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Minireview

The mystery of reactive oxygen species derived from cell respiration★✪

Hans Nohl✉, Lars Gille and Katrin Staniek

Basic Research in Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Vienna, Austria

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Mitochondrial respiration is considered to provide reactive oxygen species (ROS) as byproduct of regular electron transfer. Objections were raised since results obtained with isolated mitochondria are commonly transferred to activities of mitochondria in the living cell. High electrogenic membrane potential was reported to trigger formation of mitochondrial ROS involving complex I and III. Suggested bioenergetic parameters, starting ROS formation, widely change with the isolation mode. ROS detection systems generally applied may be misleading due to possible interactions with membrane constituents or electron carriers. Avoiding these problems no conditions reported to transform mitochondrial respiration to a radical source were confirmed. However, changing the physical membrane state affected the highly susceptible interaction of the ubiquinol/bc_1 redox complex such that ROS formation became possible.

The two outer molecular orbitals of dioxygen are each occupied by single electrons. Due to the „Pauli Principle“ the transfer of a pair of electrons to complete the vacant positions is a forbidden process. The use of dioxygen as terminal electron acceptor which is a prerequisite for optimal energy release in aerobic organisms requires therefore enzymes which reduce oxygen by mediating the transfer of single electrons. The thermo-

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✉Corresponding author: Hans Nohl, Basic Research in Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria; phone: (43 1) 2507 74400; fax: (43 1) 2507 74491; e-mail: hans.nohl@vu-wien.ac.at

Abbreviations: ROS, reactive oxygen species; $O_2^{\cdot -}$, superoxide radical.

dynamic restraint compelling single electron transfer to oxygen instead of a pair of electrons necessarily leads to odd electrons in the outer orbitals of this essential air constituent. However, dioxygen with odd electrons represents oxygen radicals. This fact in combination with the existence of enzymes removing reactive oxygen species (ROS) in aerobic tissues suggests that oxygen radicals compulsorily appear in aerobic metabolism.

The first oxygen radical occurring in aerobic organisms is the superoxide radical ($O_2^{\cdot -}$) which can be considered as the parent radical from which other oxygen radicals are derived. Some of the oxygen-reducing enzymes form stable complexes with oxygen while subsequently transferring two or even four electrons unless they release their reduction products. Examples in this respect are a couple of oxidases giving rise to the existence of H_2O_2 in aerobic systems while cytochrome oxidase of mitochondria releases water after tetravalent reduction of oxygen.

Transition metals such as iron or copper are operating in the active center of these enzymes facilitating single electron transfer to oxygen by orbital overlapping. The highest density of cooperating enzymes with iron or copper in the active center exists in the respiratory chain of mitochondria. The great majority of these redox-cycling enzymes have the capability to pass on single electrons to the respective redox partner. In addition the high rate of oxygen consumption through these mitochondrial electron carriers suggested that mitochondria are the main intracellular source of oxygen radicals.

In the early seventies Britton Chance and colleagues observed that isolated mitochondria release H_2O_2 when the respiratory chain was blocked with antimycin A (Boveris *et al.,* 1972). The presence of superoxide dismutase in the matrix of mitochondria rapidly converts $O_2^{\cdot -}$ generated in the respiratory chain to H_2O_2. Chance and his group reported that mitochondria generate $O_2^{\cdot -}$-derived H_2O_2 also in the absence of this respiratory poison (Loschen *et al.,* 1971). The authors observed H_2O_2 production under conditions of maximal membrane potential (state IV respiration) while down-regulation of the transmembraneous potential (state III respiration) stopped mitochondrial transfer of single electrons to oxygen out of sequence. From these observations it was assumed that the mitochondrial membrane potential at its highest level (state IV) triggers the deviation of single electrons to dioxygen which results in the generation of superoxide radicals (Korshunov *et al.,* 1997; Liu, 1997). These results were obtained in the presence of oligomycin to keep and preserve membrane potential at high levels (Korshunov *et al.,* 1997). There are also some opposing articles which provide evidence that a low membrane potential initiates $O_2^{\cdot -}$ generation. For example aging is reported to stimulate $O_2^{\cdot -}$ formation while the membrane potential declines (Sastre *et al.,* 2000).

Two different sources of the mitochondrial respiratory chain were considered to contribute to the univalent reduction of dioxygen. An iron-sulfur protein of complex I was reported to undergo autoxidation independent upon the height of the membrane potential (Genova *et al.,* 2001; Herrero & Barja, 1997). The identity of the complex I constituent was concluded from the use of rotenone which did not affect H_2O_2 generation in combination with selective inhibitors of iron centers. The second center suggested to be involved in mitochondrial ROS formation was reported to be activated when the membrane potential is high, e.g. under conditions of state IV respiration (Votyakova & Reynolds, 2001).

Apart from the proton-motive force considered by P. Mitchell (Mitchell, 1966; Reid *et al.,* 1966) to control mitochondrial ATP synthesis, Kadenbach & Arnold (1999) have recently described a second regulator of mitochondrial energy conservation. These authors made the observation that cytochrome oxidase has various binding centers for ATP which can be phosphorylated and dephosphorylated by hor-

Table 1. Bioenergetic parameters of isolated rat heart mitochondria respiring glutamate + malate (substrates of mitochondrial complex I) or succinate (substrate of mitochondrial complex II)

	Glutamate + Malate	Succinate
Fragmented rat heart mitochondria	4.3 ± 2.1%	
Respiratory control	5.18 ± 0.38	2.84 ± 0.16
ADP/oxygen ratio	2.23 ± 0.05	1.09 ± 0.04
Membrane potential [mV]		
State IV	–230.2 ± 9.4	–225.7 ± 1.1
State III	–200.6 ± 2.1	–187.3 ± 3.4

Data are means ± S.E.M.

mones. Dephosphorylation inhibits energy-linked respiration associated with an increase of the membrane potential (Lee *et al.,* 2002). Although evidence is still missing it is believed that dephosphorylation causes deviation of single electrons from the respiratory chain leading to mitochondrial ROS formation.

We critically reproduced all experiments taken as evidences in the past that mitochondria represent the major cellular source of reactive oxygen species.

As will be shown in this paper both isolation of mitochondria from its natural environment in the cell and the use of inadequate detection methods for ROS may contribute to false positive results. However, our investigation also show that mitochondrial ROS formation may occur under certain conditions.

FUNCTIONAL AND STRUCTURAL INTEGRITY OF ISOLATED MITOCHONDRIA

The great majority of experiments from which mitochondria are assumed to play a major role in intracellular ROS formation were performed with mitochondria removed from their natural environment in the cell. The mechanical isolation of these susceptible organelles can be expected to affect both the bioenergetic and the structural integrity. Disruption of the regular electron flow due to partial fragmentation of mitochondria, high membrane potential following dephosphorylation of cytochrome oxidase or low membrane potential resulting from destruction of the inner mitochondrial membrane must be excluded when isolated mitochondria are taken as model to study ROS formation *in vivo*.

With a novel highly sensitive method developed in our laboratory (Schönheit & Nohl, 1996) we found that the lowest amount of fragmented mitochondria adhering the stock solution was around 4% of the total. Other bioenergetic parameters such as respiratory control value, ADP/oxygen ratio and membrane potential were close to optimal data reported in the literature (Table 1).

All experiments performed in our laboratory to critically evaluate the capability of mitochondria for $O_2^{\cdot -}$-derived H_2O_2 generation were done with preparations of similar control parameters.

INVASIVE DETECTION METHODS CAN GIVE RISE TO ARTIFICIAL ROS FORMATION IN MITOCHONDRIA

Lucigenin which is recommended as a sensitive $O_2^{\cdot -}$ detector in mitochondria (Rembish & Trush, 1994) can autoxidize after being re-

duced to the respective mono-cation radical. This reduction is possible both by $O_2^{\cdot -}$ and particular constituents of the respiratory chain (Liochev & Fridovich, 1997). $O_2^{\cdot -}$ generated in the respiratory chain undergoes rapid dismutation catalyzed by the presence of superoxide dismutase in the mitochondrial matrix. Therefore, the stable dismutation product H_2O_2 is usually taken as indicator of mitochondrial ROS formation. The most frequent detection methods are based on fluorescence change of lipophilic dyes which accumulate in the membrane where electron carriers of the respiratory chain are operating.

We found that interaction of some dyes with redox-cycling electron carriers simulate the presence of H_2O_2 while fluorescence quench by particular constituents of the membrane may affect results in the opposite direction. We therefore developed a non-invasive method to identify and quantify mitochondrial H_2O_2 generation where the detection system cannot interact with components of the respiratory chain (Staniek & Nohl, 1999; 2000). The principle of this method is the accumulation of H_2O_2 in the suspension medium and separation of H_2O_2 from mitochondria prior to its determination. The method was 3–6 times more sensitive as compared to conventional procedures. Nevertheless, we were not able to confirm mitochondrial H_2O_2 formation under any conditions earlier reported except in the presence of antimycin A (Staniek & Nohl, 2000). Controversial reports were published on complex I as a possible source of $O_2^{\cdot -}$ radicals. M. Brand's group supports the idea that complex I releases $O_2^{\cdot -}$ radicals into the matrix (St-Pierre *et al.,* 2002) while Lenaz' group (Genova *et al.,* 2001) and Herrero & Barja (1997) report the flow of $O_2^{\cdot -}$ from complex I into the intermembraneous space. Since our experimental approach was focused on the measurement of H_2O_2 in intact mitochondria we expect that due to its membrane permeability H_2O_2 formation from complex I substrates can be detected independent on the sideness of $O_2^{\cdot -}$ release. In the absence of antimycin A we did not observe any H_2O_2 release with complex I substrates (Staniek & Nohl, 2000).

CONDITIONS TRANSFORMING MITOCHONDRIA TO A RADICAL SOURCE

The presence of a set of cooperating enzymes which protect mitochondria from oxidative stress clearly suggests that mitochondrial ROS formation is a realistic event. It is generally accepted that antimycin A changes the electron flow along the respiratory chain in a way which forces the leak of single electrons to dioxygen. The sensitivity of this irregular redox couple to myxothiazol reveals ubisemiquinone interacting with the bc_1 complex at the cytosolic membrane phase is the reductant of dioxygen. The well known effect of antimycin A on the diversion of the two reducing equivalents from ubiquinol to redox carriers of the bc_1 complex made us to study this redox couple more intensively. A prerequisite for a regular transfer of the first proton and electron from ubiquinol to the Rieske iron-sulfur protein (which is the oxidant) is the spatial movement of the active sites of this protein (Zhang *et al.,* 1998).

The mobility and conformational changes of a functional membrane protein are determined by the hydrophobic interaction with the surrounding phospholipids. We therefore changed the physical state of the membrane by preloading it with cholesterol or erucic acid and followed the flow rate of reducing equivalents from ubiquinol to the bc_1 complex (Gille & Nohl, 2001). In both cases the flow rate to the Rieske iron-sulfur protein was dramatically affected (more than halved) while the transfer rate to *b*-type cytochromes was less reduced. Concomitantly we observed a leakage of single electrons from the myxothiazol-sensitive ubisemiquinone to dioxygen because superoxide radicals were formed (Table 2).

Table 2. The effect of physical membrane alteration (fluidity) due to cholesterol and erucic acid insertion into mitochondrial membranes on their electron flow rates and ROS formation

Treatment of submitochondrial particles	Fluidity (Order parameter S)	Electron flow rates to (s^{-1}) Cytochrome *b*	Cytochrome c_1	Superoxide generation (nmol/min/mg)
Control	0.682 ± 0.001	0.18 ± 0.01 [a]	0.56 ± 0.06 [a]	0.11 ± 0.02
		0.18 ± 0.04 [b]	0.65 ± 0.06 [b]	
Cholesterol	0.689 ± 0.001*	0.11 ± 0.01*	0.24 ± 0.08*	0.34 ± 0.04*
Erucic Acid	0.652 ± 0.002*	0.13 ± 0.02	0.25 ± 0.07*	0.41 ± 0.02*

Data are means ± S.E.M., *P < 0.05 *vs.* Control, [a]control value for cholesterol experiments, [b]control value for erucic acid experiments. For more details see Gille & Nohl (2001).

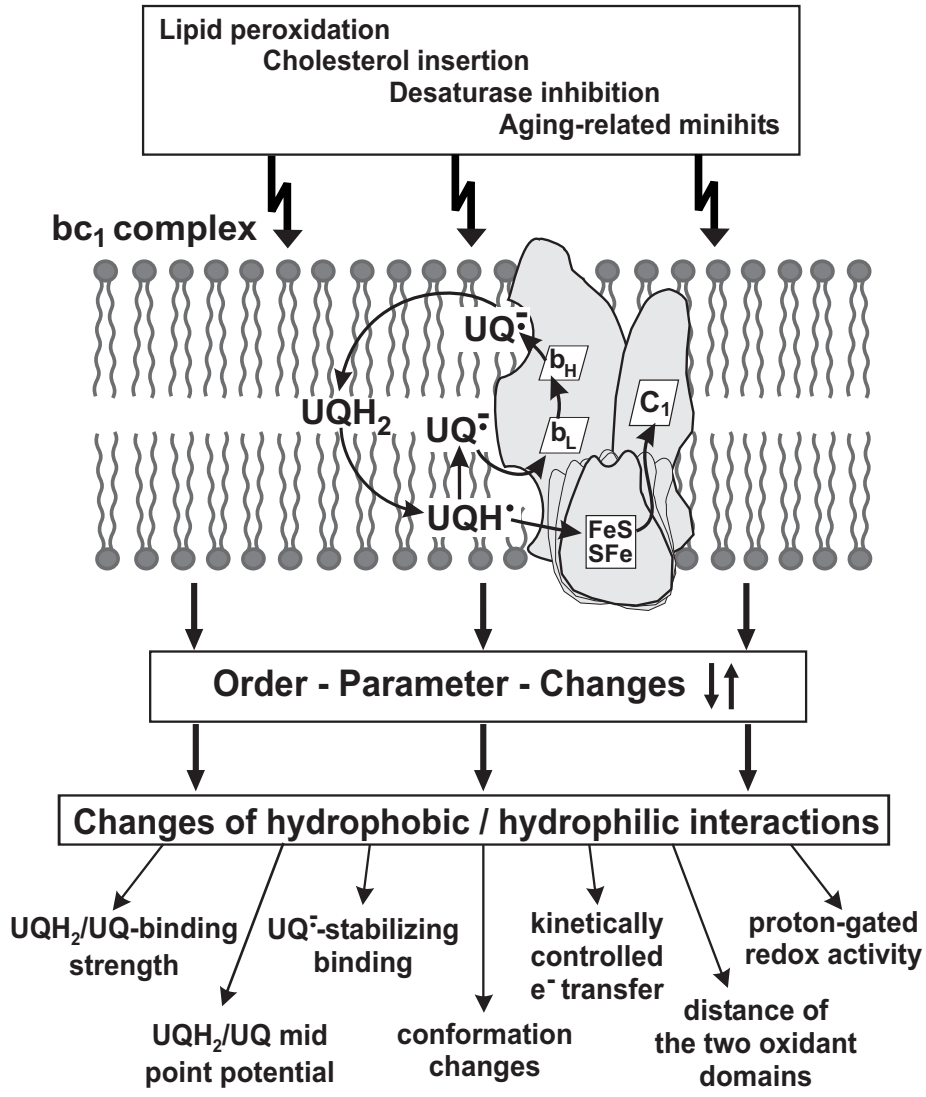

Figure 1. Tentative development of electron deviation from the UQH_2/bc_1 redox couple upon impediment of electron bifurcation.

(UQH_2 = ubiquinol, UQ = ubiquinone, $UQ^{\cdot -}$ = deprotonated ubisemiquinone, $UQH^{\cdot}$ = protonated ubisemiquinone, b_H = high potential cytochrome *b*, b_L = low potential cytochrome *b*, c_1 = cytochrome c_1, FeS = iron-sulfur protein).

Although cholesterol and erucic acid affected the physical state of the phospholipid membrane in opposite directions the transfer of the first electron from ubiquinol to its oxidant (Rieske iron-sulfur protein) was equally inhibited. It appears therefore that any impairment of the regular electron transfer at this site results in the univalent reduction of the oxygen molecule. The regular interaction of ubiquinol with its first oxidant requires a free spatial movement of the latter. It seems that the physical state of the membrane determines whether or not $O_2^{\cdot -}$ radicals are formed.

It was also reported in the literature that reversed electron flow driven by succinate respiration and high membrane potential upregulates an iron-sulfur cluster of complex I to a high reduction state which in turn autoxidizes by releasing superoxide radicals. Consequently, rotenone was found to suppress this complex I source of superoxide radicals (Votyakova & Reynolds, 2001). As mentioned above $O_2^{\cdot -}$-derived H_2O_2 formation from reversed electron flow to complex I was not observed despite using mitochondrial preparations with optimal bioenergetic parameters (see Table 1) and applying the novel non-invasive H_2O_2 detection method (Staniek & Nohl, 2000).

SUMMARY

The present study calls the established assumption in question that mitochondrial respiration is necessarily associated with the generation of superoxide radicals. Intracellular ROS provided by mitochondria was concluded from *in vitro* experiments with mitochondria mechanically removed from their natural environment. We have shown that both, the isolation procedure and the conventional invasive systems applied for ROS detection can be a cause of false conclusions. Careful isolation of mitochondria in combination with a novel non-invasive detection method for H_2O_2 revealed that $O_2^{\cdot -}$-derived H_2O_2 is not a by-product of regular respiration. We have also shown that mitochondrial respiration becomes a source of ROS when the ubiquinol/bc_1 redox couple is affected. Experimental evidence is presented demonstrating that changes of the physical state of the membrane in which electron carriers are operating triggers ROS formation. In that case redox-cycling ubisemiquinone interacting with *b*-type cytochromes becomes a source for superoxide radicals.

TAKE HOME SCHEME

All changes of the physical membrane state affect adequate conformational adaptation of the bc_1 complex such that the regular transfer of reducing equivalents from ubiquinol to the physiological oxidants becomes impeded. Alterations of hydrophilic/hydrophobic interactions affect the activity of this complex redox couple pushing electrons from the regular pathway to oxygen out of sequence. Parameters suggested to be involved are indicated by arrows in the lowest part of the scheme (see Fig. 1).

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