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EXPERT REVIEW

Inhibitors of Succinate: Quinone Reductase/Complex II Regulate Production of Mitochondrial Reactive Oxygen Species and Protect Normal Cells from Ischemic Damage but Induce Specific Cancer Cell Death

Stephen J. Ralph • Rafael Moreno-Sánchez • Jiri Neuzil • Sara Rodríguez-Enríquez

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ABSTRACT Succinate:quinone reductase (SQR) of Complex II, occupying a unique central point in the mitochondrial respiratory system as a major source of electrons driving reactive oxygen species (ROS) production, is an ideal pharmaceutical target for modulating ROS levels in normal cells to prevent oxidative stress-induced damage or increase ROS in cancer cells, inducing cell death. Value of drugs like diazoxide to prevent ROS production, protecting normal cells, while vit. E analogues promote ROS in cancer cells to kill them, is highlighted. As pharmaceuticals, agents may prevent degenerative disease; their modes of action are being fully explored. Evidence that SDH/Complex II is tightly coupled to NADH/NAD^+ ratio in all cells, impacted by available supplies of Krebs cycle intermediates as essential NAD-linked substrates, and NAD^+-dependent regulation of SDH/Complex II are reviewed, as are links to NAD^+-dependent dehydrogenases, Complex I and E3 dihiydrolipoamide dehydrogenase to produce ROS. We collate and discuss diverse sources of information relating to ROS production in different biological systems, focussing on evidence for SQR as main source of ROS production in mitochondria, particularly its relevance to protection from oxidative stress and to mitochondrial-targeted anticancer drugs (mitocans) as novel cancer therapies.

KEY WORDS mitocans · mitochondria · ROS production · SDH/Complex II · superoxide

S. J. Ralph (✉) • J. Neuzil
School of Medical Science & Health Research Institute
Griffith University
Gold Coast, QLD, Australia 4222
e-mail: s.ralph@griffith.edu.au

R. Moreno-Sánchez • S. Rodríguez-Enríquez
Departamento de Bioquímica Instituto Nacional de Cardiología
Tlalpan México DF 14080, Mexico

J. Neuzil
Molecular Therapy Group, Institute of Biotechnology
Academy of Science of the Czech Republic
Prague 4 142 20, Czech Republic

ABREVIATIONS

α-TOS	alpha-tocopheryl succinate
$\Delta\mu_H{}^+$	electrochemical H^+ gradient across the inner mitocondrial membrane
2-OG	2-oxoglutarate
2-OGDH	2-oxoglutarate dehydrogenase
3-BrPyr	3-bromopyruvate
ANT	adenine nucleotide translocator
DCA	dichloroacetate
DCPIP	dichlorophenolindophenol
DLD	dihydrolipoamide dehydrogenase
FFAs	free fatty acids
FH	fumarate hydratase
MCA	metabolic control analysis
MPTP	mitochondrial permeability transition pore
$O_2{}^{-}\bullet$	superoxide
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
PDH	pyruvate dehydrogenase
SDH	succinate dehydrogenase
SMPs	submitochondrial particles
SOD	superoxide dismutase

SQR	succinate quinone reductase
TTFA	thenoyltrifluoroacetone
UQ	ubiquinone
UQH	semiquinone
UQH_2	ubiquinol

INTRODUCTION

The study of mitochondrial respiration is as much about life's essence as it is pertinent to the regulation of disease and death. It has increasingly been linked with many different pathologies that arise due to oxidative damage, including neurodegenerative disease, aging, cancer, heart disease, muscle myopathies, and diabetes. This review highlights the roles of succinate and the succinate dehydrogenase (SDH)/Complex II of the mitochondrial respiratory system as central mediator of most oxidative damage-induced pathologies and, therefore, as a key pharmacological drug target. It draws on evidence from sources as diverse as physiology and cell biology that supports this first synopsis of the relevant research (Table I).

SDH/Complex II fulfils a unique position separate from the other complexes in the electron transport system as a pivotal regulator of ROS production, either inherently produced within or indirectly *via* Complex I, downstream Complex III, or other mitochondrial ROS producers. It is this pivotal function as regulator of ROS production affecting both the $NADH/NAD^+$ ratios and the reduced/oxidised ubiquinone pool (UQH_2/UQ ratio) that distinguishes Complex II as a target to prevent oxidative stress-mediated tissue damage and cytoprotection against ischemic/reperfusion episodes as well as a unique target for cancer therapy. Here, the properties of the SDH/Complex II system as a key redox regulator of ROS production are reviewed, together with its role in post-ischemic oxidative tissue damage and its relevance as a target for the selective killing of cancer cells by promoting extreme ROS overproduction.

SDH/COMPLEX II: BASIC STRUCTURE AND FUNCTION

As part of the Krebs cycle, SDH catalyses the oxidation of succinate to fumarate with the concomitant production of $FADH_2$. The crystal structure (1) of the SDH/Complex II comprises an inner mitochondrial membrane matrix face-bound heterodimer of SDHA and SDHB subunits as a soluble enzymatic head group, which contains the SDH activity associated with several prosthetic groups that catalyse the electron transfer from the succinate to fumarate reaction to reduce ubiquinone (UQ or Coenzyme Q) to ubiquinol (UQH_2). The components in the sequential flow of the electron transfer reactions within the two soluble catalytic subunits include subunit A covalently bound to the FAD cofactor and subunit B containing three iron-sulfur (Fe-S) clusters: [2Fe-2S], [4Fe-4S], and [3Fe-4S] in decreasing order of standard redox potential (Fig. 1).

Two other subunits, SDHC (the larger cytochrome b binding subunit or CybL) and SDHD (the smaller cytochrome b binding subunit or CybS) form the base of the enzyme as an intramembraneous complex within the inner mitochondrial membrane (reviewed in (2)). This integral membrane anchoring domain links to the soluble SDH head group on the matrix face. The membrane domain contains two UQ binding sites as hydrophobic channels for the phytyl chain, one with a pocket for the quinoid head group nearer to the matrix surface, called the proximal or Q_P site, and the second extending down the opposite side of the SDHC/SDHD structure, with its quinoid pocket nearer the intramembraneous space, known as the distal or Qd site (2).

Defining Traits of the Mitochondrial Respiratory Complex II

Physical Location and Functional Separation in Relation to Mitochondrial Respiratory System and Quinone Pool

A practical laboratory experiment commonly included in biochemistry and cell biology undergraduate courses is to isolate mitochondria from calf or rat liver or heart tissue and use different substrates to compare the differences in rates of O_2 consumption under basal (state 4 respiration) conditions or coupled to ATP synthesis (*i.e.* OXPHOS) by adding ADP + Pi to promote state 3 respiration (3). First, NAD-linked oxidisable substrates pyruvate/malate (or glutamate/malate) are added to engage the Krebs cycle and the respiratory chain complexes I, III, and IV, on electron transfer and O_2 consumption. Then, rotenone is added to specifically inhibit Complex I, and succinate is introduced into the reaction to reactivate the electron transport chain, now starting *via* SDH/Complex II. Finally, the specific Complex III inhibitor antimycin is added, and ascorbate is included to reduce cytochrome c and reactivate electron transport at the level of Complex IV. One could be forgiven for inferring that Complex II is downstream of Complex I in the respiratory chain. The well-known but surprising observation is that succinate usually gives much faster rates of O_2 consumption than other NAD-linked substrates and produces a lower P/O ratio as an indicator of ATP production to O_2 consumption (4).

Although useful as a practical teaching concept, the experiment is oversimplified in that mitochondria inside cells

Table I Pharmaceutical Agents Targeting Either SDH/Complex II Enzymatic Head Group or Succinate: Quinone Reductase (SQR).

TARGET	SDH	SQR
COMPOUND	Succinate	Ubiquinone (UQ)
	Fumarate	MitoQuinone (MitoQ)
	Oxaloacetate (OAA)	MitoVES
	Malonate	alpha-Tocopherol Succinate (α-TOS)
	3-Bromopyruvate (3-BP)	alpha-Tocopherol Phosphate (α-TOP)
	3-Nitropropionic acid (3-NPA)	Diazoxide
	Diazoxide ?	Fenretinide
		Atpenin A5
		Thenoyltrifluoroacetone (TTFA)
		Adaphostin
		Pyridoxal Phosphate

do not have restricted access to individual substrates because most substrates that ultimately feed into the respiratory chain are simultaneously available, including glutamate, 2-oxoglutarate (2-OG), pyruvate, malate, succinate, fatty acids, *etc*. However, the experiment does serve to highlight the rapid rate of O_2 consumption upon adding succinate as a substrate, and the response of the SDH/Complex II can be analysed. Nevertheless, unlike the simple linear flow implied by this practical experiment, the convergence of different available input substrates impacting simultaneously as electron donors to the electron transport system must be taken into account for a better understanding of the physiological behaviour of *in situ* mitochondria. A recent treatise reviewed convergent electron transfer as distinct from the misnomer of the linear nature implied by the term *electron transport chain* (5). Initial proposals of a random distribution (the random diffusion model or liquid state model) for the different respiratory complexes in the inner mitochondrial membrane implying a freely diffusible process of electron transfer between the complexes mediated by collisional interactions with electron carriers, UQ or cytochrome c was largely incorrect (reviewed in (6)). Rather, they seem to be more tightly connected by partial or complete channelling of electron transfer amongst the complexes (7,8).

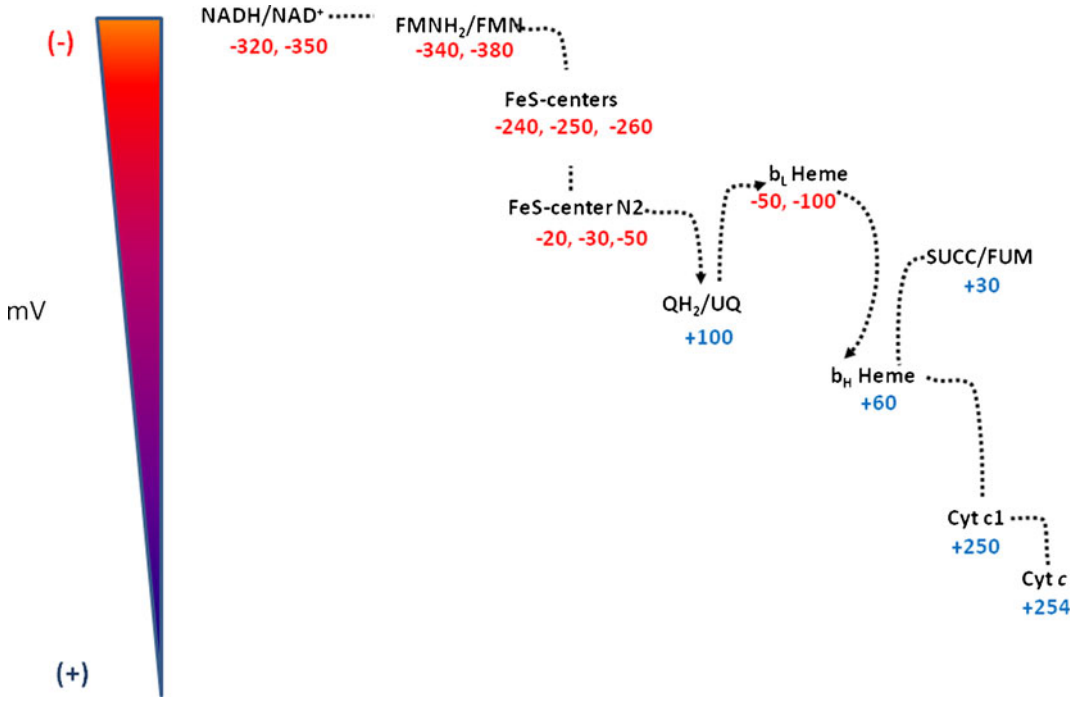

Fig. 1 Redox potencial values (in mV) for NADH/NAD^+ and $FMNH_2$/FMN ratios (281–283), FeS N-1, N-3, N-4, N-5 centers (283,284), FeS N2 center (284), SUCC/FUM and QH_2/UQ ratios (283,285), high potential b heme (b_H heme) (286), low potential b heme (b_L heme), cyt c1, and cyt c (287). Red numbers: negative redox values; blue numbers: positive redox values. cyt, cytochrome; FMN, flavin mononucleotide; FUM, fumarate; SUCC, succinate.

By contrast, studies examining the physical nature of supramolecular structural relationships displayed by respiratory chain complexes have shown that SDH/Complex II exists separated from other complexes. Thus, studies by several independent groups have established that complexes I, III, and IV commonly exist as larger supramolecular species called *respirasomes* (9,10), as demonstrated by metabolic flux control analysis and extraction from mitochondria using mild detergents (reviewed in (11)). Also unlike Complex II, the other three Complexes participate in producing the electrochemical H^+ gradient ($\Delta\mu_H^+$) as the proton motive force driving ATP synthesis by Complex V. Based on biochemical analyses and subsequent 3D mapping studies from electron microscopy of purified bovine mitochondrial respirasomes, this supramolecular complex was shown to mainly comprise a $I_1III_2IV_1$ stoichiometry that retains activity for carrying out electron transport ((12,13), reviewed in (11)). These supramolecular complexes predominantly exclude association with Complex II, raising the question "what is so special about SDH/Complex II?"

One clear distinction in function arising from the separation of Complex II that can be markedly contrasted to the linkage of other components of the respiratory chain is in their relationship to the flux of membrane electron carriers, UQH_2/UQ. A cyclic flow of these electron carriers occurs within the whole respiratory system in the mitochondrial inner membrane. Thus, starting upstream in the redox potential gradient (Fig. 1), the oxidised UQ is reduced upon interaction with Complex I or II, proceeding *via* an intermediary, the semiquinone UQH, on either complex to become the fully reduced ubiquinol (UQH_2), which is then released to carry electrons to Complex III, where UQH_2 is re-oxidised by releasing its protons and transferring its electrons to Complex III, before returning for repeated cycles (14,15). Unlike with the Complex I:III:IV/respirasome, where UQ can be channelled directly over the short distances between the physically linked Complexes I and III, Complex II exists relatively freely unattached and must exchange with the freely diffusing UQ pool. In fact, Complex II activity is the main controlling step for succinate oxidation, suggesting a distinct absence of substrate channelling to Complexes III and IV (16,17). Thus, Complex II kinetics show simple pool behaviour in mitochondrial membranes (18–20), reconstituted systems (21), and double inhibitor titration experiments in intact mitochondria (22). Evidence for a physically separated Complex II is also supported by purification studies in which discrete units containing succinate cytochrome c reductase activity were isolated and analysed (23,24).

In muscle or liver mitochondria respiring on succinate as substrate, UQ was shown to exist in different ratios distributed across at least three different pools (25): a pool for steady-state respiration, another providing a mobile reserve for maintaining energy fluxes, buffering against imbalances, and the third a non-mobile pool which did not participate in succinate-dependent respiration, presumably because it was bound up in respirasomes (25). Several other studies have established purification procedures to isolate SDH/Complex II with intact enzymatic activity, including succinate-quinone reductase (SQR) function (26–29).

It is clear from physical studies detailed here and results of flux control analysis of Complexes I and III that they can function as a single unit, with substrate channelling by UQ in transfer of electrons from Complex I to Complex III (16). Substrate channelling brings about a more efficient electron transfer between respiratory complexes. However, the rates of electron transfer and O_2 consumption catalysed by Complex II are faster than those achieved by Complex I, indicating that in the absence of I-III supramolecular respirasomes, much slower rates would be attained with NADH as the electron donor. The variety of states of complex association and compartmentalization of the electron carriers, UQ and cytochrome c, has implications for disease situations, particularly in relation to changes in cancer cells. Early studies examining the location of SDH/Complex II in the mitochondrial cristae in normal mouse liver mitochondria revealed that it forms bands/rings around the cristae, away from the contacting sites of inner and outer mitochondrial membranes, and that in state 3 respiration in the presence of ADP and succinate, Complex II underwent a geometrical rearrangement to become more widely distributed around the matrix face of swollen cristae (30). Hence, SDH/Complex II commands its own special relationship with the components of the mitochondrial respiratory chain.

Coupling of SDH/Complex II Activity to Metabolism Via Krebs Cycle: Metabolic Flux Control and Contribution to Respiration and Oxphos

A second distinguishing feature of the SDH/Complex II is its dual role providing a pivotal link as a component of the Krebs cycle and as an electron donor for the respiratory system. Studies have proposed that several enzymes of the Krebs cycle also, like the respirasome, exist in a supramolecular complex called the metabolon (31–38), attached to the matrix face of the inner mitochondrial membrane by SDH/Complex II, as the anchor site for assembly of the metabolon on the membrane (33). In addition, 2-OGDH was proposed to play a key role in metabolon formation, with two association sites on opposite sides in its interaction with the membrane. The close linkage of Krebs cycle enzymes would allow for an accelerated flux of substrates with a moderate number of intermediates, providing a means for metabolic channelling around the Krebs cycle and on to succinate as an electron donor feeding into the

respiratory chain *via* Complex II. Further studies examining the nature of metabolon complex are lacking.

Numerous studies reported during the 1960s–1970s attempting to measure SDH/Complex II activity under various conditions were made possible with the advent of tetrazolium/formazan-based dyes used for enzyme assays of cells and histochemical analyses of tissues, including tumors; these semi-quantitative histochemical methods for analysing respiration and SDH activity continue to be used (39). Although these dyes provide a useful visual indicator of SDH/Complex II activity in tissue sections, most are not accurate when used histochemically; they usually underestimate rates and lack the target specificity required for assessing particular metabolic enzyme function (40).

Given the commanding position of SDH/Complex II in the respiratory system, a large number of contributing factors will affect its activity in mitochondria. The extent and types of metabolism proceeding in the mitochondria depend on a number of factors, including the availability and accessibility of different substrates and their transport into mitochondria, the extent of activity of the major metabolic pathways (the pyruvate dehydrogenase (PDH) reaction, the fatty acid beta-oxidation system, the reactions of the Krebs cycle, the electron flow and transfer of reducing equivalents by UQ along the respiratory system, and the levels of ATP synthesis and transport reactions for ADP, ATP and Pi. Respiratory system activity depends on $\Delta\mu_H+$, degree of coupling, level and ratio of UQH_2/UQ pools, levels of substrates including ATP/ADP and $NADH/NAD^+$, and available levels of O_2, *i.e.*, whether conditions are hypoxic. For example, in cancers or ischemic tissues where hypoxia exists, relative levels of available O_2 would obviously become greatly diminished.

The control of most of the mitochondrial metabolic reactions listed above can be studied in cells or isolated mitochondria using O_2 consumption rates and/or radioactive substrate transformation steady-state rates and applying metabolic control analysis (MCA), based on the theories originally put forward by Kacser and Burns (41,42) and Heinrich and Rapoport (43,44). By determining the relative flux control coefficients, individual levels of metabolic control of each different step, or pathway segments along separate pathways of an integrated biological system such as mitochondrial respiration and OXPHOS, can be quantitatively assessed and understood. MCA allows for theoretical and experimental analysis of why an enzyme, pathway segment, or entire pathway exerts significant flux control in a pathway or cellular process or why it lacks this property. Mitochondrial respiration and OXPHOS are well suited for such studies because specific inhibitors for each reaction comprising the metabolic pathway are available. Also, the activity of well-defined pathway segments can be readily determined, such as NADH-producing (substrate transport *plus* Krebs cycle) and consuming (respiratory system *plus* OXPHOS) segments as well as $\Delta\mu_H+$–producing (Krebs cycle *plus* respiratory system) and consuming (OXPHOS *plus* heat dissipation) segments. Most often, the substrate used for these studies has been succinate (in the presence of the Complex I inhibitor, rotenone), but other systems involving physiologically more relevant substrate combinations have also been studied (45). In general, flux control is shared by a number of reactions; although the distribution of control may or may not depend on the particular prevailing set of conditions (for review, see (46)), the same controlling steps always prevail, reflecting the highly robust level of metabolic control of pathways inside living cells.

Analysis of ^{13}C-glucose as a stable isotope was used to resolve the metabolomic difference between human non-small-cell carcinoma lung cancers or rhabdomyosarcoma and their paired non-cancerous lung tissue or primary myocytes, respectively. NMR and GC-MS showed ^{13}C-enrichment in several Krebs cycle intermediates including succinate to much higher levels in cancer samples and particularly high aspartate and glutamate production, further establishing that glycolysis, together with mitochondrial anaplerotic pyruvate carboxylation and glutamine oxidative deamination, and the Krebs cycle actively function in several tumor tissues (47,48).

SDH/COMPLEX II IS A MAJOR SOURCE OF OXYGEN CONSUMPTION ASSOCIATED WITH HIGH ROS PRODUCTION

The third and most critical feature of Complex II is its predominance for driving O_2 consumption, in line with also supporting the highest rate of ROS production in mammalian mitochondria ((49), reviewed in (50) and Table 1 in (51)). Typically, ~90% of O_2 consumed by the tissues of mammals occurs in the mitochondria; of this 90%, ~70% is coupled to ATP synthesis, and the remainder is uncoupled due to the mitochondrial H^+ leak and is dissipated as heat (reviewed in (52)). Hence, mitochondria are the predominant site of O_2 consumption and ATP supply in normal cells in the body. However, few studies have specifically measured the amount of SDH/Complex II metabolic activity in cells or tissues and then used these measurements to determine the relative contribution of SDH/Complex II to the rate of overall O_2 consumption and ROS production in tissues *in vivo*, particularly cancer cells. ROS production analysed in mitochondria isolated from many tissues incubated under physiologically relevant conditions accounts for as much as 0.1–0.2% of O_2 consumed with succinate-forming ROS, thereby providing an important substrate for ROS production (53). It is hoped that more

studies aimed at addressing these issues, especially the impact of multiple simultaneous substrates, will be undertaken.

A few studies using MCA and combinations of substrates provided to the respiratory electron transport system have shown that combinations produced greater rates of O_2 consumption compared to individual substrates applied alone (20,54). Thus, beef heart submitochondrial particles (SMPs) respiring on NADH and succinate showed that the combination increased respiration levels, albeit less than additive, and that there was not a single homogeneous UQ pool for Complex I or II exchanging with Complex III. Instead, data suggested a more compartmentalized UQ pool consistent with respirasomes as one pool and a freely exchanging UQ pool for Complex II (55). Further evidence for different pathways of electron flow used by Complex I and II linking to Complex III were obtained by Gutman (54) using inhibitors such as thenoyltrifluoroacetone, TTFA (Fig. 2), for Complex II and mersalyl for Complex I, giving rise to the proposal of a reverse electron transfer from succinate-driven Complex II transferring electrons to Complex I and NAD^+ in addition to the forward electron transfer from NADH oxidation to NAD^+ via Complex I (reviewed in (10)).

Complex I is often reported to be responsible for most of the mitochondrial H_2O_2 release, low during the oxidation of the NAD-linked substrates but much higher with succinate oxidation when it was proposed to occur *via* a reverse electron flow proceeding from Complex II to Complex I (51,56,57). H_2O_2 production was physiologically relevant because it was induced at submillimolar succinate concentrations and in the presence of NAD-linked substrates with either heart (58) or rat brain mitochondria (59). Mitochondrially produced superoxide is readily converted into H_2O_2 by intramitochondrial Mn-SOD; it has for a long time been thought that the superoxide production was largely dependent on Complex I and Complex III, because although NAD-linked substrates, such as glutamate and pyruvate, are poor H_2O_2 producers compared to succinate, the much greater succinate-dependent H_2O_2 release is largely sensitive to the Complex I inhibitor rotenone. Hence, it was consequently proposed that succinate produces ROS *via* a reverse electron flow from Complex II to Complex I (60–66). However, in isolated rat brain, heart and skeletal muscle mitochondria, production of H_2O_2 is low with the NADH producing substrates (glutamate/pyruvate) and increases >10-fold with added succinate, in a sigmoidal manner (half maximal value at 290 μM, maximal H_2O_2 production at 600 μM succinate) (67). Similar results with rat skeletal muscle mitochondria and succinate also showed greater $O_2^{-}\bullet/H_2O_2$ production than with the NAD^+-dependent substrates, glutamate and pyruvate (60,62,64,66,68). Thus, the rate of H_2O_2 production is substantially greater during succinate oxidation than during NADH-linked oxidation.

Many studies have shown that $O_2^{-}\bullet$ production rate during succinate oxidation is much greater than that achieved during oxidation of succinate in the presence of rotenone (for example, see (69)) and several researchers have examined the mechanism for the perceived reverse electron flow when the SDH reaction of Complex II appears to proceed in the reverse reaction with an associated production of ROS ensuing from Complex I, because this ROS production is inhibited by rotenone (70–73).

Rotenone *vs.* Glutamate: Alternatives for Analysing Effects of Succinate on Mitochondrial Respiratory Rates and ROS Production

Rotenone is used to block the contribution of Complex I when measuring succinate-induced mitochondrial ROS production. However, using rotenone adds the complication that if physiological levels of Complex I substrates $NADH/NAD^+$ are not present or are low, when succinate is used alone, rotenone will also block NADH oxidation to NAD^+, thereby exhausting NAD^+ levels. Also, under such conditions with rotenone added, the $NADH/NAD^+$ ratio will continue to increase due to mitochondrial metabolism using up any available NAD^+ (*i.e. via* coupled oxidation of Krebs cycle NAD-linked substrates). However, NAD^+ is an essential cofactor required for several steps of the Krebs cycle, including the malate reaction to produce oxaloacetate (OAA) (Fig. 3); hence, this conversion rate will be decreased or even completely abolished when rotenone is used. Thus, rotenone addition also prevents the accumulated build up of OAA, a potent tightly bound competitive inhibitor of SDH, during state 3 respiration by maintaining a large $[NADH/NAD^+]$ ratio (74–77) whilst promoting a fully active Complex II (in the presence of added succinate); hence, rotenone is often used to inhibit succinate-driven Complex II-mediated ROS production.

An alternative and more preferred mechanism for preventing OAA build-up in mitochondria, without requiring rotenone, is to use glutamate as a supplement in the reaction system with succinate. Glutamate will also prevent build-up of inhibitory OAA by removing it *via* transamination with glutamate to produce 2-oxoglutarate (2-OG) and aspartate (77–79). An ^{1}H-NMR study showed that the highest rate of succinate synthesis could be observed by incubating rat heart mitochondria with a mixture of glutamate *plus* OAA (80). Thus, succinate was produced from 2-OG formed *via* rapid transamination of glutamate with OAA by aspartate aminotransferase (Fig. 3) or the slower glutamate dehydrogenase that is competitive for available NAD^+ and, hence, also reduces NAD^+ to NADH, as does the next step in the Krebs cycle from 2-OG to

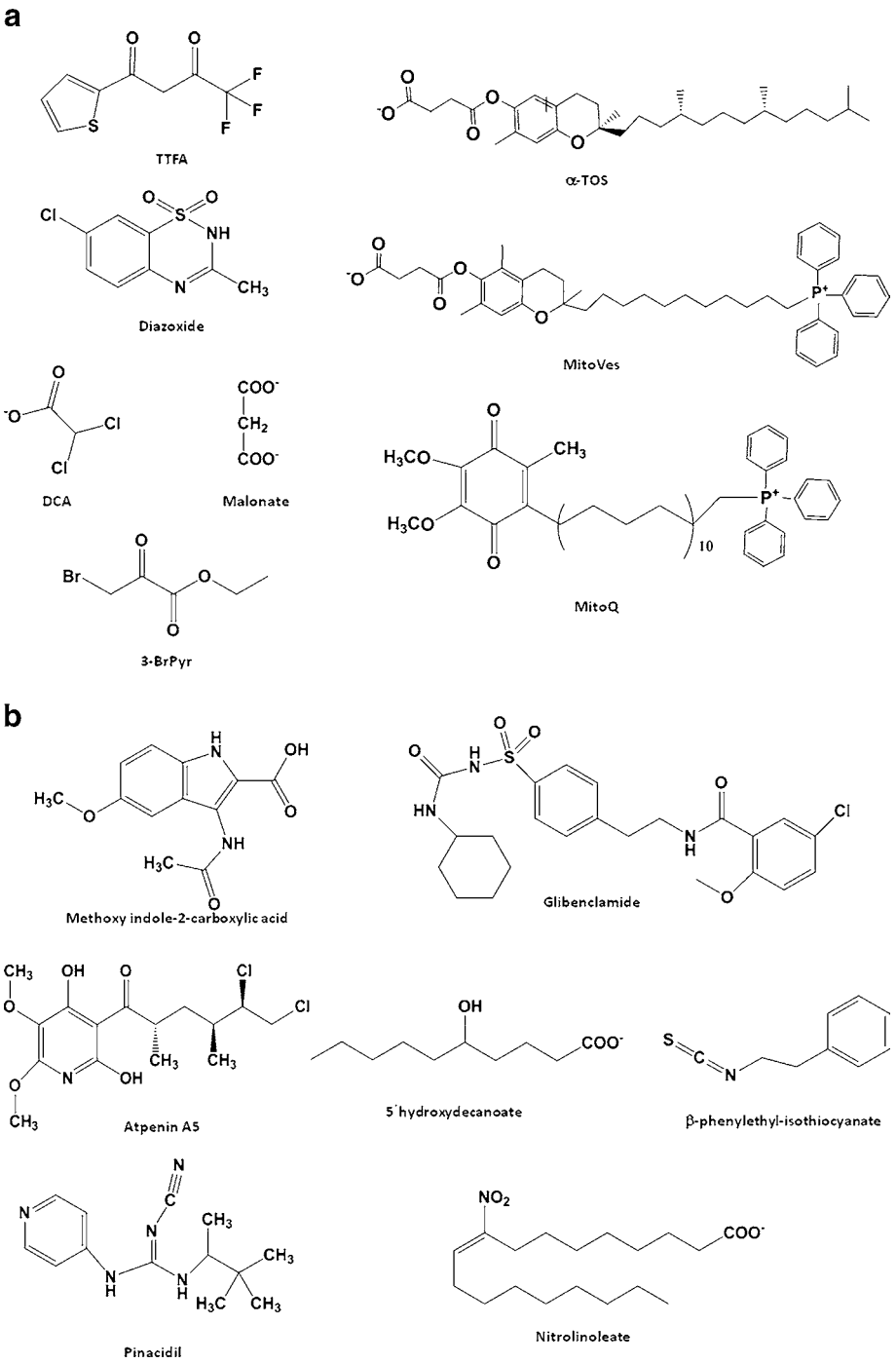

Fig. 2 Chemical structures of several SDH/Complex II inhibitors and related compounds.

succinyl CoA, catalysed by 2-OGDH (Fig. 3). As competing mitochondrial metabolic reactions, these latter two enzymes ensure that the NAD^+ available for malate to OAA conversion is sufficiently maintained at a steady level. Therefore, rotenone can be efficiently replaced with the more physiologically relevant glutamate to remove OAA through the transamination reaction, as well as slowing OAA production by keeping NAD^+ levels low, as an essential cofactor required for malate conversion to OAA. The malate dehydrogenase forward reaction is thermodynamically unfavourable (Fig. 3) and only occurs when sufficiently high ratios of NAD^+/NADH and malate/OAA exist (81). Notably, the substitution of glutamate as an alternative to rotenone has been shown not to affect the levels of succinate-supported ROS production in mitochondria, unlike rotenone, which greatly decreases it (59).

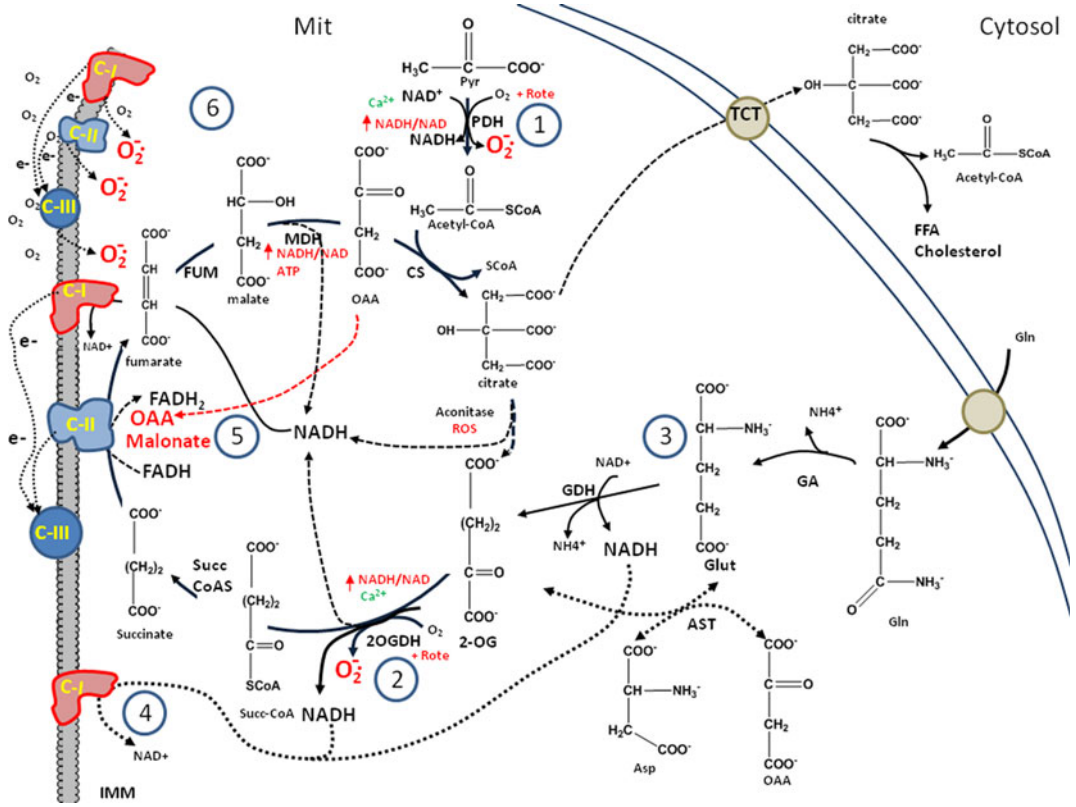

Fig. 3 Metabolic routes of Krebs cycle and anaplerotic reactions in cancer cells. (1) Pyruvate decarboxylated by PDH to generate acetyl-CoA *plus* NADH; if C-I inhibitor rotenone (rote) is added, Krebs cycle intermediates accumulate and flux of lipoamide-dependent PDH and (2) 2-OGDH stop generating $O_2^{\bullet -}$. (3) Anaplerotic reactions are highly activated in tumor cells such as glutaminolysis (*i.e.*, oxidation of glutamine towards 2-OG involving glutaminase (GA), GDH and aspartate transaminase (AST)). (4) Oxidation of glutamate and 2-OG by very active GDH and 2-OGDH, respectively, generates NADH feeding respiratory chain complex I (C-I) to ensure continuous pathway flux. (5) OAA generated in Krebs cycle as well as malonate inhibit SDH/Complex II (C-II). (6) If activities of C-I, -II and/or –III diminish, enhanced production of $O_2^{\bullet -}$ is achieved. (7) In some tumor cells, low aconitase activity promotes accumulation of citrate, which is transported by active tricarboxylate transporter (TCT). In cytosol, citrate is precursor for FFAs and cholesterol synthesis. Physiological activators (green) and inhibitors (red). Asp, aspartate; AST, aspartate transaminase; C-I, Complex I; C-II, Complex II; C-III, Complex III of the respiratory chain; FFA's, free fatty acids; GA, glutaminase; Gln, glutamine; GDH, glutamate dehydrogenase; Glut, glutamate; IMM, inner mitochondrial membrane; Mit, mitochondria matrix; OAA, oxaloacetate; Pyr, pyruvate; Succ-CoA, succinyl-CoA; Succ CoAS, succinyl-CoA synthase; TCT, tricarboxylates transporter; 2-OG, 2-oxoglutarate; 2-OGDH, 2-oxoglutarate dehydrogenase.

Moser *et al.* (77) examined changes in the levels of NAD(P)H during succinate-driven respiration and the effects of H_2O_2. Rat cardiac mitochondria respiring on succinate showed a marked decrease in NADH during state 3 respiration, a condition they concluded was consistent with NADH oxidation to NAD^+ linked with continuing Krebs cycle activity preventing build-up in OAA production levels. H_2O_2 (50 μM) did not affect the extent of NADH oxidation but did lengthen duration of the state 3 oxidative condition when respiring on succinate; it was concluded that H_2O_2 mediated an inhibitory effect on Complex II function probably by promoting inhibitory levels of OAA (77). During state 3 respiration on succinate, rotenone addition in the presence or absence of H_2O_2 increased NADH levels consistent with continuing reduction of NAD^+ by Krebs cycle dehydrogenases (MDH, IDH, 2-OGDH) and blocking of NADH consumption by Complex I.

Despite the implications of these findings and the relationship of Complex II function to Complex I via NADH/NAD^+ ratios, the commonly accepted principle has persisted for many years that the high rate of ROS production observed in mitochondria with succinate originates as $O_2^{\bullet -}$ (and/or H_2O_2) production from Complex I by driving a reverse electron flow from Complex II to I because much (but not all) of the increase in ROS is eliminated by adding rotenone. However, these findings and their impact on succinate-driven ROS production will be revisited below, but first evidence refuting reverse electron flow must be discussed.

Enigma of Reverse Electron Flow in the Direction Complex II to Complex I

Before further considering the alternative proposal to reverse electron flow and the role of SDH/Complex II,

the common enigma of reverse electron flow from Complex II to I needs further explanation. Reverse electron flow or reverse electron transport have become terms invoked to describe a mechanism whereby an electron donor with a lower redox potential uses energy supplied by utilising $\Delta\mu_H+$ to drive electrons through the electron transport chain in the reverse direction, in particular, to explain the apparent electron flow often described to occur from SDH/Complex II to Complex I under certain conditions, such as in the presence of high succinate levels. Some studies have invoked that UQH_2 from Complex II could bind to a second site on Complex I distinct from the binding site produced during the forward Complex I to Complex III UQ interaction. Furthermore, the two Complex I binding sites were proposed to become alternatively accessible depending on $\Delta\mu_H+$ magnitude (10,16).

An alternative proposal was that ATP hydrolysis-driven reverse electron flow from succinate to NAD^+ generated a high $\Delta\mu_H+$ which could trigger the structural rearrangement of the respiratory complexes so that Complex IV regulated the flow from Complex I/III but did not regulate Complex II activity (82). However, further studies under a variety of different conditions will be required to explore this proposal more thoroughly.

Resolving the Enigma of Reverse Electron Flow and ROS Production: Complex II Coupling to Complex I and NADH/NAD^+ Levels

This review presents evidence contesting the relevance of Complex I ROS production *in vivo* at the levels of FMN and UQ binding sites during Complex II to I reverse electron flow (*e.g.* in the presence of Complex III or IV inhibitors), as doubt about the underlying evidence supporting a predominance of the Complex I contribution has arisen in more recent studies. Moreover, the physiological relevance of reverse electron flow from Complex II to I in the absence of Complex III or IV inhibitors is questioned, and its contribution to ROS production is herein considered negligible or plainly non-existent, particularly in the context of intact isolated mitochondria or whole cells. An alternative rationale is proposed by invoking a more thermodynamically favourable explanation to resolve the enigma of reverse electron flow and ROS production, which involves the relationship of SDH/Complex II activity to Complex I and NADH/NAD^+ ratios, connecting the metabolic activity of the Krebs cycle to the respiratory electron transfer rates. As described above, it has long been established that succinate (in the absence of rotenone) supports by far the highest rate of ROS production in mammalian mitochondria (reviewed in (50,51)). Therefore, succinate is often used as the substrate for assessing mitochondrial ROS production *in vitro* and other functions such as in ischemia/hypoxia/reperfusion models. However, the physiological significance of using succinate to drive this ROS production has mostly gone unrecognised. It is common practice to use succinate at 5–10 mM concentrations combined with rotenone to determine the relative contribution of succinate towards OXPHOS.

During reverse electron flow, the SDH reaction of Complex II is commonly believed to proceed in association with ROS production emanating from the reverse reaction of Complex I with NAD^+ reduction to NADH (70–73). In particular, Grivennikova and Vinogradov (70), using bovine heart SMPs respiring in the presence of succinate or NADH alone or both added together in different combinations, examined $O_2^{-}\bullet$ production by Complex I and its dependency on the levels and ratio of NADH/NAD^+. Succinate-supported production of $O_2^{-}\bullet$ was measured in the presence of rotenone using acetylated cytochrome c (which accepts electrons from $O_2^{-}\bullet$ and undergoes a measurable spectral wavelength shift). $O_2^{-}\bullet$ production rate in the presence of high 1 mM NADH levels alone was much less than that obtained with succinate (10 mM) alone, and succinate-induced $O_2^{-}\bullet$ was successively decreased by increasing NADH levels from 50 μM to 1 mM. Titrating NADH alone showed a bell-shaped curve with maximal rates of $O_2^{-}\bullet$ produced by 50 μM NADH (reaching close to the high levels obtained with succinate alone), before rapidly declining to become inhibited at higher NADH levels.

Succinate-dependent Complex I-catalysed energy-linked NAD^+ reduction to NADH (reverse electron flow) could also be inhibited by NAD^+ with an apparent *Ki* close to the *Km* value for the reaction (~ 20 μM). Thus, NAD^+, at levels not affecting NADH oxidation, decreased NADH and/or succinate-induced $O_2^{-}\bullet$ production. Based on these results, Grivennokova and Vinogradov proposed that different NAD^+ binding sites were operating for NADH oxidation (forward reaction) and inhibition by NAD^+ (reverse reaction) (70). They proposed a model to explain different electron transfer pathways during NADH oxidation, NAD^+ reduction, and $O_2^{-}\bullet$ generation by Complex I (70). The model comprised a common pathway involving electrons from the UQ pool in the membrane flowing *via* Fe-S proteins to the FMN containing flavoprotein of Complex I, then splitting in separate directions to participate in either the forward reaction to NAD^+ or reverse reaction to NADH. Two structurally distinct primary binding sites for NADH (forward reaction) and NAD^+ (reverse reaction) were proposed to be located separately in the hydrophilic matrix exposed part of Complex I. However, the crystal structures of Complex I from *Thermus thermophilus* (83) and yeast (84) have shown that only one deep cavity exists capable of binding NADH, with no other binding sites detected, diminishing the likelihood of this possibility.

Moreover, the Grivennokova and Vinogradov model cannot explain why higher rates of ROS production occur with succinate (in the absence of rotenone) *versus* NAD-linked substrates (*plus* rotenone) at similar NADH levels and why rotenone only partially inhibits the succinate-dependent ROS production.

Recently, a single unified mechanism involving the reduced flavin in Complex I was proposed to explain the O_2^{-}• produced either during NADH oxidation (forward) or succinate-driven (reverse) electron transfer (56). In these studies with bovine heart SMPs, low micromolar levels of diphenyleneiodonium (DPI, a Complex I flavin-site inhibitor) were shown to inhibit O_2^{-}• production under conditions of either forward or reverse electron transfer. However, the observed rate of reverse electron transfer-induced ROS production was approximately three times slower compared to the NADH induced rate, which is not consistent with the very high levels of succinate-induced ROS production described above. Hence, reverse electron transfer does not explain nor account for the very high levels of succinate-driven ROS production under more physiological conditions of either intact mitochondria or whole cells, which are orders of magnitude higher than those produced by the forward reaction of Complex I.

Studies from Zoccarato's group (58,59,67) provide the basis for a suitable alternative and a more plausible and energetically favourable explanation for the high succinate-induced ROS production, which will be identified in detail below. First, their results need to be described in detail based on data obtained from heart or brain mitochondria respiring on both NADH and succinate (likely to reflect the more common situation as it would occur *in vivo*). In their studies, data from O_2 consumption, NADH conversion and rotenone sensitivity were suggested to indicate that the two substrates, NADH and succinate, competed as electron sources for Complex I and that succinate oxidation at Complex II was driving electrons *via* UQH_2 in the reverse direction into Complex I because the flow of succinate-driven ROS production was rotenone sensitive. Moreover, Complex I and II activities appeared to occur simultaneously, since O_2 consumption with both substrates present was greater than with either alone. However, once again, the common misinterpretation was proposed for reverse electron flow existing between Complex II and I, even allowing for the more physiological conditions (*i.e.* intact mitochondria with both substrates present and in the absence of inhibitors of Complex III or IV).

NADH promotes the transfer of electrons in Complex I *via* FMN to Fe-S centres and thence to UQ to form UQH_2. A mechanism was proposed whereby the release of UQH_2 produced *via* NADH in Complex I could be blocked by the build-up of UQH_2 from Complex II due to highly active succinate oxidation. Thus, reverse electron flow induced by high succinate levels was proposed to occur when UQH_2 originating from Complex II binds to the Q-binding site on Complex I and/or when Complex I-generated UQH_2 is retained because it cannot be released, forcing ROS production to occur there (59,65) rather than electrons seeking a different site for ROS production on Complex I (as proposed by Grivennikova and Vinogradov (70)). This proposal was consistent with oxidation of glutamate *plus* malate continuing in the presence of succinate when Complex I and the high UQH_2/UQ ratio induced by succinate oxidation are used as the major determinants of H_2O_2 production (59,67,85).

Zoccarato *et al.* (59) went on to propose that O_2^{-}• is produced by the auto-oxidation of UQH, which is in equilibrium with the UQH_2/UQ couple, whose ratio is increased when succinate oxidation increases UQH_2, thereby promoting O_2^{-}• formation directly from the Q binding site of Complex I. Similarly, NAD-linked substrates alone can also increase ROS production during flow in the forward direction from the Complex I Q site, provided that electron flow is slowed by blocking Complex I itself with the Complex I Q-site inhibitors, such as rotenone or piericidin (56) or inhibiting Complex III or IV activities (86). In addition, succinate concentrations *in vivo* can also be controlled by the activity of the NAD^+-dependent dehydrogenases in the Krebs cycle (Fig. 3). It is significant that in perfused heart, succinate concentrations oscillate between 0.3 and 1 mM (87) and mainly depend on the Krebs cycle NAD-linked dehydrogenase activity.

Lambert *et al.* (68) re-examined the proposal that Complex I-mediated O_2^{-}• production was derived from its flavin cofactor, FMN, and was dependent on NADH/NAD^+ ratio, with the reaction known to be strongly inhibited by NAD^+ (57). A high NADH/NAD^+ ratio should result in highly reduced $FMNH_2$ leading to increased rates of O_2^{-}• production, as proposed by Hirst *et al.* (56) for isolated Complex I catalysing the forward reaction. Therefore, if O_2^{-}• also originated exclusively from FMN under reverse electron flow, a direct relationship should exist between NADH redox state and O_2^{-}• production. However, a direct relationship between O_2^{-}• production rate from Complex I and the NADH reduction state did not emerge from their studies, suggesting that the Complex I FMN is not the main site of O_2^{-}• production during succinate oxidation-driven reverse electron transport. The Fe-S centres of Complex I (except for centre N1a) are maintained in near-equilibrium with the NADH/NAD^+ ratio (88) and are buried in the hydrophobic core and protected from exposure to the external microenvironment (56). Therefore, these sites are also not likely to be producing O_2^{-}• during succinate oxidation, so the most probable site was conjectured to be the Q-binding site of Complex I (68), discarding significant ROS production coming from FAD and UQ sites in Complex II.

In the studies of Zoccarato *et al.* (67), addition of malate rapidly counteracted succinate-induced H_2O_2 production, very likely by forcing partial conversion to OAA, given that OAA is a potent, tightly bound competitive inhibitor of SDH (Fig. 3). Inhibitors blocking reactions downstream of Complex II, such as Complex IV inhibitors NO, CO or cyanide (CN^-), also rendered mitochondria more sensitive to succinate-induced ROS production (67). Similarly, glutamate *plus* malate as the sole substrates induced high H_2O_2 release rates in Complex IV-inhibited mitochondria (67,86), consistent with the proposal that inhibition of the cytochrome c oxidase decreases the rate of electron flow and consequently leads to significantly greater UQH_2/UQ ratios. Based on these data and later studies discussed below (58), they proposed that succinate is the controller metabolite of mitochondrial ROS production from Complex I (67). Succinate-linked state 3 respiration and Complex II activity of rat heart mitochondria were also shown to be reversibly inhibited by low levels of H_2O_2 (25–50 μM), which was reverted by rotenone, glutamate or ATP (77).

The effects of rotenone, glutamate, or ATP can be explained by their impact on the net levels of OAA as a potent inhibitor of Complex II (74–76,78). Thus, the rotenone effect was attributed to maintenance of the NADH pool at its highest level during state 3 respiration, impeding the forward reaction of malate dehydrogenase to prevent production of the SDH inhibitory OAA, glutamate by transamination maintains OAA at a low level, while ATP was also shown to inhibit malate dehydrogenase (77) and was able to stimulate SDH activity to help decrease OAA levels (89–92). For its own part, H_2O_2-induced inhibition of Complex II is probably mediated by maintaining high OAA levels through aconitase blockade (93,94).

The succinate effect has often been considered to be linked to the non-physiological and thermodynamically unfavorable reverse electron flow occurring in the absence of Complex III or IV inhibitors, and it is assumed that the physiological situation of low succinate and large excess of NAD-linked substrates would prevent reverse electron flow-dependent H_2O_2 generation from Complex I as the source of ROS. However, the results of Zoccarato's group (58,59,67,85) revealed that these assumptions are incorrect since the co-presence of glutamate *plus* malate does not impede H_2O_2 generation by low succinate but rather acts to enhance H_2O_2 production and when glutamate *plus* malate and succinate are all being oxidized together, electrons from glutamate *plus* malate are still continuing to move through Complex I (because Complex I still continues oxidizing NADH making NAD^+ available for further 2-OG oxidation). Therefore, it appears likely that the succinate-promoted H_2O_2 production is a direct consequence of the succinate-dependent elevation of the mitochondrial UQH_2/UQ ratio and perhaps, to a lower extent, of the reduced level of the Complex I flavin. In fact, a high rate of H_2O_2 release has been reported with glutamate *plus* malate, provided that re-oxidation of UQH_2 was slowed by cytochrome c removal (86). Thus, evidence supports a high UQH_2/UQ ratio as the main common determinant in enhancing ROS production at the level of Complexes I, II, and III.

In toto, these observations suggest that although Complex I can be a highly modulated H_2O_2 generator in physiological conditions, depending on the succinate concentration, $NADH/NAD^+$ ratios, and $\Delta\mu_H^+$ acting as tight regulators (64,65), Complex I has often been misinterpreted as the major ROS source under reverse electron transport conditions. In fact, SDH/Complex II is more likely to be the major source of ROS production, but this will again depend on Complex I activity and whether it reduces NADH to NAD^+, on which the succinate link with the Krebs cycle is critically dependent. This proposal will be further developed and reinforced in the ensuing sections.

SDH/COMPLEX II INHIBITORS PROTECT AGAINST ISCHEMIA-INDUCED OXIDATIVE STRESS

Ischemic preconditioning is an experimental procedure used to induce protection against the transient loss of blood supply and ensuing damage that occurs upon restoration of oxygenated blood to many types of tissues. Ischemic preconditioning involves applying consecutive brief non-lethal periods of hypoxia (ischemic conditions) followed by reperfusion, which then provides protection to vital organs from tissue injury that would otherwise occur after prolonged periods of ischemia reperfusion. It is a useful model for studying conditions that result from cardiac arrest, stroke, or trauma-induced ischemia damage to the blood supply to tissues. Drugs that show the same effects as preconditioning are being actively sought and are likely to have considerable future application in preventing many pathologies resulting from traumatic injury, cardiac arrest, strokes, or other tissue damage following ischemic reperfusion. In addition, such drugs may have greater benefit in long-term use, much like vitamin pills, preventing oxidative damage that accrues over a lifetime and results eventually in the debilitating diseases of the aged, such as neurodegenerative diseases and cancer.

Mitochondria play an important role in protecting tissues *via* preconditioning. For example, a prolonged period of ischaemia followed by the resupply of oxygenated blood (reperfusion) irreversibly damages not just the heart, but most tissues including other vital organs resulting in long-term debilitation or death. Injury from tissue damage

that follows reperfusion involves opening the mitochondrial permeability transition pore (MPTP) by calcium overload and the high levels of oxidative stress that occur upon reperfusion (reviewed in (95)). Protection from MPTP opening and, hence, reperfusion injury can be overcome by ischemic preconditioning, in which the prolonged ischaemic period is preceded by one or more brief (2–5 min) cycles of ischemia and reperfusion.

Evidence Ruling Out a Role for MitoK$_{ATP}$ in Regulating Complex II ROS Production

It was reported that SDH/Complex II exists in a complex with the mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$) and structural associations with the mitochondrial ATP-binding cassette protein (mABC1), Pi carrier, adenine nucleotide translocator (ANT) and ATP synthase (96). This newly discovered mega-complex provided a possible mechanism whereby drugs known to inhibit SDH/Complex II function might also activate the mitoK$_{ATP}$ channel and thereby explain the ability of the Complex II inhibitors to protect cardiac and neuronal cells from ischemia-induced cell death. However, the SDH/Complex II has since been found associated with the mitoK$_{ATP}$ channel in such a way that activation of either entity is integrally linked with the loss of activity of the other, and further evidence has cast doubt on a role for the mitochondrial K^+ channels. For instance, several studies examining K^+ channel activity in isolated mitochondria and cardiac myocytes found a lack of specificity of the pharmacological agents used to implicate the mitoK$_{ATP}$ channel in ischemic preconditioning (reviewed in (95)).

Nevertheless, the proposal linking mitoK$_{ATP}$ channels to Complex II in causing ischemia/reperfusion damage has been implicated in the processes of ischemic preconditioning and apoptosis after mitochondrial matrix swelling and opening the MPTP (reviewed in (97)) for the past several years and still continues to receive support. For example, the mitoK$_{ATP}$ channels were as recently as 2007 proposed to regulate mitochondrial redox state under physiological conditions and prevent oxidative stress under pathological conditions such as ischemia/reperfusion (98).

Diazoxide: A Specific Complex II Binding Drug Inhibiting ROS Production Protects Normal Cells Against Oxidative Stress

More recent information implies direct involvement of the SDH/Complex II in ROS production, independent of mitoK$_{ATP}$, during ischemic/reperfusion-mediated tissue damage. Thus, over the last few years, an increasing number of compounds have been identified which were reported to act by opening K^+ channels, many involving studies of smooth muscle and other tissues, but these drugs are proving to directly affect SDH/Complex II function. Perhaps the most significant compound, in this regard, is diazoxide (Fig. 2), a small drug developed in the 1960s shown to inhibit succinate oxidation (99) and developed for use either as an antihypertensive or as a diabetogenic for treating hypoglycemia, initially believed to be an activator of the mitoK$_{ATP}$ channel. This led to its general use as a vasodilator in the treatment of acute or malignant hypertension as well as to decrease hypoglycemia caused by the excessive secretion of insulin in disease states such as insulinoma (a tumor producing insulin or congenital hyperinsulinism). It was proposed that drug-induced opening of K^+ channels in the plasma membrane led to cellular hyperpolarization, preventing opening of voltage-dependent Ca^{2+} channels in excitable tissues, thereby blocking neuromuscular excitation.

Hyperpolarization of the cell membrane and local relaxation in smooth muscle by increasing outward membrane permeability to K^+ switches off voltage-gated Ca^{2+} ion channels and inhibits generation of an action potential. However, biochemical properties of the mitoK$_{ATP}$ channel closely resemble those of plasma membrane K$_{ATP}$ channels, including inhibition by low concentrations of ATP and glyburide (100). Plasma membrane K$_{ATP}$ channels were initially found to be highly sensitive to a family of drugs acting as K^+ channel openers, but it soon followed that mitoK$_{ATP}$ channels existed which were similarly sensitive to these agents. By measuring K^+ flux in intact rat liver mitochondria and in liposomes containing K$_{ATP}$ channels purified from rat liver and beef heart mitochondria, it was found that K^+ channel openers completely reversed ATP inhibition of K^+ flux in both systems. In liposomes, ATP-inhibited K^+ flux was restored by diazoxide ($K_{0.5}$ or IC50 = 0.4 μM), cromakalim ($K_{0.5}$ = 1 μM), and two developmental cromakalim analogues, EMD60480 and EMD57970 ($K_{0.5}$ = 6 nM) (101). Consequently, diazoxide was proposed to bind and activate mitoK$_{ATP}$ (101,102).

Subsequent research then pointed to mitoK$_{ATP}$ channels, not their classical surface membrane counterparts, as the most likely mediators of cardioprotective effects of K^+ channel openers against ischemic reperfusion (reviewed in (103)). However, numerous studies have cast doubt on the role of mitoK$_{ATP}$ and shown that this is not the mechanism for mediating protection of tissues during ischemic reperfusion (for example, see (104)). Thus, more recently, diazoxide was shown to work independently of mitoK$_{ATP}$ channels by inhibiting mitochondrial respiration *via* Complex II, causing high levels of ROS production which mediate the tissue damage; in 2007, it was reported that diazoxide inhibition of succinate-supported respiration, independently of mitoK$_{ATP}$ channel conductance, was the mechanism underlying pharmacological preconditioning

(105). In addition, 5-hydroxydecanoate (Fig. 2), a putative mitoK$_{ATP}$ inhibitor, reversed diazoxide-induced respiratory inhibition independently of K^+ concentration. However, it is likely that 5-hydroxydecanoate was acting elsewhere. Furthermore, the cytoprotective levels of diazoxide increased ROS production independently of K^+ concentration and 5-hydroxydecanoate also inhibited this ROS production. These data implicate a direct mitochondrial respiratory inhibition-triggered ROS signalling mechanism in the post-ischemia protection of tissues by diazoxide (105).

Over the past decade, further doubt has been raised by numerous reports refuting the mitoK$_{ATP}$-dependent model (106–110) and the causative relationship of drugs such as diazoxide thought to target mitoK$_{ATP}$ to regulate ischemic preconditioning (104,111,112). Hence, studies of hypoxic pulmonary vasoconstriction showed that this process also involved mitochondrial Complex II activity (113,114). Purported inhibitors of mitoK$_{ATP}$ including glibenclamide (Fig. 2) and 5-hydroxydecanoate completely blocked hypoxia-induced vasoconstriction, whereas mitoK$_{ATP}$ activators (pinacidil or diazoxide) induced vasodilatation. These results are opposite of what would be expected from mitoK$_{ATP}$ effects, if it was responsible for regulating ischemic responses (115). On the basis of this profile of response, clearly different from those described or expected for mitoK$_{ATP}$, we can conclude that the mechanism of ischemia-based response was not *via* mitoK$_{ATP}$, but involved signalling cascades such as Complex II producing ROS and ANT-regulated MPTP responses. A clear role for redox regulation in cardioprotection has also been provided from studies of rat heart cells and mitochondria (116), which it is now possible to re-interpret as inhibitory effects of nitroxyl or nitrolinoleate (Fig. 2) on Complex II-induced ROS production, thereby protecting against ischemia-reperfusion-based tissue injury.

Additional evidence for the role of SDH/Complex II-mediated ROS production in post-ischemic tissue damage comes from detailed studies of rat heart mitochondria (117), providing possible reasons for the discrepancy of other studies and the importance of carefully considering the conditions used to determine respiration rates. Thus, with the milder "preconditioning" and depending on the mitochondrial metabolic state and $\Delta\mu_H+$, then inhibiting Complex II with diazoxide transiently increased H_2O_2 production between Complex II and III. However, during the more severe state of ischemia/reperfusion, diazoxide inhibited the more damaging ROS production that resulted from elevated succinate and hypoxic conditions (117). Several additional points arising from this study are noteworthy. First, mitoK$_{ATP}$ involvement was excluded because modifying either Na^+ or K^+ levels had no effect on results. Second, as with previous reports where succinate was used as a substrate, ensuing basal rates of ROS production were considerably higher than levels achieved using glutamate/malate. Third, it was concluded that the specific outcome from diazoxide treatment was dictated by the prevailing set of conditions, including $\Delta\mu_H+$, availability of UQH_2/UQ, and pyridine nucleotide redox status. Thus, under conditions where normal thermodynamic constraints might have been partially overcome to allow reverse electron transfer to occur, including a high $\Delta\mu_H+$, low levels of NADH, and electron flow provided by high levels of succinate, the highest rates of superoxide ($O_2^{-}\bullet$) production were proposed to be derived from the Complex II to I direction of flow, which was decreased by inhibitory effects of diazoxide on Complex II. However, when $\Delta\mu_H+$ was lower and Complex II was partially inhibited by diazoxide, the reverse electron flow-mediated ROS production from Complex I was lowered; at this point, ROS production from electron transfer was proposed to proceed in the forward direction, from Complex II to the Qo site of Complex III under conditions of 'oxidant-induced reduction' (*i.e.*, in the presence of the Qi site inhibitor, antimycin A, and adding a potent oxidant such as ascorbate or DTT to induce reduction of cytochrome b hemes in Complex III (118,119)).

The strongest supporting evidence for diazoxide directly inhibiting Complex II comes from studies of isolated rat heart mitochondria and purified succinate-cytochrome c reductase complexes using electron paramagnetic resonance (EPR) spin trapping measurements (120). These studies show that succinate oxidation-driven respiration was dose-dependently inhibited by diazoxide in intact mitochondria. In particular, electron transfer activity by SQR in purified Complex II/III supercomplexes was directly inhibited by diazoxide as measured in the ubiquinol-2 ($UQ2H_2$)-mediated dichlorophenolindophenol (DCPIP) reduction reaction used to detect electron transfer by absorption wavelength changes as an artificial electron acceptor. However, the ability of the formed $UQ2H_2$ to undergo cytochrome c reaction in the quinone cytochrome c reductase portion was not affected. This evidence strongly supports diazoxide binding to Q binding sites of Complex II to inhibit electron flow and reduction of UQ. Moreover, based on specific changes in the UV-visible spectral analysis, they concluded that diazoxide inhibited succinate-mediated ferricytochrome *b* reduction involved in the cytochrome b_{560} functioning of Complex II, and thereby diazoxide probably disrupts UQ reduction (120).

The evidence for diazoxide inhibiting Q binding sites of Complex II is also supported by the very potent Complex II SQR inhibitor, atpenin A5 (an analogue of UQ that is known to bind to Q binding sites of Complex II with high affinity (121) (Fig. 2)) with an IC_{50} of 8–9 nM, also protecting against ischemia-reperfusion injury at very low levels (1 nM) in isolated cardiomyocytes (122). Although

ROS involvement in mediating the effects of ischemia/reperfusion injury and its alleviation by atpenin A5 were not examined in this study, Drose *et al.* (123) more recently analysed different cardioprotective Complex II inhibitors, including atpenin A5, for their effects on mitochondrial ROS production when respiring on succinate. Despite different binding sites and concentrations required for half maximal inhibition—ranging from nanomolar for the Q site inhibitor atpenin A5 to millimolar for the succinate analogue malonate—all Complex II inhibitors modulated ROS production in the same ambivalent fashion, promoting generation of O_2^{-}• at the Qo site of Complex III under conditions of oxidant-induced reduction, but attenuating ROS proposed to be generated at Complex I by reverse electron transfer. With succinate driving respiration, all Complex II inhibitors (atpenin, malonate, diazoxide or TTFA (Fig. 2)) showed similar effects, independent of the presence of K^+. These findings provide additional support for direct modulation of mitochondrial ROS generation during cytoprotection *via* Complex II inhibition, negating models involving mitoK_{ATP} where Complex II would act indirectly by regulating the mitoK_{ATP} channel. Thus, diazoxide, as a direct inhibitor of Complex II (107,124,125), will thereby inhibit ROS generation in succinate-fuelled rat heart mitochondria and was proposed to decrease ROS production mediated by reverse electron transfer from Complex II to I, rather than considering the ROS generated to be directly from within the inhibited Complex II itself.

On the other hand, it was proposed that diazoxide stimulated O_2^{-}• production at the Qo site of Complex III under conditions of oxidant-induced reduction (117) by increased oxidation promoting increased UQ levels in the Q-pool (UQH_2/UQ) following Complex II inhibition. Drose *et al.* (123) showed that ROS produced at the Qo site of Complex III is maximal when the Q-pool is only partially reduced, and the bulk exists as oxidised UQ, in line with their previous proposal that O_2^{-}• is generated by reverse electron transfer from the Complex III reduced heme b_L to O_2 with UQH serving as a redox-mediator and electron donor (119). These conclusions have been confirmed by studies of several other independent groups (120,126,127).

SUCCINATE LEVELS INCREASE IN MITOCHONDRIA DURING HYPOXIA

This section reviews evidence that under hypoxic conditions, succinate levels in tissues including heart, brain, kidney, and cancer cells increase significantly, inducing greater ROS production and activation of the key transcriptional hypoxia-induced-factors, HIF-1 and HIF-2, that lead to further changes to the metabolic balance within cells. The succinate concentration is 0.2–0.5 mM in most tissues, much less than 5–10 mM typically used in experiments with isolated mitochondria. However, tissue concentrations of succinate increase several-fold to the millimolar range under ischemic and hypoxic conditions. For example, in rat brain, 5 min of ischemia resulted in an 8–10-fold decrease in concentrations of glycolytic intermediates and the mitochondrial NAD-linked oxidative substrates pyruvate, citrate, 2-OG, OAA, fumarate, and malate. In stark contrast, succinate concentrations, in turn, increased by 3-fold to the millimolar range (128,129). Furthermore, succinate remained moderately (~35%) elevated even when examined 15 min after onset of reperfusion, when all other metabolites had recovered to control levels (129).

Similar results were obtained in another study in which excess succinate found in post-ischemic brain returns to control levels only after 30 min of reperfusion (130). Another pertinent finding is that hypoxia significantly (>60%) activated succinate and glutamate oxidation by isolated rat brain mitochondria (131,132). Succinate has also been shown to inhibit oxidation of pyruvate and other NAD-linked respiratory substrates and to cause over-reduction of mitochondrial pyridine nucleotides (133). Considering these data—an accumulation of succinate during hypoxia, its slow removal upon reperfusion, and its ability to inhibit oxidation of NAD-linked substrates—it is reasonable to conclude that succinate is the major substrate oxidized *in vivo* by mitochondria during the first 30 min of reperfusion and that utilizing succinate in assays of mitochondrial functions *in vitro* is physiologically sound and most relevant to studies on the role of mitochondria in hypoxia-reperfusion-induced tissue damage, especially in experiments addressing mitochondrial ROS production.

Studies of hypoxia in isolated adult rat heart muscle cells by adding 2-OG *plus* malate induced a large (14-fold) synergistic increase in levels of succinate released by cells under hypoxic conditions (called anaerobic succinate production) but not under oxygenated conditions (134). The increase in anaerobic succinate production was inhibited significantly by rotenone, whereas antimycin A had no effect, and the mechanism was proposed to be linked to the reverse electron flow from Complex I to SDH/Complex II to form succinate.

Sources of Increased Succinate During Hypoxia

Initial experiments using radiolabelled tracer compounds revealed that 2-OG was formed *via* transamination from glutamate, whereas malate was proposed to be directly utilized to make succinate by reversal of the Krebs cycle (134). For these reactions to occur, SDH would have to become a fumarate reductase, an activity shown possible for

bovine heart SQR (135) under hypoxic (and hence high UQH_2/UQ ratio) conditions. This proposal was also consistent with highly conserved structural and catalytic properties of SDH/Complex II with quinol:fumarate oxidoreductases commonly found in anaerobic organisms (136).

Additional evidence that during hypoxia in cardiac tissue SDH/Complex II can operate as a fumarate reductase to increase succinate and protect from oxidative damage, because the forward ROS-producing reaction of Complex II is decreased, has emerged in which increased production of substrates derived from amino acid metabolism, such as glutamate and aspartate, in turn promote enhanced succinate levels (137–144). Similar findings were made with kidney proximal tubules after hypoxia-reoxygenation episodes where damage was prevented by anaerobic metabolism of 2-OG *plus* aspartate or malate and fumarate to maintain ATP production and mitochondrial $\Delta\mu_H^+$, with succinate as the end product (145). Succinate alone also rescued the tubules, and effects persisted even after its withdrawal. Again, these effects required Complex I function (146).

Truncated Krebs Cycles in Tumor Cells Eliminate Feedback Inhibition of SDH/Complex II

Some tumor cells with higher ROS levels may have a truncated Krebs cycle as a result of ROS-mediated inhibition of aconitase (93,94), which catalyzes the conversion of citrate to isocitrate (Fig. 3), and the loss of this conversion step would potentially disrupt the Krebs cycle by decreasing 2-OG levels. However, tumor cells compensate by expressing high levels of glutaminase to restore 2-OG *via* glutamine consumption (147,148). Some studies have shown that succinate-derived malate is rapidly converted to OAA and on to citrate, which may be extruded into the cytosol to form acetyl-CoA for the *de novo* synthesis of fatty acids, phospholipids and cholesterol for membrane synthesis in tumor cells (147). This conversion/extrusion process keeps the steady-state OAA concentration in the mitochondria low, thereby avoiding strong tightly bound competitive inhibition of SDH and ensuring continuous flux from 2-OG to citrate (Fig. 3).

Other studies have shown that glutaminolysis (Fig. 3) is an important energy source used by all proliferating cells including cancer cells which plays a vital role in growth under conditions of oxidative stress (reviewed in (149,150)). The highly active glutaminase in tumor mitochondria allows them to respire on glutamine by cleavage to glutamate *plus* NH_4^+. Glutamate is then either converted to alanine *via* glutamate/pyruvate transaminase or most likely to aspartate *via* glutamate/OAA transamination, either way also producing 2-OG for feeding into the Krebs cycle (151). The latter reaction also ensures that steady-state OAA levels are kept low, and either reaction restores flow around a possible truncated Krebs cycle by replenishing 2-OG and hence succinate, by-passing the aconitase step.

Under conditions of diminished glycolysis, tumor cells can switch to high levels of glutamate dehydrogenase (152). Hence, in tumor cells, glutamine and glutamate are important as alternative energy inputs into the Krebs cycle, and glutaminase has been shown as essential for cell transformation because targeting glutaminase activity with the specific inhibitory drug, 968, also inhibits oncogenic transformation and growth of cancer cells (153,154). Unlike aconitase, glutaminolysis is relatively insensitive to ROS levels.

SDH/Complex II, Hypoxia and their Importance for Tumor Development

The above studies all support increased levels of succinate occurring in cells exposed to periods of decreased O_2 supply (ischemia/hypoxia), while other studies have also linked increased succinate levels with particular genetically related cancers with mutations in the SDH and fumarate hydratase (FH) encoding genes. Ishii *et al.* (155,156) showed that a mutation in the cytochrome b large subunit (SDHC) of Complex II, called *mev-1*, caused $O_2^{-}\bullet$ overproduction, particularly when driven by succinate, leading to apoptosis and premature aging of *C. elegans* and in *mev-1* mutant mice. In addition, a significant fraction (1,000-fold higher frequency than wild type) of NIH 3T3 fibroblasts with *mev-1* mutations or mutations in the Q binding site of Complex II that survived apoptosis became transformed after prolonged culture and formed tumors when injected into recipient mice. They concluded that mutant Complex II was acting as a driver for cancer development and that SDH/Complex II has a major role in development of cancers *via* ROS production, promoting malignant cell transformation.

After genetic mutation in cell growth regulatory genes, perhaps the next most significant factor contributing to cancer cell development is the dysregulation in O_2 supply. During emergence of solid tumors, cells in the tumor microenvironment undergo intermittent periods of O_2 deprivation (157) as a result of rapid cell growth shifting cells from a state of sufficient nutrient and O_2 supply from the nearby vasculature to one of competition, relative starvation and hypoxia as the tumor volume expands. In response to hypoxic stress, tumor cells, particularly in regions more distant from the prevailing blood supply of O_2, undergo a selection process that involves activation of the transcription factor, hypoxia-inducible factor (HIF).

The presence of HIF proteins (HIF-1 or HIF-2) is a key indicator of O_2 deprivation and a regulator of energy metabolism such that when O_2 is available, HIFα subunits

have short half lives because they become modified by O_2-dependent post-translational hydroxylation, mediated by prolyl hydroxylases (PHDs 1–3) as master oxygen sensors. Prolyl hydroxylation of the O_2-dependent domain (ODD) of HIFα subunits acts as a marker for their ubiquitination by the von Hippel Lindau (VHL) E3 ligase which targets the HIF proteins to the proteasomal complex for degradation (reviewed in (158,159)). Hence, HIF proteins are not usually activated in cells under normoxic conditions. By contrast, when cells become hypoxic, the α subunit is no longer turned over rapidly and instead binds the HIFβ subunit to produce the HIFα/β heterodimer, as active transcription factor complexes (160) controlling expression of numerous genes including key enzymes that promote cell survival and increase the glycolytic pathway flux (reviewed in (148,161)). HIFs also induce vascular endothelial growth factor (VEGF) expression, resulting in formation of new blood vessels within tumor vasculature, which helps restore the supply of nutrients and O_2 (160). Amongst many genes induced by HIF activation, several are particularly important to the altered state of energy metabolism in cancer cells, limiting mitochondrial function (148,161).

Succinate and Pseudohypoxia: The HIF Connection

Studies of particular types of human neuronal malignancies such as pheochromocytomas, paragangliomas, leiomyomas as well as renal-cell carcinomas have provided insight into one protective device cancer cells use to promote their own survival. This process, known as "pseudohypoxia," is distinct from the usual hypoxic development of cancers described in the previous section. These cancers contain mutations that make cells behave as if they were hypoxic, even when normal levels of O_2 are present. In pseudohypoxic cancers, inherited or somatic mutations are commonly found in SDH or FH (Fig. 3). Inherited or somatic mutations in *SDHB, SDHC* or *SDHD* genes encoding Complex II subunits are associated with phaeochromocytomas or paragangliomas. A paraganglioma is a rare neuroendocrine neoplasm that can develop in different body sites (including head, neck, thorax and abdomen). About 97% are benign and cured by surgical removal; the remaining 3% are malignant because they produce distal metastases. Mutations in the *FH* gene are associated with leiomyomas, leiomyosarcomas or renal cell cancers (reviewed in (162)).

As a consequence of SDH or FH mutations, build-up in succinate and/or fumarate is induced. Both metabolites then inhibit prolyl hydroxylase (PHD3/ENGL3) by competition with 2-OG (163–168), and HIFα is no longer modified by PHDs to facilitate its ubiquitination and degradation. This situation resembles the state occurring during hypoxia (hence the name "pseudohypoxia") in that levels of activated HIF-1α and HIF-2α subunits become elevated, despite continued O_2 presence. Downstream effects of higher HIFα levels then promote malignancy of the cells as described for the hypoxic situation in the previous section, including high levels of aerobic glycolysis and lower or normal OXPHOS associated with changes in gene expression of metabolic enzymes.

This last process may certainly not explain the origin and development of all paragangliomas. The SDHB mutation and its association with expression of hypoxia (HIF)-regulated genes has been examined (169), and SDHB mutant phenotype has been linked with a poorer prognosis and highly metastatic tumors, but no correlation was found with increased classic hypoxia signalling as detected by HIF-1α, CA-9 or GLUT-1 immunostaining, although other HIF family members were not excluded. In these tumors, the lack of association with classical hypoxia signalling phenotype suggests that the hypoxia hypothesis in malignant phaeochromocytomas or paragangliomas is not complete, and other factors must be involved. The heavily vascularised nature of these metastatic malignancies suggests that increased VEGF production might be one potent factor (170), although a mechanism remains unclear. Further analyses of transcriptional differences in these tumors (171) may help define their nature and explain the lack of hypoxic signalling association.

Individuals with PTEN mutations have Cowden syndrome, associated with breast, thyroid, and endometrial neoplasias. However, a fraction of Cowden syndrome patients do not have PTEN mutations; of these, 20% show high level expression of Mn^{2+}-SOD, of which 13.5% have germline SDHB or SDHD mutations, associated with increased frequencies of breast, thyroid, and renal cancers (172). It remains unclear precisely how SDHB or D mutations contribute to Cowden syndrome, as does the contribution that elevated levels of succinate and fumarate in cells containing mutant SDH and FH will make to the ROS production levels in tumors like phaeochromocytomas or paragangliomas. Two studies found increased HIF-1 and greater succinate levels but no signs of redox stress (165,173,174). More detailed studies are required to determine whether ROS generation plays a role in such tumors (reviewed in (166)), although mutations in SDHC were shown to result in increased $O_2^{-}\bullet$, oxidative stress and genomic instability in fibroblasts (175).

DIHYDROLIPOAMIDE DEHYDROGENASE (DLD): LINKING 2-OGDH, PDH, AND SDH/COMPLEX II WITH ROS PRODUCTION

Recently, Grivennikova *et al.* used a soluble crude protein fraction derived from bovine heart mitochondrial matrix to

resolve the apparent contradiction in Complex I NAD binding sites and discovered an additional function as an NADH-dependent NH_4^+ stimulated enzyme producing significant H_2O_2 (71,176). They identified this novel H_2O_2-producing enzyme was the free form of 50kD dihydrolipoyl dehydrogenase (DLD), which also functions in supramolecular complexes as the E3 component of 2-OGDH and PDH complexes. DLD is a mitochondrial flavoprotein enzyme constituent of these enzyme complexes that transforms lipoamide, producing dihydrolipoamide (177) (Fig. 4).

In the presence of high NH_4^+ (5–30 mM) and low NADH (50 μM), the matrix-soluble/free form of DLD produced significant H_2O_2 levels in permeabilized rat heart mitochondria (in the presence of rotenone), revealing that >70% of the total produced was catalyzed by protein(s) other than Complex I (71,73,176). When stimulating levels of NH_4^+ were present, almost all (> 95%) H_2O_2 was produced and most likely derived from DLD activity (71,73). DLD stimulation of reverse NADH oxidation for ROS production by NH_4^+ could be involved in cellular toxicity induced by NH_4^+ as a by-product from amino acid catabolism. Intriguing is the similarity between properties of Complex II to I, reverse electron transfer-mediated ROS production and those of NH_4^+-dependent ROS production by DLD. Resemblance arises when properties of these two enzyme systems, DLD and succinate-driven reverse electron transfer, are compared in that both are regulated by the NADH/NAD^+ ratio with inhibition occurring at high levels of NADH (> 1 mM) or NAD^+ (> 50 μM), suggesting they are one and the same identity.

Grivennikova *et al.* (73) questioned the physiological relevance of such high levels of NH_4^+, although levels of NH_4^+ in the mitochondrial matrix have not been analysed. However, it may be more relevant to cancer where evidence exists that NH_4^+ is elevated in tumor cells (150) and in mitochondria heavily oxidizing glutamine, proline and glutamate, as occurs in cancer cells, where glutaminolysis is elevated; it is highly plausible that NH_4^+ concentrations could easily reach higher levels sufficient to activate free DLD and produce ROS. Another feature is that DLD is part of the glycine cleavage system in mitochondria that also produces NH_4^+ as a final product of glycine breakdown, raising another link with NH_4^+ levels (178).

2-OGDH and PDH have three central subunits, E1-3, as the decarboxylase, lipoyl transferase, and DLD. Enzyme complexes use a central E2 core with other subunits surrounding this core to form the complex. In the gap between E2 and DLD E3 subunits, the lipoyl domain transfers intermediates between active sites. For its part, DLD forward reaction reoxidizes reduced lipoamide (dihydrolipoamide, L(SH)2) in the E2 active site to disulfide and 2 e^- + 2 H^+ are transferred to a disulfide on E3 itself (*via* disulfide interchange). Resulting E3 dithiol is then reoxidized by transferring the 2 e^- + 2 H^+ to FAD to produce $FADH_2$ which, in turn, is then reoxidized by electron transfer to NAD^+, yielding NADH + H^+. The forward reaction (rate of reduction) depends on the NADH/NAD^+ ratio and is stimulated by substrates which raise the NAD^+ content of mitochondria. Methoxyindole-2-carboxylic acid (Fig. 2) is a specific DLD inhibitor (Fig. 4).

The DLD reaction is reversible and involves two reaction centres: a thiol pair that accepts electrons from dihydrolipoamide and a non-covalently bound FAD moiety that transfers electrons to NAD^+. The DLD reaction catalyzed by purified pig heart enzyme is strongly inhibited by Zn^{2+} (Ki=0.15 μM); steady-state kinetic studies revealed that Zn^{2+} competes with oxidized lipoamide for the two-electron-reduced enzyme (Fig. 4). Incidentally, Complex III activity can also be potently inhibited by similar levels of Zn^{2+} (Ki=0.1 μM) (179). Purified heart DLD also catalyzed the reverse NADH oxidation reaction by O_2, producing H_2O_2 as the major product and O_2^{-}• as a minor product. Zn^{2+} (activation constant, K_A of 90 nM) accelerated the NADH oxidase reaction up to 5-fold and stimulated ROS production by approximately 2-fold even in the presence of inhibitory NAD^+ (177). Activation of the NADH oxidation reaction is a consequence of Zn^{2+} binding to the reduced catalytic thiols of DLD, which prevents delocalization of reducing equivalents between catalytic disulfide and FAD. Distinct effects of Zn^{2+} on different DLD activities represent a novel example of a reversible switch of the enzyme, suggesting Zn^{2+} will interfere with mitochondrial antioxidant production and may also stimulate ROS production.

NAD^+ inhibition of NADH oxidation reaction by DLD in the absence of Zn^{2+} is mixed-type, with the competitive component suggesting binding of NAD^+ and NADH to the same enzyme form, *i.e.* two electron-reduced enzyme. In the presence of saturating Zn^{2+} and low NADH, NAD^+ behaves as an uncompetitive inhibitor indicating formation of a dead-end (non-productive) NADH-DLD-NAD^+ complex with cofactor binding at different sites (177). The observed degree of NAD^+ inhibition strongly depends on available O_2, so the inhibition by NAD^+ is more severe in low O_2 conditions. Thus, in the presence of NAD^+ and low O_2, ROS production by DLD will be heavily suppressed, while the presence of Zn^{2+} will switch on ROS production.

Properties of the DLD Enzyme, ROS Production, and Evidence of a Link with Complex II

The free radical theory of aging and involvement of mitochondria have gathered much support in recent years (reviewed in (180,181)). In yeast, DLD was shown to be involved in aging, where it is a major source of ROS production that is inhibited by calorie restriction (182).

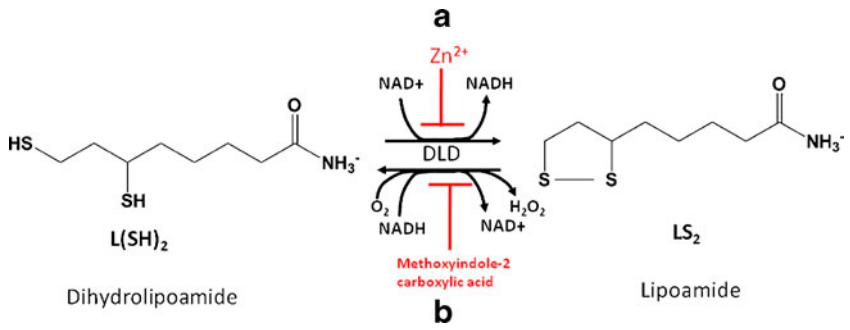

Fig. 4 Reaction of dihydrolipoamide dehydrogenase (DLD) in the forward (**a**) and (**b**) reverse reactions. High NADH/NAD^+ ratios promote the reverse reaction, forming reactive oxygen species (ROS). Inhibitors are shown in red.

Previously, it was proposed that increased survival in response to calorie restriction resulted from NAD^+-dependent histone deacetylase activity of the sirtuin family protein, Sir2p, (reviewed in (183)), and changes in pyridine nucleotide redox state were not considered important. However, yeast strains defective for NAD^+ synthesis and salvage pathways had lower O_2 consumption and increased mitochondrial H_2O_2 release, which was reversed over time by calorie restriction, promoting long-term survival. Calorie restriction enhances efficient mitochondrial OXPHOS and respiratory O_2 consumption rates and concomitantly decreases mitochondrial ROS formation and glutathione oxidation with a strong inverse correlation between respiratory rates and ROS release (182). In contrast, decreased NAD^+ synthesis inhibits respiration, enhances mitochondrial ROS production, and diminishes lifespan with the major source of ROS being DLD, not the electron transport chain. These observations are consistent with the free radical theory of aging (184) and strongly implicate mitochondria as the major source of ROS impacting cellular lifespan limitation.

The above studies with yeast are consistent with other studies of mammalian cells in which DLD-containing enzymes, including the family of alpha-oxo-acid dehydrogenases, 2-OGDH in particular, and PDH were also shown capable of producing high levels of ROS. Thus, under conditions of maximum respiration induced with ADP or an uncoupling agent (*i.e.* H^+ ionophore) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CCCP), 2-OG was found to induce the highest rate of H_2O_2 production (185). Based on the assumption that PDH and 2-OGDH produced O_2^{-}• as their primary ROS and that two O_2^{-}• molecules dismutate to produce one H_2O_2 molecule, the authors calculated that mitochondria produced a maximum of 560 pmol of H_2O_2/min/mg protein with 2-OG as substrate and 330 pmol of H_2O_2/min/mg protein with pyruvate, whereas minimum rates (in the presence of NAD^+) were 70 and 90 pmol of H_2O_2/min/mg protein, respectively. These values were remarkably similar to actual rates of H_2O_2 production observed with rat brain mitochondria, particularly for 2-OG (185). Altogether, results indicated that the DLD E3 component of 2-OGDH, and to a lesser degree PDH, were an important constitutive source of ROS in mitochondria.

These results are consistent with other studies showing that 2-OGDH is a target of oxidative stress. For instance, whereas 50 μM H_2O_2 completely inhibited aconitase, as the most sensitive point in the Krebs cycle, 2-OGDH was only partially inhibited at ≥100 μM H_2O_2 (94). As a result, it was proposed that when aconitase is inactivated, glutamate becomes the major fuel for the Krebs cycle, and NADH production is unchanged, but under stronger oxidative stress where 2-OGDH becomes affected, NADH levels for the respiratory chain become limiting. In the absence of ADP or in the presence of rotenone, H_2O_2 production rates correlated with decreased NADH levels in mitochondria respiring on various substrates, with the exception of 2-OG. In addition, rotenone inhibition of Complex I induced much higher H_2O_2 production in the presence of 2-OG, even compared with succinate-supported H_2O_2 production (185). In these experiments, rotenone completely inhibited NADH oxidation and caused NADH levels to build up, so the 2-OG induced ROS production was unlikely to be only derived from Complex I or any other respiratory component, as succinate formation was also fully blocked. Furthermore, isolated mitochondrial 2-OGDH or PDH complexes were shown to produce O_2^{-}• and H_2O_2 (50,94,185), and the fact that they contain E3 DLD reinforces the suggestion that the H_2O_2 produced is generated by E3. NAD^+ also inhibited ROS production by isolated enzymes and permeabilized mitochondria, consistent with ROS-producing activity mediated by E3/DLD.

H_2O_2 production by brain mitochondria obtained from heterozygous knock-out mice deficient in DLD has also been measured, and these mitochondria produced significantly less H_2O_2 than mitochondria isolated from their littermate wild-type mice (185). Data strongly indicate that 2-OGDH and, in particular, associated component enzyme DLD is a primary site of ROS production in normally functioning mitochondria.

Tretter and Adam-Vizi further characterised the properties of 2-OGDH, establishing NAD^+ as a powerful inhibitor of 2-OGDH-mediated H_2O_2 formation, switching the H_2O_2 forming mode of the enzyme off and activating it to the forward catalytic (reducing NAD^+ to NADH formation) reaction. In contrast, NADH stimulated H_2O_2

formation by 2-OGDH, and the $NADH/NAD^+$ ratio determined H_2O_2 production rate with higher $NADH/NAD^+$ ratios promoting increasing rates of H_2O_2 production. Both reverse (H_2O_2-producing NADH oxidation) and forward (NADH-producing) 2-OGDH reactions were activated by Ca^{2+} (186). Using cortical brain synaptosomes and 2-OG as respiratory substrate, H_2O_2 production rate increased 2.5-fold compared to glucose media, and aconitase activity decreased, indicating that 2-OGDH is capable of producing sufficiently high levels of H_2O_2 in mitochondria for the inhibitory effect on aconitase to occur. The $NADH/NAD^+$ ratio as a key regulator of H_2O_2 production by 2-OGDH suggests that ROS production could be significant not only in the respiratory chain but also in the Krebs cycle when NADH oxidation from the respiratory system is impaired and, hence, $NADH/NAD^+$ ratios increase.

Purified DLD catalyzes NADH oxidation by O_2 with concomitant H_2O_2 formation (Fig. 4) (177,187). Studies of other flavoenzymes have also demonstrated H_2O_2 formation, although they show a slower rate of re-oxidation of reduced enzyme (from flavin-hydroperoxide to flavin) by O_2 to mainly produce H_2O_2 with some $O_2^{-}\bullet$ (188). Production of ROS as by-products in the 2-oxo acid dehydrogenase reaction has also been detected using EPR and was accompanied by enzyme inactivation (189). Data pointed to a dual pro-oxidant action of the complex-bound dihydrolipoamide, propagated through the first (E1) and third component enzymes (E3) and controlled by thioredoxin and the ($NADH/NAD^+$) pool. Mechanistically, these latter results are consistent with those obtained by Starkov *et al.* (185) and suggest that reducing equivalents for ROS formation by E3/DLD subunit of 2-OGDH originate during substrate oxidation in the forward reaction, 2-OG to succinyl CoA. However, in the absence of NAD^+ and presence of high levels of NADH, the reverse DLD reaction reduces O_2 to form H_2O_2, with the obvious source of electrons being NADH in agreement with the mechanism proposed for isolated DLD by Gazaryan *et al.* (177). Results are also consistent with findings of Kareyeva *et al.* (73); it can be surmised that the extent of DLD activity and, hence, mitochondrial ROS production are related to the extent of Complex I activity, as a major regulator of mitochondrial $NADH/NAD^+$ ratios.

In addition, reverse electron flow from Complex II to I could impact on NAD^+ to NADH conversion by Complex I. It is tempting to speculate that this reverse respiratory enzymatic reaction does not in fact occur, but instead, the apparent ROS production from Complex I is related to processivity of the DLD reaction linked to Complex I/II coupling and $NADH/NAD^+$ ratios (affecting Complex I and Krebs cycle activity). This coupling would regulate the DLD reverse reaction level, $NADH + H^+ + O_2 -> FADH_2 -> NAD^+ + H_2O_2$, proceeding when NADH levels become high.

2-OGDH, Complex II, DLD, and Redox Regulation of the Krebs Cycle

The family of 2-oxo-acid dehydrogenase complexes are key participants in redox regulation, because their function is modulated by the $NADH/NAD^+$ ratio and complex-bound dihydrolipoate/lipoate ratios. The Redox state of bound lipoate reflects availability of reaction substrates (2-oxo acid, CoA and NAD^+) and thiol-disulfide status, while accumulation of the dihydrolipoate intermediate causes inactivation of decarboxylase E1 enzyme in the complexes. With mammalian PDH, feedback inhibition by the product acetyl-CoA and phosphorylation by pyruvate dehydrogenase kinase (PDK) are the major forms of enzyme regulation along with lipoate-dependent regulation. However, 2-OGDH has a much greater sensitivity to direct redox regulation either by the ratio of enzyme-bound dihydrolipoate/lipoate or external SH/S-S, including mitochondrial thioredoxin. Consequently, PDH, unlike 2-OGDH, can be ruled out as being directly redox regulated, as it is insensitive to H_2O_2 inhibition (50–500 μM) (94).

Tretter and Adam-Vizi (94) showed in cortical samples that aconitase > 2-OGDH > SDH were inhibited by H_2O_2, although overall rate of the Krebs cycle has been mainly considered to be controlled by PDH, citrate synthase, isocitrate dehydrogenase (IDH), and 2-OGDH activities (190–192). For example, in an elaborate study of nerve terminals, Yudkoff *et al.* (193) compared fluxes over two different halves of the Krebs cycle, from 2-OG to OAA, and from OAA back to 2-OG. They suggested the overall rate-controlling reaction of the cycle involved either citrate synthase or PDH (193), whereas results of Tretter and Adam-Vizi (94), who included redox involvement, showed neither enzyme was influenced by H_2O_2, and only aconitase was particularly vulnerable to inhibition by H_2O_2, with a complete inactivation at ≥50 μM H_2O_2. Hence, flux control of the Krebs cycle can be strongly influenced by the mitochondrial pyridine nucleotide redox status and levels of ROS production.

In the segment between 2-OG and OAA, 2-OGDH is the slowest (and very likely controlling) enzyme, and to a degree, thioredoxin can help protect 2-OGDH from self-inactivation during catalysis at low NAD^+ so 2-OGDH continues to supply succinyl-CoA for phosphorylation of GDP or ADP (Fig. 3) under conditions of restricted NAD^+ availability. This may be essential at either end of the spectrum where NADH levels accumulate or during exhaustion of the pyridine nucleotide pool. However, DLD in 2-OGDH will produce ROS as a side effect of 2-oxo-acid oxidation when NAD^+ reaches low threshold levels for DLD activation at <50 μM (reviewed in (194)).

In a recent study of murine heart tissue, the relationship between 2-OG levels and its regulation by SDH/Complex II was examined (195). Mice, including wild-type and a

heterozygous mutant strain for SDH (SDHD+/−) with decreased SDH function, were housed under normoxic or hypoxic conditions for prolonged periods before cardiac tissue was analysed for SDH/Complex II activity and 2-oxo-acid levels. In wild-type and mutant SDH animals, hypoxia was associated with decreased 2-oxo-acid levels, although the relative decrease was much greater in wild-type compared to SDH mutant mice. As a result, the authors proposed that SDH/Complex II participates in normoxic and hypoxic regulation of 2-oxo-acid levels, including 2-OG and branched chain 2-oxo-acids (195). These differences were not attributable to changes in blood flow, cardiac tissue remodelling, or HIF-1α expression. Therefore, it is highly likely that hypoxia-increased levels of succinate are linked to DLD (as E3 of 2-OGDH) to induce ROS production, which, in turn, inhibits 2-OGDH to slow down the Krebs cycle input to succinate, as a feedback regulation.

Electron flow during succinate oxidation by Complex II proceeds *via* $FADH_2$ as an intermediate product, which is then re-oxidized to FAD by Fe-S clusters within SDH/Complex II. Next, Fe-S clusters transfer electrons onto the UQ pool. It is possible a connection exists linking Complex II with the free or E3 form of DLD. One possibility is that 2-OGDH and SDH/Complex II form another supercomplex, with succinylCoA synthetase, which senses and is modulated by $NADH/NAD^+$ and succinate/fumarate ratios. Another mechanism could be that DLD, as a reversible enzyme, may also use UQ as an electron acceptor, which will help to regulate the balance of UQH_2 to oxidized UQ (Fig. 5). However, blocking SDH/Complex II and/or Complex I will cause build-up in UQ and $FADH_2$ or $FMNH_2$, whereas increased rates of succinate oxidation will produce higher UQH_2/UQ ratio. No studies have yet reported examining effects of UQH_2/UQ ratio on DLD-mediated ROS production and are eagerly awaited. Zn^{2+}, although having no effect on NADH-dependent UQ reduction reaction, increased the rate of NADPH-dependent UQ reduction by DLD >10-fold (196,197). It should also be recalled that Zn^{2+}, and probably other divalent heavy metal ions with high affinity for -SH groups, such as Cd^{2+} and Hg^{2+}, switch DLD to become an NADH-dependent oxidase that can react with O_2 to produce H_2O_2 (Fig. 5). Whether high levels of UQH_2 can act in a similar manner remains to be determined.

FREE FATTY ACIDS AND FATTY ACYL-COA INHIBITORS OF DLD

Additional evidence relevant to the regulation of redox status and ROS production by the mitochondrial respiratory system has emerged from studies with free fatty acids (FFAs) and their CoA derivatives showing that they inhibit DLD, respiration, and the Krebs cycle. These studies have a significant bearing and implications for degenerative pathological diseases associated with oxidative damage, including Alzheimer's, diabetes, Parkinson's, cardiopulmonary disease, neurotoxicity, and cancer. FFAs provide one of life's metabolic fuels, especially during times of stress or prolonged fasting. Most FFAs used in catabolism have chain lengths of 12–20 carbon atoms. Complete oxidation of FFAs in mitochondria involves four consecutive steps: cell uptake, activation in cytosol to acyl-CoA esters, transport across the inner mitochondrial membrane *via* a carnitine shuttle (carnitine palmitoyl transferase I (CPT I), carnitine/acylcarnitine translocator and carnitine palmitoyltransferase II (CPT II)), and beta-oxidation *via* a further four consecutive reactions: dehydrogenation, hydration, dehydrogenation again, and lipoic acid cleavage. Degree of unsaturation and position of double bond(s) do not play a major role in their inhibitory potential on OXPHOS (198–200), and patients deficient in long-chain FFA oxidation exhibit early in life more severe features of debilitation than patients with medium-chain FFA oxidation deficiency. This may be related to a more toxic effect of long-chain fatty acid derivatives 14–20 carbons in length.

Genetic Conditions Affecting Fatty Acid Beta Oxidation Support Complex II Role in ROS Production

Additional support for Complex II and ROS production comes from studies of the role of fatty acyl-CoAs and their impact in certain genetic diseases where defects in enzymes of FFA beta oxidation result in build-up in fatty acyl-CoAs that indirectly impair function of the mitochondrial respiratory complexes. Given the evidence that acyl-CoAs are potent inhibitors of DLD-containing enzymes, it is not surprising that OXPHOS, Complex II function, and mitochondrial respiration are arrested under these conditions and that analysis of FFA metabolic pathways may help explain why FFA oxidation disorders, such as medium chain acyl-CoA dehydrogenase (MCAD) deficiency and respiratory system defects, have certain clinical features in common, such as myopathy, cardiomyopathy, neurological symptoms, and hepatopathy, and why some share biochemical abnormalities, such as hypoglycaemia and lactic acidosis.

Ventura *et al.* (200) used digitonin permeabilized human fibroblasts to examine the effect of different acyl-CoA esters on OXPHOS. When succinate in the presence of rotenone was used as a respiratory substrate, palmitoyl-CoA was shown to inhibit succinate-driven OXPHOS in a concentration-dependent manner, while further studies showed inhibitory effects on OXPHOS of 3-hydroxyacyl-CoAs and other long-

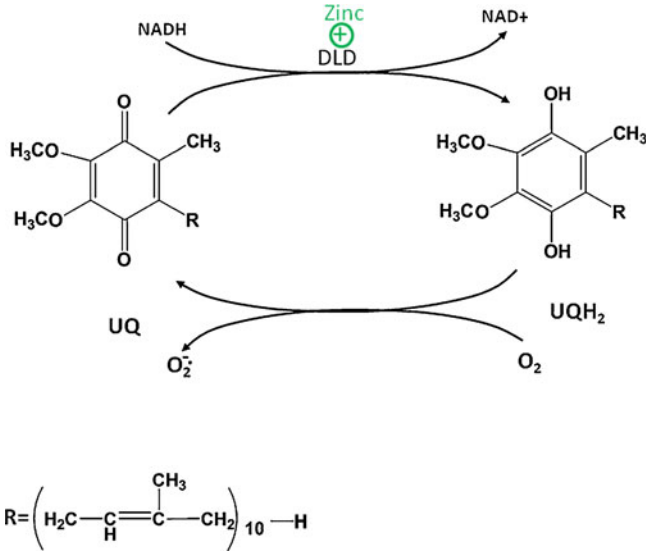

Fig. 5 Dihydrolipoamide dehydrogenase (DLD) oxidizes ubiquinone (UQ) to ubiquinol (UQH_2) in a zinc-dependent reaction. Oxygen is required to react with UQH_2 (or UQH) to generate $O_2^{\bullet -}$.

chain FFA beta-oxidation intermediates. Their evidence strongly implicated the adenine nucleotide (ATP/ADP) transporter (ANT) as the target for inhibitory effects (198).

More recent studies with isolated rat liver mitochondria incubated under state 3 conditions (199) showed that long-chain acyl-CoA esters and their beta-oxidation intermediates inhibited ATP synthesis and O_2 consumption with succinate and rotenone or glutamate. When an uncoupler (2,4-dinitrophenol) was used instead of ADP to stimulate respiration maximally, the various CoA esters showed differential effects on oxidation of succinate and glutamate, respectively. When succinate was used as substrate, palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA strongly inhibited O_2 consumption in coupled as well as uncoupled mitochondria. On the other hand, with glutamate as substrate, inhibition was only observed under coupled conditions, possibly reflecting the requirement of OXPHOS for ANT activity.

The finding that acyl-CoA esters inhibit uncoupler-induced respiration with succinate as substrate but not with glutamate was proposed to indicate that the inhibition observed was acting at the level of succinate transport by the dicarboxylate carrier across the mitochondrial membrane, because glutamate can use other inner membrane transporters to access the mitochondrial matrix. This conclusion was believed to be supported by mitochondrial swelling studies associated with inhibition of succinate transport by different acyl-CoA esters, whereas no effect was observed with Pi/hydroxyl and glutamate/hydroxyl carriers. Furthermore, long-chain acyl-CoA esters potentiated inhibitory effects of n-butylmalonate (a known competitive inhibitor of the dicarboxylate carrier) on O_2 consumption driven by succinate *plus* rotenone. Thus, it was concluded that inhibitory effects of long-chain acyl-CoA esters on OXPHOS depended on the type of substrate used, with ANT and dicarboxylate carrier as targets for inhibition (199,201).

It has been long established that the DLD E3 containing PDH complex is inhibited by acetyl-CoA (competitive *versus* CoA; and allosterically on E1), NADH (competitive *versus* NAD^+; and allosterically on E1), and acetoin (competitive *versus* pyruvate) (202–208) and that the mechanism of inhibition by acetyl-CoA *plus* NADH may involve lipoate acetylation (207). Consequently, the substantial rise in acetyl-CoA/CoA ratio induced by FFAs, ketone bodies, or alloxan-induced diabetes mediates their inhibitory effects on PDH in rat heart (202,208,209). For example, with acetate, acetyl-CoA/CoA ratio can increase some 60-fold and suppress pyruvate oxidation almost completely within 1 min of entry of acetate into the coronary circulation (210). FFA and ketone-body metabolism inactivate and inhibit PDH through allosteric feedback inhibition of E1 and typical hyperbolic E3 DLD inhibition by elevating acetyl-CoA/CoA ratio. Studies analysing mitochondria isolated from adipocytes of fed *versus* fasted rats also showed elevated long-chain acyl-CoA and inhibition of PDH activity by 50% with 30 μM palmitoyl-CoA (211). Octanoic acid is also a potent inhibitor of PDH activity (207) consistent with feedback inhibition as the precursor for *de novo* synthesis of lipoic acid, required for PDH function. In addition, Butyryl-, octanoyl-, and palmitoyl-CoAs are potent inhibitors of the other E3 containing enzyme, 2-OGDH, in brain mitochondria, with IC50 values of 11, 20, and 25 μM, respectively. Inhibitory effects of fatty acyl-CoAs and NH_4^+ on 2-OGDH enzyme activity were additive and at pathophysiological levels (2 mM) NH_4^+ alone in these studies was found to be a potent inhibitor of brain mitochondrial 2-OGDH (212). It should be recalled that Grivennikova *et al.* (described above) showed that NH_4^+ is a potent activator of soluble DLD-mediated reverse NADH oxidation to stimulate ROS production. These results provide some support for the hypothesis that selective inhibition of a rate-limiting and regulated enzymatic step (*i.e.*, 2-OGDH) by NH_4^+, and fatty acyl-CoAs may be a major underlying cause of neurotoxicity by ammonia and FFAs (212), although the role of ROS production in this situation has not been established.

A more specific study examining effects of short- and medium-chain FFA, acylcarnitines, and acyl-CoAs on mitochondrial energy metabolism has since been reported (213). No significant effects were found on the individual respiratory Complexes I–V, except for the well-established malonate competitive inhibition of SDH/Complex II and the surprising finding that octanoyl-CoA inhibited Complex III noncompetitively *versus* UQH_2 and uncompetitively

versus cytochrome c. However, the major finding was that to varying degrees, all short- and medium-chain acyl-CoAs examined over 250–1000 μM had significant inhibitory effects on PDH and 2-OGDH activities. This is of interest given the high likelihood that these effects, as with redox regulatory effects of NADH/NAD^+ ratios and inhibitors of PDH and 2-OGDH, all operate through their common DLD E3 enzyme. It is pertinent that 0.5–1 mM levels of the epigenetic drug valproic acid and its metabolites, and particularly the CoA adducts, directly inhibit DLD activity associated with inhibition of O_2 consumption and OXPHOS with glutamate or 2-OG as respiratory substrates (214). In contrast, no inhibitory effect of OXPHOS was observed when succinate (*plus* rotenone) was used as substrate. Thus, the action of fatty acyl-CoAs would be to impair 2-OG-driven OXPHOS when 2-OG, glutamate, or glutamine are used as respiratory substrates and, hence, would restrict the flux of these substrates through the Krebs cycle to form NADH and succinate. Unfortunately, effects of fatty acyl-CoAs on ROS production levels in this study were not examined.

Fatty Acyl CoA Derivatives Inhibit SDH/Complex II ROS Production

Although these studies showed that fatty acyl-CoAs inhibit mitochondrial respiration and OXPHOS and they block the transport of essential substrates into mitochondria, these are not their only actions. More recent evidence suggests that long-chain fatty acyl-CoAs also directly inhibit ROS production by inhibiting mitochondrial matrix enzyme complexes (58). Thus, long-chain fatty acyl-CoAs, but not FFAs, acted as strong inhibitors of succinate-dependent H_2O_2 release. This inhibitory effect of acyl-CoAs was independent of their oxidation, because it was relieved by carnitine (by promoting the reverse exchange acyl-$carnitine_{in}$/$carnitine_{out}$ and then diminishing matrix content of inhibitory acyl-CoA) and was unaffected or potentiated by malonyl-CoA (which by blocking carnitine-acyl CoA transferase I can induce increased matrix levels of acyl-CoA). Hence, long-chain fatty acyl-CoAs arrested PDH and 2-OGDH activities, most likely *via* DLD inhibition. On its own, FFA oxidation did not significantly affect H_2O_2 release but greatly decreased succinate-stimulated H_2O_2 generation, as did long-chain acyl-CoAs independently of mitochondrial oxidation. These effects also occurred in brain mitochondria that do not oxidize FFAs and were therefore not due to downstream metabolites (58). Hence, it can be concluded that long-chain acyl-CoAs can act as strong inhibitors of SDH/Complex II-derived ROS production.

Zoccarato's group (58) analysed the role of FFAs as major substrates used by heart mitochondria with the understanding that acyl-CoAs were known inhibitors of the enzyme energy-dependent transhydrogenase (which catalyses under physiological conditions the synthesis of NADPH for anabolic reactions having as driving force the consumption of mitochondrial H^+ gradient in the exchange reaction, $NADP^+ + NADH + H_{out}{}^+ <=> NADPH + NAD^+ + H_{in}{}^+$). Thus, long-chain fatty acyl-CoA esters were proposed to inhibit succinate-supported reverse electron transfer at Complex I, since 10–50 μM palmitoyl-CoA inhibited succinate-dependent ROS production as well as NADH-dependent acetoacetate reduction (58). However, it is more likely that the net outcome here is to establish a similar set of conditions to those obtained when rotenone is used to inhibit Complex I. Thus, palmitoyl-CoA inhibits the ketone body 3-hydroxybutyrate dehydrogenase reaction, acetoacetate + $NADH + H^+ <=>$ 3-hydroxybutyrate + NAD^+, which in turn would support succinate oxidation by substituting as a coupled reaction for the normal Complex I-mediated NADH oxidation, to provide necessary NAD^+ for continued Krebs cycle activity. Rather than adopting this explanation, Zoccarato *et al.* (58) resort to reverse electron flow and rationalized their results by proposing that if long-chain acyl-CoAs were decreasing the affinity of UQH_2 binding to Complex I, then a strong decrease of NAD^+ reduction from succinate (*i.e.* reverse electron flow) would occur in the presence of palmitoyl-CoA. Decreased NADH concentration was proposed to inhibit H_2O_2 removal *via* GSH (or thioredoxin) peroxidase. Hence, it was suggested that long-chain acyl-CoAs on one hand inhibit succinate-mediated O_2^{-}• formation but on the other hand diminish the H_2O_2 removing capacity of mitochondria. Thus, metabolic cellular conditions and levels of succinate or other substrates would determine whether the rate of mitochondrial ROS production is to be increased or decreased.

Solution to the Enigma of Reverse Electron Flow and Relationship Of Complex II to Complex I and NAD^+

An alternative and more appealing interpretation of the above results of Zoccarato *et al.* (58) is that acetoacetate acts as an effective substitute for Complex I-mediated NADH to NAD^+ requirements in mitochondria. Hence, acetoacetate is a direct replacement for coupling of Complex II to Complex I activity whereby NADH is converted to NAD^+ in the acetoacetate to 3-hydroxybutyrate reaction. Consequently, the NAD^+ produced will then meet requirements for coupling succinate to the Krebs cycle, allowing malate conversion to OAA to 2-OG and thence to succinate to proceed (preventing potent SDH inhibitory OAA levels from rising), by using the alternative NAD^+ supplied from 3-hydroxybutyrate dehydrogenase reaction rather than from Complex I (although the origin of acetyl-CoA supply remains to be solved in this scheme).

Additional support for this alternative proposal is that in the case of anaerobic succinate production, this exact relationship has been shown to occur. Thus, although

normally negligible in liver mitochondria, anaerobic succinate production becomes markedly increased by replacing malate with acetoacetate, as a result of coupling $NAD^+ \rightarrow$ NADH-linked 2-OG oxidation (to succinyl CoA) to NADH $\rightarrow$ NAD^+-linked acetoacetate reduction (to 3-hydroxybutyrate) (215). Moreover, conditions reported in Bortolami *et al.* (58) present yet another situation that is proposed as succinate-supported reverse electron transfer taking place in the presence of acetoacetate, but which is more likely to represent direct effects on Complex II-associated ROS production and its regulation by NADH/NAD^+ ratios. Although the authors proposed that the effects of fatty acyl-CoAs could act *via* the energy-dependent transhydrogenase reaction, which would eliminate the possibility of a homeostatic regulatory mechanism for maintaining the $NADP^+ < = > NAD^+$ balance in mitochondria, it is also highly likely that DLD was inhibited as well. Either way, this situation will result in an inability to remove ROS in the form of the reverse reaction of DLD being blocked and, consequently, elevated ROS production levels induced by fatty acyl-CoAs.

The question arises as to why SDH/Complex II depends on and requires NAD^+ for its function. The reason is the SDH enzyme activity is regulated by mitochondrial NAD^+-dependent deacetylase, SIRT3 (216). Thus, SDHA is extensively deacetylated by SIRT3 in mitochondria, and, most likely, reversible acetylation of SDHA regulates accessibility and entry of succinate as substrate into the active site. When acetylation levels of SDH build up in the absence of the NAD^+-activated sirtuin, SIRT3, then the activity of SDH/Complex II becomes inhibited. SIRT3 has now been established as the major deacetylase activity modulating mitochondrial function in response to changes in NADH/NAD^+ ratios and promotes OXPHOS by deacetylating and activating SDH/Complex II and Complex I (reviewed in (217)). In addition, SIRT3-mediated deacetylation activates several other key enzymes involved in regulating flux through the Krebs cycle including not only SDHA, but also long-chain acyl-CoA dehydrogenase (LCAD), promoting stimulation of FFA oxidation and acetyl-CoA synthetase 2 (AceCS2) to increase acetyl-CoA production, as well as isocitrate dehydrogenase 2 (IDH2) and glutamate dehydrogenase (GDH) promoting conversion of isocitrate and glutamate, respectively, to 2-OG (reviewed in ((217)). The net effect of SIRT3 activity in this manner will be to increase carbon flows through the Krebs cycle to succinate.

Thus, evidence discussed throughout the previous sections on the enigma posed by reverse electron flow from Complex II to I would suggest the possibility that involvement of reverse electron transfer reaction and its relationship to ROS production in intact mitochondria or whole cells is less likely. Rather, we can conclude that NAD^+ levels in mitochondria are an essential regulator of SDH/Complex II throughput and, hence, will also reflect the role of this respiratory enzyme as a source of ROS and which is further supported by studies detailed in the next sections.

MITOQUINONE, COMPLEX II AND ROS PRODUCTION

Greater insight into the events occurring at Complex I and II may be gained from studies of their interactions with a modified ubiquinone, Mitoquinone (MitoQ) (Fig. 2), a mitochondrially targeted form of UQ proposed to bind specifically to Q sites on Complex II (reviewed in (218)). Thus, ROS production by bovine aortic endothelial cell mitochondria was analysed using MitoQ and ROS measured with fluorescent probes, DCFDA, or Amplex Red (a peroxidase substrate that sensitively detects H_2O_2) or by electron paramagnetic resonance (EPR, to detect superoxide *in situ*) (219). Consistent with reports of other mitochondrial sources discussed in previous sections, succinate (alone in the absence of MitoQ) as substrate was again shown to substantially increase H_2O_2 levels, and this increase was inhibited by rotenone. Although MitoQ inhibited succinate-mediated increase in H_2O_2, it promoted much greater H_2O_2 production with the alternative substrates, glutamate *plus* malate, which was also inhibited by rotenone or stigmatellin (the Complex III inhibitor). These results were interpreted to be consistent with the proposal of reverse electron flow but are equally consistent with the relationship to changes in NADH/NAD^+ levels and Krebs cycle activity as proposed above.

A closer analysis using EPR to determine production of $O_2^{-}\bullet$ by mitochondria showed that succinate-increased $O_2^{-}\bullet$ levels were not inhibited by rotenone and were only slightly reduced by MitoQ, indicating production of $O_2^{-}\bullet$ from succinate was probably directly emanating from Complex II. No effects on $\Delta\mu_H{}^+$ were noted, ruling out involvement of effects on this thermodynamic parameter. By EPR analysis, MitoQ also increased $O_2^{-}\bullet$ production with Complex I-driving substrates glutamate *plus* malate or with pyruvate. From these findings and studies with inhibitors, it was proposed that $O_2^{-}\bullet$ production involved MitoQ acting at the rotenone-sensitive Q site on Complex I, blocking $O_2^{-}\bullet$ production induced by reverse electron transport but increased $O_2^{-}\bullet$ and significantly increased H_2O_2 production *via* the forward electron flow of Complex I (219,220).

However, evidence analysed in the present review indicates that ROS production by reverse electron flow is not significant from Complex I but is much more likely to be derived from Complex II. Moreover, it has been demonstrated that MitoQ is a poor substrate for Complexes I and III, whereas it is indeed an effective redox

substrate of Complex II (221), *i.e.*, the MitoQ quinone moiety can only access the UQ binding site of Complex II but not I. Hence, it is more likely that MitoQ was binding to Q sites of Complex II in the studies of O'Malley *et al.* (219,220) and promoting ROS production from there, not Complex I. This last notion is supported by the results of Plecita-Hlavata *et al.* (222). Here, MitoQ and a range of respiratory inhibitors were used with HepG2 human hepatoma cells to examine ROS production. Intact cells were cultured in glucose or galactose *plus* glutamine or isolated rat liver mitochondria were incubated with combined glutamate/malate/succinate to drive respiration (222). For the whole cell study, the fluorescent probe MitoSOX Red measured ROS as O_2^{-}• production. Addition of rotenone significantly elevated cellular O_2^{-}• production, and subsequent addition of MitoQ decreased O_2^{-}• production to basal levels of control cells. Addition of MitoQ alone increased O_2^{-}• production to about two-thirds the level induced by rotenone. However, when rotenone was added with the Q site Complex II specific inhibitor, thenoyltrifluoroacetone (TTFA), MitoQ no longer induced O_2^{-}• production. Results indicated that MitoQ could act as a pro-oxidant and was likely binding to Complex II because TTFA (as a high affinity inhibitor of Complex II Q binding Ki of 1.6–14 μM (223,224) that does not appear to promote ROS production) prevented the MitoQ-induced ROS increase. Inhibiting the Complex I proton pump with amiloride did not alter the effect of MitoQ in suppressing rotenone-enhanced ROS production, ruling out effects on the proton pump and $\Delta\mu_H^+$. Hence, these results provide solid support for binding of MitoQ to SDH/Complex II, where it can act as a pro-oxidant, and are consistent with ROS produced directly and intrinsically from SQR.

MitoQ also significantly accelerated galactose/glutamine or succinate *plus* rotenone-supported state 3 and 4 respiration rates of isolated HepG2 mitochondria. Again, MitoQ effects were inhibited by TTFA or Complex III inhibitors antimycin or stigmatellin. MitoQ also showed increased respiration with isolated rat liver mitochondria in the same fashion as Hep G2 mitochondria. Further analyses of H_2O_2 production by isolated rat liver mitochondria respiring on combined glutamate, malate, and succinate were determined using Amplex Red peroxidase assay rather than MitoSOX Red used to measure O_2^{-}• production. Results showed H_2O_2 was increased several fold by MitoQ and inhibited by rotenone or TTFA, whereas TTFA did not inhibit H_2O_2 production induced by rotenone, and MitoQ did not affect ROS production in the presence of rotenone and TTFA (222). In this situation, TTFA acts as a specific Complex II Q site inhibitor that blocks ROS production, whereas MitoQ promotes ROS production from this Q site, and rotenone promotes ROS production from the Complex I Q site. These data strongly support specific binding of MitoQ to Q sites of SDH/Complex II as a pro-oxidant promoting ROS production specifically and directly from SQR.

ALPHA-TOCOPHEROL SUCCINATE (α-TOS)

Advances in molecular medicine have produced a clearer understanding of cancer initiation and progression leading to improved means of treatment, sometimes resulting in beneficial outcomes. However, cancer remains a major disease. Cancer cells show chromosomal instability generating novel mutations that can often render anti-cancer drug therapies ineffective as they acquire drug resistance. In this regard, some cancer types, like malignant mesothelioma or hepatocellular and pancreatic carcinomas, remain very difficult to treat. Other cancers, such as HER2-positive breast carcinomas, rely on treatments with relatively expensive Herceptin therapy or Avastin that show considerable cardiotoxicity and only partial long-term responses. Thus, novel drugs are still actively sought that can overcome complications associated with current therapeutics.

Recently, the novel concept has emerged that targeting cancer cell mitochondria may offer a more effective approach to selectively kill cancer cells (225–228). We have proposed the term *mitocans* (Fig. 2) to refer to molecules with anti-cancer activity that induce apoptosis by destabilizing mitochondria in cancer cells (228–235). Based on their mode of action, known mitocans currently include several groups, each comprising reagents with distinct activities that directly or indirectly destabilize mitochondria and induce the intrinsic apoptotic pathway (230,231,234). Several mitocans are proving promising for treatment of cancer since they are potent and selective, some with little effect on normal cells in short-term studies. Prime examples of such drugs include α-tocopheryl succinate (α-TOS, Fig. 2), which induces selective apoptosis of a variety of cancer cell types (236), 3-bromopyruvate (3BP) (237), and dichloroacetate (DCA)(238). 3BP inhibits hexokinase, a controlling enzyme of the glycolytic pathway that is predominantly bound to the external face of mitochondria in cancer cells, and 3BP also inhibits SDH as well as other glycolytic and Krebs cycle enzymes (161) suppressing cellular ATP production and mitochondrial respiration. DCA selectively targets cancer cells by inhibiting mitochondrial PDK. In addition, β-phenylethyl isothiocyanate (PEITC) (Fig. 2) selectively kills cancer cells by causing mitochondrial generation of ROS (239). These examples epitomise an emerging group of anti-cancer compounds offering new directions for developing improved and highly selective anti-cancer drugs.

The advantage of mitocans as anti-cancer drugs is supported by recent evidence that individual types of cancers are complex and can differ considerably in their array of DNA mutations, harbouring different sets of genetic defects (240,241). This complexity suggests it will be very difficult to cure cancers using drugs that target only one or a few gene products or single signalling or metabolic pathways that are involved in tumor survival (242). What is essential is an invariant target, common to all cells, but which is only affected by drugs selectively delivered and active when inside cancer cells. Mitocans, epitomised by α-TOS or the mitochondrially targeted vitamin E (VE) succinate, MitoVES (see Fig. 2 and below), may meet these criteria.

α-TOS is a mitocan with dual actions involving ROS generation and BH3 mimicry (148). In particular, α-TOS acts as a BH3 mimetic, inhibiting anti-apoptotic function of Bcl-2 and Bcl-xL by blocking their BH3-binding domains (243). BH3 mimetic action partly explains how α-TOS sensitizes cancer cells to killing by other drugs. However, α-TOS, an esterified analogue of vitamin E, also causes rapid production and accumulation of ROS in all cancer cell lines tested, triggering extensive apoptosis (244–247) by interfering with mitochondrial respiratory chain. Anti-oxidants like SOD or MitoQ prevent α-TOS-induced accumulation of ROS in malignant cells, negating the apoptotic action of drug (245,246), and cancer cells that accumulate low levels of ROS in response to α-TOS are less susceptible to the drug (244,247–249). Hence, α-TOS kills cancer cells not by BH3 mimicry, but primarily by inducing mitochondrial ROS production and apoptosis.

Selectivity of α-TOS for Cancer Cells

Additional evidence indicates that cancer cell-specific action of α-TOS and lack of toxic effects on normal cells occur because normal cells are endowed with greater anti-oxidant defences and/or contain high levels of esterases that inactivate pro-oxidant α-TOS by releasing the succinate moiety (converting the pro-vitamin to non-apoptogenic α-tocopherol, α-TOH) (250–254). In addition, the more acidic microenvironment developed by cancer cells favors protonation of the carboxylic group, which in turn promotes enhanced internalization of α-TOS and its derivatives (Fig. 6). These features make agents like α-TOS excellent candidates for cancer therapy, as it is inactivated in and protects normal cells and tissues. α-TOS also acts *in vivo* to suppress a large range of experimental cancers (reviewed in (255)).

Compelling evidence for the anti-cancer potential of vitamin E analogues also arises from our recent studies showing that α-TOS and its analogues caused diminution in size of erbB2-high and low breast carcinomas (256). Thus, α-TOS shows considerable promise for treatment of fatal mesotheliomas and breast carcinomas in mouse models (244,256,257).

SDH/Complex II UQ Sites as Mitochondrial Targets of α-TOS for ROS Generation

Although it has been established that α-TOS induces generation of ROS essential for apoptosis of cancer cells, until recently, the exact target was unknown. Therefore, we investigated the mechanism by which α-TOS caused generation of ROS leading to cancer cell death, focussing on mitochondrial electron transport chain and particularly the role of SDH/Complex II, since it was known that induction of apoptosis by α-TOS was suppressed by MitoQ (218,244). Support for interaction of α-TOS with Complex II and more specifically its Q-binding sites was obtained in experiments showing that SDH activity was inhibited by α-TOS and the other known inhibitors, 3BP and TTFA. However, unlike 3BP, inhibition of SDH activity by α-TOS and TTFA was reversed by MitoQ (258). It was known that 3BP acts on Complex II at the level of the catalytic centre in the SDHA subunit, whereas TTFA displaces UQ from proximal (Q_P) and distal (Q_D) Q-binding pockets in the transmembrane region comprising subunits SDHC and SDHD (1). Thus, α-TOS acts like TTFA, except TTFA is highly toxic to all cells, while α-TOS is not, although it inhibits SDH activity in both isolated liver mitochondria and bacteria (258).

Recently, the crystal structure of mammalian (porcine) Complex II was reported (1), and since the 4 subunits of SDH show 95–97% homology between pig and human, we used the published coordinates to model interaction of α-TOS with Q_P and Q_D sites of human enzyme. AutoDock and ligand-binding programmes revealed a very good fit for the drug in the Q-binding pockets. Strong hydrogen bonding occurs between S68 of SDHC and oxygen atoms of succinate moiety of α-TOS in Q_P, and succinate oxygen atoms and K128 and K135 in the Q_D site of SDHD with interaction energies for α-TOS similar or greater than those for the natural substrate, UQ. To solidly establish Complex II as a target for α-TOS, transformed Chinese hamster lung fibroblast B9 cells deficient in SDHC were used (259). These Complex II-dysfunctional (SDHC-mutant) cells failed to accumulate ROS or undergo apoptosis with α-TOS, although α-TOS did induce apoptosis when the defective Complex II gene in B9 cells was replaced with a normal human SDHC gene (258). Knocking down SDHC using siRNA also rendered normally sensitive breast cancer MCF7 cells resistant to α-TOS (258). These results are consistent with a report that SDHC-mutant cancer cells were resistant to apoptosis induction by TTFA and a variety of other agents (260).

Based on the above evidence, we propose that α-TOS displaces UQ from its binding site(s) in SDH/Complex II, thereby blocking electron transfer. As a consequence, electrons that would normally be channelled from the

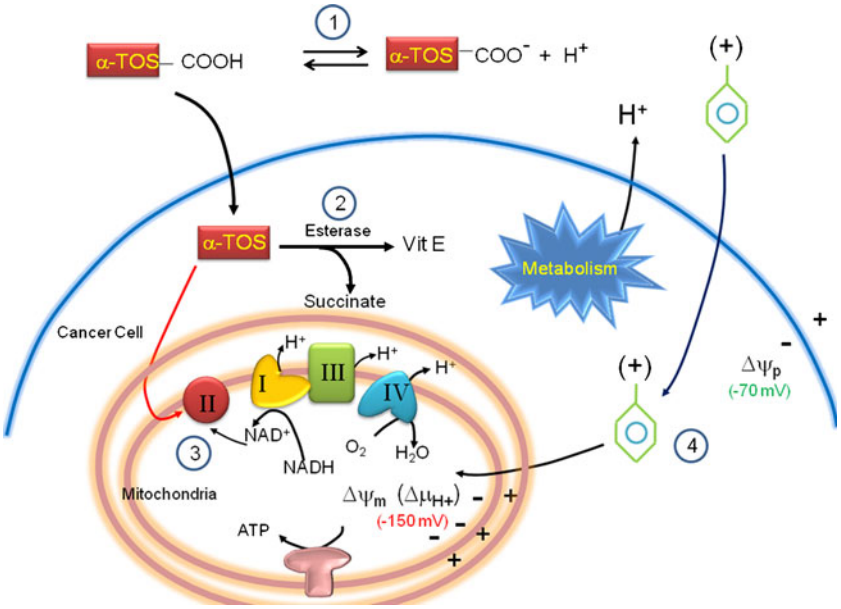

Fig. 6 Targeting cancer cells and mitochondria by α-tocopherol-succinate (α-TOS) and vitamin E (Vit. E) analogues like MitoVES. (1) Tumor acidic microenvironment promotes protonation of α-TOS carboxylic group, enhancing cellular internalization in cancer *versus* normal cells. Once in cytosol, α-TOS (2) is actively hydrolysed by active unspecific esterases generating succinate and Vit. E in normal cells, whereas α-TOS and derivatives remain unmodified in cancer cells because they have negligible esterase activity. Once inside cancer cells, α-TOS and derivatives (3) may access mitochondrial inner membrane and perhaps even mitochondrial matrix inhibiting SDH/Complex II. NADH -> NAD^+ is required to promote SDH/Complex II activity via Sirt-3 NAD^+-dependent deacetylation. NAD^+ is also required for Krebs cycle enzyme function (not shown). (4) Tumor cells maintain higher electrical potential difference across inner mitochondrial membrane ($\Delta\Psi_m$, as a function of $\Delta\mu_{H+}$) and plasmamembrane ($\Delta\Psi_p$) than normal cells and mitochondria, favoring entrance and enhanced accumulation of delocalized lipophilic cations, such as MitoVES, that affect functionality of different vital processes such as OXPHOS.

catalytic site in the SDH head group of Complex II to Q site(s) cannot bind to their natural acceptor (261,262) and, instead, react with O_2 to produce high levels of O_2^{-}• (263,264) leading to apoptosis induction. Either O_2^{-}• or ROS derived from it triggers apoptosis predominantly by Bak-dependent mitochondrial permeabilisation, which relies on ROS-mediated, FOXO-regulated transcriptional upregulation of the BH3-only protein Noxa, FOXO being activated by redox-active kinase Mst1 (265,266). Thus, generation of ROS is pivotal to subsequent events that result in apoptosis. Understanding molecular mechanisms involved in apoptosis promotion downstream of ROS generation is now a current focus of our research.

Enhancing the Anti-Cancer Action of α-TOS by Mitochondrial Targeting with Mitoves

Mitochondrially targeted vitamin E succinate (MitoVES) is a modified form of α-TOS, designed with a triphenylphosphonium (TPP^+) adduct as a delocalized cation so it is preferentially localized to functional mitochondria, greatly enhancing its pro-apoptotic and anti-cancer activity. Using genetically manipulated cells, MitoVES caused apoptosis and generation of ROS in Complex II-proficient malignant cells but not their Complex II-dysfunctional counterparts (267,268). MitoVES inhibited SDH activity of Complex II with an IC_{50} of 80 μM, whereas electron transfer from Complex II to III was inhibited with an IC_{50} of 1.5 μM. The agent had no significant effect on NADH dehydrogenase enzymatic function of Complex I or electron transfer from Complex I to III at this concentration level.

Over 24 h, treatment of cancer cell lines expressing O_2-dependent destruction domain of HIF-1α fused to GFP (GFP-ODD) (269,270) in culture or growing as tumors *in vivo*, MitoVES caused stable expression of GFP-ODD, indicating promotion of pseudohypoxia in treated cancer cells (268). Molecular modelling predicted that the succinyl group anchored into the Q_P Complex II UQ-binding site and successively reduced interaction energies for serially shorter phytyl chain homologs of MitoVES correlated with their lower effects on apoptosis induction, ROS generation, and SDH activity. Mutation of UQ-binding Ser68 within the Q_P site of Complex II SDHC subunit (S68A or S68L) suppressed both ROS generation and apoptosis induction by MitoVES (268). We hypothesise that MitoVES with an 11-carbon chain localizes the agent into an ideal position across the interface of mitochondrial inner membrane and matrix, optimizing its biological effects as an anti-cancer drug specifically targeting Q_P site of Complex II in cancer cells to induce high level ROS production (268) (Fig. 6).

DIFFERENTIAL SENSITIVITY OF SDH/COMPLEX II TO DRUG INHIBITION AND ROS PRODUCTION

A number of different compounds have now been identified which modulate the function of SDH/Complex II in mitochondria (Table 1). The range of different properties that these drugs show as pharmaceutical agents in different situations suggests their mechanisms of action can be quite different. For example, although drugs such as TTFA and atpenin A5 bind strongly to UQ sites of Complex II and can completely inhibit electron flow from succinate; unfortunately, they are also highly toxic substances to normal cells. However, drugs such as diazoxide, α-TOS and MitoVES (Fig. 2) are relatively non-toxic to most normal cells. In this regard, it will be highly interesting to determine whether the two mitocans, α-TOS and MitoVES, also act like diazoxide to protect normal cells from oxidative damage. Diazoxide, on the other hand, has been shown to inhibit tumor cell growth (271,272).

Diazoxide has been used in humans for many years as an anti-hypertensive (273), but has more recently been proposed to block oxidative stress-induced damage of tissues during post-ischemic reperfusion (274–277). As outlined in previous sections, diazoxide is a potent inhibitor of ROS formation following hypoxia-induced elevation in succinate levels in cells. This contrasts with the specific effects of drugs such as α-TOS and particularly the mitochondrially targeted version of this drug, MitoVES. Both compounds are specific for Q binding sites in Complex II, and MitoVES appears to preferentially localize to the Qp site of Complex II (258,267). Both drugs promote significant increases in ROS production in cells when visualised by stabilisation of GFP-ODD domain in tumors growing in mice only when treated with the drugs (258,267). Diazoxide may not only affect SQR at the Q binding sites, but may also act as a succinate-competing mimetic (99,124,278,279) and therefore have distinct properties from mitocan drugs such as α-TOS and MitoVES, which with their hydrophobic phytyl groups and delocalised lipophilic cation in the case of MitoVES TPP^+ group, are rapidly transported across mitochondrial inner membrane and accumulated into mitochondrial matrix (Fig. 6), where they are more specific for Q binding site interactions than diazoxide.

Mitocans may differ from diazoxide because they only block electron flow from Complex II to III, causing $O_2^{-}\bullet$ production from Complex II. Further studies are required to determine whether Complex II ROS production is sensitive to rotenone inhibition of NADH to NAD^+ conversion and the role of DLD. It will be interesting to compare properties of mitocans and other drugs with targets in Complex II and their relationship to DLD enzyme activity as a major ROS-producing agent inside mitochondria, particularly tumor mitochondria with highly elevated NH_4^+ levels. Evidence discussed above suggests that an intimate connection exists between DLD and Complex II function in ROS production, which could explain much of the current confusion with regard to NADH/NAD^+ levels and reverse electron flow, previously attempting to link rotenone sensitivity of ROS production from Complex II and identify DLD as a major ROS-producing entity in the mitochondrial matrix.

Despite current shortcomings in understanding its direct involvement in apoptosis of cancer cells and mechanism(s) of ROS production mediated *via* Complex II, it is eminently clear that the SDH/Complex II system is a key source of electrons for ROS production in all eukaryotic cells (280). It is therefore very likely that it will provide considerable drug development opportunities in the not-too-distant future.

CONCLUDING REMARKS

This review has drawn from a considerable volume of research to bring together all available information relating to ROS production from the SDH/Complex II of the mitochondrial respiratory system. From this study has emerged the proposal that the SDH/Complex II acts as a major source of electrons for ROS production under many highly relevant physiological states relating to disease scenarios such as cancers or oxidative-stress-associated tissue damage in stroke, cardiac arrest, or related conditions. Proposals put forward may have an important bearing on development of pharmaceutical interventions to alleviate these disorders and prevent degenerative disease processes.

A key enigma and misplaced emphasis has been previously assigned to the situation of ROS production by reverse electron flow between Complexes II and I, purported to have a predominant Complex I contribution at the level of FMN, as clearly demonstrated by Hirst and coworkers for Complex I forward reaction (51,56). ROS production rate by Complex I forward reaction is 10–100 times lower than that attained by reverse electron transport from Complex II to I. In the presence of rotenone, a condition under which full reduction of FMN is reached, ROS production by Complex I reaches its maximal possible rate but is still 2–3-fold lower than that dependent on succinate and Complex II during reverse electron transport. Why should higher ROS production be generated under the presumed reverse electron transport? The level of reduction in Complex I FMN is lower under reverse electron transport than under forward reaction with rotenone. Therefore, FMN cannot be the main site for ROS production under reverse electron transport. Although rotenone strongly inhibits ROS production by

reverse electron transport, approximately 10–15% remains, which cannot be catalysed by Complex I $FMNH_2$. In addition, this ROS fraction is significantly higher (1.3–15 times) than ROS produced by the Complex I forward reaction. What redox component could be generating rotenone-insensitive ROS during reverse electron transport? The answer would be either Complex I UQ site and/or Complex II redox components. It is surprising to find that Complex II contribution to ROS production has been mostly ignored.

This issue of ROS production by reverse electron transport has been an unfortunate undertaking, much like the proposal that mitoK_{ATP} regulated oxidative damage, because it has led the focal point away from the real source of electrons for ROS production, emanating from SQR of Complex II. One of the main focuses of this review is to highlight the unlikely nature of (i) the predominant contribution of Complex I to ROS production by reverse electron flow proceeding from Complex II to I and (ii) the reverse electron flow in absence of Complex III or IV inhibitors (*e.g.* under physiological conditions), by presenting evidence from both sides with a different perspective and pointing out a much more energetically favourable and essential relationship of NAD-linked dehydrogenases regulating $NADH/NAD^+$ levels to control Krebs cycle flows and SQR of Complex II function directly as the source of ROS. Precise mechanisms that lead to ROS production from Complex II have yet to be fully defined at the molecular level, although present indications suggest an essential role for O_2 and disruption of electron flows affected by the balance in levels of UQH_2/UQ and UQH as important mediators. Specific interactions of drugs such as MitoQ, diazoxide, and vitamin E analogues with Q binding sites should help provide greater insight into these mechanisms and how reactions to produce O_2^{-}• proceed. At present, they are very useful tools that directly implicate SDH/Complex II as the key source of electrons for ROS production, regardless of whether ROS emerges from Complex I, II, or III. These drugs may yet prove highly effective in their particular applications to prevent oxidative damage in normal cells leading to degenerative illnesses or as anti-cancer therapies.

It is hoped that this review will now redirect the focus of research back to the source of ROS to further define the nature of supramolecular enzymatic complexes of the Krebs cycle that couple to SDH/Complex II and the relationship to important ROS-producing enzymes such as free or E3 bound form of DLD. The impact of diet and fat intake resulting in increased long-chain fatty acyl-CoA levels contributes significantly to levels of DLD or SDH/Complex II ROS production; more studies are required in this area to further define the nature of links and changes in ROS production induced by fatty diets with associated increased fatty acyl-CoA levels. Future studies should lead to development of improved pharmaceutical agents as interventions that help ameliorate ravages of oxidative-stress induced damage on health, counteracting ROS damage, improving longevity, and providing more effective means for targeting and eliminating cancer cells when they arise.

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