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Review

Superoxide generation by complex III: From mechanistic rationales to functional consequences☆

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ABSTRACT

Apart from complex I (NADH:ubiquinone oxidoreductase) the mitochondrial cytochrome bc_1 complex (complex III; ubiquinol:cytochrome *c* oxidoreductase) has been identified as the main producer of superoxide and derived reactive oxygen species (ROS) within the mitochondrial respiratory chain. Mitochondrial ROS are generally linked to oxidative stress, aging and other pathophysiological settings like in neurodegenerative diseases. However, ROS produced at the ubiquinol oxidation center (center P, Q_o site) of complex III seem to have additional physiological functions as signaling molecules during cellular processes like the adaptation to hypoxia. The molecular mechanism of superoxide production that is mechanistically linked to the electron bifurcation during ubiquinol oxidation is still a matter of debate. Some insight comes from extensive kinetic studies with mutated complexes from yeast and bacterial cytochrome bc_1 complexes. This review is intended to bridge the gap between those mechanistic studies and investigations on complex III ROS in cellular signal transduction and highlights factors that impact superoxide generation. This article is part of a Special Issue entitled: Respiratory complex III and related *bc* complexes.

1. Introduction

Mitochondria are generally regarded as a major source of reactive oxygen species (ROS) in eukaryotic cells [1]. Within mitochondria, respiratory chain complexes I (NADH:ubiquinone oxidoreductase) and III (cytochrome bc_1 complex; ubiquinol:cytochrome *c* oxidoreductase) are considered as the main producers of superoxide and derived reactive oxygen species [2–4]. So far, an elevated ROS production of mitochondria has been mainly associated with pathophysiological settings including neurodegenerative diseases [5], oxidative damage during ischemia/reperfusion injury [6] and the aging process [7,8]. However, recent findings and novel concepts imply a role of (mitochondrial) ROS as regulatory agents in a number of biological phenomena [9–11]. Especially complex III derived ROS have been linked to cellular redox signaling pathways, *e.g.* hypoxic stabilization of HIF-1α [12–15], but a detailed understanding of the molecular mechanisms that trigger the generation of 'signaling ROS' is missing. Insights into the molecular mechanism of superoxide production came from studies aiming to elucidate the bioenergetic function of the cytochrome bc_1 complex to pump protons across inner mitochondrial and bacterial cytoplasmic membranes. The protonmotive Q-cycle first proposed by Peter Mitchell [16,17] is generally accepted as the fundamental mechanism of proton pumping [18–22]. It has been shown a long time ago [23,24] that superoxide is formed at the ubiquinol oxidation center (Q_o site, center P) of the cytochrome bc_1 complex and a semiquinone as an intermediate of ubiquinol oxidation has been proposed as the immediate electron donor for oxygen [25–27]. However, recent studies [28–30] have shown that superoxide production from the Q_o site is stimulated by the presence of oxidized ubiquinone suggesting that the electron might be transferred onto oxygen from reduced cytochrome b_L *via* ubiquinone in a reverse reaction rather than during the forward Q-cycle reaction. This finding might have some implications for the production of 'signaling ROS' [31]. This review summarizes the current knowledge of superoxide production by the cytochrome bc_1 complex and also intends to build a bridge between mechanistic studies and investigations on cellular redox signaling including factors/proteins that influence mitochondrial ROS generation and release.

2. Mechanism of superoxide generation by complex III

2.1. Bioenergetic background: the protonmotive Q-cycle

2.1.1. A brief overview

The pioneering concept of Peter Mitchell on the molecular mechanism of the H^+-pumping cytochrome bc_1 complex (complex III) known as the protonmotive Q-cycle [16,17] has been approved by numerous studies (reviewed in [18–22]), though a few refinements

Abbreviations: H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide; Q, ubiquinone; QH_2, ubiquinol; $Q^{\bullet -}$/SQ, semiquinone; Q_i site/center N, ubiquinone reduction site; Q_o site/center P, ubiquinol oxidation site; ROS, reactive oxygen species; SMPs, submitochondrial particles

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and modifications had to be introduced. The initial theory-based model is supported by the crystal structures of cytochrome bc_1 complexes from bovine [32,33], chicken [34], yeast [35–37] and bacteria [38–40] that define the positions of the electron-transferring cofactors and reveal possible proton conduction pathways and the structural organization of the two ubiquinone binding sites. However, the exact position of the ubiquinone(s) at the Q_o site has not been resolved yet [41,42]. Three transmembrane subunits of complex III that are conserved from bacteria to mammals contain all prosthetic groups involved in the redox reactions: the diheme cytochrome *b*, cytochrome c_1 and the so-called Rieske protein containing a unique [2Fe–2S] cluster in which one of the two iron atoms is coordinated by two histidines rather than two cysteines. The cytochrome bc_1 complexes of the photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* contain only one additional subunit or none, respectively and can be regarded as a minimal functional form of the proton pump [43]. Both bacteria and the yeast *Saccharomyces cerevisae* [44] have been widely used as model organisms for mutagenesis and functional studies on the cytochrome bc_1 complex. Mitochondrial complex III of higher eukaryotes contains up to 8 additional accessory subunits that are not essential for catalysis.

The spatial arrangement of the redox centers is the basis for vectorial H^+-transport [41,42]. The two ubiquinone-reaction centers are located on opposite sides of the membranes and are mainly formed by cytochrome *b*. The ubiquinol-oxidation center (called center P or Q_o site) resides on the positive side of the membrane and the ubiquinone-reduction center (center N or Q_i site) faces the negative side of the membrane (Fig. 1). Furthermore, the structures demonstrated an obligate homodimeric organization of the cytochrome bc_1 complexes [41]. The dimeric organization has functional implication as shown by extensive kinetic and mutational investigations with bacterial and yeast complex III that indicate electron equilibration between the cytochrome *b* subunits [45–49] and conformational communication between center P and center N [50,51]. It has been proposed that such a 'bus bar' organization [48] would allow fast electron exchange between all four *b*-hemes and diminish leak of electrons and generation of superoxide [48,52–54]. However, it is still controversially discussed whether both monomers operate independently or function cooperatively [55,56]. Anyway, the overall protonmotive Q-cycle starts with the oxidation of ubiquinol to ubiquinone at the Q_o site, whereupon the two electrons are fed into a high-potential and low-potential chain in a bifurcated manner [18,22]. One electron (the 'first electron' in sequential models, see Section 2.1.2) is transferred to the binuclear iron–sulfur cluster of the Rieske protein (Fig. 1). The flexible head of the reduced Rieske protein then moves from the 'b-position' (close to cytochrome *b*) to the 'c_1-position' (close to cytochrome c_1) and the electron is transferred *via* the heme of cytochrome c_1 to soluble cytochrome *c*. Simultaneously, two protons are released on the positive side of the membrane (intermembrane space in the case of mitochondria). The other (second) electron enters the low-potential chain at heme b_L (low potential heme *b*) and is transferred *via* heme b_H (high potential heme *b*) onto ubiquinone bound to the Q_i site, leading to the formation of a stabilized semiquinone that can be detected by EPR spectroscopy [57]. To complete the Q-cycle a second ubiquinol is oxidized at the Q_o site, two more protons are released at the positive side of the membrane and a second cytochrome *c* is reduced *via* the high potential chain, while a second electron transfer through the low potential chain eventually reduces the semiquinone to ubiquinol at the Q_i site. This is accompanied by the uptake of two protons from the negative side of the membrane (matrix in the case of mitochondria). In summary, the net translocation of $2H^+/2\,e^-$ is achieved by a directed uptake and release of protons at topologically separated ubiquinol-oxidation and ubiquinone-reduction sites, located at opposite sides of the membrane, and the vectorial transport of electrons through cytochrome *b* towards the negative side of the membrane (Fig. 1).

2.1.2. Mechanism of electron bifurcation during ubiquinol oxidation

Understanding the exact molecular mechanism of ubiquinol oxidation at the Q_o site is indispensable as it is the most critical step in the protonmotive Q-cycle for a number of reasons. First of all, it is the energy conserving reaction and is the prerequisite to vectorial proton translocation. Second, it is generally accepted that (unwanted) short circuits at this site would result in unproductive or even deleterious aberrant electron transfer including the formation of superoxide (for a review see [22]). Such short circuits or bypass reactions are largely suppressed in the native (not inhibited) complex III and the production of superoxide is low. To understand the molecular mechanism of ubiquinol oxidation it would be beneficial to know the exact position of the substrate at the Q_o site, but none of the crystal structures have revealed it yet [41,42]. Nevertheless, cytochrome bc_1 complexes from different sources [32–36,58] have been crystallized with competitively acting Q_o site inhibitors giving a hint where substrate molecules may bind. The structural and kinetic data [58] indicate that binding of these inhibitors is mutually exclusive, suggesting overlapping distal and proximal binding sites towards heme b_L. Accordingly a double-occupancy Q_o site model has been proposed by Dutton and coworkers for the *R. capsulatus* cytochrome bc_1 complex in which two ubiquinones cooperate at the primary steps of the catalytic reaction [59–62]. In addition, binding of two ubiquinones at the Q_o site has been shown for the bovine cytochrome bc_1 complex with a NMR-based method [63]. On the other hand a number of single occupancy models have been proposed which include simultaneous, as well as sequential electron transfer to the primary acceptors, *i.e.* the Rieske iron–sulfur protein and heme b_L (Fig. 2) [18,19,21,22,64–66]. Despite fundamental differences (Fig. 2) all models include a mechanistic constraint or gating that suppresses potential short circuits or bypass reactions. Brandt [64] explained the obligate bifurcation of electron flow at the Q_o site (center P) by combination of a 'catalytic switch' of the Rieske iron–sulfur protein between two positions and a chemical control of ubiquinol oxidation that forbids the simultaneous occurrence of a (negatively charged) semiquinone species and a reduced heme b_L at the Q_o site. Highlighting the complete reversibility of the Q-cycle, Osyczka et al. [22,52] suggested a 'double-gating' mechanism incorporating a semiquinone as intermediate that allows ubiquinol oxidation when the Rieske FeS-cluster and heme b_L are both oxidized, as well as ubiquinone reduction in the reverse mode, when FeS-cluster and heme b_L are both reduced, but forbids quinone electron transfer when the FeS-cluster is oxidized and heme b_L is reduced. A semiquinone as a true intermediate in a sequential mechanism of ubiquinol oxidation has been also included in other models [21]. Even such models imply that ubisemiquinone is formed only transiently at the Q_o site, and never accumulates to significant amounts in the functional enzyme. Hence the occupancy of this redox intermediate is expected to be extremely low and formation of a Q_o site-associated semiquinone has been proven very difficult to show experimentally — even under conditions that should promote its occurrence, *i.e.* in the presence of the Q_i site inhibitor antimycin A or in a heme b_H knockout mutant [67–69]. In contrast, Yu and co-workers could not detect a semiquinone in *R. sphaeroides* mutants lacking heme b_H or heme b_L [70]. In models proposing a concerted electron transfer from ubiquinol onto both redox centers, the participation of a semiquinone intermediate is completely excluded [22,52,65,66] (Fig. 2). Such a scenario is supported by investigations of pre-steady state reduction kinetics of the bc_1 complex redox prosthetic groups (cytochromes and the Rieske protein) [66,71]. The fundamental question, whether a semiquinone is a true intermediate during ubiquinol oxidation, has immediate implications for the mechanism of superoxide formation at the Q_o site (Fig. 2). Very recently, it was proposed that molecular oxygen acts as a mediator for the electron transfer from QH_2 to heme b_L during the bifurcated oxidation of ubiquinol [72]. However, this suggestion disregards the fact that the catalytic mechanism of

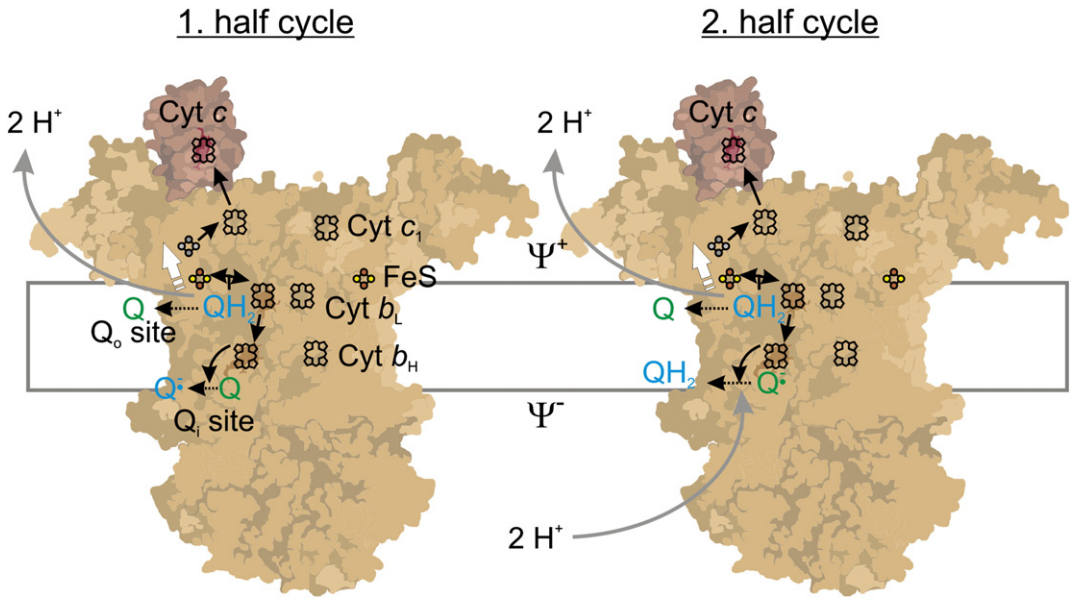

Fig. 1. The protonmotive Q-cycle of the cytochrome bc_1 complex. Schematic model illustrating the approximate positions of the redox cofactors and the ubiquinone binding sites of the functional dimer. Electron transfer within one monomer is indicated by solid black arrows, protonation/deprotonation by solid gray arrows. The oxidized form of ubiquinones in a partial redox reaction is shown in green and the reduced form is shown in blue. A white arrow indicates the movement of the flexible head of the Rieske protein from the b-position (colored symbol) to the c_1-position (gray). Bifurcated ubiquinol oxidation at the Q_o site directs one electron to the high-potential chain (FeS, Cyt c_1), and the other to the low-potential chain (Cyt b_L, Cyt b_H). In the first half cycle, one cytochrome *c* (Cyt *c*) is reduced at the positive side of the membrane in addition to the reduction of ubiquinone to ubisemiquinone at the Q_i site. During the second half cycle, a second Cyt *c* is reduced in addition to the reduction of the ubisemiquinone to ubiquinol at the Q_i site. The complete cycle translocates 2 charges/2 electrons across the inner membrane. For further details see text.

cytochrome bc_1 and related cytochrome b_6f complexes must have been evolved in ancestral enzymes in a low oxygen environment [54].

2.2. Molecular mechanism of superoxide production at the ubiquinol oxidation site

2.2.1. Superoxide is produced at the ubiquinol oxidation site (Q_o site, center P)

That superoxide is indeed produced at the Q_o site and not at the Q_i site has been shown by the use of specific inhibitors that bind to the two distinct ubiquinone binding sites. A vast number of studies (*e.g.* [25–29,73–78]) that used differentially complex preparations (intact mitochondria, submitochondrial particles, purified cytochrome bc_1 complexes) from diverse sources (photosynthetic bacteria, mitochondria from yeast and mammals — mainly from heart) have shown that the superoxide production at complex III is enhanced upon addition of the Q_i site inhibitor antimycin A. The rate is strongly increased under conditions of so-called oxidant-induced reduction, *i.e.* if the presence of sufficient amounts of reducing equivalents leads to the reduction of the *b*-type hemes and the downstream respiratory chain (cytochromes c_1 and *c*) is oxidized at the same time [79]. Importantly, the antimycin A induced superoxide production is blocked by the addition of Q_o site inhibitors [26–28,76,77]. It emerged that the 'distal' Q_o site inhibitor stigmatellin completely abolishes the superoxide production while binding of a 'proximal' Q_o site inhibitor like myxothiazol caused only a partial reduction and can even *per se* induce superoxide production at a lower rate [26,27,80]. This might be explained by the fact that both types of inhibitors bind to different niches of the Q_o site which have independent functions [27] as implicated by the double-occupancy Q_o site model proposed by Dutton and coworkers. It may be also attributed to the fact that stigmatellin is a much more 'tight' Q_o site inhibitor than myxothiazol, because it raises the midpoint potential of the Rieske protein by 250 mV [81] and arrests its flexible head domain in the 'b-position' [82]. It was shown that binding of stigmatellin to only one monomer per dimer is sufficient to abolish catalytic activity of the cytochrome bc_1 complex [81,83,84]. However, it was observed that stigmatellin binding to the fully oxidized cytochrome bc_1 complex from *R. sphaeroides* induces reduction of the Rieske FeS-protein and superoxide production even in the absence of the substrate ubiquinol [84]. The authors concluded that the Rieske FeS-protein becomes a strong oxidant upon stigmatellin binding and extracts electrons from an organic compound, likely an amino acid residue leading to the formation of organic radicals that can be detected by EPR. The strong increase of the midpoint potential of the Rieske FeS-protein implies that such a reaction is not reversible, *i.e.* that the Rieske FeS protein stays reduced, indicating that stigmatellin binding would not lead to a continuous production of radicals.

The maximal rates of antimycin A-induced superoxide generation by purified cytochrome bc_1 complexes from yeast and bovine heart are around one molecule superoxide per second and cytochrome bc_1 complex [26–28,85]. Sun and Trumpower [86] found about 10-times higher rates, but these results might be misleading because the applied method (cytochrome *c* reduction ± SOD) even gave high rates for the uninhibited complexes from yeast and bovine heart. In any case, studies with the purified enzymes have to be interpreted with caution since electrons have to be supplied by artificial hydrophilic ubiquinols that can *per se* cause substantial background rates, especially in the presence of detergents and cytochrome *c* [28]. Direct ubiquinol oxidation by cytochrome *c*, accompanied by the production of superoxide, has been observed in protein-free phospholipid vesicles [87]. This implies that any reduction of the catalytic activity – either by inhibition, mutation or by a preparation-dependent impaired structural integrity – may promote non-catalytic superoxide production in a system that uses extrinsic cytochrome *c* and short-chain (comparably hydrophilic) ubiquinol derivatives, because substrate concentrations will be kept high. Furthermore, the supplied substrates (ubiquinols and cytochrome *c*) can give background rates with dyes that are commonly used for ROS detection. For instance ubiquinols may react with components of the Amplex Red/HRP assay [28] while cytochrome *c* can directly oxidize dichlorodihydrofluorescein (H_2DCF) [88,89]. It has also to be mentioned that in contrast to complex I, a downstream block at

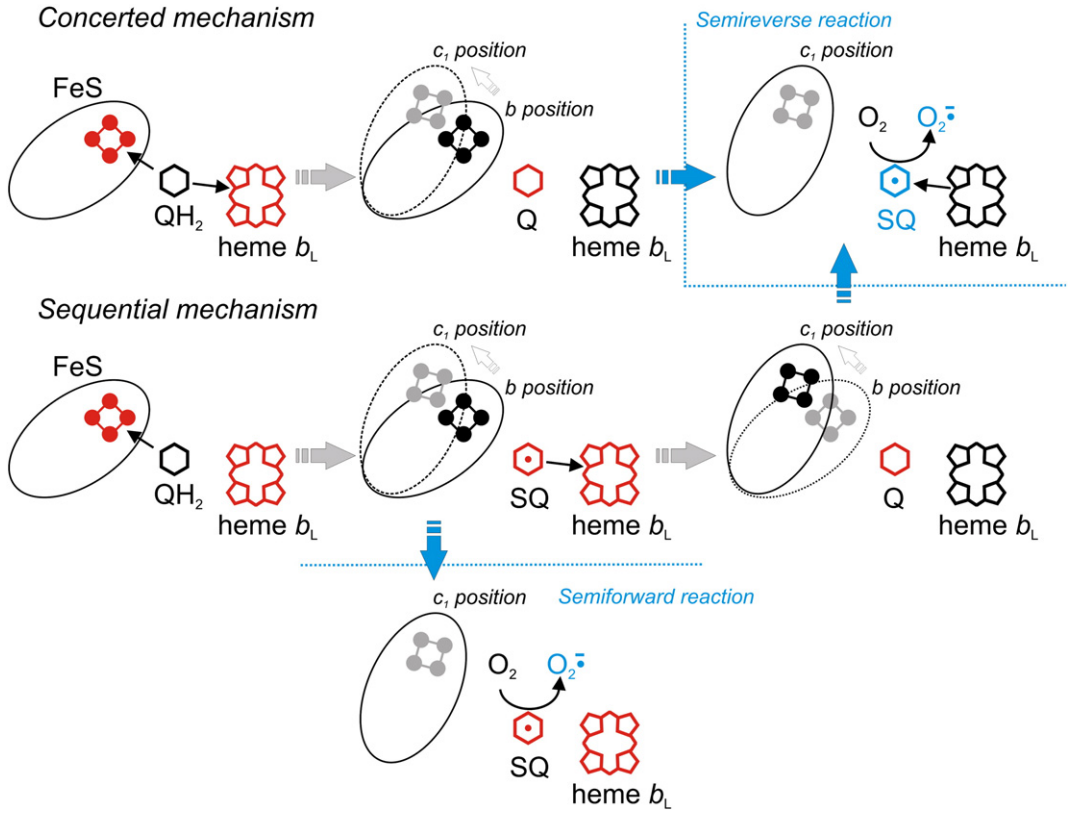

Fig. 2. Different mechanisms describe the bifurcation of electrons during ubiquinol oxidation and the generation of superoxide at the Q_o site. Reduced cofactors are shown in black, oxidized cofactors and the semiquinone (SQ) that is generated by the catalytic forward reactions are shown in red, while the very transiently occurring semiquinone that is formed by reverse electron transfer from reduced heme b_L is shown in blue. Electron transfer is indicated by solid black arrows, white arrows indicate the movement of the flexible head of the Rieske protein. Side reactions that lead to the formation of superoxide are indicated by blue arrows. In the concerted mechanism, both electrons are transferred at the same time onto the FeS protein and the heme b_L, respectively. A semiquinone intermediate does not occur in the catalytic forward reaction and – as a consequence – superoxide production is only possible by the semireverse reaction. Oxidized Q that is very transiently reduced to semiquinone serves as a redox mediator for the reverse electron transfer from heme b_L to molecular oxygen. In the sequential mechanism of ubiquinol oxidation, the first electron is transferred onto the FeS protein which leaves a semiquinone intermediate at the Q_o site and then the second electron is transferred to heme b_L. Superoxide production could occur (i) by a semiforward mechanism from the semiquinone intermediate or (ii) by a semireverse mechanism after the second electron has been transferred to heme b_L. For further details see text.

complex IV (by azide or cyanide) does not increase the superoxide generation from the Q_o site [77]. This can be explained by the fact that a tight inhibition of complex IV would result in an almost complete reduction of cytochrome *c* and the Q-pool suppressing superoxide generation by either a semiforward mechanism or a semireverse mechanism (see Section 2.2.3). In accordance, a reduction of the antimycin A-induced superoxide production by cyanide has been observed [90].

2.2.2. The membrane potential and the redox state of the Q-pool have major impact on superoxide production

Since superoxide production of native cytochrome bc_1 complexes is only substantial during inhibition by Q_i site inhibitors like antimycin A [75,91], one has to critically ask which factors could increase ROS generation at the Q_o site under physiological conditions. Rottenberg and colleagues [92] could show in experiments with purified reconstituted yeast cytochrome bc_1 complex that the superoxide formation strongly depends on the applied membrane potential and can reach values similar to those promoted by antimycin A. The rate of superoxide generation increased exponentially with the membrane potential (Fig. 3). This suggests that the membrane potential severely slows down electron transfer from heme b_L to heme b_H thereby promoting a more reduced state of heme b_L which then increases superoxide generation. This can be predicted since the electron transfer from heme b_L to heme b_H is the only transmembrane electrogenic step in the Q-cycle. Furthermore, the investigation clearly showed that the membrane potential alone and not the protonmotive force determines the rate of superoxide production [92]. A strong effect of the membrane potential on complex III superoxide production has already been suggested by an early study using succinate-fueled isolated mitochondria [93]. Yet ROS generation under the chosen conditions can be largely attributed to complex I due to reverse electron transfer from complex II [94–96] and presumably also to specific dehydrogenases of the TCA cycle due to an increased NADH/NAD^+ ratio [97].

The redox state of the Q pool is another factor with major impact on the superoxide production at the Q_o site (Fig. 3) —at least for the antimycin A-inhibited complex III [28]. By analyzing ROS production of submitochondrial particles (SMPs) from bovine heart with succinate as the sole electron source, we observed an increase of the antimycin A-induced superoxide production rate when the electron supply was reduced by addition of the competitive complex II inhibitors malonate or oxaloacetate [28] or inhibitors that bind to the ubiquinone binding site of complex II [31,98]. ROS generation was maximally increased by a factor of ~3 when approximately 75–85% of the succinate oxidase activity was inhibited, whereas with further inhibition superoxide generation decreased again. Such a stimulating effect of complex II inhibitors on the antimycin A-induced ROS generation by complex III has also been observed by others [75,76,99–102] and an impact of the Q-pool redox state has been proposed. Similarly, titrating the redox status of the Q-pool by varying succinate:fumarate ratios resulted in a bell-shaped curve for antimycin A-dependent superoxide generation of alamethicin-permeabilized rat heart mitochondria [80]. In intact mitochondria antimycin A-induced ROS generation is also maximal at intermediate redox states of the Q-pool that can either be titrated by inhibiting complex II in succinate-fueled

mitochondria [31,78,98] or by varying the substrate supply of complex I or II [78]. By far the highest rates of antimycin A-induced ROS production of intact mitochondria were observed in the presence of 'optimal' complex II inhibitor concentrations and an uncoupler [31,98], whereas an uncoupler alone increased the rate to a lesser extent [31,75,90,94]. Uncoupling probably impedes the antioxidative defense of mitochondria which at least partly depends on the ΔpH-dependent transhydrogenase for regeneration of reducing equivalents [103] and hence increases the detectable ROS 'release', but not the actual production. Alternatively/additionally it was shown that even in the presence of antimycin A and the complex II inhibitor malonate, an uncoupler further oxidized the Q-pool, while the *b* hemes remained reduced [75].

To test whether partial oxidation of the Q-pool indeed stimulates the antimycin A-induced superoxide production by cytochrome bc_1 complex, we directly titrated the ubiquinone to ubiquinol ratio by mixing oxidized and reduced forms of decylubiquinone [28]. In submitochondrial particles and in the purified, lipid-activated complex III from bovine heart mitochondria the rate increased proportionally with increasing ratios of the oxidized form until 25–30% was reached. We concluded that the generation of superoxide at the Q_o site occurs by reverse electron transfer from reduced heme b_L onto molecular oxygen. While our model also includes a semiquinone as a short-lived electron donor for superoxide production, this semiquinone is not the intermediate formed during normal catalytic forward reaction thought to 'accumulate' during antimycin A inhibition as the source for superoxide [25,26,69,78,104,105] (Fig. 2). Rather ubiquinone serves as a 'redox mediator' that transfers electrons from reduced heme b_L onto molecular oxygen. In a detailed analysis with rat skeletal muscle mitochondria Quinlan and colleagues [78] not only confirmed that the maximal antimycin A-induced superoxide generation rate occurs at an intermediate redox state of the Q-pool, but they could further correlate this with the redox state of heme b_L, showing that Q_o site ROS production peaks at about 70–80% heme b_L reduction. An additional applied membrane potential entailed a reduced maximal rate of superoxide generation, which occurred at complete reduction of heme b_L and was also influenced by the redox state of heme b_H [78]. In conclusion this thorough study suggests that the Q-pool redox state and the membrane potential modulate superoxide production at the Q_o site by affecting the reduction states of cytochromes b_L and b_H.

2.2.3. Mechanistic considerations: a semiforward or semireverse mechanism?

There is still an ongoing debate whether superoxide is generated at the Q_o site *via* an 'accumulation' or stabilization of a semiquinone that also occurs during normal catalytic turnover – representing the 'classical view' [25,26,69,78,104,105] (Fig. 2) – or by a direct [106] or indirect (*via* oxidized ubiquinone) 'reverse' electron transfer from reduced heme b_L onto molecular oxygen [28,30]. Using the words of Osyczka and colleagues, the question is whether superoxide is formed by a *semiforward* or a *semireverse* mechanism [29,30]. This has also important implications for the mechanism of ubiquinol oxidation at the Q_o site and *vice versa*, since a concerted reaction excludes superoxide generation by a *semiforward* mechanism. Osyczka and colleagues [29,30] who carefully analyzed the superoxide production of different cytochrome bc_1 mutants of *R. capsulatus* came to the conclusion that the superoxide production is dominated by a *semireverse* mechanism whereas the contribution of the *semiforward* mechanism is likely to be minor since only the *semireverse* model matched the experimental data for the antimycin A-inhibited wild type enzyme and all analyzed mutants with a remarkable degree [30]. In contrast, a kinetic model of Quinlan and coworkers based on their analysis of antimycin A-induced superoxide production, implemented a semiquinone generated in the *semiforward* reaction as an indispensable component [78]. Like in other mathematical models describing superoxide generation by the cytochrome bc_1 complex [107–109], the outcome is clearly influenced by the favored fundamental mechanism that may not consider some proposed mechanistic restrictions, *e.g.* that a reduced heme b_L and a semiquinone cannot coexist at the Q_o site [2]. The superoxide generation at the Q_o site is intrinsically tied to the mechanism of electron bifurcation during ubiquinol oxidation. While a *semireverse* reaction of superoxide generation works with a concerted and a sequential mechanism of ubiquinol oxidation, a concerted mechanism does not allow a *semiforward* reaction of superoxide generation. Hence, the *semireverse* mechanism appears more likely.

2.3. Residues/subunits of complex III that affect superoxide production

Several studies with cytochrome bc_1 complexes from *S. cerevisiae* and *Rhodobacter* species indicate that mutations which severely impede the electron flow through the low potential or the high potential

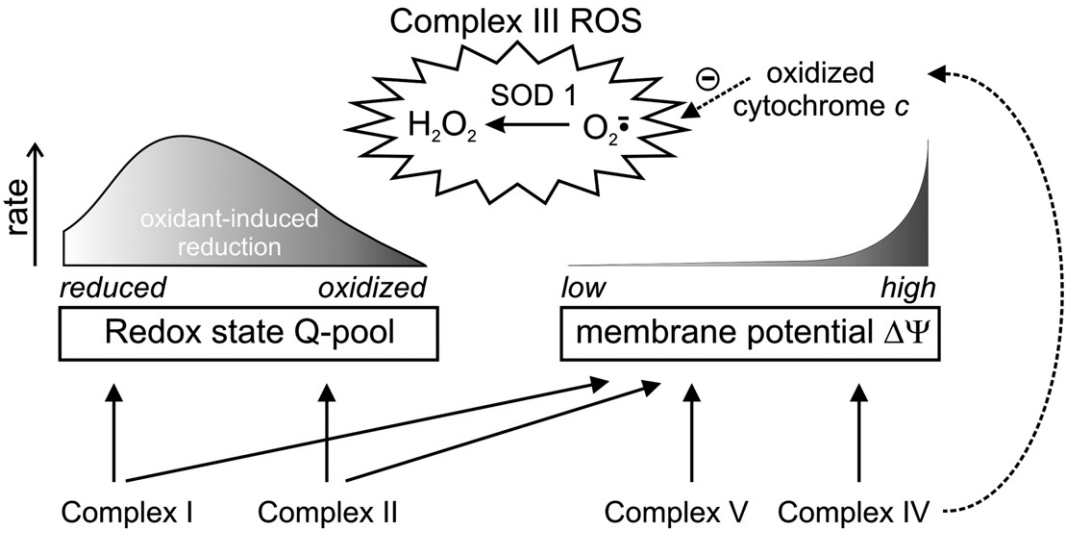

Fig. 3. Factors and respiratory chain complexes with an impact on the superoxide generation by complex III. The rate of superoxide production is increased when the redox state of the Q-pool is at intermediate levels (at least under conditions of oxidant-induced reduction, *e.g.* in the presence of antimycin A) and when the membrane potential is high. The redox state of the Q-pool is mainly determined by the activities of upstream respiratory chain complexes (especially complexes I and II) while the membrane potential is determined by the concerted activity of complexes I, II, III, IV and V (and presumably other proteins like the uncoupling proteins that are not shown). A completely reduced cytochrome *c* pool will attenuate the superoxide production at the Q_o site (see text), while oxidized cytochrome *c* is an effective superoxide scavenger thereby reducing the release of ROS.

chain and increase the reduction of cofactors (heme b_L and/or the FeS cluster of the Rieske protein) result in superoxide production at the Q_o site. In contrast to most of the work described above (see Section 2.2.1) an increased superoxide production was also observed in the absence of antimycin A. In most instances these mutants have a drastically reduced catalytic turnover, but this seems not to be a general prerequisite for increased superoxide generation [30]. Mutations in the Q_o site of yeast [110,111] and bacterial (*R. capsulatus*) [112] enzymes including a conserved tyrosine residue (Tyr^{279} of yeast, Tyr^{302} of *R. capsulatus* cytochrome *b*) that affect binding and oxidation of ubiquinol showed transfer of an increased fraction of electrons from ubiquinol onto molecular oxygen with regard to the catalytic electron transfer to cytochrome *c*. Interestingly, a mutation of the homologue tyrosine (Tyr^{278} of cytochrome *b*) in human cytochrome bc_1 complex causes 'multisystem disorder' (deafness, mental retardation, growth retardation, *etc.*) [113]. Yet some of the mutants featured a low turnover number, meaning that observed increases of the relative rates do not necessarily reflect an increase of absolute superoxide production compared with the wild type enzymes. Yu and coworkers analyzed the superoxide production of *R. sphaeroides* cytochrome bc_1 complexes lacking the cofactors heme b_L or heme b_H, respectively [70]. The purified complexes of both mutants produced as much superoxide as the antimycin A-inhibited wild type complex. Inhibition by antimycin A had little effect on the superoxide production of the mutant complexes, indicating that it is indeed produced at the Q_o and not at the Q_i site. The finding that the complex lacking heme b_L produces superoxide supports a *semiforward* rather than a *semireverse* mechanism. On the other hand, a semiquinone radical could not be detected by EPR measurements and the authors suggested that molecular oxygen may share quinol electrons with the Rieske protein when heme b_L is not available [70]. Bacterial mutant complexes lacking the Rieske protein [84] or containing a sterically arrested FeS domain close to the Q_o site ('FeS motion knockout') [29,30] do not produce significant amounts of superoxide, even in the presence of antimycin A [29]. Osyczka and coworkers concluded from their studies with different 'FeS motion knockouts' that the generation of superoxide only occurs when the FeS head domain transiently leaves the Q_o site (moves to the c_1 position) during the catalytic cycle, while when arrested at the b position the FeS head effectively diminishes the probability of superoxide formation [29,30]. Mutations of cytochrome c_1 (Met^{183} in *R. capsulatus*) causing severely impeded electron flow through the high potential chain by dramatically slowing the oxidation of the FeS center lead to increased electron transfer onto molecular oxygen given that the ubiquinol concentration is high or the cytochrome c_1/cytochrome *c* interaction is attenuated by high ionic strength [29,30].

3. Factors influencing the release of superoxide generated by complex III

3.1. Complex III releases ROS into different mitochondrial compartments

At physiological pH, superoxide exists mainly in its anionic charged form [114] that is highly membrane-impermeable [115,116]. Thus, mitochondrial superoxide production is characterized by a distinct membrane sideness or topology of release, respectively [117], which might have important implications for the onset of pathophysiological processes or cellular redox signaling. However, the dismutation product hydrogen peroxide (H_2O_2) can readily cross membranes. The fundamental studies of St. Pierre et al. [91] and Muller et al. [117] with isolated mitochondria revealed that the cytochrome bc_1 complex releases superoxide at least partially into the intermembrane space (and the cytoplasm), while complex I releases superoxide exclusively into the matrix. These findings are in agreement with the available structures of the two mitochondrial complexes: the Q_o site of complex III is immediately adjacent to the intermembrane space [41,42] whereas the proposed site(s) of superoxide generation of complex I [2], the FMN [118,119] and possibly also the ubiquinone binding site [120] are both located in the peripheral arm that protrudes into the matrix [121]. Muller et al. [117] concluded from their studies that ~50% of the superoxide produced at the Q_o site is released into the intermembrane space and that the remaining ~50% have to be released into the matrix. Drawbacks of this and other studies are that (i) the portion of superoxide released into the two main mitochondrial compartments (intermembrane space and matrix) was determined indirectly as an effect of added SOD and/or (acetylated) cytochrome *c* which cannot enter the intermembrane space and cristae lumen of intact mitochondria and (ii) the spontaneous rate of superoxide dismutation ($10^5\ M^{-1}\ s^{-1}$ [122]) was not taken into account. Considering the mitochondrial ultrastructure with cristae deeply folded into the matrix [123] we think that the superoxide production was underestimated and propose that superoxide is exclusively released into the intermembrane space as suggested by St. Pierre et al. [91]. However, after (spontaneous or SOD-catalyzed) superoxide dismutation, a significant fraction of the emerging H_2O_2 diffuses from the cristae lumen to the matrix and potentially oxidizes protein thiols (Fig. 4; Bleier et al., unpublished results). Cadenas and colleagues noted that the intermembrane space appears to possess a less efficient peroxidase activity than the mitochondrial matrix [124] and that superoxide (and derived H_2O_2) generated at the intermembrane space side of the inner membrane might be more effectively released into the cytoplasm *via* porins than ROS released to the matrix [124,125]. These considerations might be relevant for the role of ROS as 'second messengers' during cellular redox signaling.

3.2. Cytochrome c and antioxidative systems

One factor that can attenuate the release of superoxide from the Q_o site is cytochrome *c* that is present in high concentration in the intermembrane space [126]. Oxidized cytochrome *c* [127,128] and cytochrome *c* oxidase form an efficient scavenging system for superoxide [126]. Besides, some SOD1 is present in the intermembrane space [129] that accelerates the dismutation of superoxide and increases the rate of H_2O_2 generation (Fig. 4). After dismutation H_2O_2 can diffuse to the cytoplasm or into the matrix, where it is very efficiently removed by the mitochondrial antioxidative system [130–133] encompassing the glutathione/glutathione peroxidase and thioredoxin/peroxiredoxin systems which were shown to affect the release of Q_o site derived ROS [134].

3.3. Complex III activity influencing ROS generation at other mitochondrial sites

It has to be noted that inhibition of complex III activity – either by inhibitors of the Q_o site (*e.g.* myxothiazol or stigmatellin) or the Q_i site (antimycin A) or by mutations that reduce complex III activity – will induce superoxide production at complex I if NADH is present [28,31,77,135]. From studies with bovine heart SMPs it can be deduced that in the presence of NADH and antimycin A superoxide is generated in equal parts at complex I and the Q_o site of complex III since the rate is reduced to 50% by the addition of stigmatellin [28]. Furthermore, the complex I inhibitor DQA and stigmatellin both induced a similar rate of ROS production. Very recently, Quinlan and colleagues could show that complex II also produces superoxide in the presence of low succinate concentrations if complex III is blocked by myxothiazol [136].

4. Are complex III derived ROS involved in cellular redox signaling?

Meanwhile mitochondrial ROS are not only considered as damaging by-products from respiration, but also as redox signaling molecules in various physiological and pathophysiological processes [9–11]. While numerous studies indeed report mitochondrial ROS as

integral part of cellular signal transduction in various processes, considerably less studies deal with the actual ROS generating source. Nevertheless, a regulatory role of complex III-generated ROS in the cellular adaptive response to hypoxia, as well as in related cardioprotective ischemic preconditioning has been suggested by numerous studies (see Table 1).

In particular a crucial role for complex III-derived ROS in stabilization of the hypoxia-inducible factor HIF-1α in response to hypoxia has been proposed (for reviews see [15,137,138]). HIF-1α is stabilized by impeded hydroxylation of prolyl-residues which suppresses proteasomal degradation and hence enables nuclear translocation of the HIF-heterodimer to induce transcription of various gene products involved in angiogenesis, metabolism and erythropoiesis (for review see [139]). Initial evidence of a functional involvement of mitochondria came from Schumacker's group showing that mtDNA depleted ρ^0 cells failed to activate hypoxia-induced transcription [140]. Subsequently, the requirement of mitochondrial ROS, more precisely hydrogen peroxide as signaling molecule for HIF-1α stabilization and mitochondrial complex III as the superoxide-generating site was demonstrated. Much of the information was gained by cell culture preparations and the use of specific mitochondrial ETC inhibitors, other inhibitors and enzymatic or chemical antioxidants or oxidants [12,13,141–144]. Genetic data obtained from knockdown of complex III subunits, like the Rieske [13,142] and cytochrome *b* [144] or downstream components of the electron transfer chain like cytochrome *c* [143], as well as from overexpression of components of the antioxidative system [12,13,142], confirmed an involvement of complex III ROS and disproved earlier controversial reports [145,146]. Yet, some conclusions drawn from a study [144] using cybrid cells deficient of cytochrome *b* [147] barely withstand from a bioenergetic point of view, as it was proposed that those cells still generate Q_o-site ROS though the Q_o site is located within the cytochrome *b* [41,42]. A general problem in studying the actual underlying ROS generator site appears to be the necessity to apply specific inhibitors or to knockdown subunits of the complex. Complex III inhibition in particular, as electrons cannot bypass downstream, inevitably affects other ROS-modulating factors like the membrane potential, ratios of ADP/ATP and $NAD(P)^+$/NAD(P)H or induces a shift in metabolism. Nonetheless, any contribution to HIF-1α stabilization from complex I, the other main mitochondrial ROS source seems unlikely since a fully reduced downstream electron chain evoked by complex III inhibition should promote complex I ROS generation, though hypoxic signaling was found diminished under these conditions in all studies. Yet the role of Q_o site ROS generation in response to hypoxia is still controversially discussed as studies using isolated mitochondria observed decreased mitochondrial ROS generation with decreasing oxygen partial pressure [148]. Direct oxygen sensing by mitochondrial complex III, as suggested before, is challenged by this finding and a different triggering mechanism must be assumed. Interestingly, a study showed that terpestacin, a small molecule targeting complex III subunit UQCRB, abrogated hypoxia-induced ROS generation without affecting respiration [149]. This shows that in principle an accessory subunit can regulate superoxide generation by complex III. Recently, Bell and coworkers suggested that acetylation of complex III subunits might alter its ROS production, as they could show that knockdown of mitochondrial deacetylating sirtuin SirT3 augmented ROS levels and HIF-1α stabilization in response to hypoxia [150].

Apart from HIF-1α stabilization mitochondrial complex III-generated ROS have been implicated in hypoxia-induced calcium increase and subsequent pulmonary vasoconstriction (for reviews see [151–153]). Hypoxic pulmonary vasoconstriction (HPV) as an important physiological response to hypoxia ensures sufficient oxygenation of the blood by optimizing the matching of alveolar ventilation to pulmonary perfusion (for review see [154]). Likewise to their role in HIF-1α stabilization, evidence for the involvement of Q_o-site-generated ROS in HPV was mostly gained by use of specific pharmacological inhibitors, antioxidants or oxidants, as well as by genetic manipulation [155–160]. Yet, elevated ROS levels for hypoxic adaptation in pulmonary artery smooth muscle cells are controversially discussed as other groups favor a decrease in ROS levels as signaling mechanism for HPV [161–166].

Further, complex III-generated ROS seem to act as second messengers in the context of cardioprotective ischemic and pharmacological preconditioning (for reviews see [6,167]). Cardioprotective ischemic preconditioning refers to short, intermittent ischemic periods prior to prolonged cardiac ischemia thereby diminishing downstream cardiac damage by ischemia reperfusion injury. This phenomenon can be mimicked by pharmacological drugs such as diazoxide. Early studies observed that antioxidants prevented cardioprotection by preconditioning suggesting a signaling role for ROS [168–172]. Subsequent support came from studies showing a modulatory effect of diazoxide on mitochondrial ROS generation [31,101,173]. Two early studies indicated an involvement of Q_o site-generated ROS demonstrating that the Q_o site inhibitor myxothiazol diminished ROS production and cardioprotection [168,174]. Yet the underlying triggering mechanisms are not fully understood. Based on observations that diazoxide also inhibits mitochondrial complex II [175–177], as well as other complex II inhibitors exert cardioprotective effects [178,179] a link between modulation of Q_o site ROS generation by complex II activity and cardioprotection seems plausible. It is a matter of debate whether opening of a putative mitoK$_{ATP}$ channel is also involved in evoking Q_o site ROS generation [179,180]. Recently, we showed that partial inhibition of complex II augments complex III Q_o site ROS generation *via* a more oxidized Q-pool [28], and in a potassium independent manner

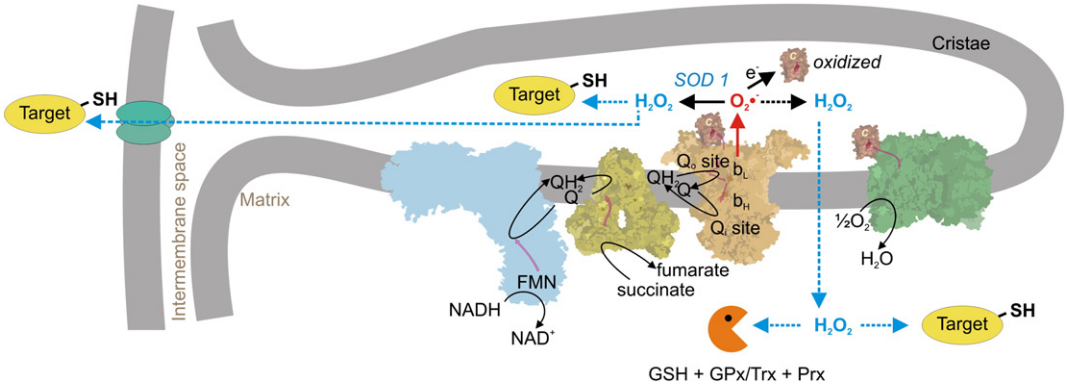

Fig. 4. Topology of ROS release from the Q_o site and implications for redox signaling. In our model, the primarily formed superoxide is almost completely released from the Q_o site into the intermembrane space and the cristae lumen that encompass the main portion of the inner membrane in intact mitochondria. The membrane-impermeable superoxide can either react with oxidized cytochrome *c* or be readily dismutated to hydrogen peroxide. H_2O_2 can diffuse across membranes thereby reaching the matrix where the bulk is probably consumed by the antioxidative system (especially by the thioredoxin (Trx)/peroxiredoxin (Prx) system and glutathione (GSH)/glutathione peroxidases (Gpx)). Nevertheless, our unpublished results show an oxidation of thiols groups of proteins located in the intermembrane space and the matrix by complex III derived ROS (Bleier et al., unpublished results).

Table 1
Selected studies on complex III-derived ROS in cellular signaling.

Biological process	Cell culture (and other) preparations[a]	Evidence obtained by use of	ROS detection[b]	Ref
Response to hypoxia				
HIF-1α stabilization	Hep3B; Hep3B ρ^0	ETC inhibitors; antioxidants	DCF(H)-DA	[140]
	Hep3B; Hep3B ρ^0; 293; 293 ρ^0	ETC inhibitors, antioxidants, oxidants, genetic approaches (catalase overexpression)	DCF(H)-DA	[12]
	A549; A549 ρ^0; HT1080; HT1080 ρ^0; 293; CCL16-B2; CCL16-NDI1	ETC inhibitors	CM-H_2DCF-DA	[141]
	A549; 293; SURF-1 null and WT[c]; C2C12; CCL16-B2; CCL16-NDI1	ETC inhibitors, genetic approaches (Rieske knockdown; SOD1, SOD2, GPx1 and catalase overexpression)	N/A	[142]
	Hep3B; Hep3B ρ^0; 293; 293 ρ^0; 143B; 143B ρ^0; murine embryonic cells; cyt *c* null murine embryonic cells	ETC inhibitors, antioxidants, oxidants, genetic approaches (generation of cytochrome *c* null embryonic cells)	DCF(H)-DA; carboxy-H_2DCF-DA	[143]
	Hep3B; 293; 143B; 143B ρ^0; murine embryonic cells, chick embryonic cardiomyocytes	ETC inhibitors, antioxidants, oxidants, genetic approaches (Rieske knockdown; SOD1, SOD2, cytos. and mito-targ. catalase overexpression)	ROS-sensitive HSP-FRET probe	[13]
	A549; 143B; 143B ΔCyt b cybrids	ETC inhibitors, antioxidants, oxidants, genetic approaches (Rieske knockdown)	Amplex Red; CM-H_2DCF-DA	[144]
Non-hypoxic HIF-1α stabilization (Angiotensin II-induced)	Rat vascular smooth muscle cells	ETC inhibitors, genetic approaches (Rieske and HIF-1α knockdown)	MitoSOX	[185]
Hypoxic pulmonary vasoconstriction (HPV)	Rat small intrapulmonary arteries (IPAs)	ETC inhibitors	N/A	[155]
	Rat pulmonary artery myocytes, pulmonary artery myocytes ρ^0, perfused rat lungs	ETC inhibitors, antioxidants	DCF(H)-DA	[156]
	Rat pulmonary artery myocytes	ETC inhibitors, oxidants, genetic approaches (catalase overexpression)	Xylenol orange assay	[157]
	Rat PASMCs	ETC inhibitors, antioxidants, oxidants, genetic approaches (SOD1, SOD2, cytos. and mito-targ. catalase, GPx1 overexpression)	ROS-sensitive HSP-FRET probe	[158]
	Mouse PASMCs; isolated, perfused and ventilated rabbit lungs	ETC inhibitors, simultaneous plotting of respiration and mitochondrial cytochromes redox state	MitoSOX	[159]
	Rat PASMCs; rat SASMCs	Antioxidants, oxidants, genetic approaches (cytos. and mito-targ. catalase overexpression)	RoGFP	[160]
Cardioprotective preconditioning	Embryonic ventricular cardiac myocytes	ETC inhibitors, antioxidants, oxidants	DHE; DCF(H)-DA	[168]
	Adult rabbit ventricular myocytes	ETC inhibitors and antioxidants	MitoTracker™ Red	[174]
Adipocyte differentiation	Human mesenchymal stem cells (MSCs)	Antioxidants, oxidants, genetic approaches (Rieske, UQCRB and Raptor knockdown; PPARγ overexpression)	Amplex Red	[181]
Apoptosis induction by matter air pollution	Mammalian cell culture (A549; A549 ρ^0; rat alveolar type II epithelial cells); C57BL/6 mice	Antioxidants, oxidants, genetic approaches (Rieske knockdown; SOD1 and SOD2 overexpression)	Mito-Ro-GFP	[182]

[a] Hep3B, human Hep3B hepatoma cells; 293, kidney HEK 293 cells; A549, human A549 lung epithelial cells; HT1080, HT1080 human fibrosarcoma cells; 143B, 143B osteosarcoma cells; murine embryonic cells; PASMCs, pulmonary artery smooth muscle cells; SASMCs, systemic artery smooth muscle cells; CCL16-B2, Chinese hamster lung fibroblast lines CCL16-B2; CCL16-NDI1, Chinese hamster lung fibroblast lines CCL16-NDI1; C2C12, C2C12 mouse myoblasts.

[b] Carboxy-H_2DCF-DA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescin diacetate; CM-H_2DCF-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescin diacetate acetyl ester; cytos., cytosolic; DCF(H)-DA, 2′,7′-dichlorofluorescin diacetate; DHE, dihydroethidium; MitoSOX, MitoSOX Red mitochondrial superoxide indicator; N/A, not available.

[c] SURF-1 null fibroblasts from a patient with Leigh's syndrome, which is associated with cytochrome *c* oxidase deficiency [186].

questioning a contribution of the putative mitoK$_{ATP}$ channel [98]. Hence we proposed as a potential triggering mechanism for cardioprotection direct modulation of mitochondrial ROS generation *via* altered complex II activity [98].

A so far unknown signaling function of complex III-derived ROS in adipocyte differentiation was recently found by Chandel's group [181]. The authors reported that complex III ROS initiate adipocyte differentiation by induction of the PPARγ transcriptional machinery in an mTORC1-dependent manner. Further, a signaling role of complex III-derived ROS in apoptosis induction by matter air pollution has been suggested by Soberanes et al. [182].

It can be concluded that growing evidence draws a convincing picture of Q_o site ROS as second messengers in cellular signal transduction. Yet the research field faces methodical challenges and various questions remain to be clarified in order to completely understand the relevant events and mechanisms: (i) A major technical limitation surely is the reliable detection of ROS, as most ROS detection methods are highly susceptible to side reactions, lack selectivity or suffer from pH sensitivity, autooxidation or uncontrolled probe distribution or reaction with antioxidants [183]. Besides, while a variety of different ROS detecting assays exist, there are so far no reliable approaches to pinpoint the actual ROS-generator. Hence to fully unravel the function of complex III-generated ROS in cellular signal transduction, 'biomarkers' for the different ROS generator sites allowing differentiation of the variably contributing mitochondrial superoxide sources would be helpful. Thus we studied the redox status of protein thiols in isolated mitochondria subjected to different ROS generating conditions by Redox DIGE (difference gel electrophoresis) [184]. We observed that ROS generated at mitochondrial complex I or complex III targeted a distinct subset of proteins and identified potential candidates for such 'biomarkers' (Bleier et al., unpublished results). (ii) Mostly, the underlying triggering events of Q_o site signaling ROS are not fully understood. The molecular mechanisms of signal communication to mitochondria and thus enhanced Q_o site ROS generation need to be further investigated. (iii) Existing ambiguity regarding the exact mechanism of superoxide generation by complex III further impedes progress. (iv) For most cases the biological outcome is well documented, but the direct biological targets of Q_o site signaling ROS remain elusive. Yet, considering the rapidly advancing field of redox proteomics numerous

biological targets of signaling ROS will soon be identified. This will promote our understanding of redox signaling and will hold major implications for various physiological and pathophysiological processes, *e.g.* for cardiac damage and tumorigenesis.

5. Conclusions and outlook

In conclusion it can be noted that the exact mechanism of superoxide generation at the Q_o site still remains a matter of debate. Moreover, significant amounts of Q_o site-derived superoxide have been measured so far only under non-native conditions, *e.g.* in the presence of the Q_i site inhibitor antimycin A. Thus, the fundamental question that needs to be addressed is which physiological factors or processes promote Q_o site superoxide generation *in vivo*. This is of particular importance considering the reported regulatory role of complex III derived ROS in cellular redox signaling. In this regard possible post-translational modifications of complex III subunits, *e.g.* acetylation and their influence on Q_o site superoxide production should be further investigated as it seems plausible that they may serve as physiological mechanisms to control superoxide generation.

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