain the mechanism of the Warburg effect in cancers with upregulated EGFR signaling, and it remains to be seen if a similar sequence of events wherein the receptor is translocated to the mitochondria followed by phosphorylation and partial inhibition of COX also applies to other receptor tyrosine kinases.

The presence of kinases targeting COX implies the presence of phosphatases to reverse such signaling. Protein tyrosine phosphatase Shp-2, which is part of the Ras pathway, was the first identified tyrosine phosphatase that localizes to the mitochondrial intermembrane space and the outer mitochondrial membrane in addition to the cytoplasm [68], and we speculated that it might target COX or Cyt c, the only two components of the ETC with mapped tyrosine phosphorylation sites (Table 1). Mutations in the Shp-2 encoding gene *PTPN11* account for about 50% of cases with Noonan syndrome [69], an autosomal dominant disorder characterized by dysmorphic

facial features, congenital heart disease, webbed neck, short stature, chest deformity, and mental retardation. In these Noonan patients Shp-2 is constitutively active [70]. We analyzed patient and mouse cell lines and observed significantly increased COX activity [71]. Among OxPhos complexes, only COX and Cyt c protein levels were down-regulated in the mutant cells, suggesting a compensatory mechanism to counterbalance increased COX activity. Additional work is needed to establish COX and Cyt c as possible direct or indirect targets of Shp-2. Clearly, dephosphorylation of Tyr48 and Tyr97 on Cyt c and Tyr304 on COX subunit I would result in increased respiration rates, and all three epitopes are located in the intermembrane space, as is Shp-2. Alteration in COX/Cyt c activity and their total amount could affect mitochondrial energy and ROS production. Indeed, in the Shp-2 mutant cells ATP levels were reduced by 30% and ROS levels were increased. Both reduced ATP and increased ROS may interfere with organ development as is observed in the patients (for a more detailed discussion see [13]).

Another important mechanism related to cell signaling that acts on COX may be through the direct and indirect action of calcium. Calcium is the proposed strongest signal for mitochondrial activation [72]. Mammalian COX contains a calcium-sodium exchange site in subunit I [73,74], which may affect COX activity. In addition, calcium was shown to cause dephosphorylation of most mitochondrial proteins [75], likely through calcium-activated phosphatases. As described above, dephosphorylation of COX (at Tyr304, subunit I) and Cyt c (Tyr 48 and Tyr97) would result in increased ETC activity. Calcium also plays a key role during conditions of cellular stress, where it leads to hyperactive ETC complexes and increased  $\Delta\Psi_m$  levels, leading to excessive ROS production as discussed in Section 3.

### 3. The connection between ETC activity, $\Delta\Psi_m$ , energy, and ROS in human disease: acute inflammation and ischemia/reperfusion injury as two distinct mitochondrial pathologies

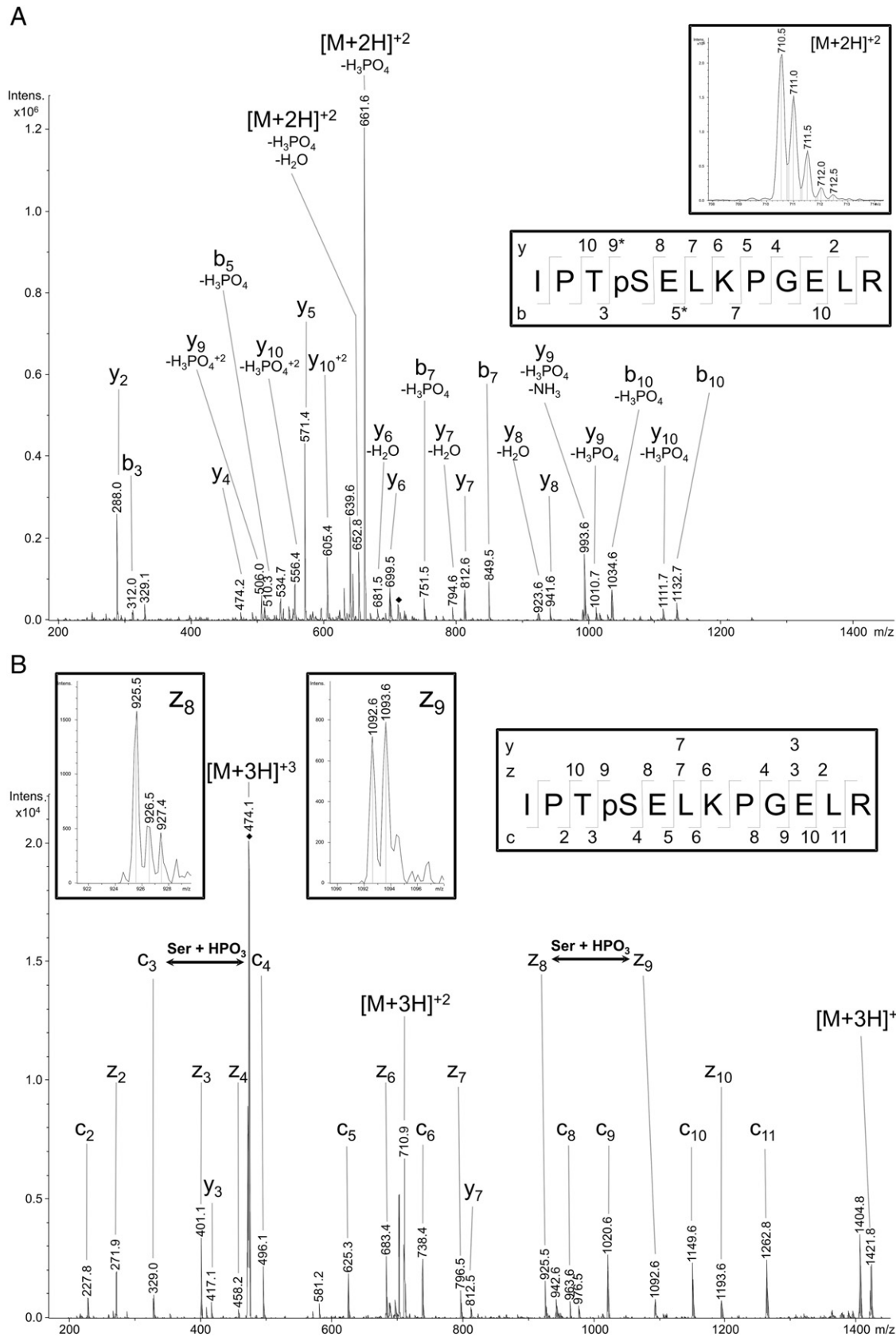
The role of OxPhos in human disease can be distilled into two buzzwords, energy and ROS. Many pathologies present with decreased ATP or increased ROS levels, and sometimes a combination of both. We have previously proposed a model that links ETC activity,  $\Delta\Psi_m$ , and the production of ATP and ROS [13]. Based on our model there are three states, 'hypoactive', 'healthy', and 'hyperactive' (Fig. 3), which may explain many pathophysiological conditions where mitochondria are involved. In the basic, 'healthy' state the OxPhos complexes are phosphorylated, which reduces their activity. Importantly, this prevents the generation of  $\Delta\Psi_m$  levels larger than 140 mV; i.e., physiologically lower  $\Delta\Psi_m$  levels are sufficient to inhibit further proton pumping. This regulation ensures that  $\Delta\Psi_m$  is maintained around 120 mV under healthy conditions, which is sufficient for efficient ATP production [76] but prevents the formation of ROS, which are generated exponentially at  $\Delta\Psi_m$  levels exceeding 150 mV [77].

A further fine-tuning of the regulation of mitochondrial respiration under healthy conditions is mediated via the allosteric regulation of COX and Cyt c by ATP and ADP discussed above. Respiration rates can be high if ADP is not limiting and no allosteric ATP-inhibition of COX occurs [21,23]. Under these conditions the high rate of proton uptake by ATP contributes to the maintenance of lower  $\Delta\Psi_m$  levels. When ADP becomes limiting the allosteric ATP-inhibition inhibits respiration and further proton pumping. This occurs already at healthy  $\Delta\Psi_m$  levels, because maximal rates of ATP synthesis by ATP synthase occur at  $\Delta\Psi_m$  = 100–120 mV [76]. It should be noted, however, that phosphorylation of OxPhos components appears to be the overarching regulatory mechanism preventing unhealthy high  $\Delta\Psi_m$  values even in the presence of ADP. This is so because  $\Delta\Psi_m$  values during state 3 reported for isolated mitochondria, with presumably dephosphorylated OxPhos complexes, are often higher (> 140 mV) than those reported for intact cells. For example, a  $\Delta\Psi_m$  value of 172 mV was

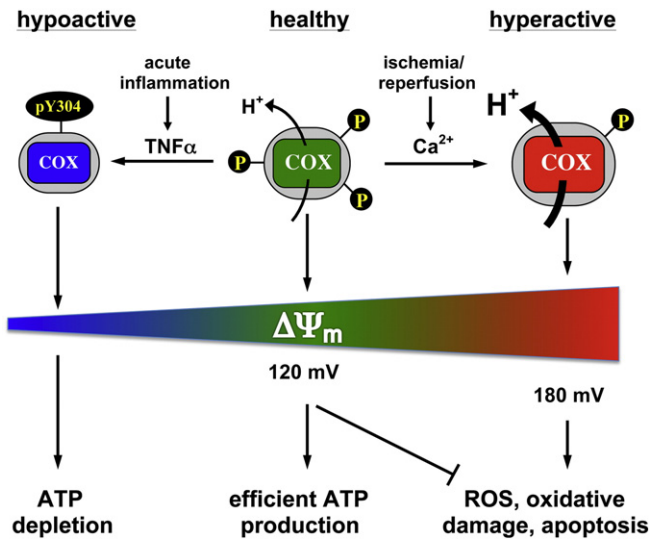
reported for rat liver mitochondria during state 3 respiration [78]. During state 4, mitochondria isolated according to traditional protocols with dephosphorylated OxPhos complexes can generate  $\Delta\Psi_m$  levels of 200 mV and above [13]. Isolated mitochondria are morpho-

logically and functionally distinct from mitochondria in intact cells and show unphysiologically high COX activities [79].

To exemplify the two distinct pathological states of our model, ‘hypoactive’ and ‘hyperactive,’ we will discuss acute inflammation and







**Fig. 3.** Proposed model linking COX activity with the mitochondrial membrane potential, ATP and ROS production under healthy conditions and in two distinct pathological states. Under healthy conditions (middle) COX and Cytc are phosphorylated leading to a partial inhibition of respiration (Cytc is not shown). This generates healthy mitochondrial membrane potentials  $\Delta\Psi_m$  of about 120 mV, sufficient for efficient ATP generation but too low for ROS production. Two distinct pathological conditions are shown, both of which can lead to cell death: 1) acute inflammatory signaling via TNF $\alpha$  causes COX subunit I Tyr304 phosphorylation (pY304), leading to strong COX inhibition, decreased  $\Delta\Psi_m$  levels, and eventually energy depletion. In patients with sepsis this model would explain organ failure and death through energy failure; 2) the opposite mode of action takes place during ischemia/reperfusion. During ischemia, nutrients and oxygen become depleted. This stress causes excessive calcium release leading to changes in the phosphorylation pattern and/or dephosphorylation of Cytc and COX. The electron transport chain is now 'primed' for hyperactivity. During reperfusion, in the presence of oxygen and metabolites, OxPhos resumes to rebuild  $\Delta\Psi_m$  and ATP. Since the ETC complexes are in a hyperactive state, flux is maximal, which leads to a hyperpolarization of  $\Delta\Psi_m$ , reaching levels at which excessive ROS are generated. ROS cause extensive damage to the cell and trigger apoptosis.

ischemia/reperfusion injury. The hypoactive state seen in acute inflammation results in decreased  $\Delta\Psi_m$  and energy levels. In contrast, the hyperactive state during reperfusion following ischemia results in  $\Delta\Psi_m$  hyperpolarization and ROS production. Both states can result in cell death through these distinct pathways.

### 3.1. Role of cytochrome c oxidase and cytochrome c in acute inflammation: the hypoactive state and the energy crisis

Sepsis is an acute inflammatory condition that affects the entire body and represents a potentially life-threatening systemic inflammatory state. Sepsis is sometimes referred to as blood poisoning in the non-medical literature. In the United States, sepsis develops in 750,000 people annually with more than 210,000 deaths, which makes it the leading cause of mortality in patients in intensive care units [80]. Sepsis was formerly attributed to a pathogenic infection of the blood, but the definition has changed in modern times to reflect the body's inflammatory response to severe insult or injury, including the development of multiple organ dysfunction syndrome (MODS) [81]. Therefore, the combination of an infection with an overly active immune response can lead to organ dysfunction and eventual death.

Except for mutations in the pathogen recognition toll-like receptor 4 (TLR4) [82], the only other known genetic predictor for survival after sepsis is a particular mitochondrial DNA composition, the haplogroup H. Mitochondrial haplogroups are grouped based on a relatively small number of mitochondrial DNA polymorphisms that are preserved in certain populations, for example from different continents. Those subtle changes have been linked to certain phenotypes and pathologies, such as longevity or male fertility [83]. Patients belonging to haplogroup H, which is common in Europeans, have a more than twofold increased chance of survival after sepsis compared to patients in other haplogroups [84]. This finding suggests that mitochondria play a key role in acute inflammation.

Given the role of COX and Cytc in providing energy for all cellular functions and the role of Cytc in the regulation of cellular survival and apoptosis, it is no surprise that both enzymes have been implicated in the pathology of sepsis. The terminal step of the ETC is a logical functional target: it is characterized by poor tissue oxygen extraction and utilization as seen in patients, suggesting that the ETC is a target of inflammatory signaling. In fact, it was hypothesized in the past that dysfunction of mitochondrial electron transport may be responsible for some of the symptoms of sepsis because direct systemic delivery of oxygen during the course of sepsis was shown to be ineffective [85]. This indicated that oxygen utilization rather than uptake is impaired, a model referred to as cytopathic hypoxia [86].

Lipopolysaccharide (LPS, or endotoxin) is often used in animal experiments to cause a septic state and leads to the production of pro-inflammatory cytokines, including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) through the activation of TLR4. TNF $\alpha$  is a key cytokine that is induced as part of the inflammatory cascade that leads to the septic state. TNF $\alpha$  affects metabolism in that it induces the production of lactate *in vitro* and *in vivo* [87,88], indicating a switching from aerobic to glycolytic energy metabolism. In LPS-treated rats, ETC complexes I, II, and IV were down-regulated both at the transcript and protein level within 24 h after treatment [89]. It was further demonstrated that early in sepsis oxidation of Cytc by COX is competitively and reversibly inhibited; in later stages (at 48 h), it becomes noncompetitive and irreversible [90]. Rats subjected to cecal ligation and puncture, another animal model for sepsis, showed unchanged muscle tissue oxygen levels between sham control and septic animals but the septic animals had reduced ATP levels [91]. Another study performed by the Kozlov group showed that induction of endotoxin shock through LPS treatment resulted in an early increase in blood levels of TNF $\alpha$  and 70% decreased ATP levels after 8 h, a critical time at which about 30% of the animals are dead and most of the remaining animals surviving [92].

All the above reports indicate that metabolic changes occur at the level of OxPhos during sepsis, but the molecular mechanism remained unclear. To gain more insight into this question we investigated the effect of TNF $\alpha$  on cow and mouse liver tissue and mouse hepatocytes in culture. TNF $\alpha$  treatment of bovine and murine liver homogenates produced a 60% reduction of COX activity within 5 min after treatment [93]. Importantly, isolation of cow COX revealed phosphorylation of tyrosine 304 of subunit I after TNF $\alpha$ , which was identified with a phospho-epitope-specific antibody. This is the same site that is targeted for phosphorylation by the cAMP-dependent pathway in liver discussed above. TNF $\alpha$  treatment decreased the mitochondrial membrane potential and resulted in 35% and 64% reduction of cellular ATP levels in mouse hepatocytes and H2.35 cells [93]. It will be interesting to see if Cytc is also targeted by TNF $\alpha$  signaling, which could augment the effect of COX phosphorylation.

**Fig. 2.** Nano-LC-ESI-MS/MS analysis of Blue Native-PAGE-separated, BrCN and trypsin cleaved COX subunit II isolated from cow heart. Prior to analysis by tandem mass spectrometry (MS/MS) the phosphopeptides were enriched with titanium dioxide as described [54,120]. The MS/MS spectrum unambiguously identifies the peptide IPTpSELKPGELR. The localization of Ser126 as the site of phosphorylation was possible after fragmentation of the doubly charged precursor ion (710.5 Th) with collision-induced dissociation (CID) and the triply charged ion (474.3 Th) with electron transfer dissociation (ETD). A, the CID-MS/MS spectrum contains a CID-typical neutral loss of phosphoric acid from the phosphoserine of the precursor ion. The  $y_{10}$  and  $b_3$  fragment ions as well as the neutral loss ions  $y_9$  and  $b_5$  (both  $-H_3PO_4$ ) indicate phosphoserine. B, the ETD fragmentation resulted in a spectrum specifying the peptide amino acid sequence including the phosphosite-specific C-terminal  $z_8$  and  $z_9$  ions and the corresponding N-terminal  $c_3$  and  $c_4$  ions. Furthermore, on the basis of the fragmentation pattern phosphorylation of Thr125 was excluded by the presence of ions  $c_2$ ,  $z_9$ , and  $z_{10}$ .



A question that emerges is: why did such a response evolve, which can lead to death in humans? A likely explanation is that, in a day-to-day context, the organism constantly has to defeat pathogens at a more local setting, e.g., after a small skin cut. Some pathogens seize control of the host infrastructure and energy production system. For example, chlamydiae express a number of nucleotide transporters that facilitate the uptake of molecules such as ATP [94]. Therefore, shutting down OxPhos locally at the site of infection slows down pathogenic growth because essential metabolites are no longer provided by the host. In the rare situation of a systemic inflammation this response can get out of control, leading to MODS and death due to energy failure of entire organs.

In summary, our model proposes that acute inflammatory signaling leads to phosphorylation of COX and strong enzyme inhibition, reduced membrane potentials, and consequently decreased energy levels (Fig. 3). If ATP levels drop below a threshold, such as 30% in liver tissue [92], organ function cannot be maintained, leading to organ failure.

### 3.2. Role of cytochrome c oxidase and cytochrome c in brain ischemia/reperfusion injury: the hyperactive state and excessive reactive oxygen species

Cerebral ischemia/reperfusion injury is caused by interruption of blood flow to the brain. This can occur in multiple pathologies, the most common being ischemic stroke. Ischemic stroke occurs when blood flow to a region of the brain is prevented by occlusion of a cerebral blood vessel. Restoration of blood flow to ischemic tissue is necessary to limit tissue damage; however restoration of oxygen and nutrients to stressed tissue leads to additional damage. This damage is called reperfusion injury and contributes greatly to the overall severity of tissue damage. The brain also undergoes ischemia/reperfusion injury in the setting of cardiac arrest followed by resuscitation. Cardiac failure results in complete cerebral ischemia, and resuscitation of the arrested patient initiates reperfusion of the brain. Both ischemic stroke and cardiac arrest/resuscitation result in extensive morbidity and mortality throughout the population. Stroke is the 3rd leading cause of death and disability in the US and only 10% of resuscitated patients leave the hospital neurologically intact and can resume their former lifestyles [95]. These statistics demonstrate the dramatic need for greater understanding of ischemia/reperfusion injury in the brain and for targeted therapies to treat these disease processes.

Mitochondria play multiple roles in the setting of cerebral ischemia/reperfusion injury both as a cause of neuronal damage and a site of intracellular injury. We propose that there are three distinct phases of altered COX activation during the progression of ischemia/reperfusion injury: 1) the ischemic starvation phase, 2) the reperfusion-induced hyperactivation phase, and 3) the mitochondrial dysfunction phase. The role of COX and Cyt c phosphorylation in these distinct activation profiles remains largely unknown. However, many studies have identified critical alterations in COX activity during the progression of reperfusion injury, suggesting an important role for cell signaling in these changes.

During the ischemic starvation phase, tissue becomes oxygen deprived, thereby depleting COX of its terminal substrate for respiration. In the ischemic brain, this is rapidly followed by ATP depletion, elevated ADP, and, importantly, an increase in mitochondrial calcium [96]. There are multiple cell signaling pathways activated by ischemia that signal to the mitochondria to increase ATP production in order to compensate for the rapid loss of energy. Many phosphorylation/dephosphorylation events have been shown to occur on COX that could, in theory, increase ATP production in ischemic tissue if oxygen were present. Of particular interest is the role of mitochondrial calcium during ischemia and early reperfusion. Mitochondrial calcium concentrations increase during ischemia when intracellular pumps fail, and mitochondria actively

sequester calcium during early restoration of blood flow. This results in dramatically increased mitochondrial calcium concentrations [97]. As discussed above, calcium is the most important signal for mitochondrial activation [72] and induces dephosphorylation of most mitochondrial proteins [75]. Calcium has been shown *in vitro* to lead to hyperactivation of COX through a higher basal activity and loss of the allosteric inhibition by ATP [43]. This is likely caused by changes in the phosphorylation state of COX, which is further supported by the finding that dephosphorylation of COX makes the enzyme insensitive to ATP and ADP [13]. Thus, the loss of allosteric control does not appear to be a direct effect of calcium on COX, but is caused indirectly by dephosphorylation of COX, presumably by a calcium-dependent phosphatase [43]. Increased basal COX activity in combination with a loss of allosteric inhibition by ATP could cause profound problems when reperfusion of the tissue is initiated, as this would lead to hyperpolarization of mitochondrial  $\Delta\Psi_m$  (Fig. 3). It was shown that treatment of brain mitochondria with calcium increased state 4 respiration by 141% [98], which may be explained by COX dephosphorylation. These events would render COX “primed” for hyperactivity resulting in deleterious effects when oxygen and nutrients are restored upon reperfusion.

Based on our model (Fig. 3), upon restoration of blood flow, oxygen and nutrients reach stressed mitochondria and a COX enzyme that has been post-translationally modified to increase its activity. These alterations aid in restoration of  $\Delta\Psi_m$ , and reestablishment of cellular energy levels [99]. In fact, mitochondria rapidly become re-energized and  $\Delta\Psi_m$  is quickly restored within 5 min [99]. However, in this hyperactive state, the ETC generates pathologically high  $\Delta\Psi_m$  levels, and mitochondrial hyperpolarization leads to an exponential increase in ROS generation at membrane potentials exceeding 140 mV [77]. Additionally, measurements of ROS following ischemia indicate that the majority of mitochondrial ROS are created during this early reperfusion interval [100], and the ETC is a primary source of ROS during reperfusion [101]. Indeed, Liu and coworkers demonstrated that reperfusion of ischemic brain results in a rapid restoration of  $\Delta\Psi_m$ , followed by a transient hyperpolarization of  $\Delta\Psi_m$ , which lasts for about 8 min in the mouse brain [102]. This effect is lost when animals undergo ischemic preconditioning, a method shown to produce a robust effect in reducing ROS generation by the mitochondria, which makes it profoundly neuroprotective. These findings position COX as a potential regulatory site that can indirectly control ROS generation by regulating overall ETC flux, thereby controlling  $\Delta\Psi_m$ . This also suggests that regulation of ETC activity and/or  $\Delta\Psi_m$  hyperpolarization provides targets for therapeutic intervention. Neuroprotective therapies designed to prevent  $\Delta\Psi_m$  hyperpolarization have been effective at both preventing ROS generation by the mitochondria and preventing neuronal cell death [103]. In agreement with this hypothesis, mild mitochondrial uncoupling with FCCP reduces  $\Delta\Psi_m$  hyperpolarization, prevents ROS generation, and minimizes cell death in models of cardiac ischemia [104], traumatic brain injury [105], and peroxide-induced neuronal death [106]. Additionally, the converse is also true; promoting hyperpolarization during reperfusion by knocking out mitochondrial uncoupling protein 2 (UCP2) results in increased ROS generation and neuronal damage [103].

The final phase in the progression of brain ischemia/reperfusion injury is known as mitochondrial dysfunction or secondary energy failure. In this phase, COX activity becomes dramatically reduced throughout the progression of reperfusion injury, eventually culminating with energy failure and cell death [107–109]. The role of COX phosphorylation in reduced COX activity during this late stage is currently unknown. Multiple studies have suggested that diminished mitochondrial respiration is likely caused by oxidative damage to the mitochondria [110,111]. Interestingly, while COX appears to be a major site of depressed respiratory capacity [107], COX is particularly resistant to direct oxidative damage [111]. Oxidative damage to the mitochondria can occur on proteins involved in respiration as well as

lipids critical for respiratory protein function. Specifically, the inner mitochondrial membrane lipid cardiolipin, which is required for proper membrane insertion and function of COX and other OxPhos components, can become oxidized [112]. Oxidative damage to the mitochondria results in eventual failure of OxPhos. In addition, ROS production during the reperfusion phase and subsequent mitochondrial dysfunction activate the intrinsic apoptotic pathway, thereby committing the cell to death. In summary, this model puts forth the hypothesis that ischemia-induced stress leads to changes in the phosphorylation state of COX. This renders COX hyperactive and leads to mitochondrial ROS generation during the initial stages of reperfusion. ROS can trigger apoptosis, and they cause damage that initiates ETC and COX hypoactivity and mitochondrial dysfunction during later reperfusion. It is thus possible that therapeutic intervention at the level of COX may be a potent neuroprotective therapy in the context of brain reperfusion injury. If the alterations, i.e., phosphorylations, to COX that increase its activity can be targeted to prevent mitochondrial hyperpolarization and subsequent ROS generation, it may be possible to prevent the downward spiral of mitochondrial dysfunction and cell death.

The role of Cytc in ischemia/reperfusion injury is twofold. The loss of Cytc function is a contributing factor to mitochondrial dysfunction or secondary energy failure, and Cytc release from mitochondria is an initiating event in mitochondrial type II apoptosis and cell death. Recent identification of phosphorylation sites on Cytc discussed above that regulates its activity in respiration and apoptosis raises the possibility that Cytc is actively targeted by stress signaling during ischemia/reperfusion. Because phosphorylation of Cytc partially inhibits respiration, if stress-induced signaling also targets Cytc for dephosphorylation, this would lead to increased ETC flux and further contribute to the hyperpolarization of  $\Delta\Psi_m$ . In support of this hypothesis, we found that Cytc isolated from ischemic brain showed no signs of phosphorylation (unpublished data). In contrast to phosphorylated Cytc, this dephosphorylated Cytc would have the full capability to bind to Apaf1 and trigger downstream caspase activation [10].

In the progression of brain reperfusion injury, mitochondrial respiration begins to diminish [108,113], and mitochondrial dysfunction eventually culminates in cell death. Diminished respiratory capacity precedes the release of Cytc into the cytosol. Therefore, a lack of Cytc availability cannot account for impaired respiration during early reperfusion. One possible factor in mitochondrial dysfunction is peroxidation of cardiolipin by Cytc, an important step in the execution of the apoptotic cascade [114]. Cardiolipin tethers Cytc to the inner mitochondrial membrane, whereas peroxidation of cardiolipin allows disassociation of Cytc and liberates it into a free-floating pool [114]. Extensive peroxidation of cardiolipin has been demonstrated during brain reperfusion, and is associated with initiation of apoptosis. Interestingly, cardiolipin peroxidation might be regulated by phosphorylation of tyrosine sites on Cytc, as suggested by studies using phosphomimetic mutant Cytc [115].

During the final stages of reperfusion injury, Cytc participates in cell demise by initiating apoptosis. Multiple studies have demonstrated the neuroprotective and antiapoptotic effect of therapies designed to activate cell survival signaling and prevent apoptotic release of Cytc [116,117]. However, the idea that stimulation of cell signaling could lead to phosphorylation of Cytc, thereby suppressing apoptosis, has yet to be investigated. In summary, phosphorylation events that regulate COX and Cytc may be novel targets for therapeutic intervention to limit the damage caused by ischemia/reperfusion.

#### 4. Conclusion

COX and Cytc show all three main regulatory features found in key metabolic enzymes: isoform expression, allosteric control, and phosphorylation. This points to the importance of the regulation of the terminal step of the ETC, and supports its suggested rate-limiting

role for overall ETC flux in intact cells. This has direct implications for energy and ROS production, which are dysregulated in numerous human diseases.

In higher organisms cell signaling cascades function as communication networks that allow adjustment of cell, organ, and organismal function to varying internal and environmental conditions, ranging from complete rest (e.g., hibernation) to strenuous exercise. To study the effect of signaling cascades on mitochondrial function important precautions have to be taken to preserve posttranslational modifications. To maintain the physiological phosphorylation state, phosphatase inhibitors have to be included at all steps during the isolation of mitochondrial proteins, and detailed protocols are available for both Cytc and COX [38,118].

Another crucial aspect that deserves special attention is that signaling must not be generalized, and instead has to be carefully evaluated in a tissue-specific context. We have touched on this topic discussing cAMP-dependent signaling that, depending on the tissue, can lead to distinct and sometimes opposing effects. The expression of tissue-specific isoforms further underlines this notion since it can provide tissue-specific targets for phosphorylation as is the case for COX subunit VIa and likely others that have yet to be identified.

Phosphorylation sites have been mapped in all OxPhos complexes and it is therefore possible that strong regulatory effects on enzyme activity, e.g., as seen for COX subunit I Tyr304 phosphorylation, will be reported for complexes I, II, III, and V in future work. Identification of kinases and phosphatases that directly act on OxPhos would also make possible the utilization of genetic methods to further pinpoint the role of posttranslational regulation. This research direction holds tremendous translational potential because it would allow targeted therapeutic intervention in conditions such as sepsis, ischemia/reperfusion injury, cancer, and many others.

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