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Review

Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning☆

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ABSTRACT

The production of reactive oxygen species by the mitochondrial complex II (succinate:ubiquinone oxidoreductase) recently has gained broad scientific interest. Depending on the (patho)physiological situation, ROS produced or triggered by complex II can have either beneficial or deleterious effects. This ambivalence can be explained mechanistically by the diverse role of complex II on mitochondrial ROS production: it can be a source as well as a suppressor or enhancer of ROS generation by other respiratory chain complexes. Since complex II directly links the respiratory chain to the tricarboxylic acid (TCA) cycle, the TCA-cycle intermediates – especially oxaloacetate that acts as a high affinity endogenous inhibitor – have major impact on complex II-related ROS release. The review relates the diverse effects of complex II activity on the mitochondrial ROS production that have been observed during cardioprotective ischemic or pharmacological preconditioning and the oxidative burst that occurs during ischemia/reperfusion. This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

1. Introduction

Mitochondrial complex II (succinate:ubiquinone oxidoreductase; succinate dehydrogenase, SDH) oxidizes succinate to fumarate and reduces ubiquinone, thereby creating a direct link between the tricarboxylic acid (TCA) cycle (also known as citric acid or Krebs cycle) and the respiratory chain [1] (Fig. 1). The respiratory chain provides the main portion of ATP in eukaryotic cells, but is also a major source of reactive oxygen species (ROS) [2–6]. An increased mitochondrial ROS production has been associated with numerous pathophysiological settings including neurodegenerative diseases [7], oxidative damage during ischemia/reperfusion injury [8,9] and the aging process [10,11]. Within the respiratory chain, complexes I (NADH:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome *c* oxidoreductase; cytochrome bc_1 complex) are generally regarded as the main ROS sources, while the contribution of intact complex II seems to be negligible [3–6]. However, some mutations in complex II subunits that have been linked to specific forms of cancer or neurodegeneration can lead to an increased ROS generation (reviewed in [12–14]). Furthermore, it is well known that complex II activity can have major impact on the ROS production by complexes I and III [15,16]. With isolated mitochondria it has been shown that a high succinate concentration and a high membrane potential induce reverse electron transfer from complex II into complex I that is associated with a high rate of superoxide production [17–23]. Under these conditions inhibition of complex II leads to a reduction of complex I related ROS generation [15,16,19,20,24]. On the other hand, inhibition of complex II can increase the generation of superoxide at the ubiquinol oxidation site (Q_o site) of complex III under specific conditions [25], *i.e.* when succinate is the predominant substrate and when the electron transfer between heme b_L (low potential heme) and heme b_H (high potential heme) is slowed down additionally, *e.g.* when the ubiquinone reduction site of complex III (Q_i site) is blocked by inhibitors like antimycin A [15,16,26–31]. This ROS-promoting effect of complex II inhibitors can be related to a partially oxidized Q-pool that maximizes ROS production by complex III upon antimycin inhibition [25,32,33]. In addition, it has been proposed that complex II controls mitochondrial ROS production *via* a succinate-dependent interaction with complex I [34,35] or that complex II *per se* is a major source of ROS which under physiological conditions is linked to disease scenarios such as cancers or tissue damage associated with oxidative stress [36]. Very recently, Quinlan and colleagues could show that mitochondrial complex II can indeed produce high levels of ROS when (i) succinate concentration is low and

Abbreviations: DLD, dihydrolipoamide dehydrogenase; I/R, ischemia/reperfusion; (mito)K_{ATP}, (mitochondrial) ATP-dependent K^+ channel; Q, ubiquinone; QH_2, ubiquinol; QFR, quinol:fumarate reductase; Qd site, distal ubiquinone binding site of complex II; Qp site, proximal ubiquinone binding site of complex II; Q_i site, ubiquinone reduction site of complex III; Q_o site, ubiquinol oxidation site of complex III; RET, reverse electron transfer; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SMP, submitochondrial particles; SQ, semiquinone; SQR, succinate:quinone oxidoreductase; TCA cycle, tricarboxylic acid cycle; TTFA, 2-thenoyltrifluoroacetone

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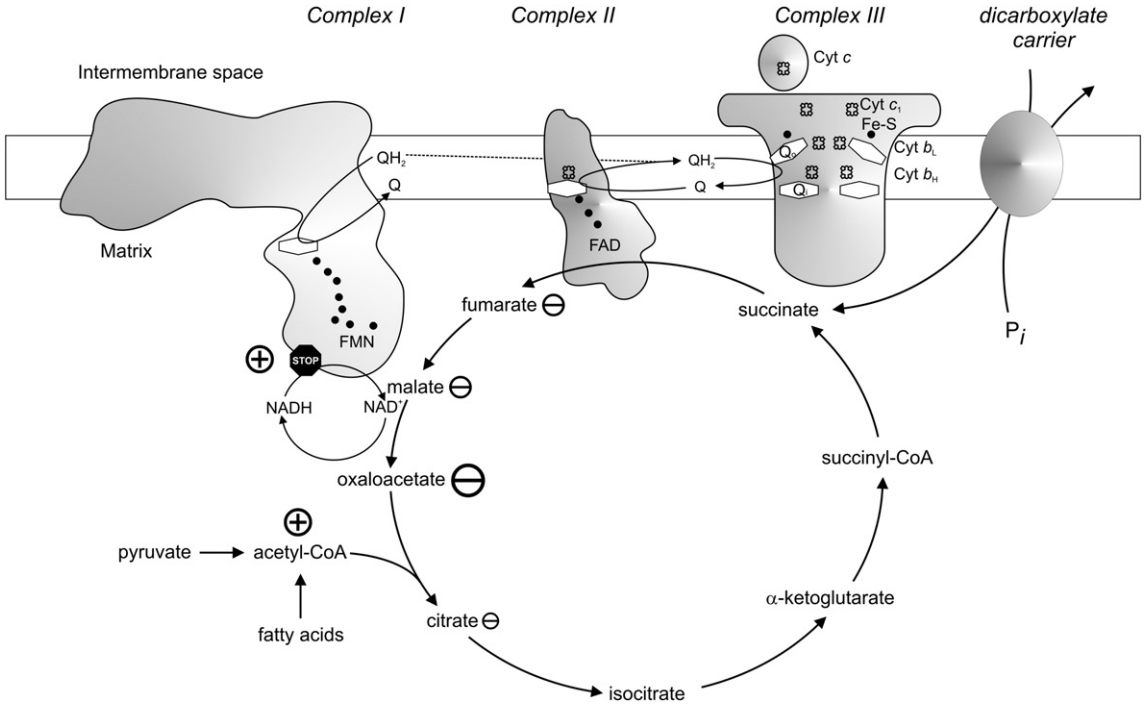

Fig. 1. Complex II links the respiratory chain to the TCA cycle. Complex II oxidizes succinate to fumarate and transfers two electrons onto ubiquinone that fuels the respiratory chain complexes III and IV (not shown). The activity of complex II is regulated by the concentration of succinate and some intermediates of the TCA cycle (especially oxaloacetate), that have been shown to competitively inhibit complex II (indicated by a circled minus sign; the size of the sign reflects the inhibitor potency). The activity can also be positively regulated (indicated by a circled plus sign) by (1) acetyl-CoA generation reactions that will promote removal of accumulated oxaloacetate, which in turn releases complex II inhibition. And (2) by inhibition of complex I, since this will shift the NADH/NAD^+ ratio completely to the reduced form and oxaloacetate can no longer be generated from malate.

(ii) a downstream block of the electron flow in the respiratory chain occurs [37]. Hence, it is not surprising that complex II inhibitors show ambivalent effects on mitochondrial ROS production [15,16] depending on substrate supply, membrane potential and overall activities of respiratory chain and TCA cycle enzymes.

An ambivalence of mitochondrial ROS production has been also detected in the context of ischemia/reperfusion and ischemic or pharmacological preconditioning: while reactive oxygen species are among the main determinants of cellular damage during longer periods of ischemia and reperfusion, there is also ample evidence that mitochondrial ROS production is involved in cardioprotective signaling during ischemic and pharmacological preconditioning (overviews in [8,38–40]). In this context, complex II inhibitors have been shown to exert protective effects in different model systems during preconditioning phases or when they were present during reperfusion [41–44]. It has been proposed that the production of 'deleterious ROS' during reperfusion can be related to complex I, while the generation of 'signaling ROS' during preconditioning occurs at the Q_o site of complex III [15]. The protective effects of complex II inhibitors could then be explained by their differential effects on complex I and complex III related ROS production: they reduce ROS that are generated at complex I by reverse electron transfer (RET) during reperfusion while they promote production of ROS at the Q_o site of complex III by shifting the redox state of the Q-pool to an intermediate state [15]. This review intends to summarize the intricate influence of complex II on mitochondrial ROS production and its relation to ischemia/reperfusion and preconditioning.

2. Composition, catalytic activity and physiological function of mitochondrial complex II

Mitochondrial complex II is unique among the respiratory chain complexes in several ways: it does not contribute directly to the generation of the proton motive force (however it contributes to the reduction of ubiquinone to ubiquinol that fuels the downstream complexes III and IV), it does not contain subunits that are encoded by mitochondrial DNA and it is an indispensable component of another major energy-converting pathway, the TCA cycle. In addition, mitochondrial respiratory chain complexes I, III and IV are organized in 'supercomplexes' or 'respirasomes', whereas complex II was identified as an isolated entity in mildly solubilized mitochondrial membranes [45–47]. One report implicates that complex II is associated with 'supercomplexes' [48], but a detailed analysis of the complexome of purified rat heart mitochondria did not confirm an association of complex II with other respiratory chain complexes [49].

2.1. Subunit composition and redox groups

Mitochondrial as well as bacterial succinate:quinone oxidoreductases (SQRs) catalyze the oxidation of succinate to fumarate and transfer the two electrons derived from this reaction *via* four prosthetic redox-groups (one covalently bound flavin adenine dinucleotide (FAD) asnd three iron–sulfur clusters) onto membrane-bound ubiquinone under aerobic conditions [1,50,51]. The structural organization of complex II is well known (for recent reviews see [14,52]), since the X-ray crystal structures of the enzymes from *Escherichia coli* [12,53,54], as well as porcine [55] and avian [56] mitochondria have been determined. In addition, the structures of closely related bacterial fumarate reductases (quinol:fumarate reductases, QFRs) [57,58] and further members of the 'complex II superfamily' have been solved (overview in [52], this issue). In bacteria, fumarate reductase replaces the succinate:quinone oxidoreductase under anaerobic conditions [59,60]. In *E. coli*, SQR and QFR are capable of replacing each other functionally *in vivo i.e.* both catalyze succinate oxidation and fumarate reduction, albeit with different affinities and catalytic rates [52]. It is important to note that the overall structural and functional similarity of *E. coli* SQR and QFR to mammalian complex II has made them useful models systems for structure–function investigations and the analysis of disease-related human mutations [14].

Most eukaryotic SQRs are composed of two hydrophilic subunits, a flavoprotein SdhA and an iron–sulfur protein subunit (SdhB), and two hydrophobic membrane anchored subunits (SdhC, SdhD), which contain one heme *b* and the binding site(s) for ubiquinone [12,14,52,55,56] (Fig. 2A). However, a number of bacterial SQRs (*e.g.* the Gram-positive *Bacillus subtilis*) only have a single membrane anchor subunit [1,50]. SdhA is the largest subunit and contains a covalently attached FAD in the dicarboxylate binding site, where succinate is oxidized to fumarate. SdhB contains three linearly aligned iron–sulfur clusters (in the order 2Fe–2S, 4Fe–4S, 3Fe–4S towards the membrane) that transfer the electrons derived from succinate oxidation to the ubiquinone binding site located at the interface of SdhB and the integral membrane proteins SdhC and SdhD. In addition to this 'proximal' Q-binding site (Qp), a second 'distal' Q-binding site (Qd) near the intermembrane space side has been proposed based on biochemical studies [50] and co-crystals of the porcine complex II with the competitive Q-site inhibitor TTFA [55]. However, the functional significance of these findings has been questioned [51]. SdhC and SdhD contain a *b*-type heme that is located off-pathway from the electron transferring cofactors and does not contribute to the electron transfer between the two substrate binding sites [12,52]. Since both FAD semiquinone [61,62] as well as ubisemiquinone radicals [63,64], have been detected by EPR, a stepwise removal of electrons from succinate followed by a stepwise electron transfer onto ubiquinone can be deduced.

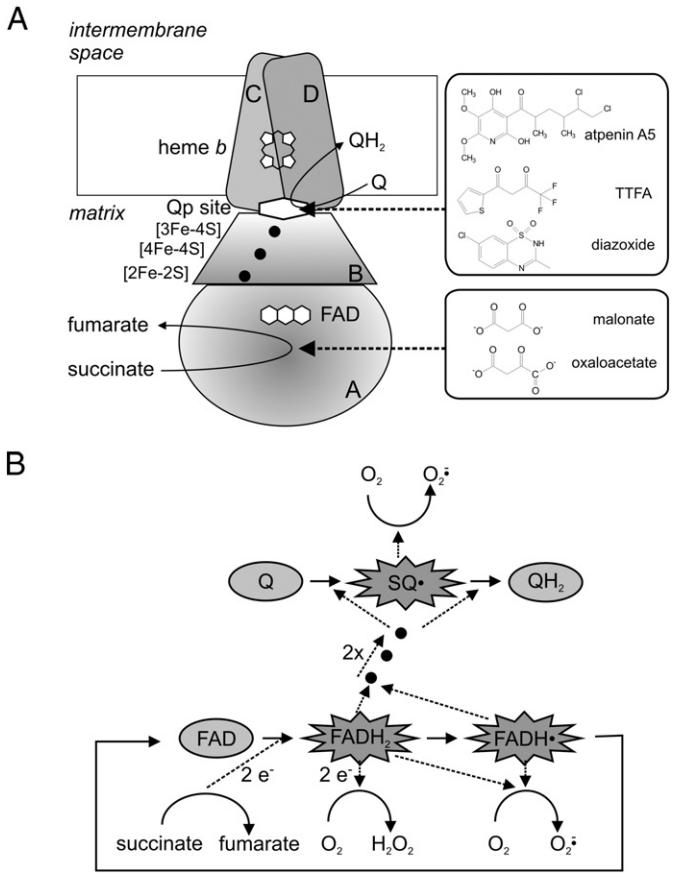

Fig. 2. Structure and function of complex II. A) Composition and arrangement of redox centers. The four subunits (Sdh A-D) are indicated by capital letters, the relative positions of the prosthetic groups are indicated as black circles ([FeS]-clusters), a tricyclic structure (FAD), a stretched hexagon (Qp site) and a schematic porphyrin ring (heme *b*). The structures and binding sites of some inhibitors mentioned in this review are also indicated. B) Electron transfer and potential sources of ROS within complex II. Intermediates that were shown or have the potential to produce reactive oxygen species are highlighted by a star-shaped edge. Electron transfer reactions are indicated by dotted arrows. 2 e^-, indicates a sequential or concerted transfer of two electrons; 2×, indicates that the path through the iron–sulfur clusters (black circles) subsequently transfers two single electrons – the first supplied by the fully reduced flavin, the second by the FAD semiquinone (FADH•) – stepwise to ubiquinone (Q), yielding semiquinone (SQ•) and ubiquinol (QH_2), respectively. For details, see text.

2.2. Complex II links the TCA cycle to the respiratory chain

As a component of the respiratory chain and the TCA cycle complex II provides a direct link between these two major energy-converting pathways in the mitochondria (and bacteria). Besides the supply of electrons for the respiratory chain, the TCA cycle also provides precursors and redox equivalents for the synthesis of various organic substances including certain amino acids. Since several catabolic pathways converge on the TCA cycle, it is the final common pathway for providing energy from carbohydrates, fatty acids and proteins and as such requires a strict regulation. The activity of the eight enzymes involved is regulated by the general metabolic situation (*i.e.* energy charge) and the concentration of the respective metabolites that are controlled by the enzyme activities, anaplerotic reactions and the activity of specific transporters located in the inner mitochondrial membrane (Fig. 1). The activity of complex II is directly influenced by the concentration of other TCA cycle metabolites, since malate, fumarate, citrate and especially oxaloacetate [65–67] have been shown to inhibit competitively with succinate. Besides a direct competition between succinate and malate, it has been observed that complex II can oxidize malate to the enol form oxaloacetate [67,68], which binds to the dicarboxylate binding site with high affinity [66]. Oxaloacetate formation is also the reason why succinate alone is only supporting weak state 3 respiration of isolated mitochondria [65]. However, oxaloacetate formation can be indirectly suppressed by the addition of complex I inhibitors like rotenone that block reoxidation of NADH to NAD^+ thereby preventing the oxidation of malate by the malate dehydrogenase. On the other hand, uncouplers and other factors that impede the removal of accumulated oxaloacetate can also reduce succinate supported respiration [69]. Another important factor that determines complex II activity is the succinate concentration itself. The K_m values of complex II and the dicarboxylate carrier that catalyzes transport of succinate into the mitochondria are around 1 mM [70,71]. The succinate concentration in different tissues and mitochondria is in the submillimolar range under aerobic conditions [22,72,73], indicating that complex II is not working at maximal velocity under 'standard conditions'. However, several studies have shown that the succinate concentration increases under hypoxic conditions up to values of several millimolar [22,72,74–78]. This increased succinate pool under hypoxia might be supplied either from glutamate through α-ketoglutarate by transamination [76,77] or by a partial reversal of the TCA cycle from malate through fumarate, which requires that complex II would become a fumarate reductase under hypoxia [36,79].

2.3. Inhibitors of complex II

Inhibitors of respiratory chain complexes have proven to be useful tools for investigations of their functions and mechanisms, including the production of reactive oxygen species. Specific inhibitors of complex II can be divided into two subgroups: (i) inhibitors that bind to the carboxylate (succinate) binding site and (ii) inhibitors that bind to the Q site (Fig. 2A). The first group comprises molecules that structurally resemble succinate (overview in [14]) including the above mentioned oxaloactate, malonate and the toxin 3-nitropropionate [80], that has also been used for co-crystallization with complex II from porcine heart mitochondria [55]. This complex II has also been co-crystallized with the TTFA (2-thenoyltrifluoroacetone), a well known inhibitor of the Q-binding site [81,82]. TTFA binds to the Qp site close to the matrix and to a putative second ubiquinone binding site (Qd) [55], whose functional significance has been questioned [51]. The most potent inhibitors

of complex II so far are the atpenins [83,84] that have been shown to bind to the Qp site [53]. Another interesting group of complex II Q-site inhibitors are vitamin E analogues, including α-tocopheryl succinate (α-TOS) [85] and MitoVES (mitochondrially targeted vitamin E succinate) [86,87] that have been used in model systems to specifically induce cell death of cancer cells. Covering this topic is beyond the scope of this review and the interested reader is referred to recent overviews [36,88]. Furthermore, complex II activity can also be regulated by post-translational modification, *e.g.* by acetylation of lysine residues that is controlled by the activity of the mitochondrial NAD-dependent deacetylase SIRT 3 [89,90].

3. Complex II and reactive oxygen species, a complex scenario

3.1. Complex II as a source of ROS

Up to the recently published investigation by Quinlan and colleagues [37], intact mammalian complex II has hardly been recognized as a major source of ROS [3–6]. In most studies with isolated mitochondria and submitochondrial particles (SMP), a saturating succinate concentration (usually around 5 mM) has been applied. In coupled mitochondria and SMP, high ROS production is observed under these conditions, but this can be largely attributed to complex I due to reverse electron transfer from complex II, since (i) it is sensitive to complex I inhibitors like rotenone [18–20,24,91,92], (ii) requires a high membrane potential, *i.e.* is sensitive to uncouplers and ionophores [15,18–20,91] or ADP (that induces state 3 respiration) [17], and (iii) does not occur in uncoupled submitochondrial particles (SMP) [32,93]. This was also confirmed by Quinlan et al. [37], however these authors also observed that complex II in isolated rat skeletal mitochondria produced ROS at a significant rate with succinate concentrations below 5 mM (with a peak at around 400 μM) and when the downstream respiratory chain was inhibited. A significant contribution of complexes I and III to the detected ROS generation could be excluded by the addition of rotenone (that inhibits ROS production by RET at complex I) and myxothiazol (that inhibits ROS production at the Q_o site of complex III and at the same time served as the downstream block). At succinate concentrations below 400 μM, ROS production decreased again. Furthermore, this ROS production was completely sensitive to the competitive carboxylate site inhibitor malonate and was only partially repressed by the Q-site inhibitor atpenin A5. Therefore, the authors concluded that the ROS must have been produced at the flavin site of complex II [37]. Furthermore, Quinlan et al. could show that complex II also produced ROS in the reverse reaction, *i.e.* when the electrons are provided from the reduced ubiquinone pool [37]. They proposed a mechanism for ROS production by complex II that relies upon the occupancy of the carboxylate binding site and the reduction state of the enzyme, *i.e.* ROS are only generated when the flavin is reduced and the carboxylate binding site is not occupied. Finally, it was shown that the complex II related rates approach or exceed the maximum rates of complexes I and III indicating that complex II may be an important contributor to physiological and pathological ROS production [37].

It has been observed that loss of function of complex II can lead to the accumulation of succinate and increased ROS generation in mammalian cells (overview in [14,36]). This seems to be especially the case for mutations in complex II subunits C and D located in the vicinity of the Qp site that have been associated with specific forms of cancer such as hereditary paragangliomas and pheochromocytomas (overview in [13,14,36]) and in SdhA that can cause Leigh syndrome, a progressive neurodegenerative disease [14,94]. However, investigations showing unambiguously that a disease associated mutation directly causes an increased ROS generation by mammalian complex II are scarce. Ishii and coworkers observed that a mutation in the SdhC subunit in transgenic mouse fibroblasts and conditional transgenic mice induces mitochondrial respiratory chain dysfunction and increased ROS production [95]. However, conclusive experiments that would exclude other respiratory chain complexes or matrix dehydrogenases as ROS source are not shown. This also applies to an investigation that revealed an increased ROS production by a SdhD mutant in hamster fibroblasts [96]. Ishii and coworkers have used non-mammalian model organisms for the analysis of oxidative stress associated with complex II mutations (overview in [97]). They found that a mutation in SdhC in complex II from *Caenorhabditis elegans* (*mev-1(kn1)*) caused hypersensitivity to increased oxygen concentrations [98] and induced an increased ROS production [99] that was observed in succinate-fueled mitochondria and SMP from the (*mev-1(kn1)*) mutant [99]. The experiments with (most likely uncoupled) SMP largely exclude that the observed ROS production was due to RET-induced superoxide production by complex I. A detailed investigation was performed with complex II from the parasitic helminth *Ascaris suum* that functions in adult worms as the terminal oxidase of the anaerobic respiratory chain and catalyzes fumarate reduction [100]. The succinate-dependent ROS generation of SMP from adult worms, that do not contain complex III and IV activities in their respiratory chain, was not influenced by a complex I inhibitor and was completely and partially suppressed by malonate or atpenin A5, respectively. This indicates that complex II is the exclusive ROS source under these conditions. Also the ROS generation of succinate-fueled SMP from *A. suum* L_3 larvae, that contain a respiratory chain similar to mammalian mitochondria, was only affected by complex II inhibitors and not by inhibitors of the other respiratory chain complexes.

Detailed analysis of the ROS production has also been performed with bacterial SQRs and QFRs from *E. coli* [101] featuring structural and functional similarity to mammalian complex II [14]. Both enzymes produced ROS with a linear dependence on the dissolved oxygen concentration, although SQR produced ROS at a much lower rate. Furthermore, both enzymes showed maximal rates of ROS at intermediate succinate concentrations between 0.1 and 1 mM and decreased rates at lower and higher substrate concentration, which resembles the dependence found later for mammalian [37] and nematode [100] complex II. *E. coli* SQR exclusively produces superoxide. In contrast, *E. coli* QFR produces mainly superoxide at lower succinate concentrations and comparably more hydrogen peroxide at higher substrate concentrations [101]. A similar effect was observed in a mutant containing a proximal [2Fe–2S] cluster with lower redox potential. Messner and Imlay concluded that QFR releases superoxide from the fully reduced flavin if the [2Fe–2S] cluster is available to sequester the semiquinone electron; otherwise this electron is rapidly transferred to the nascent superoxide and hydrogen peroxide is released [101]. Yankovskaya et al. [12] proposed that the key difference in the ROS production by *E. coli* SQR and QFR lies in the differential arrangement of redox potentials among the redox centers. In SQR the high potential centers ([3Fe–4S] and heme *b*, which attract electrons and pull them away from the dicarboxylate site) are close to the Q site, while in QFR, FAD and the [2Fe–2S] clusters have the highest redox potentials and hence accumulate electrons close to the dicarboxylate binding site. While these arrangements favor the respective physiological activity (*i.e.*, ubiquinone reduction for SQR and fumarate reduction/menaquinol oxidation for QFR) they also presuppose a differential disposition for superoxide production under aerobic conditions. This suggests that an increased reduction of FAD in the dicarboxylate binding site – either by differential intrinsic properties and arrangements of the redox centers, or mutations/inhibition of downstream located redox centers – causes an increased risk for ROS production under aerobic conditions. Mutants of *E. coli* SQR that do not contain heme *b* showed an unchanged ROS generation, indicating that this prosthetic group is not serving as a 'sink' for excess electrons [102,103].

There is an ongoing discussion whether the Q site or the FAD is the primary source of ROS within complex II. As mentioned above (Section

2.1), ubisemiquinone and FAD semiquinone radicals have been detected by EPR, which are both in principle suitable as electron sources for the generation of superoxide (Fig. 2B). The Q site has been proposed as the site of ROS production based on studies in *Saccharomyces cerevisae* [104,105] and a mixed contribution of FAD and Q site was suggested for complex II from *A. suum* [100]. While a contribution of the Q site might occur in the respective mutants, the most likely source of ROS in wild-type complex II is the fully reduced FAD [37,101]. Especially the suppression of ROS generation by higher substrate concentrations (which leaves the redox state of the redox centers largely unaffected) strengthens the latter model indicating that complex II generates ROS when the FAD is reduced and the dicarboxylate binding site is not occupied. In principle, fully reduced FAD can either transfer one electron onto molecular oxygen which leads to the formation of superoxide (probably the major reaction in complex II, but only confirmed for *E. coli* SQR [101]) or it may also transfer both electrons onto O_2 which leads to the formation of H_2O_2 (as shown for *E. coli* QFR [101]) (Fig. 2B). FAD is also supported as the primary ROS source by studies with Q-site inhibitors like α-TOS or MitoVES that induce an increased ROS generation linked to selective cell death in some cancer forms [85–87,106] (detailed overview in [36]). A ROS production at the Q site should rather be blocked by these inhibitors, which leaves only the FAD as the redox group involved — provided that complex II is indeed the ROS source under the applied conditions. It has to be noted that this class of inhibitors only functions in cancer cells with an unaffected ubiquinone binding ability of complex II [85–87].

3.2. Complex II as a modulator of the ROS production by complexes I and III

Apart from the direct generation of ROS (Section 3.1), complex II has been recognized as a modulator of the superoxide production by other mitochondrial enzymes, especially the respiratory chain complexes I and III [15,16,25] that are generally regarded as main ROS sources in the mitochondria [3–6]. It is well known that isolated mitochondria produce large quantities of ROS when they are incubated with succinate as the sole or equally supplied substrate source [17–23]. Under some specific conditions (*i.e.* in presence of pyruvate and glutamate), a supplementation of only 15–150 μM succinate is enough to increase ROS release from isolated mitochondria significantly [34]. While alternative explanations for the source or mechanism of ROS production under these conditions have been proposed [34,36], the prevalent view is that under these conditions ROS are produced by complex I due to RET from complex II [18–20,23]. Whereas a contribution of other components – especially the DLD (dihydrolipoamide dehydrogenase) component of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [22,107] – cannot be excluded, the strongest argument for the 'ROS by RET' hypothesis is that ROS production is highly sensitive to even small changes in membrane potential [18]. In a detailed analysis, Votyakova and Reynolds determined that a drop in $\Delta\Psi$ by only 5% reduces the hydrogen peroxide release of isolated mitochondria by 95%. This indicates that a large membrane potential is prerequisite to drive the electrons against the gradient of redox potentials from complex II into complex I. Furthermore, investigations with different experimental systems (intact mitochondria and coupled SMP) revealed that different complex I inhibitors like the Q-site inhibitor rotenone or inhibitors that compromise the primary electron acceptor FMN or the NADH binding site suppressed ROS generation under conditions of reverse electron transfer [18–20,24,91,92]. The ROS production is also attenuated by complex II inhibitors that bind either to the dicarboxylate site or the Qp site if succinate is the prevalent substrate [15,16,19,20,24]. Under these conditions complex II activity is necessary for the reduction of ubiquinol that fuels the respiratory chain complexes III and IV to generate the proton-motive force that is required for RET (Fig. 3A). Inhibition of complex II reduces the membrane potential by diminishing the electron supply for the downstream respiratory chain complexes. A high rate of ROS production is also observed when NADH-generating substrates and succinate are concomitantly supplied to isolated mitochondria [21,23,34,35]. Zoccarato and colleagues have shown that under these conditions electrons can flow downstream through complex I, which should attenuate RET [21]. Accordingly, they have suggested that the ROS production is not realized by RET, but by a direct interaction between complexes I and II. However, such a specific interaction still has to be demonstrated. Furthermore, other studies demonstrated that different NADH-generating substrates have an opposing effect on ROS generation [23,35,108] and it was suggested by Muller et al. that the differential contribution of TCA cycle enzymes and the conditional removal of the endogenous complex II inhibitor oxaloacetate are key factors that control succinate-dependent ROS generation [23]. They have proposed that the reduction of the succinate-dependent ROS formation by malate is due to an accumulation of oxaloacetate that occurs unless acetyl-CoA is available. Furthermore, it can be concluded that the oxaloacetate inhibition of complex II has developed as a mechanism to minimize RET-mediated superoxide production [23]. However, experimental evidence for an oxaloacetate-dependent inhibition of complex II under anoxic conditions is lacking [109].

Under very specific conditions, complex II can also modulate the superoxide production at the Q_o site of complex III [15,16,33] (Fig. 3B). One prerequisite for this is that succinate is the predominate substrate, another that conditions of 'oxidant-induced reductions' are applied to complex III. Experimentally, this can be achieved by the addition of the Q_i site inhibitor antimycin A, but a corresponding physiological scenario has not been identified yet. Anyway, the reduction of complex II activity leads to an increase of the ROS generation by the antimycin A-inhibited complex III [15,16,26–31,33]. This can be explained by an increased oxidation of the Q-pool, since it was shown that the superoxide generation at the Q_o site is maximal at intermediate reduction states of the ubiquinone pool if the Q_i site is blocked by antimycin A [25,32,33].

In summary, the differential effects of complex II on mitochondrial ROS production seem to be influenced by the succinate concentration and the activity of the other respiratory chain complexes. It can be assessed by the distinct effects of complex II inhibitors (Fig. 3). Complex II predominantly produces ROS from reduced FAD at low succinate concentrations and when the downstream electron transfer chain is blocked (Fig. 3C). The block can occur either at the Qp site or further downstream, *e.g.* at the Q_o site of complex III. Also inhibition of the Q_i site by antimycin A should increase ROS generation by complex II under these conditions, but this will be not immediately evident since antimycin A will also induce superoxide at the Q_o site of complex III. Importantly, inhibitors of the dicarboxylate binding site will attenuate ROS production at complex II. In this regard, oxaloacetate could act as a physiological suppressor or regulator of the ROS production by complex II. In contrast, Qp site inhibitors will induce ROS production at low succinate concentrations. However, atpenin A5 induced ROS generation by complex II was somewhat lower than ROS generation induced by a further downstream block (at the complex III Q_o site by myxothialzol), which has been explained by an alteration of the succinate dehydrogenase activity upon atpenin A5 binding [37]. A high succinate concentration and elevated complex II activity will suppress ROS generation from FAD, but will induce superoxide production at complex I due to RET (Fig. 3A). The activities of complexes III and IV are necessary for the generation of a high membrane potential that is prerequisite for ROS by RET. This has not been considered in recent publications that have challenged the existence of a reverse electron transfer from complex II into complex I [36,110]. ROS production under these conditions is therefore not only attenuated by both classes of complex II inhibitors, but also by uncouplers, ionophores and complex I inhibitors. Also complex III inhibitors like stigmatellin, myxothiazol or antimycin A will inhibit succinate-dependent ROS by RET, because the membrane potential collapses upon complex III inhibition. However, inhibition by antimycin A will increase the superoxide generation at the Q_o site. Hence, the overall ROS release

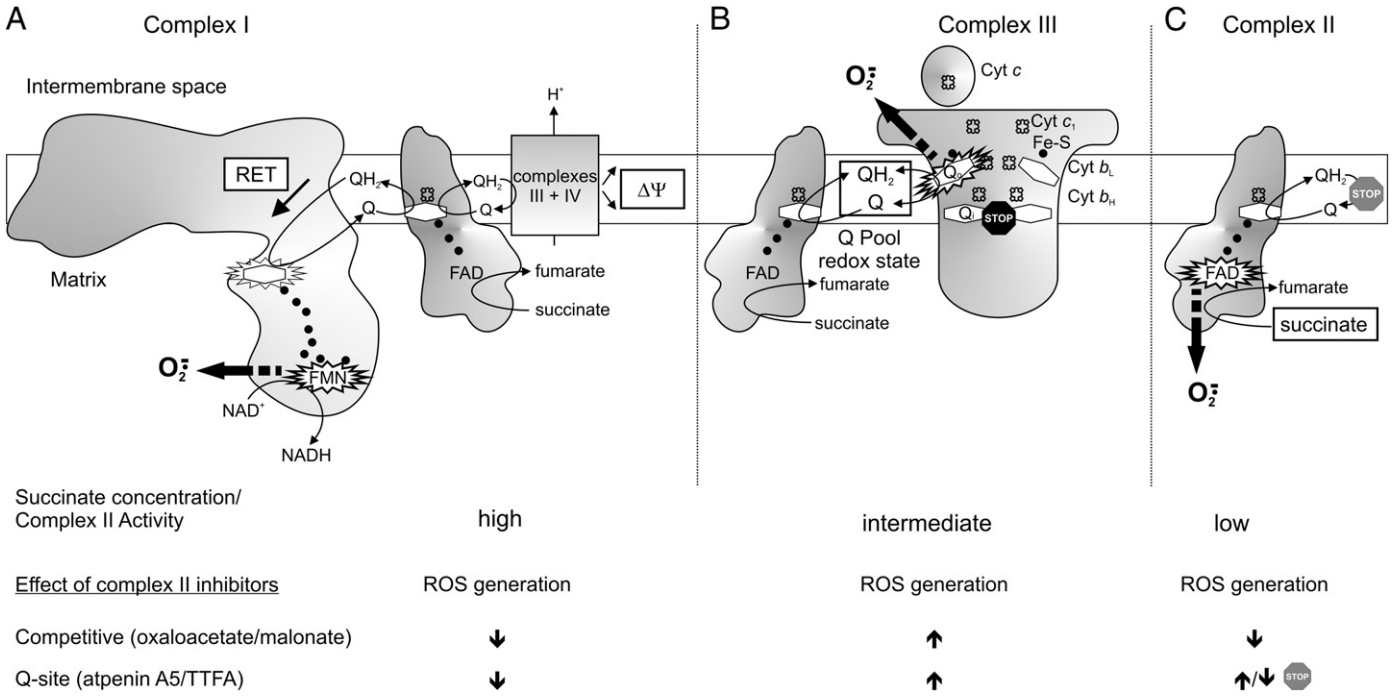

Fig. 3. Differential effects of complex II on the mitochondrial ROS production. The succinate concentration/complex II activity that promotes the respective mode of action and the effect of the two groups of complex II inhibitors are indicated below the schematic representation of the respiratory chain complexes involved. [FeS]-clusters are shown as black circles, ubiquinone binding sites are stretched hexagons, heme groups as schematic porphyrins. Other factors influencing ROS production are highlighted by white boxes. A stop sign indicates a requirement for a downstream block in the electron transfer chain. A) At high succinate concentrations, ROS are generated at complex I due to reverse electron transfer (RET) from complex II. This requires the activity of complexes III and IV for the generation of a high membrane potential ($\Delta\Psi$). Potential sites that generate superoxide are either the FMN or the Q-binding site of complex I. B) Under conditions of 'oxidant-induced reduction', a moderate complex II activity stimulates superoxide production at the Q_o site of antimycin A inhibited complex III, which is favored by a partially oxidized Q-pool (for details see text). C) Complex II produces ROS (presumably mainly superoxide) at low succinate concentrations and when the electron transfer to the Qp site is inhibited/slowed down, which can be attained by Qp-site inhibitors like atpenin A5 or TTFA or by a block located further downstream (*e.g.* at complex III). Under these conditions, ROS are produced from the flavin site. Some mutations in the Qp site might also cause increased ROS generation from this site (not shown, for details see text).

from isolated mitochondria might appear almost unchanged, yet the ROS production is shifted from complex I to complex III. Finally, both classes of complex II inhibitors will increase superoxide production at the Q_o site of complex III under conditions of 'oxidant-induced reduction', *i.e.* when the Q_i site of complex III is blocked and when succinate is the predominate substrate (Fig. 3B). Under these specific conditions, an intermediate succinate concentration/complex II activity results in an intermediate reduction of the Q-pool that favors superoxide production by complex III [32].

3.3. Complex II and the antioxidative defense

Recent investigations indicate that mitochondria are not only an important source of ROS, but can also scavenge large quantities of internally and externally produced reactive oxygen species, especially hydrogen peroxide [22,111–116]. Therefore, it has been proposed that mitochondria are rather a sink for cellular ROS than a main producer [22]. The highly effective mitochondrial antioxidative defense system encompasses the thioredoxin/peroxiredoxin system as well as the glutathione/glutathione peroxidase system (overview in [22,117]). In mammalian cells, these two major antioxidative defense systems rely on the availability of NADPH. In this context, it is important to note that the H_2O_2 scavenger activities of isolated mitochondria are lower with succinate as the sole substrate than with NADH-generating substrates like malate/glutamate or malate/pyruvate [111,112]. This might be explained by the fact that one of the main $NADP^+$-reducing enzymes in the mitochondria, the membrane-bound transhydrogenase [118], depends on a constant NADH supply. Alternatively, the substrate flow through the TCA cycle differs depending on the substrates, which might affect the activity of the other two mitochondrial NADPH-regenerating enzymes the $NADP^+$-dependent isocitrate dehydrogenase [119,120] and the malic enzyme [118,121]. While *in vivo* succinate will hardly be the only available substrate in the mitochondria, the differential ROS scavenger activities with different substrates have certainly an impact on *in vitro* investigations with isolated mitochondria.

4. Modulation of complex II activity in the context of cardioprotective pre- and postconditioning

4.1. Brief introduction to ischemia/reperfusion and the phenomena of pre- and postconditioning

It is well known that a prolonged period of ischemia followed by reperfusion irreversibly damages the heart and other tissues (overview in [8,9]). Mitochondria seem to play a central role in reperfusion injury, since the induction of the mitochondrial permeability transition pore (mPTP) upon calcium-overload and oxidative stress leads finally to apoptotic and necrotic cell death [8,122]. It has been recognized that the mitochondria are both targets and sources of injury during cardiac ischemia and reperfusion [123]. However, the damage occurring during ischemia/reperfusion (I/R) can be reduced by ischemic preconditioning where the prolonged ischemic period is preceded by one or more brief (2–5 min) cycles of ischemia and reperfusion [8,39,124]. Ischemic preconditioning can be mimicked by the K_{ATP} channel opener diazoxide and various other substances, a phenomenon termed pharmacological preconditioning [39]. Diazoxide as well as several very brief ischemic intervals (10 s) during the early phase of reperfusion also improve the recovery of the heart and reduce infarct size, a phenomenon termed postconditioning [8]. Covering all aspects of this extensive topic is

beyond the scope of this review and I will focus on the putative role of complex II in the generation of mitochondrial ROS during reperfusion, pre- and postconditioning.

4.2. Ambivalent roles of complex II and ROS in cardioprotective preconditioning and I/R injury

Reactive oxygen species are a main factor contributing to the damage during I/R [8]. During ischemia, only a small increase of ROS has been detected, but a burst occurs upon reperfusion [43,125]. The source of these ROS is still a matter of debate [8,123]. Considering the accumulation of succinate under hypoxic/ischemic conditions (Section 2.2), the RET-dependent ROS production by complex I seems to be a likely candidate (Fig. 3A). Chen and colleagues summarized how respiratory chain-modulating substances protect cardiac mitochondria and decrease myocardial injury during ischemia and reperfusion [123]. They identified uncouplers and complex I inhibitors as compounds that attenuate the ROS-induced damage in I/R-hearts. It seems plausible that both substance groups exert their protective effects by suppressing the RET-induced superoxide production by complex I (Section 3.2), but Chen et al. suggested complex III as the source of ROS [123]. Anyway, there is also evidence that complex II inhibitors can suppress the oxidative burst when they are present during reperfusion, which can be related to postconditioning. It has been shown that malonate and diazoxide, a well known opener of ATP-gated K^+-channels (K_{ATP} channels), can attenuate the ROS generation that occurs after and during an ischemic period and upon reperfusion in isolated rat heart mitochondria [41–43]. In addition to the interaction with K_{ATP} channels, diazoxide has been shown to inhibit mitochondrial complex II [15,126–129]. Accordingly, inhibition of complex II and attenuation of the succinate supported ROS generation have been shown by Dzeja et al. [130]. Mechanistically, the ROS-reducing properties of complex II inhibitors during reperfusion might be explained by the reduction of the membrane potential (Fig. 3A) that is required for the generation of superoxide at complex I *via* reverse electron transfer. Such a mechanism is supported by several studies with isolated mitochondria [15,16,19,20,24].

Diazoxide and other complex II inhibitors like malonate and atpenin A 5 can also exert protective effects in different model systems (isolated cardiomyocytes, Langendorff-perfused rat hearts) when they are added prior to the long damage-inducing ischemic/reperfusion period [42–44]. Diazoxide transiently induces increased ROS production [43,131–133], which is in agreement with the view that mitochondria-derived reactive oxygen species are involved in cardioprotective signaling during ischemic and pharmacological preconditioning (overview in [8,39,40]). Importantly, diazoxide and ischemic preconditioning associated ROS production is attenuated by myxothiazol [132,134], which clearly indicates that the Q_o site of complex III is the source. This site has been considered as a source for mitochondria-derived 'signaling ROS' in several cellular processes including the stabilization of the hypoxia-inducible factor 1-α [135–137]. In isolated rat heart mitochondria, complex II inhibitors can increase the superoxide production at the Q_o site of complex III only under the specific conditions indicated in Section 3.2 and Fig. 3B [15,16,33], *i.e.* when succinate is the predominant substrate and under conditions of 'oxidant-induced reduction'. This might explain why a ROS-increasing effect of diazoxide has not been detected in the presence of NADH-generating substrates or when complex III was not inhibited [129,138,139]. However, it has to be pointed out that a physiological correlate of the experimentally induced antimycin A inhibition has not been identified yet [25]. Hence, it is uncertain whether this mechanism correctly describes ROS generation *in vivo*. Alternatively, diazoxide that probably binds to the Qp site of complex II (unpublished results) might directly induce ROS generation at the FAD at relatively low succinate concentrations (Fig. 3C). But this also has still to be shown experimentally.

4.3. Is a mitochondrial K_{ATP} channel involved in cardioprotective preconditioning?

It was concluded from many investigations that diazoxide targets a mitochondrial form of ATP-dependent K^+ channels (mitoK_{ATP}) and that this channel plays a central role in cardioprotection by ischemic and pharmacological preconditioning and by ischemic postconditioning [140–142]. However, the existence of such a mitoK_{ATP} channel is controversial [8,39,142–144]. Especially, the fact that the K_{ATP} channel subunits could not be identified so far in highly purified mitochondria challenges its existence [145,146]. Furthermore, the selectivity of the drugs that are generally used for the pharmacological characterization of the putative mitoK_{ATP} channel is limited. Diazoxide inhibits complex II [128,129,147], while the K_{ATP} channel antagonist 5-HD can be metabolized by the ß-oxidation pathway of the mitochondria [148–150]. It has been pointed out that the effects of diazoxide on mitochondrial respiration occur at concentrations that are above the concentrations that are usually applied for mitoK_{ATP} opening [151]. However, there is general uncertainty whether the applied concentration in various test systems will result in similar effective concentrations, which might be largely influenced by the partition coefficient and relative membrane permeability of the applied drug. Therefore, several investigators have concluded that diazoxide and related substances exert their effect independently of a mitoK_{ATP} channel by altering mitochondrial respiration [42,128–130,133,152–155]. Recently, a model was presented that merges these two conflicting views, *i.e.* that complex II may be a component or regulator of the mitoK_{ATP} channel [44,156,157]. Importantly, the results of Wojtovich and Brookes indicate that the highly potent and specific complex II inhibitor atpenin A5 had the same cardioprotective effects in cardiomyocytes and Langendorff-perfused rat hearts as ischemic preconditioning, diazoxide, and malonate [44]. The effect of atpenin A5 was independent of complex II inhibition, and the authors suggested that opening of the putative mitoK_{ATP} channel is the underlying mechanism. However, higher concentrations of atpenin A5 – that were shown to inhibit complex II activity – had to be used in Langendorf-perfused hearts to induce a cardioprotective effect [44]. Hence, an alternative hypothesis was presented that relates the cardioprotective effects of atpenins to the modulation of mitochondrial ROS production [16]. We could show in investigations with isolated rat heart mitochondria, that the succinate-driven superoxide production at complex I by RET can be abolished completely by atpenin A5 concentrations of 10 nM, a concentration that only partially inhibited succinate-dependent respiration [16]. This can be explained by the pronounced sensitivity of RET toward small changes in $\Delta\Psi$ [18]. In addition, similar atpenin A5 concentrations stimulated the superoxide production at the Q_o site of antimycin A inhibited complex III, which may represent the source of 'signaling ROS' during ischemic and pharmacological preconditioning [15]. Importantly, the modulation of mitochondrial ROS production was independent of K^+-ions, indicating that a putative mitoK_{ATP} channel was not involved.

5. Conclusions and outlook

There is increasing evidence that complex II (succinate:ubiquinone oxidoreductase) can be a major regulator of mitochondrial ROS production under physiological and pathophysiological circumstances. In doing so, complex II can adapt different roles as a producer or modulator of mitochondrial ROS depending on substrate supply and the activities of the other respiratory chain complexes and TCA cycle enzymes. The multifaceted function requires a careful analysis of the complex II related ROS production with the available tools

(respiratory chain inhibitors, uncouplers, substrates) to unambiguously identify the source of ROS. This should help to understand the role of complex II in different pathophysiological settings, including ischemia/reperfusion injury. Future investigations should focus more on the dual functions of complex II as a component of the respiratory chain and the TCA cycle, since especially oxaloacetate might be a main endogenous regulator of complex II activity. Finally, basic mechanisms of ROS production by complex II can be further analyzed in different model organisms including bacterial SQRs and QFRs that have already been used for structure–function investigations and the analysis of disease-related human mutations.

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