Chapter 10
Phosphorylation of Mammalian Cytochrome *c* and Cytochrome *c* Oxidase in the Regulation of Cell Destiny: Respiration, Apoptosis, and Human Disease

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Abstract The mitochondrial oxidative phosphorylation (OxPhos) system not only generates the vast majority of cellular energy, but is also involved in the generation of reactive oxygen species (ROS), and apoptosis. Cytochrome *c* (Cytc) and cytochrome *c* oxidase (COX) represent the terminal step of the electron transport chain (ETC), the proposed rate-limiting reaction in mammals. Cytc and COX show unique regulatory features including allosteric regulation, isoform expression, and regulation through cell signaling pathways. This chapter focuses on the latter and discusses all mapped phosphorylation sites based on the crystal structures of COX and Cytc. Several signaling pathways have been identified that target COX including protein kinase A and C, receptor tyrosine kinase, and inflammatory signaling. In addition, four phosphorylation sites have been mapped on Cytc with potentially

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large implications due to its multiple functions including apoptosis, a pathway that is overactive in stressed cells but inactive in cancer. The role of COX and Cytc phosphorylation is reviewed in a human disease context, including cancer, inflammation, sepsis, asthma, and ischemia/reperfusion injury as seen in myocardial infarction and ischemic stroke.

10.1 Introduction

The mitochondrial oxidative phosphorylation machinery (OxPhos) is essential for cell function, maintenance, and survival. OxPhos in mammals provides more than 90% of cellular energy. OxPhos consists of the electron transport chain (ETC), which generates the mitochondrial proton motive force by pumping protons across the inner mitochondrial membrane, and ATP synthase (complex V), which couples the backflow of protons into the matrix with the synthesis of ATP from ADP and phosphate. The ETC consists of three proton-pumping complexes, NADH dehydrogenase (complex I), bc_1-complex (complex III), and cytochrome *c* oxidase (COX; complex IV), and nonproton pumping succinate dehydrogenase (complex II), as well as the nonprotein two-electron carrier ubiquinone and the small one-electron carrier cytochrome *c* (Cytc). Electrons enter the ETC mainly through complex I from NADH. In addition, complex II feeds electrons derived from succinate directly into the ubiquinone/ubiquinol pool, linking the Krebs cycle with OxPhos.

OxPhos dysfunction is devastating as can be seen in patients with "traditional" mitochondrial diseases, caused, for example, by mutations in the mitochondrial DNA or nuclear encoded assembly factors of OxPhos complexes. The tissues that are most prominently affected are those that rely most heavily on aerobic energy production including skeletal muscle, brain, and the visual system. More recently, mitochondrial dysfunction has been implicated in an increasing number of human diseases, including the most common pathologies such as cardiovascular disease, diabetes, cancer, and ischemia/reperfusion injury as seen in myocardial infarction and stroke. These "nontraditional" mitochondrial diseases can be better understood when viewed in light of cellular signaling pathways and regulatory control mechanisms, which are often dysregulated in those pathologies. Recent studies have begun to establish a connection between cell signaling and OxPhos, and more than 20 phosphorylation sites have been mapped on the OxPhos complexes (Hüttemann et al. 2007). There is strong evidence that additional sites are phosphorylated, and given the hydrophobic nature of the many membrane-spanning subunits of the OxPhos complexes, several additional phosphorylation sites will likely be revealed in the future due to technological improvements, specifically in mass spectrometry (MS) and related methodologies such as enrichment of phospho-peptides (Thingholm et al. 2006; Walther and Mann 2010).

10.2 Composition and Function of Cytochrome *c* Oxidase and Cytochrome *c*

This chapter focuses on the regulation of cytochrome *c* oxidase (COX) and cytochrome *c* (Cytc) by cell signaling pathways. To gain a better insight into this topic, we will first briefly discuss the basic properties of both enzymes.

Mammalian COX contains 13 subunits per monomer, and cow heart COX has been crystallized as a dimer suggesting that this is the functional form in mammals (Tsukihara et al. 1996). Each COX monomer contains two heme and two copper redox centers, which are located in catalytic subunits I and II. Of the 13 subunits, three are encoded by mitochondrial DNA and the remaining ten by nuclear DNA (Fig. 10.1).

Mammalian Cytc is a one-electron carrier that shuttles electrons from bc_1-complex to COX. It contains 104 amino acids and a heme group, which is covalently attached to cysteines 14 and 17 (Fig. 10.1, top left). Cytc is highly positively charged with a p*I* of 9.6 and is located in the mitochondrial intermembrane space,

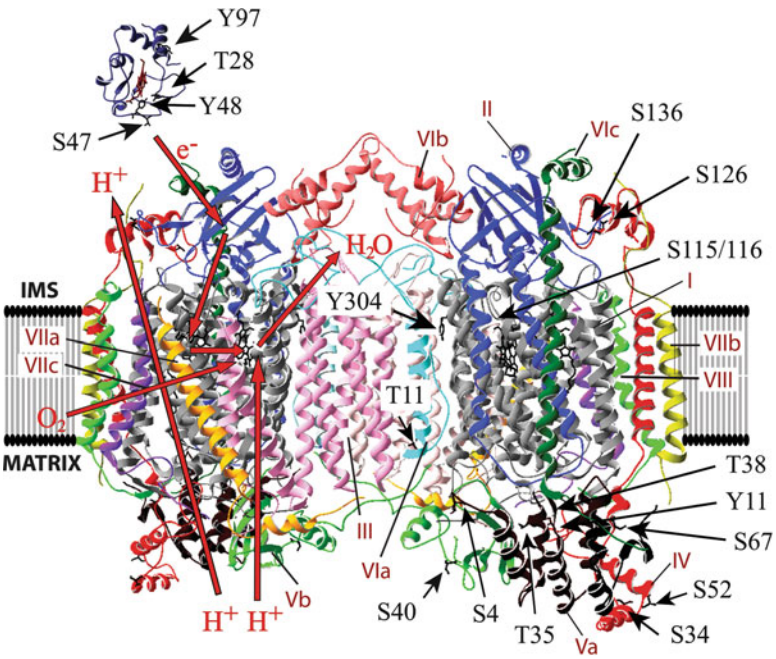

Fig. 10.1 Identified phosphorylation sites on cytochrome *c* and cytochrome *c* oxidase. Crystal structure data of horse heart cytochrome *c* (Sanishvili et al. 1995) and cow heart COX (Tsukihara et al. 1996) were processed with the program Swiss-PDBViewer 3.7. Identified phosphorylated amino acids in mammals are shown in sticks. See Table 10.1 for a detailed description of the sites including phospho-epitopes and references. Note that subunit IV residue Thr52 in rabbit corresponds to Ser52 in cow

where it is associated with negatively charged phospholipids of the inner membrane, in particular cardiolipin.

Cytc and COX represent the last step of the ETC, and catalyze the successive transfer of four electrons to molecular oxygen, which is reduced to water. At the same time, COX pumps protons across the inner mitochondrial membrane, with a stoichiometry of $1H^+/1e^-$ under normal conditions. The precise mechanism of how electron transfer is coupled to proton pumping and the location of the proton exit pathways remain unclear and are a matter of heated debate (von Ballmoos et al. 2011; von der Hocht et al. 2011; Yoshikawa et al. 2011). In addition to the pumped protons, the consumption of "chemical" protons from the matrix side for the water formation reaction contributes to the generation of the proton motive force.

The reaction catalyzed by COX with a free energy of $\Delta G^{\circ\prime} = -100$ kJ/mol (Hinkle et al. 1991) is essentially irreversible. In mammals, it is the proposed rate-limiting step of the ETC in intact cells (Villani and Attardi 1997; Villani et al. 1998; Acin-Perez et al. 2003; Piccoli et al. 2006; Dalmonte et al. 2009; Pacelli et al. 2011), but not in isolated mitochondria. The latter phenomenon may be explained by unsuitable mitochondria isolation methods, which disrupt cellular structures and signaling networks, leading to a loss of regulatory properties of COX and Cytc, likely via dephosphorylation reactions. It has been shown that not only mitochondrial morphology changes dramatically after isolation but also that, in contrast to the other ETC complexes, specifically COX activity is significantly increased (Picard et al. 2011), which may account for a loss of ETC flux control by COX in isolated mitochondria.

Based on the central role of the terminal step of the ETC, it is not surprising that a multitude of regulatory mechanisms are in place in addition to reversible phosphorylation. One other mechanism is the expression of tissue-specific and developmentally-regulated isoforms. Cytc occurs as somatic and testis-specific isoforms in rodents (Goldberg et al. 1977), but not in humans, where the genomic region syntenic to the testes-isoform in rodents now contains a nontranscribed pseudogene (Hüttemann et al. 2003; Zhang and Gerstein 2003). The slight differences in amino acid composition between the isoforms affect its function. In comparison with the somatic isoform, testes Cytc shows a threefold increased activity to reduce hydrogen peroxide; however, it also shows a fourfold increased ability to trigger apoptosis (Liu et al. 2006).

For COX, six subunit isoforms have been identified in mammals to date. Those are heart/skeletal-muscle-specific isoforms of subunit VIa, VIIa, and VIII, a lung-specific isoform of subunit IV, a testes-specific isoform of subunit VIb, and a third isoform of subunit VIII [see Hüttemann et al. (2011) for a current review]. All isoforms are encoded by separate genes. Expression of tissue-specific isoforms may not only directly affect the activities of COX and Cytc but it can also provide a platform for tissue-specific cell signaling. For example, a phosphorylation site was mapped in the heart/skeletal muscle-specific subunit VIa of COX (Table 10.1) (Tsukihara et al. 2003). The corresponding epitope in the liver-type isoform is distinct suggesting that cell signaling is adapted to tissue-specific signals and needs.

Table 10.1 Identified phospho-epitopes in mammalian cytochrome *c* and cytochrome *c* oxidase[a]

Enzyme	Species and tissue	Phosphorylated amino acid[b]	Phospho-epitope[c]	Reference	Method
Cytc	Human skeletal muscle	Thr28	EKGGKHKTGPNLHGL	Zhao et al. (2010)	HT-MS[d]
Cytc	Human skeletal muscle	Ser47	TGQAPGYSYTAANKN	Zhao et al. (2010)	HT-MS
Cytc	Cow liver	Tyr48	GQAPGFSYTDANKKN	Yu et al. (2008a)	MS
Cytc	Cow heart	Tyr97	EREDLIAYLKKATNE	Lee et al. (2006)	MS
COX	Cow liver	Tyr304, SU I	MDVDTRAYFTSATMI	Lee et al. (2005)	MS, cAMP-dependent
COX	Cow liver	Tyr304, SU I	MDVDTRAYFTSATMI	Samavati et al. (2008)	Phospho-epitope-specific antibody; TNFα-dependent
COX	Rabbit heart	Ser115 and Ser116, SU I	SLHLAGVSSILGAINF	Fang et al. (2007)	MS; after I/R[e]
COX	Cow heart	Ser126, SU II	DSYMIPTSELKPGEL	Hüttemann et al. (2012)	MS
COX	Cow liver	Tyr11, SU IV-1	SVVKSEDYALPSYVD	Lee et al. (2006)	MS
COX	Cow heart	Ser34, SU IV-1	VAHVKNLSASQKALK	Helling et al. (2008)	MS
COX	HeLa cells	Ser67, SU IV-1	YRIKFKESFAEMNRG	Olsen et al. (2010)	HT-MS
COX	Rabbit heart	Thr52, SU IV-1	KAPWGSLTRDEKVEL	Fang et al. (2007)	MS; after I/R
COX	HeLa cells	Ser136, SU IV-1	NPIQGLASKWDYEKN	Olsen et al. (2010)	HT-MS
COX	Cow heart	Ser4, SU Va	SHGSHETDEEF	Helling et al. (2008)	MS
COX	Cow heart	Thr35, SU Va	ELRKGMNTLVGYDLV	Helling et al. (2008)	MS
COX	HeLa cells	Thr35 and Thr38, SU Va	LRKGINTLVTYDMVPE	Olsen et al. (2010)	HT-MS
COX	Rabbit heart	Ser40, SU Vb	MLPPKAASGTKEDPN	Fang et al. (2007)	MS; after I/R
COX	Cow heart	Thr11, SU VIa	AKGDHGGTGARTWRF	Tsukihara et al. (2003)	Crystal structure

[a]Identified phosphorylation sites are presented in an ascending amino acid and subunit (SU) order
[b]Numbering according to mature peptide based on cow
[c]Sequence is based on the species where the phosphorylation site was identified
[d]High-throughput mass spectrometry sequencing
[e]Ischemia/reperfusion

Another mechanism is allosteric regulation via binding of ATP and ADP, a built-in energy sensor in COX that adapts COX activity to energy demand (see Chap. 11). Interestingly, ATP also binds to Cytc, contributing to the inhibition of the reaction between Cytc and COX (Ferguson-Miller et al. 1976) but no such regulation has yet been reported for the other OxPhos complexes. Additional regulatory mechanisms acting on COX include (1) competitive binding of nitric oxide, making COX one of the most important targets for NO signaling within the cell (Martinez-Ruiz et al. 2011); (2) binding of small molecules such as thyroid hormone T2, leading to an activation of COX even in the presence of allosteric inhibitor ATP (Arnold et al. 1998) and the fatty acid palmitate, which reduces the H^+/e^- stoichiometry in liver COX, making the COX reaction less efficient (Lee and Kadenbach 2001); and (3) direct protein–protein interactions with nitric oxide synthase (Persichini et al. 2005), the androgen receptor (Beauchemin et al. 2001), the epidermal growth factor receptor (EGFR) (Boerner et al. 2004), and Smad4, a downstream executer of TGF-β signaling, which binds to COX during apoptosis (Pang et al. 2011).

10.3 Regulation of Mitochondrial OxPhos via Phosphorylation of Cytochrome *c* Oxidase and Cytochrome *c*

Single-celled and particularly multicellular organisms employ mechanisms that allow the exchange of information within and between cells and with the cellular environment. Posttranslational modifications such as phosphorylations allow rapid modification of protein function, adapting it to cellular changes. Since the mitochondrial OxPhos system is a central functional unit primarily linked to energy production it is a logical target of cell signaling. Other functions such as ROS production and the participation of Cytc in apoptosis also require tight regulation.

Phosphorylation sites have been identified on all mammalian OxPhos complexes. For the vast majority, signaling pathways, kinases and phosphatases mediating these phosphorylations remain unknown or uncertain (Hüttemann et al. 2007). Future work should focus on the functional consequences of individual phosphorylations, their dynamics, i.e., regarding both extent and change over time, and the identification of kinases and phosphatases acting on OxPhos. Among OxPhos complexes, phosphorylation of COX and Cytc have been studied more deeply and will be discussed here, including their potential role in pathological conditions.

10.3.1 Phosphorylation of Cytochrome c

Cytc is a pivotal component of both apoptosis and electron transfer. There are good reasons to believe that such an important molecule would be the target of cell signaling pathways, but such pathways had never been discovered, even though Cytc

had been studied for more than a century. To discover phosphorylations of Cytc, if they existed, it would be necessary to isolate Cytc from animal tissue under stringent conditions that would preserve the physiological phosphorylation state. Carrying out isolation of Cytc from cow heart tissue using such stringent conditions, we found by mass spectrometry (MS) that it was phosphorylated on Tyr97 (Fig. 10.1) (Lee et al. 2006). We then investigated the effects of that phosphorylation. Previous investigators had found that the heme iron-Met80 absorption band (normally at 695 nm) is an important indicator of functional intactness of Cytc. We found that this band was shifted to 687 nm upon phosphorylation of Tyr97. Phosphorylated Cytc showed functional differences: a shifted K_m of COX for Cytc from 2.5 μM compared to 5.5 μM and enhanced sigmoidal kinetics indicating an inhibition in the reaction with COX.

Cytc isolated from cow liver tissue, we later found, is phosphorylated too, but on a different residue, Tyr48 (Fig. 10.1) (Yu et al. 2008a). This finding surprised us at first, but is consistent with what is known about differential regulation of COX from tissue type to tissue type. The effects of this phosphorylation were distinct from those found in Tyr97 phosphorylation in cow heart in that in liver Cytc there were no spectral changes, and Tyr48 phosphorylation produced a hyperbolic response, similar to the unphosphorylated Cytc. However, at maximal turnover COX activity was more than 50% reduced with Tyr48-phosphorylated Cytc. Thus, phosphorylation in both cases (Tyr97 in heart, Tyr48 in liver) causes partial, but not full, inhibition of the reaction between Cytc and COX.

The functional explanation for inhibition of mitochondrial respiration by Cytc phosphorylation may be that it provides ample mitochondrial membrane potential $\Delta\Psi_m$ for the production of ATP without the production of excessive free radicals that are concomitant with high $\Delta\Psi_m$ levels. However, excess capacity is needed in certain conditions as a trigger of type II apoptosis, which is initiated by a transient hyperpolarization of $\Delta\Psi_m$, followed by a burst of ROS as a signal to commit to cell death, a model that we will revisit below.

Experiments with phosphomimetic mutant Cytc indicate that Cytc phosphorylation at Tyr48 may have important functional effects for apoptosis, the second key role of Cytc. We replaced Tyr48 with Glu, which mimics the negative charge of the phosphate group (Pecina et al. 2010). The mutant Cytc showed a 45 mV reduction of its midpoint redox potential compared to wild-type unphosphorylated Cytc. The reaction kinetics of phosphomimetic Cytc with COX were similar to that of the Tyr48 Cytc with COX, suggesting that the phosphomimetic form is a good model for Tyr48-phosphorylated Cytc. Strikingly, the ability of Cytc to trigger downstream caspase activation, a requirement of apoptosis, was completely lost in the phosphomimetic mutant. The possibility that phosphorylation of Cytc regulates programmed cell death has potentially important therapeutic implications for diseases like cancer, in which apoptosis is inhibited. Once the kinases and phosphatases are identified that act on Cytc, these enzymes could be specifically targeted to promote apoptosis by dephosphorylation of Cytc in conditions such as cancer. In addition, phosphorylation of Cytc could be induced in conditions of stress such as ischemia/reperfusion injury where cell survival strategies would be beneficial.

A fraction of Cytc is normally bound to the mitochondria-specific lipid cardiolipin, tethering Cytc to the inner mitochondrial membrane. It has been suggested that the release of Cytc from cardiolipin is one of the first steps of the participation of Cytc in apoptosis, and that this is mediated via cardiolipin peroxidase activity of Cytc (Kagan et al. 2009). Upon oxidation of cardiolipin by Cytc in the presence of ROS or lipid peroxides that serve as substrates, Cytc binding affinity is reduced, leading to a dissociation of Cytc from cardiolipin. Interestingly, peroxidase activity of Tyr48Glu phosphomimetic Cytc was inducible only at high cardiolipin concentrations, unlike controls, suggesting that Cytc phosphorylation may suppress the cardiolipin oxidation reaction (Pecina et al. 2010). This could be a second safeguard mechanism to ensure that apoptosis is well regulated, through modulation of Cytc attachment to the inner mitochondrial membrane and thus its release.

Two more phosphorylation sites (Thr28 and Ser47; Fig. 10.1, Table 10.1) have recently been mapped on Cytc in skeletal muscle tissue by high-throughput phosphoproteomic MS analysis (Zhao et al. 2010). Although their function is unknown, these phosphorylations suggest regulation by a different pathway than those operating in heart and liver.

It should be kept in mind that there are other functions of Cytc than the well-known roles of Cytc in electron transport and apoptosis. Cytc also scavenges ROS under healthy conditions (Korshunov et al. 1999; Wang et al. 2003), and generates ROS through the $p66^{shc}$ pathway (Giorgio et al. 2005).

10.3.2 Phosphorylation of Cytochrome c Oxidase

The first indication of phosphorylation of COX was presented by Steenaart and Shore (1997), who found that COX was phosphorylated on subunit IV-1 by labeling mitochondrial proteins with radioactive ATP. Since then 14 phosphorylation sites have been mapped on COX (Table 10.1; Fig. 10.1) and several signaling pathways have been studied in some depth. Although many pieces of the puzzle are still missing it is clear that phosphorylation of COX can decisively regulate its activity.

10.3.2.1 The cAMP-Dependent Pathway

In comparison to other signaling pathways PKA signaling has received the most attention. In vitro work first suggested that in cow heart COX incubated with PKA, cAMP, and γ-^{32}ATP, subunit Vb, perhaps subunit II or III, and subunit I are all phosphorylated (Bender and Kadenbach 2000; Lee et al. 2002). However, in vitro work does not usually include various auxiliary components such as scaffolding proteins (e.g., A-kinase anchoring proteins for PKA), and therefore phosphorylation may lack specificity (Welch et al. 2010). Based on our experience, from observation of phosphorylation patterns of COX after in vitro incubation with several

kinases, which did not match results obtained in vivo after stimulation or inhibition of the corresponding signaling pathway, we caution the reader against pursuing an in vitro approach.

Our laboratory pursued an in vivo approach to elucidating the issue of cAMP-dependent phosphorylation of COX in liver tissue, where cAMP is a starvation signal triggered by the hormone glucagon. High cAMP levels were generated by treatment with phosphodiesterase inhibitor theophylline. Vanadate and fluoride, two unspecific tyrosine and serine/threonine phosphatase inhibitors, respectively, were included during the purification of mitochondria and subsequently of COX. The treated enzyme, but not the untreated control, showed phosphorylation on Tyr304 of subunit I (Lee et al. 2005). Tyr304 is located adjacent to the oxygen-binding center on COX, and structural modifications near that site can be expected to exhibit functional consequences. Experiments revealed that Tyr304-phosphorylated COX was inhibited strongly to completely at up to 10 μM Cytc substrate concentrations, even in the presence of allosteric activator ADP (Lee et al. 2005). A similar inhibitory effect was seen with other agents that increase cAMP levels, i.e., the adenylyl cyclase activator forskolin and the physiological starvation hormone glucagon. Functionally, this finding makes sense because in starved conditions it would be adaptive for energy production via the electron transport chain to be decreased. Mechanistically, since PKA does not phosphorylate tyrosine residues it appears that a tyrosine kinase downstream of PKA phosphorylates COX.

A similar inhibitory effect along with lowered ATP levels resulted when cow lung tissue was treated with theophylline (Lee et al. 2005). The concentrations of theophylline were those used in asthma therapy, for which theophylline has long been used effectively. The efficacy of theophylline, then, may be in part related to its effect on cAMP, which would then lead to COX phosphorylation. The general mechanism of action would be the following. Theophylline treatment increases cAMP levels, which causes COX phosphorylation, thus reducing COX activity and decreasing ATP production. Decreased ATP production would lower the ability of the airway to constrict, which is an energy-intensive process, and thus would counter the airway constriction that is a hallmark of asthma. If the energy hypothesis is correct, mitochondrial OxPhos could be specifically targeted for asthma treatment, preferably by agents that act only on lung.

It is known that the response of COX to cAMP signaling is tissue-specific. In neuronal tissue, theophylline treatment leads to COX activation (Hüttemann et al. 2010), whereas in heart theophylline and also 3-isobutyl-1-methylxanthine (IBMX, a heart-specific cAMP inducer) had no significant effects on COX activity in our hands. Other phosphorylations have been found in ischemic rabbit heart, and the possibility was raised that these could be a result of cAMP/PKA signaling (Prabu et al. 2006) because the phosphorylations were not found in the presence of kinase inhibitor H89. However, H89 does not affect only PKA and instead inhibits multiple kinases (Bain et al. 2007). The sites were later identified by MS and are Ser115 and Ser116 of subunit I, Thr52 of subunit IV, and Ser40 of subunit Vb (Fang et al. 2007). Since the above sites are not PKA consensus sequences PKA is probably not directly involved in these phosphorylations.

Although COX subunit I Tyr304 phosphorylation, located toward the intermembrane side, inhibits the enzyme in mammalian liver, a distinct effect was found by the Manfredi group in their investigation of a carbon dioxide/bicarbonate-regulated adenylyl cyclase, which localizes to the mitochondrial matrix (Acin-Perez et al. 2009). This enzyme links nutrient availability to OxPhos activity through sensing of CO_2 generated by the citric acid cycle. The study was performed in HeLa cells, and COX subunits I and IV were phosphorylated. The authors proposed that this phosphorylation is mediated by a matrix-localized PKA, although the phosphorylation sites need to be mapped to further confirm PKA involvement. The effect of such signaling is activation of COX (Acin-Perez et al. 2009). Another example of matrix-localized PKA signaling to COX had been reported earlier: the Riα regulatory subunit of PKA can bind to matrix-localized subunit Vb of COX (Yang et al. 1998).

10.3.2.2 Other Signaling Pathways

Various other studies suggest that COX is a target of signaling pathways, without identification of the phosphorylation sites. For example, in addition to its primary cytosolic localization, nonreceptor tyrosine kinase Src has been shown to localize to the mitochondrial intermembrane space (Salvi et al. 2002). In osteoblasts Src targets COX subunit II for phosphorylation, leading to increased COX activity (Miyazaki et al. 2003).

10.3.2.3 Signaling Pathways Unknown

Additional phosphorylation sites have been mapped on COX for which the signaling pathways involved and the functional consequences are unknown. Those sites are Tyr11 of subunit IV-1 in isolated cow liver (Lee et al. 2006), Ser67 and Ser136 of subunit IV-1, Thr35 and Thr38 of subunit Va in human HeLa cells by high-throughput MS (Olsen et al. 2010), Ser34 of subunit IV-1 and Ser4 and Thr35 of subunit Va in cow heart (Helling et al. 2008), Thr11 of subunit VIa heart isoform, which was identified in the cow heart crystal structure (Tsukihara et al. 2003), and Ser126 on catalytic subunit II, which was identified in three independent COX isolations (Hüttemann et al. 2012) (Table 10.1; Fig. 10.1).

10.3.3 Calcium Signaling

Finally, a promising avenue of research would be to investigate the direct or indirect action of calcium on COX and Cytc phosphorylation. Calcium has been proposed to be the strongest signal for mitochondrial activation (Robb-Gaspers et al. 1998). 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 Sect. 10.4.1.1. Mammalian COX contains a calcium–sodium exchange site in subunit I (Kirichenko et al. 1998, 2005). In addition to a possible direct functional effect on COX, which remains to be studied, calcium may indirectly affect COX and Cytc through changes in the phosphorylation state. It was shown in pig heart mitochondria that calcium leads to dephosphorylation of most mitochondrial proteins (Hopper et al. 2006). Since dephosphorylation of COX at subunit I Tyr304 and of Cytc at Tyr48 and Tyr97 results in increased ETC activity, this may explain some of the activating effects of calcium signaling.

10.3.4 Mitochondrial Tyrosine Phosphatase Shp-2 and Noonan Syndrome

Protein tyrosine phosphatase Shp-2 was the first identified tyrosine phosphatase with mitochondrial co-localization. It was identified in the intermembrane space and the outer mitochondrial membrane in addition to the cytoplasm in rat brain (Salvi et al. 2004). Shp-2 is mostly associated with Ras/mitogen-activated signaling, where it acts as a positive modulator. Mutations in the gene encoding Shp-2 (*PTPN11*) account for about half of the cases of Noonan syndrome (Tartaglia et al. 2001), a relatively common autosomal dominant disorder characterized by congenital heart defects, dysmorphic facial features, webbed neck, short stature, chest deformity, and variable cognitive deficits (Tartaglia et al. 2011). In these Noonan patients with *PTPN11* mutations, Shp-2 shows increased basal phosphatase activity (Neel et al. 2003). Because COX and Cytc are currently the only OxPhos components with mapped tyrosine phosphorylation sites, three of which localize to the IMS (Fig. 10.1) as does Shp-2, they might be targets of Shp-2. We thus analyzed patient and mouse cell lines with Noonan syndrome mutations and observed significantly increased COX activity (Lee et al. 2010). COX and Cytc protein levels were downregulated in the mutant cells, suggesting a compensatory mechanism to counterbalance increased COX activity. In addition, mutant cells showed 30% decreased ATP and increased ROS levels, both of which may interfere with organ development. Currently it is unknown if any of the identified tyrosine phosphorylation sites on COX or Cytc are targets of Shp-2, but the observation above, i.e., the combination of significantly increased COX activity with reduced COX and Cytc protein levels, suggests changes in protein phosphorylation as a mechanistic explanation.

10.4 Role of Cytochrome *c* Oxidase and Cytochrome *c* in Human Disease

COX and Cytc have been implicated in numerous diseases, some of which we briefly discussed above. This final section focuses on three distinct pathological conditions, ischemia/reperfusion injury, cancer, and inflammation, all of which involve COX and/or Cytc in a highly specific and distinct manner.

10.4.1 Cytochrome c Oxidase and Cytochrome c in Ischemia/Reperfusion Injury and the Role of Protein Kinase C Signaling

Loss of blood flow and thus delivery of oxygen and nutrients to tissues causes extensive damage. Different cell types have varying sensitivities or resistance to ischemic damage, depending on the extent to which the tissue relies on OxPhos for ATP production. While tissue damage caused by ischemia can be extensive, of greater clinical interest is the additional damage induced by restoration of blood flow, or reperfusion injury (Oliver et al. 1990). Currently, the only treatment for ischemia is prompt restoration of blood flow. However, paradoxically, the act of restoring oxygen supply to tissue that has undergone ischemic stress causes profound damage (Campbell et al. 1986; Zweier 1988; Aronowski et al. 1997; Fellman and Raivio 1997; Tilney and Guttmann 1997). This, in turn, results in worsened morbidity and mortality in a host of clinical disorders, including stroke, cardiac arrest/resuscitation, myocardial infarction, acute tubular necrosis, and neonatal hypoxic/ischemic encephalopathy (Zweier 1988; Schumer et al. 1992; Aronowski et al. 1997; Fellman and Raivio 1997; Roger et al. 2011). Importantly, reperfusion injury can, in theory, be treated therapeutically.

10.4.1.1 The Connection Between Respiratory Activity, the Mitochondrial Membrane Potential, and the Production of Reactive Oxygen Species

Mitochondria play a central role in reperfusion injury, primarily as a major source of ROS. As we discussed in detail previously (Hüttemann et al. 2008, 2011), mitochondrial ROS are generated by OxPhos, specifically at high $\Delta\Psi_m$. One of the proposed mechanisms of increased ROS at high $\Delta\Psi_m$ is an overall reduction of electron flux in the ETC because high $\Delta\Psi_m$ levels inhibit the proton pumping activity of complexes I, III, and COX from further proton pumping. This causes an increased half-life of the ubisemiquinone radical intermediates, which now have more time to transfer their unpaired electron to oxygen (Liu 1999). As the proposed rate-limiting step of the ETC in intact cells (see Sect. 10.1), Cytc and COX can be considered primary regulators of mitochondrial ROS generation (Piantadosi and Zhang 1996). Here we put forth a model of ROS generation during reperfusion in multiple tissues, and present studies suggesting the validity of this model. Specifically, we propose that altered phosphorylation of COX and Cytc, caused by ischemia, results in high "uncontrolled" respiratory rates, high $\Delta\Psi_m$ levels, and thus a reperfusion-induced ROS burst upon restoration of blood flow (Fig. 10.2a).

Our model proposes that cells modify the activity of OxPhos by posttranslational modifications on COX and/or Cytc under normal physiologic conditions. Under conditions of energy depletion, cells attempt to augment their energy production by

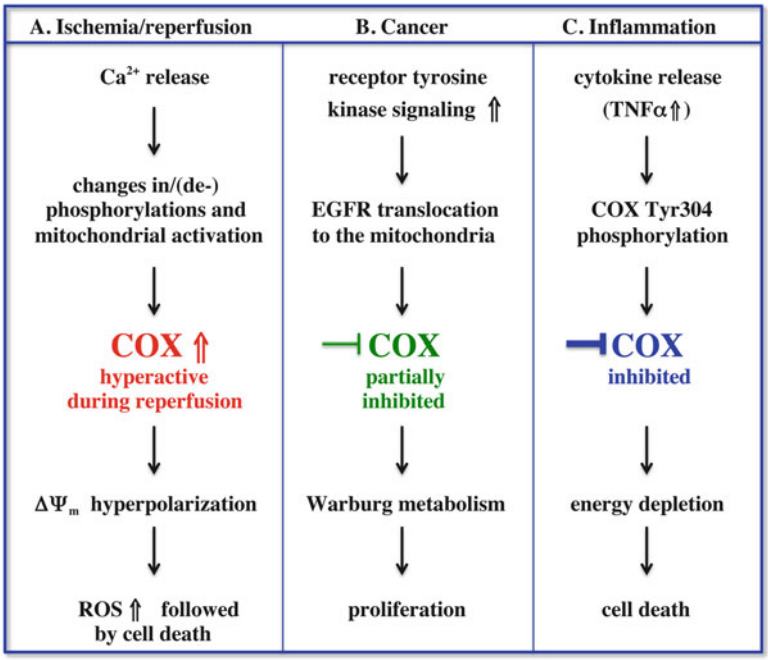

Fig. 10.2 Role of COX in human disease. In our proposed model, the activities of COX and Cytc are regulated by cell signaling pathways. Under healthy conditions both proteins are phosphorylated in vivo leading to controlled respiration. This generates healthy mitochondrial membrane potentials ($\Delta\Psi_m$), allowing efficient energy production but preventing the production of ROS, which are only generated at pathologically high $\Delta\Psi_m$ levels. During pathological conditions the phosphorylation patterns change, leading to an imbalance of ROS and ATP production. (**a**) Ischemic stroke and myocardial infarction are common human pathologies and the only current treatment is rapid resumption of blood flow, i.e., reperfusion. Reperfusion causes the major part of ischemia/reperfusion-related injury through the following sequence of events. During episodes of ischemia, nutrients and oxygen become depleted causing excessive calcium release and alterations of PKC signaling. This results in changes of phosphorylation and/or dephosphorylation of COX and/or Cytc (Cytc is not shown). In the ischemic phase COX does not turn over due to lack of substrates. In the reperfusion phase, oxygen and nutrients are reintroduced leading to a rapid reestablishment of $\Delta\Psi_m$. Because COX is still in a hyperactive state, $\Delta\Psi_m$ increases further, leading to a $\Delta\Psi_m$ hyperpolarization and the production of excessive ROS. ROS in turn serve as a signal for triggering apoptosis leading to cell death. (**b**) Many cancers are characterized by hyperactive receptor tyrosine kinase signaling including EGFR signaling. Here, activation of the pathway leads to an internalization of the receptor and translocation to the mitochondria where EGFR directly interacts with and phosphorylates COX subunit II, leading to a partial inhibition of COX activity. This step allows shifting of aerobic energy metabolism to Warburg metabolism: increased glycolytic and pentose phosphate pathway activity now provide essential building blocks for the cell, enabling rapid proliferation. (**c**) Acute inflammation as in sepsis leads to the release of cytokines including TNFα resulting in phosphorylation of Tyr304 on catalytic subunit I. COX is strongly inhibited leading to decreased $\Delta\Psi_m$ levels, and eventually depletion of ATP. In septic patients, this model would explain organ failure and death through energetic failure

the OxPhos system by altering the phosphorylation status of these complexes. However, under conditions of pathologic energy depletion, such as the ischemic state, energy exhaustion is coupled to oxygen deprivation. In this state, the terminal substrate for OxPhos is absent, and no aerobic ATP production is possible. We propose that the apparent dysfunction of this normal response to energy depletion is the proximal cause of ROS generation upon reperfusion.

The specific role of COX and Cytc phosphorylation in the setting of ischemia/reperfusion remains largely unknown at present. However, many studies have identified alterations in COX activity during the progression of reperfusion injury suggesting a role for cell signaling that alters OxPhos activity. During ischemia, intracellular calcium concentrations increase when ATP-dependent pumps fail (Rosenthal et al. 1987), and mitochondria actively sequester calcium during early restoration of blood flow (Zaidan and Sims 1994). Increased mitochondrial calcium is a potent signal for phosphatase activation, and calcium induces dephosphorylation of most mitochondrial proteins (Robb-Gaspers et al. 1998; Hopper et al. 2006). Interestingly, multiple studies have observed increased mitochondrial respiration, and COX activity, upon calcium sequestration by mitochondria (Rosenthal et al. 1987; Fiskum et al. 2004). In vitro, calcium causes COX hyperactivation and loss of allosteric inhibition by ATP, and the Kadenbach group showed that calcium did not act directly on COX (Bender and Kadenbach 2000). In contrast, they observed an indirect effect, most likely through changes in posttranslational modifications of COX induced by calcium. Additionally, mitochondria treated with calcium had increased state 4 respiration (Vlessis et al. 1990), which may be explained by COX dephosphorylation. Dephosphorylation of COX in vitro results in loss of the ability of ATP to allosterically inhibit COX (Hüttemann et al. 2008). This effect, when occurring in the setting of ischemia/reperfusion injury would result in a condition where COX could generate high $\Delta\Psi_m$ that subsequently leads to the generation of ROS, triggering death processes (Fig. 10.2a).

As discussed above, all functionally studied phosphorylations of Cytc inhibit respiration. Therefore, if calcium-induced dephosphorylation of Cytc were to occur, this would contribute to increased OxPhos flux and thus the hyperpolarization of $\Delta\Psi_m$. Recent studies from our group found that Cytc phosphorylation is lost when brain is rendered ischemic (unpublished). This dephosphoryated Cytc would also have the full capability to induce apoptosis (Pecina et al. 2010).

10.4.1.2 Protein Kinase C

In the context of ischemia/reperfusion injury, PKC signaling has been studied in some detail. PKC is a stress-activated kinase involved in modulating calcium uptake by mitochondria, production of free radicals, and the induction of apoptosis. Importantly, several PKC subtypes have been implicated in regulating these deleterious events following ischemia/reperfusion injury in multiple tissues. It is generally believed that PKC signaling serves to protect the cells during episodes of ischemia/reperfusion but the intricate connection between calcium and PKC signaling may complicate the picture.

There is clear evidence of a direct action of PKC on mitochondria. For example, following ischemia/reperfusion in the heart, PKCε translocates to the mitochondrial inner membrane (Budas et al. 2010). In a model of global brain ischemia, PKCβ translocates to the mitochondria in neurons resistant to cell death, while in brain regions that go on to die (the CA1 hippocampus), no translocation of PKCβ was seen (Kowalczyk et al. 2012). Interestingly, PKCβ was found in the mitochondria, associated with components of the electron transport chain. Indeed, inhibition of PKC family kinases leads to increased $\Delta\Psi_m$ and ROS production (Lu et al. 2011). An important protective effect of PKCε in ischemia/reperfusion injury may be due to its interaction with the calcium-sensing receptor, leading to a reduction of calcium release (Dong et al. 2010). This would prevent calcium-activated dephosphorylation and hyperactivation of mitochondrial proteins. Interestingly, administration of compounds that induce PKC activation provides protection from ischemia/reperfusion injury of the heart (Sivaraman et al. 2009) and brain (Della-Morte et al. 2011). The compound tribulosin protects the heart from ischemia/reperfusion injury through activation of PKCε and parallel activation of superoxide dismutase (Zhang et al. 2010). Another selective PKCε activator, psivarepsilon-RACK, demonstrated significant neuroprotection from reperfusion-induced neuronal cell death (Della-Morte et al. 2011). Translocation of PKCδ to the mitochondria does not occur during ischemia, but was observed within 5 min of reperfusion after 30 min ischemia in rat hearts, followed by a decrease in mitochondrial respiration and an increase in superoxide radical production (Churchill and Szweda 2005).

The above studies underscore the potential contribution of PKC signaling in reversible phosphorylation of OxPhos complexes, and the central role PKC may play in the pathophysiology of ischemia/reperfusion injury. As discussed in Sect. 10.3.2.1, ischemia directly affects COX and leads to phosphorylation of several subunits (Prabu et al. 2006), and it is possible that this phosphorylation is mediated by a yet-to-be-identified PKC isozyme. In rat heart, ischemia/reperfusion resulted in changes of the immunoreactivity of several COX subunits, especially subunit I, and the authors proposed that those subunits are lost from the holoenzyme (Yu et al. 2008b). An alternative and perhaps more likely explanation might be masking of the epitope recognized by the antibody by phosphorylation.

The ischemia-triggered phosphorylations lead to a partial inhibition of COX activity (Prabu et al. 2006), which would be protective during reperfusion. However, other studies have shown that the opposite effect, i.e., activation of COX, is possible. In rat neonatal cardiac myocytes, activation of PKC with diacylglycerol or 4β-PMA caused phosphorylation of COX subunit IV in vitro and resulted in about two- to fourfold increased COX activity (Ogbi et al. 2004; Ogbi and Johnson 2006). PKCε is a possible candidate for this phosphorylation because the authors showed that it co-immunoprecipitated with COX (Guo et al. 2007).

Based on the extent of the stress impact, calcium signaling may prevail leading to an overall activation of the ETC proton pumps. Based on our model (Fig. 10.2a), upon restoration of blood flow, if oxygen reaches a COX enzyme that has been posttranslationally modified to increase its activity, these alterations would initially aid in the restoration of $\Delta\Psi_m$, and the reestablishment of

cellular energy levels (Ekholm et al. 1993). However, in this hyperactive state, the proton pumps would not be inhibited by physiological $\Delta\Psi_m$ levels <140 mV and would continue to generate pathologically high $\Delta\Psi_m$ levels >140 mV, which would lead to ROS generation (Liu 1999). The majority of mitochondrial ROS are created during this early reperfusion interval (Fabian et al. 1995), and the ETC is a primary source of ROS during reperfusion (Piantadosi and Zhang 1996). Reperfusion of ischemic brain results in a rapid restoration of $\Delta\Psi_m$, followed by a transient hyperpolarization of $\Delta\Psi_m$ and substantial ROS generation (Liu and Murphy 2009). These findings position COX and Cytc as potential regulatory sites that can indirectly control ROS generation by regulating overall ETC flux, thereby controlling $\Delta\Psi_m$ and ROS. It is thus possible that therapeutic interventions targeting COX and/or Cytc phosphorylations may be neuroprotective in the context of ischemia/reperfusion injury.

In summary, we propose a model of reperfusion-induced ROS generation and cell death where posttranslational modifications of Cytc and/or COX play a critical role in controlling the eventual fate of the cell. Specifically, we propose that cell signaling systems, most notably PKC signaling, retain Cytc and/or COX in a phosphorylated "controlled" state. However, ischemic stress can result in excessive calcium release and subsequent dephosphorylation of OxPhos complexes, thus tipping the balance from controlled respiration to $\Delta\Psi_m$ hyperpolarization, subsequent ROS generation, and cell death (Fig. 10.2a). Therefore, regulation of OxPhos phosphorylation may represent a novel method to minimize reperfusion injury following ischemic events in multiple tissues.

10.4.2 Cancer and Inflammation: Cytochrome c Oxidase and Cytochrome c as Functional Targets

In this section, we will discuss mechanisms underlying cancer and inflammation and discuss the emerging link between the two (Fig. 10.2b, c). We will start at the macroscopic level by identifying similarities between cancer and inflammation and eventually focus on COX and Cytc at the molecular level.

10.4.2.1 Inflammation as a Promoter of Cancer

Metabolism changes during carcinogenesis, and most solid tumors show a 25–60% reduction of mitochondrial mass compared to healthy differentiated tissue (Pedersen 1978). During carcinogenesis, cells shift their metabolism from aerobic energy production to glycolysis. The shift takes place even in the presence of oxygen and is therefore referred to as aerobic glycolysis, and it is known as the Warburg effect (Warburg et al. 1924; Warburg 1956). Since Warburg's discovery, considerable work has been done on the role of mitochondria in cancer. Generally, two primary mitochondrial cancer-promoting factors have been tied to cancer, via metabolic switching

to provide building blocks for the growing cells (Weinberg and Chandel 2009), and/or via increased mitochondrial ROS production resulting in the emergence of some cells with oncogenic mutations (Ralph et al. 2010).

Inflammation is an immune response initiated by the vascular system to fight various compounds including pathogens, irritants, and even cells of the organism itself. It is involved acutely and chronically in numerous pathological conditions, such as sepsis, asthma, and rheumatoid arthritis. More recently, inflammation has also become a widely accepted component in different stages of tumor development (Rakoff-Nahoum 2006; Mantovani et al. 2008). Grivennikov et al. (2010) proposed a two-step model in which reactive oxygen and nitrogen species (RONS) produced by inflammatory cells first cause mutations in neighboring cells. Tumor initiation is further amplified by cytokine-mediated increased RONS production in premalignant cells. The second step, tumor promotion, is accompanied by immune cell-mediated cytokine production, which activates key transcription factors in premalignant cells, including NF-κB and STAT3. This induces pro-tumorigenic processes, including survival, proliferation, growth, angiogenesis, and invasion. Since it has turned out that key factors such as ROS are found in *both* inflammation and cancer and are connected to mitochondria, this has stimulated examination of the links that may tie them together (Kamp et al. 2011).

The observations that tumors often arise at a site of chronic inflammation, and that they contain inflammatory cells, are more than 100 years old. Recent interest in this topic was promoted by several types of observations, including substantial epidemiological evidence, such as the beneficial effect on cancer prevalence of chronic use of nonsteroidal anti-inflammatory drugs, and the unraveling and manipulating in animal models of the molecular pathways. The considerable epidemiological evidence includes a wide array of chronic infections, such as by *Helicobacter pylori*, exposure to a wide range of irritants that trigger inflammation, such as tobacco smoke, and autoimmune conditions. Strong evidence also comes from studies of inflammatory bowel disease. Patients with ulcerative colitis, an inflammatory bowel disease, have a five- to sevenfold increased risk of colorectal cancer; this risk is reduced by 80% by administration of cyclooxygenase-2 inhibitors (Kamp et al. 2011).

Germ-line mutations are rare causes of cancer; about 90% of cancers results from a combination of environmental factors and somatic mutations. Detailed studies have uncovered two pathways of inflammatory connection to cancer. An extrinsic pathway, which raises cancer risk, is provided by a chronic site-specific inflammatory condition such as pancreatitis or inflammatory bowel disease (Mantovani et al. 2008). In the intrinsic pathway oncogenes are activated, which is the driving force in an environment provided by the extrinsic pathway. Studies of *RAS* family mutations and *MYC* show the early induction of chemokines and inflammatory cytokines as part of the remodeling of the tissue microenvironment.

Inflammation has been shown to participate in all of the recognized stages in tumorigenesis—initiation, promotion, and metastasis. Initiation usually consists of accumulation of multiple mutations in the same cell. This is more likely in an inflammatory microenvironment, either via RONS produced by activated

inflammatory cells or via cytokines produced by inflammatory cells that stimulate RONS production in neighboring epithelial cells. In tumor promotion, an initiated cell transforms into a tumor. Initiated cells are aided by inflammation both to proliferate and to survive, such as via the ROS-stimulated production of HIF-1α (Hamanaka and Chandel 2010). In addition, inflammatory mediators such as STAT3 and NF-κB promote the HIF-1α stimulation of angiogenesis that is needed by growing tumors to provide adequate blood supply (Grivennikov and Karin 2010). Lastly, since more than 90% of cancer mortality results from metastasis, this component is of the greatest clinical importance. Metastasis is clearly linked to inflammation. Many initiated cells express chemokine receptors on their surface (Balkwill 2004). During metastasis, such cells utilize chemokines to aid their migration to distant sites, and to aid their survival upon arriving (Kim et al. 2005, 2009). Furthermore, both autocrine and paracrine signaling by cytokines like TNFα upregulate receptor expression and thereby increase invasive capacity and facilitate metastasis (Kulbe et al. 2005).

The epidemiological connection between inflammation and cancer stimulated numerous studies designed to elicit the mechanistic basis. As signaling pathways were unearthed, it became clear that central mediators of inflammation-associated cancer are RONS. Among those, ROS are the most studied reactive species and arise primarily from NADPH oxidase in phagocytes but elsewhere largely (>90%) from metabolism via the mitochondrial electron transport chain.

Work being pursued from another direction has turned out to be germane. The Warburg hypothesis connected metabolism and cancer in the original version by noticing that tumors are more glycolytic than normal tissues and that, therefore, metabolism was a component of cancer. This observation has also stimulated numerous studies, which have led to a more nuanced picture than the original hypothesis that takes into account a number of metabolic adaptations made by tumors to promote their growth. Except for the situation where hypoxia is found centrally in solid tumors, glycolysis appears not to be utilized for ATP production but for producing intermediates through the pentose phosphate pathway for nucleotide and phospholipid synthesis (Hamanaka and Chandel 2010; Weinberg et al. 2010). Mitochondrial metabolism per se in normoxic conditions may be dispensable, as suggested by a study that utilized a mutation in a complex III gene to block electron transport. Cells containing this mutation reduced but did not abolish a proxy for tumor growth, whereas ρ° cells did not show anchorage-independent growth in a Kras tumor model (Weinberg et al. 2010). An important function of mitochondria appears to be ROS production as a signal for cell proliferation.

A provisional consolidated picture that emerges from considering these dual connections is that inflammation-stimulated ROS, produced in mitochondria under stimulation by cytokines and hypoxia, can act as a tumor promoter. Furthermore, such ROS would be amplified by ROS already produced as growth stimulators by emerging tumors. The increased ROS level could therefore act also as a mutagen to initiate feed-forward cycles of cellular decline, thereby playing a role in both the extrinsic and the intrinsic pathways (Mantovani et al. 2008).

10.4.2.2 Cancer Signaling Targets Cytochrome *c* Oxidase

In addition to metabolic changes, cancers manage to evade apoptosis, and both changes are likely caused, at least in part, by receptor tyrosine kinase signaling that is upregulated in many cancers. Increased epidermal growth factor receptor (EGFR) signaling is implicated in numerous cancers including breast, colon, and lung cancers, and it is the first example of a tyrosine kinase receptor with a direct effect on COX. After stimulation with EGF it was shown in breast cancer cell lines that EGFR translocates to the mitochondria where it physically interacts with COX subunit II (Boerner et al. 2004). Only the activated, Tyr845-phosphorylated EGFR receptor binds to COX together with Src kinase leading to an increase in COX subunit II phosphorylation in vitro as was shown after incubation with [γ-^{32}P]ATP (Demory et al. 2009). Although the phosphorylation site remains to be identified, those findings are in line with the Warburg effect because COX activity was decreased by 60% after cells were treated with EGF (Fig. 10.2b). It is possible that other receptor tyrosine kinases may follow a similar mechanism and that they target other OxPhos components. The only other OxPhos complex where tyrosine phosphorylation has been shown to date is ATP synthase (Ko et al. 2002): NIH3T3 and kidney cells treated with platelet-derived growth factor (PDGF) displayed tyrosine phosphorylation of the δ-subunit of ATP synthase.

Future studies of receptor tyrosine kinase signaling on multiple OxPhos components might reveal a concerted mode of action, i.e., the parallel targeting of several enzymes for phosphorylation to adapt OxPhos activity to cancer-specific energy metabolism. Specifically, Cytc might be targeted for phosphorylation for two reasons: since the functionally studied Cytc phosphorylations on tyrosines 48 and 97 both lead to an inhibition of respiration as discussed above, increased phosphorylation would contribute to the Warburg effect. In addition, Cytc phosphorylation may interfere with apoptosis as suggested by studies with phosphomimetic Cytc, which was not able to trigger any measureable caspase activation (Pecina et al. 2010). Since cancers manage to evade apoptosis, increased phosphorylation of Cytc via cancer signaling might provide a mechanism for the suppression of apoptosis.

10.4.2.3 Inflammatory Signaling Targets Cytochrome *c* Oxidase

The effect of acute inflammation on mitochondrial function has been studied in some detail. Acute inflammation as seen in sepsis is a major medical problem and leading cause of mortality in intensive care units with 210,000 deaths annually in the USA alone (Hotchkiss and Karl 2003). It can be caused by pathogenic infections of the blood and is therefore often referred to as blood poisoning. Sepsis can affect various organs such as the brain, heart, and liver, and it can result in multiple organ dysfunction syndrome (MODS) (Ruggieri et al. 2010).

Genetic evidence strongly suggests that mitochondria play a key role in sepsis. The mitochondrial DNA composition is a genetic predictor for survival after sepsis. Mitochondrial DNA can be grouped into evolutionarily related DNA

families, i.e., mitochondrial DNA haplogroups. Interestingly, septic patients belonging to haplogroup H, which is common in Europeans, have a more than twofold higher chance of survival compared to patients with other haplogroups (Baudouin et al. 2005).

Somewhat similarly to cancer, acute inflammation is accompanied by metabolic changes and a suppression of mitochondrial respiration. Thus septic patients show increased rates of lactate production and blood lactate levels (Revelly et al. 2005). Increased systemic delivery of oxygen during the course of sepsis does not improve outcome (Hayes et al. 1994), suggesting that oxygen consumption rather than uptake and delivery is impaired, a condition referred to as cytopathic hypoxia (Fink 2002). Therefore, the ETC and specifically COX seem to be a logical target of inflammatory signaling. Indeed, in endotoxin-treated rats, a commonly used animal model for sepsis, ETC complexes I, II, and COX were downregulated both at the transcript and protein levels within 24 h after treatment (Callahan and Supinski 2005). A recent study with 96 septic patients analyzed COX in platelets. The authors demonstrated a highly significant positive correlation between survival and COX activity and amount (Lorente et al. 2011). The Levy group demonstrated in a cecal ligation sepsis animal model that oxidation of Cytc by COX was competitively and reversibly inhibited, whereas in later stages it became irreversible and noncompetitive (Levy et al. 2004). Others have further shown that cellular energy levels are significantly reduced in septic animals (Astiz et al. 1988), which can be explained with the suppression of mitochondrial respiration. In septic rats, a 70% reduction of tissue ATP appears to be a critical threshold for cellular survival, since a further reduction appears to be incompatible with sustaining cellular functions, resulting in death (Duvigneau et al. 2008).

The above reports point to alterations in COX function during the course of sepsis. To gain a better molecular understanding, we tested the effect of tumor necrosis factor α (TNFα) on COX. TNFα is a proinflammatory cytokine that is strongly induced during sepsis and is a promoter of the septic state (Duvigneau et al. 2008). TNFα alters cellular metabolism by inducing lactate production in vitro and in vivo (Lee et al. 1987; Tracey et al. 1987), indicating a metabolic switch from respiration to glycolysis, as seen in septic patients.

We therefore analyzed the effect of TNFα on cow and mouse liver tissue as well as mouse hepatocytes in culture. TNFα treatment of liver homogenates caused a 60% reduction of COX activity within 5 min after treatment (Samavati et al. 2008). To identify the molecular mechanism explaining this effect, we isolated COX from cow liver with and without TNFα treatment. Further analysis revealed phosphorylation of subunit I tyrosine 304 after TNFα treatment, the same site that was targeted for phosphorylation by the cAMP-dependent pathway in liver as discussed above (Fig. 10.1). TNFα treatment resulted in a reduction of $\Delta\Psi_m$ and a 35 and 64% decrease of cellular ATP levels in mouse liver tissue and H2.35 cells, respectively (Samavati et al. 2008).

The reader may ask, why does such an inflammatory mechanism with potentially disastrous consequences exist in humans? We recently proposed the following scenario

(Hüttemann et al. 2012): The septic state is an extreme inflammatory condition that affects major parts of or an entire organism, and thus is not localized to a restricted small area, a much more common inflammatory situation. Sepsis is very rare compared to conditions of localized inflammation, such as small wounds that may occur on a daily basis. Here shutdown of cellular processes and specifically OxPhos is understandable because several pathogens take over the host infrastructure and energy production system. For example, Chlamydiae bacteria express several nucleotide transporters that facilitate the uptake of molecules such as ATP (Knab et al. 2011). As a result cutting off essential metabolites locally at the infected area will help the organism fight the pathogen. If inflammation gets out of control and becomes a systemic reaction, MODS and death can occur due to energy failure of entire organs (Fig. 10.2c).

10.5 Conclusion

In contrast to the other ETC complexes, COX and Cytc show all three main regulatory mechanisms found in key metabolic enzymes: isoform expression, allosteric control, and phosphorylation. This supports the suggested rate-limiting role of this step in the ETC and thus makes COX and Cytc prime target candidates for therapeutic interventions in the future in the numerous pathological conditions where mitochondrial energy and ROS production are dysregulated. Identification of kinases and phosphatases that act on COX and Cytc will be a central step in this endeavor and allow specific manipulation of signaling pathways.

Functional consequences of cell signaling also have to be carefully analyzed in order to gain a better understanding of the structure–function relationships and the effect at the physiological or organismal level. For example, from what is known about inflammatory and cancer signaling to date, both pathways affect COX at the molecular level and both pathways lead to an inhibition of COX activity (Fig. 10.2b, c). COX is a target via phosphorylation on subunits I or II triggered by TNFα and EGFR, respectively. The resultant changes in COX kinetics are different, however, since TNFα causes a shift from hyperbolic to sigmoidal kinetics with very low COX activities at low Cytc substrate concentrations (Samavati et al. 2008), whereas EGFR signaling does not change the hyperbolic kinetics but decreases maximal turnover by 60% (Demory et al. 2009). Thus, inflammatory signaling can function as an off-switch whereas growth factor signaling leads to a general partial inhibition of COX, in support of the Warburg hypothesis.

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