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ScienceDirect
Mitochondrion 7 (2007) 13–16
Mitochondrion
www.elsevier.com/locate/mito

Review

Detoxifying function of cytochrome *c* against oxygen toxicity

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Received 23 February 2006; accepted 19 October 2006
Available online 5 December 2006

Abstract

The detoxifying function of cytochrome *c* to scavenge $O_2^{-\cdot}$ and H_2O_2 in mitochondria is confirmed experimentally. A model of respiratory chain operating with two electron-leak pathways mediated by cytochrome *c* is suggested to illustrate the controlling mechanism of ROS level in mitochondria. A concept of mitochondrial radical metabolism is suggested based on the two electron-leak pathways mediated by cytochrome *c* are metabolic routes of $O_2^{-\cdot}$. Two portions of oxygen consumption can be found in mitochondria. The main portion of oxygen consumed in the electron transfer of respiratory chain is used in ATP synthesis, while a subordinate part of oxygen consumed by the leaked electrons contributes to ROS generation. It is found that the amount of electron leak of respiratory chain is not fixed, but varies with age and pathological states. The models of respiratory chain operating with two cytochrome *c*-mediated electron-leak pathways and a radical metabolism of mitochondria accompanied with energy metabolism are helpful to comprehend the pathological problems caused by oxygen toxicity.

Keywords: Oxygen toxicity; Cytochrome *c*; Reactive oxygen species (ROS); Respiratory chain; Mitochondria

1. Oxygen toxicity is a driving force of organic evolution

Reduction of O_2 to H_2O is a four steps reaction of sequential single electron additive process. The intermediates of O_2 reduction, such as $O_2^{-\cdot}$, $^{\cdot}OH$ and H_2O_2, are toxic to organisms. Historically, the organisms are derived from the reduced atmosphere of young globe. Emergence of oxygen let the original life face to serious damage of oxygen toxicity. Survivals have to be evolved with an ability to reduce oxygen toxicity and the cytochrome chain is the naturally selected apparatus for this purpose. Cytochrome *c* oxidase of respiratory chain plays a key role in reducing oxygen toxicity. It fastens the toxic intermediates at the active site of oxidase until O_2 transforms to H_2O by accepting four electrons. Whereas, the finding of electron-leak bypass of respiratory chain reveals that the oxygen toxicity is not fully controlled, damages still occur once the respiratory chain leaks more electrons.

2. Respiratory chain leaks electron to produce $O_2^{-\cdot}$ and H_2O_2 in mitochondria

In the very early stage of Keilin's lab two projects had been performed with mitochondria, one is the ATP generation and the other is the H_2O_2 generation. The former was performed normally and carried forward as the mechanism of mitochondrial oxidative phosphorylation. The later was paused in the very beginning due to the lack of sensitive method to detect H_2O_2 in biological system. The book named as "The History of Cell Respiration and Cytochrome" written by Joan Keilin for memory her father David Keilin recorded this story (Keilin, 1966). One paragraph at page 230 of the book depicted that "*Although we have failed so far to discover a reaction between cytochrome and hydrogen peroxide, the experiments with hydrogen peroxide were not devoid of interest*". This record reveals that the generation of H_2O_2 was a question to be asked very early in Keilin's lab, but it was not well promoted at that time.

The studies on this project had been not feasible until Chance developed a method to detect H_2O_2 generation in

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doi:10.1016/j.mito.2006.11.011

isolated mitochondria in 1970th. Chance found that about 2% of oxygen consumed in mitochondria is spent in the production of H_2O_2 in the normal physiological conditions (Chance et al., 1979). This result implies that the H_2O_2 is a byproduct of ATP synthesis in mitochondria. Chance's finding set up a new project of searching the way of H_2O_2 generation in mitochondria. It has been recognized so far that the precursor of H_2O_2 is the superoxide anion ($O_2^{-\cdot}$) and the $O_2^{-\cdot}$ is produced through a single electron reduction of O_2 (Koppenol et al., 1976; Turrens and Boveris, 1980). The complex I and III of respiratory chain are thought to be the main position where leak electrons , and complex II is also reported recently to be involved in electron leakage (Jezek, 2005).

3. Two electron-leak pathways mediated by cytochrome *c* in the respiratory chain

Chance tries to elucidate for the way of $O_2^{-\cdot}$ and H_2O_2 generation in mitochondria, we are interested in how mitochondria dispose of the potential dangerous $O_2^{-\cdot}$ and H_2O_2. In answering the later question, a hypothesis of cytochrome *c* leaks electron to reduce H_2O_2 was suggested based on the redox potential data and an experiment carried out with the purified succinate-cytochrome *c* reductase (SCR). It was observed that the transferred electrons from succinate through SCR to cytochrome *c* can be forth delivered to H_2O_2 (Xu, 1988). Combine the finding of reduction of H_2O_2 by ferrocytochrome *c* with the finding of generation of H_2O_2 by the leaked electrons from substrate side, an electron-leak bypass of respiratory chain could be constructed (Xu et al., 1996). In proving the operation of electron-leak bypass, a redox-shift experiment was carried out with a system containing SCR and cytochrome *c*. The expected observation, the shift of cytochrome *c* from reduced state to the oxidized state occur simultaneous with the accumulation of H_2O_2, was obtained when adding an inhibitor which blocks the electron transfer from succinate to cytochrome *c* but not affect the electron leak to generate H_2O_2 (Xu et al., 1996). In order to further affirmation the ability of cytochrome *c* to scavenge H_2O_2, an experiment of cytochrome *c* depletion and reconstitution was carried out in all kinds of preparations containing respiratory chain enzymes, such as Heart Muscle Preparation (HMP), Submitochondria preparation (SMP), Mitochondria (Mit) and purified SCR. It was observed that the generation of $O_2^{-\cdot}$ and H_2O_2 is 7–8 times greater in the cytochrome *c* depleted preparations than that in normal preparation, and the enhanced $O_2^{-\cdot}$ and H_2O_2 can be exponentially decayed with the reconstitution of cytochrome *c* to the cytochrome *c* depleted preparations (Wang et al., 2003; Zhao et al., 2003; Zhao and Xu, 2004). These experiments set up a model of respiratory chain operating with two cytochrome *c*-mediated electron-leak pathways, the ferricytochrome *c* disposes of $O_2^{-\cdot}$ and the ferrocytochrome *c* disposes of H_2O_2 (Zhao et al., 2003; Zhao and Xu, 2004) shown as in Fig. 1.

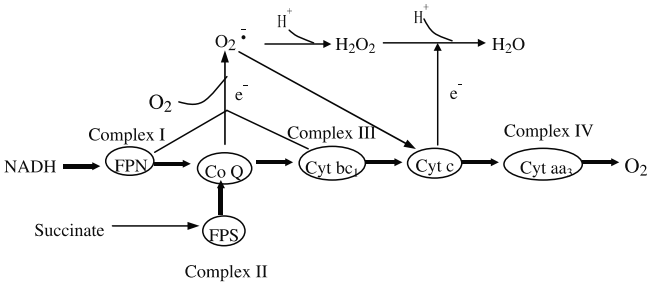

Fig. 1. Two electron-leak pathways mediated by cytochrome *c* in mitochondrial respiratory chain (Copied from JBC 278:2356–2360, 2003).

4. What we could learn from the model of Fig. 1

4.1. The detoxifying function of cytochrome c

The first realized function of cytochrome *c* is function as an electron carrier in the respiratory chain. 1996 in Wang's lab it was found that the cytochrome *c* plays a central role to stimulate cell apoptosis in cytosol (Liu et al., 1996). Our result shows that the cytochrome *c* has a detoxifying function to dispose of ROS in mitochondria. Therefore, it can be hypothesized that cytochrome *c* plays different roles in different locations of the cell and a functional migration of cytochrome *c* occur during the life span of cell.

4.2. The mechanism of cytochrome c down regulate ROS level in mitochondria

The model of respiratory chain operating with two cytochrome *c* mediated electron-leak bypass could be one of the regulating mechanisms of mitochondria which keep ROS level in normal physiological condition. Cytochrome *c* would on duty to eliminate $O_2^{-\cdot}$ and H_2O_2 when ROS were over-generated. It is rational that the less cytochrome *c* on duty to transfer electrons in the respiratory chain would cause more electrons leak out and generate more ROS. A vicious circle between ROS generation and cytochrome *c* release from respiratory chain is ambuscaded in mitochondria. A burst of ROS generation would occur once cytochrome *c* less than a "threshold" value on duty to transfer electrons in the respiratory chain. The ROS burst could be the earlier event to drive cell down to the programmed death.

4.3. Radical metabolism of mitochondria

Fig. 1 tells us that the $O_2^{-\cdot}$ and H_2O_2 is in a steady state of the generation at the substrate side of respiratory chain and the elimination at the oxygen side cytochrome *c*. Cytochrome *c* plays a role down regulate mitochondrial ROS for keeping them in the normal physiological concentrations. It is obvious that the two pathways of cytochrome *c* to dispose of $O_2^{-\cdot}$ and H_2O_2 are the metabolic routes of $O_2^{-\cdot}$, therefore it is reasonable to induce a concept of radical metabolism of mitochondria for describing the function of

mitochondria owing to the electron leakage of respiratory chain (Xu, 2004). Four reactive pathways of $O_2^{-\cdot}$ were collected and linked to the electron leak positions of respiratory chain and described as radical metabolism of mitochondria, shown as in Fig. 2. Two of the four pathways linked with cytochrome *c* are the same as in the Fig. 1, they play a role down regulate ROS in protecting mitochondria from the damage. The other two pathways, the way of $O_2^{-\cdot} + H^+ \rightarrow HOO^{\cdot}$ and the way of $O_2^{-\cdot} + NO \rightarrow ONOO^-$, may have their own pathophysiological roles. Such as the way of $O_2^{-\cdot} + H^+ \rightarrow HOO^{\cdot}$ may have a role in keeping animal heat based on the heat release reaction of $HOO^{\cdot}$ with the unsaturated fatty acids of the membranes described by Bielski (Bielski et al., 1983). The role of $O_2^{-\cdot} + NO \rightarrow ONOO^-$ is an open question to be understood. The binding of $O_2^{-\cdot}$ to NO could play a role to regulate the rate of ATP synthesis because NO could occupy the bind site of O_2 in the terminal oxidase retarding the ATP synthesis. The toxicity of $ONOO^-$ makes the way of $O_2^{-\cdot} + NO \rightarrow ONOO^-$ more pathological meanings. As $ONOO^-$ can move pass through membranes it could be a disseminator of $O_2^{-\cdot}$ and NO for long distance transportation. What is the real role of the different pathways of radical metabolism needs to be further searching. Besides the four radical pathways, Fenton reaction could cause more harmful $^{\cdot}OH$ generated in case of the electron leak of respiratory chain is abnormal higher.

Recently the research on the electron leakage of respiratory chain is promoted even further. The sites of electron leak in complex I and complex III are validated experimentally (Zhang et al., 1998; Genova et al., 2004). The precise generation position of $O_2^{-\cdot}$ is also discussed based on the orientation of the electron leak sites in the mitochondrial membrane. It is believed that the electrons leaked from complex I fall into the *matrix* of the mitochondria, while those from complex III goes to both the *matrix* and the space of mitochondrial inner- and out-membranes (Brand et al., 2004). The $O_2^{-\cdot}$ generated in the *matrix* is disposed of by SOD and GSH-peroxidase, while the $O_2^{-\cdot}$ in the space of mitochondrial membranes is disposed of by cytochrome *c*. The radical metabolism formed by the leaked electrons occur in both the *matrix* and the space of mitochondrial membranes.

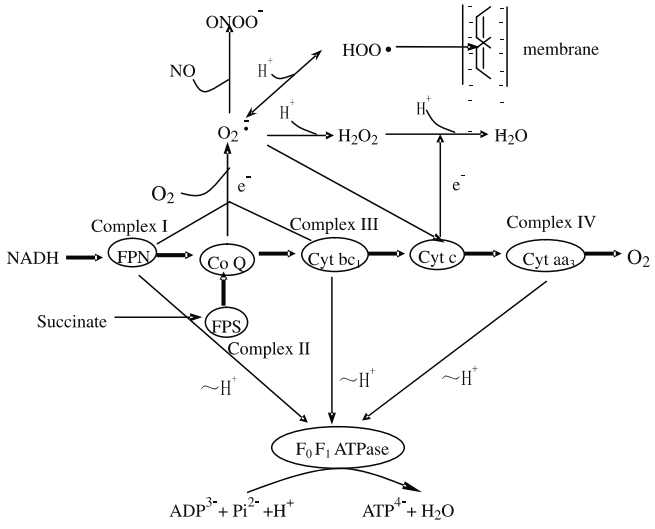

Fig. 2. Radical metabolism is partner to energy metabolism in mitochondria (Copied from Ann. N. Y. Acad. Sci. 1011: 57–60, 2004). The radical metabolism (upper) linked by the electron leakage of respiratory chain and the energy metabolism (lower) coupled with electron transfer in mitochondria. The cycle in the middle linked by arrow is the respiratory chain enzymes, The upper part shows the generation of $O_2^{-\cdot}$ and it's four reactive pathways. The lower part shows the coupled ATP synthesis.

4.4. Two potions of oxygen consumption in mitochondria

A more integrate description on the function of mitochondria is shown in Fig. 2. Mitochondria not only produce ATP, but also produce ROS. Two pathways of oxygen consumption can be found in mitochondria. The KCN inhibitory respiration achieved by the transferred electrons is coupled with ATP synthesis and the KCN insensitive respiration achieved by the leaked electrons is related to the ROS generation. Mitchell's theory is applicable in the part of KCN inhibitory oxygen consumption that is responsible for ATP synthesis (Mitchell, 1961). The oxygen consumed in the electron-leak pathways, which is not concerned in ATP synthesis, can be a logical contribution to the warp of Mitchell's theory to the experimental data.

As both the ATP synthesis and ROS generation sharing the electrons of respiratory chain, it is reasonable to describe mitochondria dysfunction as the disorder of mitochondrial metabolism. The disorder of radical metabolism of mitochondria can be described as higher level of electron leak or abnormal ratio of the different metabolic pathways of $O_2^{-\cdot}$. The higher of electron leakage of respiratory chain the more of ROS generated in mitochondria. The deteriorated effect of ROS on biological macromolecule indicated that the electron leakage of respiratory chain has the pathological sense.

4.5. The reason for the existence of cytochrome c mediated electron-leak pathways

Whereas, there is still in dearth of the proper explanation to the existence of cytochrome *c* mediated electron-leak pathways in the respiratory chain. One of the hypotheses is that the cytochrome *c* could be the protein naturally selected by the original organism for reducing oxygen toxicity. This assumption results from the experiments of studying on the mechanism of the reaction of cytochrome *c* with H_2O_2. It is observed that an automatic generation of H_2O_2 in the solution of ferricytochrome *c* through an aerobic reaction is observed. Store the solution of ferricytochrome *c* resulted in H_2O_2 generated in a time- and dose-dependent manner when exposure the solution to air. If carefully store the solution anaerobic, such as sealed by paraffin oil, no H_2O_2 can be generated (data is in publishing). This observation implies an auto-

matic reaction between cytochrome *c* and oxygen is happen. This automatic reaction supports an idea of cytochrome *c* as the candidate to reduce oxygen toxicity in the early stage of organic evolution. It has been known that the complex I and II are the original apparatus to generate ATP in the original prokaryotic cell. The O_2 can be reduced to toxic $O_2^{-\cdot}$ by the leaked electrons from complex I and II. If cytochrome *c* is in solution, the reaction $(O_2^{-\cdot} + \text{cyt}.c^{3+} \rightarrow O^2 + \text{cyt}.c^{2+})$ can proceed according to Forman and Azzi (Forman and Azzi, 1997) and ferrocytochrome *c* would dispose of toxic H_2O_2 (Xu et al., 1996). This is that the reactions were involved in the two electron-leak pathways mediated by cytochrome *c* in Fig. 1. The electron-leak pathways mediated by cytochrome *c* could be the living fossil of the evolution of cytochrome chain. It is found that in vitro experiments ferrocytochrome *c* eliminate H_2O_2 is not a simple redox reaction and one molecule ferrocytochrome *c* can scavenge about 500 molecule H_2O_2, indicating that ferrocytochrome *c* can act as a peroxidase to eliminate H_2O_2 (data is in publishing). It has been known that in the yeast mitochondria there exists the cytochrome *c* peroxidase to eliminate H_2O_2 accompanied with cytochrome *c*, while in mammal there is no cytcohrome *c* peroxidase, indicating that the peroxidase was eliminated through the evolution. In other words the cytochrome *c* mediated electron-leak pathway could be the result of evolutionary selection for reducing oxygen toxicity in original organisms, and during the evolution cytochrome *c* might gain some peroxidase activity and play an important role in the regulation of ROS generation.

Acknowledgements

This work is supported by the fund from National Science Foundation and the Ministry of Science and Technology of China.

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