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The operation of the alternative electron-leak pathways mediated by cytochrome *c* in mitochondria

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Received 8 March 2004

Abstract

Low (1×10^{-9} M) concentrations of cytochrome *c* inhibit H_2O_2 production in cytochrome *c*-depleted mitochondria, purified succinate-cytochrome *c* reductase (SCR) and antimycin A inhibited cytochrome *c*-depleted HMP. At higher concentration (2×10^{-6} M), cytochrome *c* eliminates pre-existed H_2O_2 if feeding electrons to it by succinate. Cytochrome *c* also decreases the OH· produced by succinate-cytochrome *c* reductase oxidizing succinate. We conclude that the alternative electron-leak pathway mediated by cytochrome *c* operates very well. In the presence of antimycin A, ferrocytochrome *c* can suppress the generation of H_2O_2 in SCR system, but ferricytochrome *c* cannot. Similar results are obtained on the elimination of pre-existed H_2O_2 by cytochrome *c*. For hydroxyl radical, antimycin A abolishes the suppression caused by both ferrocytochrome *c* and ferricytochrome *c*. These results indicate that the reductive state of cytochrome *c* caused by electron-flow is necessary and sufficient for the operation of cytochrome c-mediated electron-leakage pathway.
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Keywords: Cytochrome *c*; Hydrogen peroxide; Hydroxyl radical; Electron transfer; Alternative electron-leak pathway

Oxygen free radicals are highly reactive species which are known to be the major factor in oxidative cell injury. The mitochondria electron transport chain has been recognized as a major intracellular source of reactive oxygen species (ROS) [1,2]. In the study on ROS generation it is acknowledged that over 90% oxygen is consumed in mitochondria and 2% of them are expended in the production of $O_2^{-\cdot}$ and H_2O_2 in the normal physiological condition [1]. Although molecular oxygen is reduced to water in the terminal complex IV by a sequential four-electron transfer, a minor proportion of oxygen can be reduced to $O_2^{-\cdot}$ by a one-electron addition in complex III [3,4] and complex I [2]. $O_2^{-\cdot}$ is a short-lived molecule, which can rapidly evolve to H_2O_2 through three pathways: the catalyzation of superoxide dismutase; chemical dismutation; and HOO·, in equilibrium with $O_2^{-\cdot}$, reacting with membrane polyunsaturated fatty acids to produce heat and form H_2O_2 [5].

Although the level of ROS is very much lower in physiological conditions, they are hazardous to cells in case it is higher. So the identity and regulation of ROS in mitochondria is of fundamental interest for the understanding of the physiological and the pathophysiological role of ROS. Organisms have developed an efficient defense system that includes primary and secondary defense. The primary defense system is antioxidant enzymes that remove ROS before damage occurs. Mitochondria contain Mn-superoxide dismutase and glutathione peroxidase in the matrix, which maintain superoxide anion and H_2O_2 at relatively low steady-state concentrations under normal physiological conditions [1]. In addition, rat heart mitochondria also contain catalase in the matrix, possibly to keep H_2O_2 at low concentrations particularly when glutathione peroxidase is overwhelmed, preventing H_2O_2 from causing mitochondrial injury and reaching the extramitochondrial space [6]. These enzymes are located in the aqueous phase, whereas the formatting site of ROS is localized between the inner and outer mitochondrial membranes. There should be an antioxidative mechanism around the

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.144

respiratory chain to remove the generated $O_2^{-}\cdot$ and H_2O_2 from complex I and III rapidly.

From 1988 to 2003, some papers [7–11] have been published by our laboratory to suggest a new free radical metabolic pathway—the alternative electron-leak pathway mediated by cytochrome *c*—which acts as an antioxidative mechanism to maintain the normal level of ROS in mitochondria. Not long ago some data obtained with Keilin–Hartree heart muscle preparation (HMP) revealed that the cytochrome *c* is a powerful scavenger of $O_2^{-}\cdot$ and H_2O_2 in mitochondria, the ferrocytochrome *c* disposes $O_2^{-}\cdot$, and ferricytochrome *c* disposes H_2O_2 [10,11]. The level of $O_2^{-}\cdot$ and H_2O_2 is in the steady state between the generation in the substrate side—complex I and III and the elimination in the oxygen side—cytochrome *c*. The generation of $O_2^{-}\cdot$ and H_2O_2 is about 7–8 times higher in the cytochrome *c* depleted HMP (c-dHMP) than in normal HMP. The reconstitution of cytochrome *c* to c-dHMP causes the generation of $O_2^{-}\cdot$ and H_2O_2 exponential decrease to the lower normal level. In order to explain the downregulative effect of cytochrome *c* on ROS generation, the cytochrome *c* mediated electron-leak bypass model was presented [10,11].

In this paper, more evidence is presented to show the real operation of the alternative electron-leak pathways mediated by cytochrome *c* in mitochondria. The continuous reducing of cytochrome *c* is believed to be the necessary and sufficient condition for the operation of cytochrome *c*-mediated electron-leakage pathway.

Materials and methods

Chemicals. Luminol (3-aminophthalhydrazide) was obtained from Arcos Organics. Scopoletin (7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one), horseradish peroxidase (HRP), bovine serum albumin (BSA), antimycin A, dimethyl-1-pyrroline-*N*-oxide (DMPO), TTFA, PMSF, and mannitol were purchased from the Sigma Chemical. Cytochrome *c* was from Koch-Light Laboratories. 1,10-Phenanthroline anhydrous and ADP were from ICN Biomedicale. Succinic acid disodium salt was from Aldrich Chemical. Hepes was from Boehringer. NADH was from Amresco. Aprotinin was from Roche. All other reagents were of analytical grade.

Materials. HMP was prepared according to the method of Keilin and Hartree [12]. Cytochrome *c* depleted heart muscle preparation (c-dHMP) was prepared according to the method of Tsou [13]. Rat liver mitochondria were prepared as described by Rickwood et al. [14]. The cytochrome *c* was depleted using the method of Turrens et al. [15]. Succinate-cytochrome *c* reductase (SCR) was prepared according to the procedure of Yu et al. [16], but the pig heart muscle preparation was used as start materials instead of bovine heart muscle preparation. SCR contained 7.718 nmol cytochrome *b*/mg protein and 2.692 nmol cytochrome c_1/mg protein. The SCR activity was 2.133 μmol cytochrome *c* reduced/min/mg protein.

Preparation of ferrocytochrome c and ferricytochrome c. Pig heart cytochrome *c* was dissolved in 0.5% NaCl solution to 0.5 mM. Excess sodium dithionite was added in the ratio 10:1 by weight. After shaking vigorously, desalting was done to remove excess reducing agent using a High Trap size exclusion column (Pharmacia) prepacked with Sephadex G-25 superfine. The columns were delivered in 0.2 M NaCl with 20% ethanol as a bacteriostatic agent, hence initially it was pre-equilibrated with 0.5% NaCl solution. The concentration of the reduced cytochrome *c* was determined with UV–VIS spectrophotometer using a molar absorptivity for reduced cytochrome *c* of 29,500 M^{-1} cm^{-1} at 550 nm.

Preparation of ferricytochrome *c* is the same as the above procedure but with potassium ferricyanide instead of sodium dithionite.

Assay for generation and elimination of H_2O_2. Two methods were used. Luminol plus HRP-derived chemiluminescence (LDCL) is assayed with BPCL Ultra-weak luminescence analyzer at 37 °C. The reaction mixtures contain 500 μM luminol, 2.5 U HRP, 50 mM Na-phosphate buffer, pH 7.4, 4 mg/ml c-dMit, and different concentrations of cytochrome *c*, total volume is 1 ml. The LDCL was initiated by adding 200 μM NADH as substrate. The relation between cytochrome *c* concentration and the H_2O_2 formation is plotted as the integral area of the peak on the ordinate with the cytochrome *c* concentration on the abscissa. Another method is scopoletin-horseradish peroxidase spectrophotometric assays [17]. Measurements were performed at 360 nm (excitation wavelength) and 460 nm (emission wavelength). The reaction mixtures consist of 50 mM Na-phosphate buffer, pH 7.4, 0.4 mg/ml c-dHMP or 1.2 mg/ml SCR, 2.5 U HRP, 0.6 μg/ml scopoletin, and some cytochrome *c*. The reaction was initiated by adding 20 mM succinate. The decrease of the intensity means the generation of H_2O_2 and the increase of intensity means the elimination of H_2O_2.

Assay for hydroxyl radical. Spin trapping with DMPO was used to detect hydroxyl radical. The reaction system contains cytochrome *c* (0–50 μM), SCR (6.2 mg/ml), and DMPO (400 mM) in the Krebs' buffer. The initiator is succinate (20 mM).

Results

Cytochrome c decreases H_2O_2 generation in both intact mitochondria and parts of respiratory chain

It has been reported by our laboratory that the generation of $O_2^{-}\cdot$ and H_2O_2 in c-dHMP is 7–8 times higher than that in normal HMP, and the reconstitution of cytochrome *c* to the c-dHMP causes the enhanced ROS to exponentially decay [10]. HMP is a physically broken mitochondrial membrane fragment containing all the components of respiratory chain. Intact mitochondria are different from HMP, for intact mitochondria have membrane and are in the common state for energy production. Does this downregulative effect of cytochrome *c* operate in the intact mitochondria? To answer this question cytochrome *c* depleted mitochondria (c-dMit) were prepared for the examination. Fig. 1 shows the effects of cytochrome *c* on the H_2O_2 generation by c-dMit oxidizing NADH using LDCL assay. The normal mitochondria did not produce LDCL signal, but the c-dMit gave a strong signal which shows that the lack of cytochrome *c* in the respiratory chain caused greater H_2O_2 generation. Reconstitution of cytochrome *c* to the c-dMit causes the H_2O_2 generation to be exponentially decreased. When the concentration of the reconstituted cytochrome *c* reaches 24.4 nM, the generation of H_2O_2 is abolished. A similar result was also obtained by detecting the generation of superoxide anion, but the concentration of cytochrome *c* is about 200 nM to

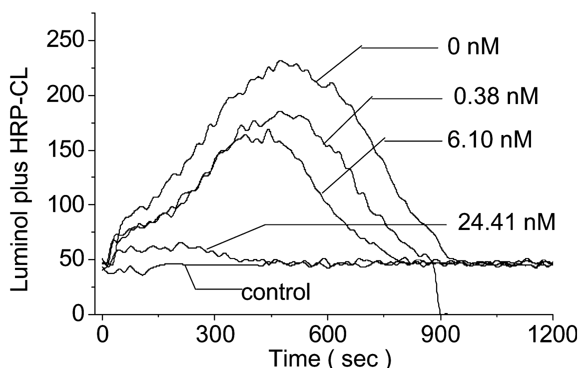

Fig. 1. The suppressive effect of cytochrome *c* on H_2O_2 generation in intact mitochondria. The method is LDCL assay. Reaction condition: 500 μM luminal, 2.5 U HRP, 50 mM Na-phosphate buffer, pH 7.4, 4 mg/ml c-dMit, 200 μM NADH, cytochrome *c* concentration is labeled on the line.

abolish $O_2^{-\cdot}$ signal (data not given). This result implies that the cytochrome *c* plays a role to regulate the generation of H_2O_2 and its precursor $O_2^{-\cdot}$ in mitochondria. Enough cytochrome *c* is necessary for keeping a lower physiological H_2O_2 concentration and the release of cytochrome *c* from mitochondria causes the H_2O_2 generation increased remarkably.

The downregulative effect of cytochrome *c* on the H_2O_2 generation in SCR and AA inhibited c-HMP also can be examined by scopoletin-horseradish peroxidase spectrophotometric assays. Shown as Fig. 2A, adding succinate to SCR causes the great increase of H_2O_2. The H_2O_2 generation can be abolished by adding both ferrocytochrome *c* (5 μM) and ferricytochrome *c* (5 μM). Fig. 2B shows that ferrocytochrome *c* can decrease the H_2O_2 generation in AA inhibited c-dHMP and there is a dose–effect relationship between the decreasing level of H_2O_2 and the concentration of reconstituted cytochrome c. When the ferrocytochrome *c* concentration reaches 18.8 μM, the H_2O_2 generation signal disappeared completely. It is suggested that cytochrome *c* also can suppress the H_2O_2 concentration when respiratory chain is blocked by AA.

Some paper reported that cytochrome *c* strongly inhibits H_2O_2 production in rat heart mitochondria under conditions of reverse electron transfer from succinate to NAD^+ [18]. According to Fig. 2A, the reverse electron transfer does not contribute to the antioxidation of cytochrome *c* in our system for SCR does not contain complex I. Because the cytochrome *c* which added to reactive system is reductive (Fig. 2A, line 3), the direct ROS elimination pathway caused by oxidative cytochrome *c* does not work. In Fig. 2A, line 2 the oxidative cytochrome *c* can be reduced by electron transfer. In Fig. 2B, in the presence of AA, the H_2O_2 generation of c-dHMP can be decreased by ferrocytochrome c. AA is an inhibitor of complex III, which blocks the electron transfer from substrate to cytochrome *c*, but not stop the generation of H_2O_2 by complex III. In the presence of AA the complex III generated H_2O_2 can be disposed by ferrocytochrome *c*, but not by ferricytochrome *c*. The latter only can dispose $O_2^{-\cdot}$, the precursor of H_2O_2, but it cannot dispose H_2O_2 directly.

Therefore, the alternative electron-leak pathway mediated by cytochrome *c* operates very well to decrease H_2O_2. The electron offered by substrate can transfer to cytochrome *c*, and reductive cytochrome *c* gives electron to H_2O_2.

Suppression of hydroxyl radical by the alternative electron-leak pathway mediated by cytochrome c

Both $O_2^{-\cdot}$ and H_2O_2 are the primary sources of ROS, hydroxyl radical ($OH^\cdot$) would be produced through Fenton reaction if $O_2^{-\cdot}$ and H_2O_2 were not removed in time. Hydroxyl radical is the main harmful factor for lipids, protein, and DNA. Using ESR spin trapping with DMPO the generation of $OH^\cdot$ can be detected. It is seen in Fig. 3A (0 μM) that hydroxyl radical is formed when SCR was incubated with succinate. The $OH^\cdot$ signal can be decreased when ferrocytochrome *c* is added to the reactive system (Fig. 3A), and can be eliminated completely by 150 μM ferrocytochrome *c*. Along with the increase of ferrocytochrome *c* adding to system, the generation of $OH^\cdot$ decreased gradually. The amount of ferrocytochrome *c* is anti-proportional to the hydroxyl radical signal (Fig. 3B). In the control experiment, it is confirmed that cytochrome *c* cannot react with hydroxyl radical directly, for the signal of Fenton reaction is not abolished by higher concentration of cytochrome *c* (data not shown). So the decrease of $OH^\cdot$ in our experiment is caused by the elimination of precursory ROS, such as H_2O_2 and $O_2^{-\cdot}$.

The elimination of pre-existed H_2O_2 by the alternative electron-leak pathway mediated by cytochrome c

Cytochrome *c* also related to the ability of elimination of pre-exited H_2O_2 by respiratory chain itself. This ability is detected via scopoletin-horseradish peroxidase spectrophotometric assay. Scopoletin is a fluorescence sensor with excitation wavelength at 360 nm and the emission wavelength at 460 nm. The elimination of fluorescence is caused by the reaction with H_2O_2 mediated by HRP. So the decrease of fluorescent intensity means the generation of H_2O_2 and the increase of it means the elimination of H_2O_2 in system.

There are two electron-leak pathways in mitochondria, one can image the H_2O_2 generation and the other disposed generated H_2O_2. Adding succinate to the reactive system containing c-dHMP and different concentrations of cytochrome *c*, the change of fluorescence intensity is recorded as panel A of Fig. 4. Sometimes after reaction beginning, there is the signal of hydrogen

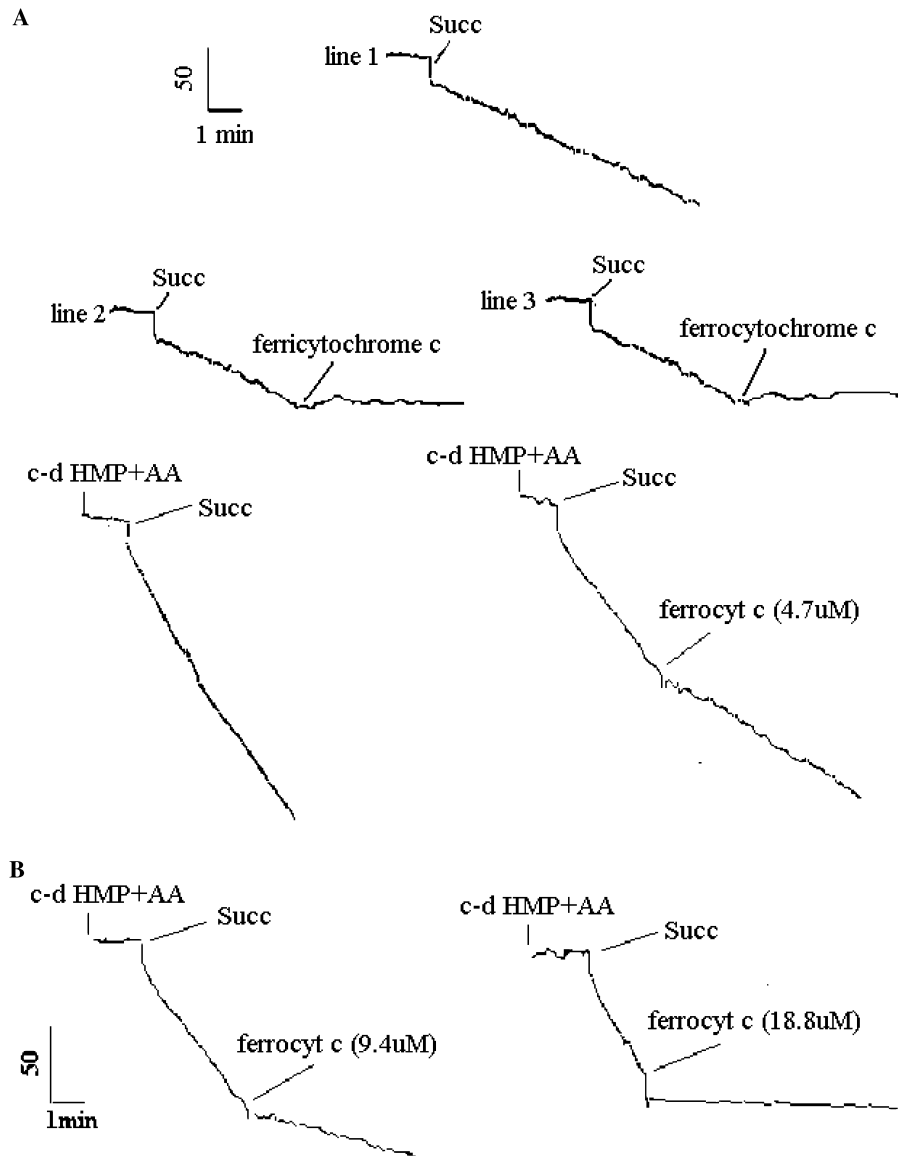

Fig. 2. The suppressive effect of cytochrome *c* on H_2O_2 generation in parts of respiratory chain. The method is scopoletin-horseradish peroxidase spectrophotometric assay. The excitation wavelength is 360 nm and the emission wavelength is 460 nm. (A) The H_2O_2 abolishment caused by both ferrocytochrome *c* and ferricytochrome *c* in SCR plus succinate system. Reaction condition: 20 mM Na-phosphate buffer, pH 7.4, 20 mM succinate, 2 mg/ml SCR, 6 U/ml HRP, 0.6 μg/ml scopoletin, no cytochrome *c* (line 1) and 5 μM cytochrome *c* (lines 2 and 3). (B) The dose–effect relationship between H_2O_2 generation and ferrocytochrome *c* added to c-dHMP system. Reaction condition: 20 mM Na-phosphate buffer, pH 7.4, 0.6 μg/ml scopoletin, 6 U/ml HRP, 0.5 mg/ml HMP, 2 μM AA, 20 mM succinate, and reductive cytochrome *c* (different concentrations).

peroxide generation indicated by the decrease of intensity (Δh). The increase of fluorescent intensity (ΔH) implies the disposal of H_2O_2 when oxygen is exhausted in the system. The addition of KCN also causes the increase of fluorescent intensity (ΔH). The lasting time between the Δh and ΔH is the steady state of H_2O_2 generation and elimination, during which time the oxygen is consumed by the electron transfer from succinate to oxygen. Under the condition of no cytochrome *c*, the value of Δh is large and that of ΔH is zero. Checking the relationship between Δh or ΔH and the concentration of reconstituted cytochrome *c*, the result is described as panel B of Fig. 4. The value of Δh decreased along with the increase of the concentration of cytochrome *c* reconstituted to c-dHMP, and stabilized at the point of 12 μM. On the other hand, the value of ΔH (ability of elimination of H_2O_2) increased along with the increase of the concentration of cytochrome *c*. The amount of cytochrome *c* reconstituted into the respiratory chain is anti-proportional to the generation of H_2O_2 and proportional to the elimination of H_2O_2. Using c-dMit as material, similar results were obtained (data not shown). This experiment indicates that the cytochrome *c*-mediated electron-leak pathway is operating alone with the electron transfer of respiratory chain. Normal electron leakage at complex I and III causes the accumulation of

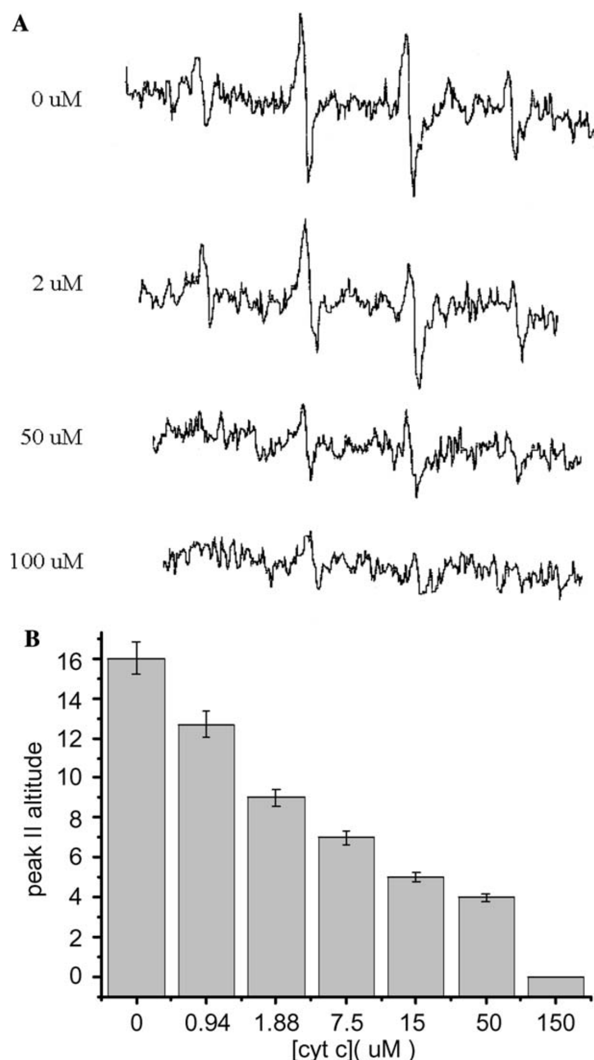

Fig. 3. Reductive cytochrome *c* decreases hydroxyl radical production in SCR plus succinate system. (A) The reactive curves. (B) The relation between reconstituted cytochrome *c* concentration and hydroxyl radical generation (peak II altitude). Reaction condition: 6.2 mg/ml SCR, 20 mM succinate, and 400 mM DMPO. ESR measurement: CF 3385 G, SW 200 G, CT 200 mS, ST 200 S, SP 20 mW, GN 4×10^5, MA 1 G, *f* 9.47–9.48 GHz.

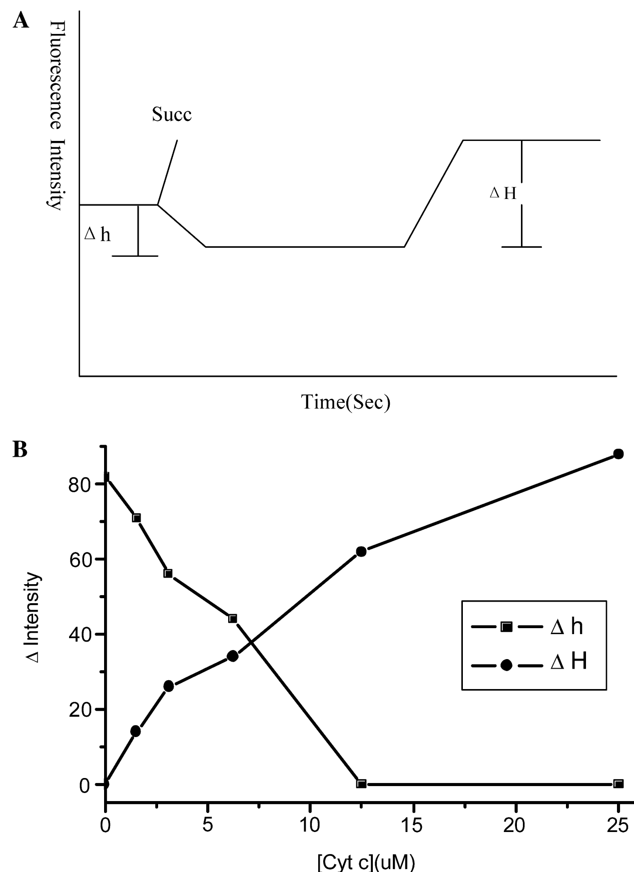

Fig. 4. The relationship between the concentrations of cytochrome *c* reconstituted to respiratory chain and Δintensity using c-depleted HMP as materials. (A) Simulative reactive curve. (B) The relationship between the concentrations of cytochrome *c* reconstituted to respiratory chain and Δintensity. ΔH is the amount of the increase of intensity, it means the elimination of H_2O_2 in system; Δh is the amount of the decrease of intensity, it means the generation of H_2O_2 in system. Reaction condition: 50 mM Na-phosphate buffer, pH 7.4, 0.4 mg/ml c-dHMP, 25 U HRP, 100 nM scopoletin, 50 mM succinate, and different concentrations of cytochrome *c*. Temperature: 37 °C. The amount of cytochrome *c* reconstituted into the respiratory chain is anti-proportional to the generation of H_2O_2 and proportional to the elimination of H_2O_2.

H_2O_2, the alternative electron-leak pathway mediated by cytochrome *c* reduced H_2O_2 to water and decreased cytotoxic effects.

The redox state of cytochrome c and the operation of the alternative electron-leak pathway mediated by cytochrome c

The above data show that the alternative electron-leak pathway is operating in all kinds of preparations that contain respiratory chain. It means this pathway operates in both physiological and pathological conditions. Cytochrome *c* is the executor of this pathway. Some observations indicate that the redox state of cytochrome *c* is very important for the operation of the alternative electron-leak pathways.

Fig. 5 shows that the ferrocytochrome *c* (5 μM) and ferricytochrome *c* (5μM) all can abolish H_2O_2 produced by SCR oxidizing succinate (lines 2 and 3). But AA, a inhibitor of complex III, makes a difference between ferrocytochrome *c* and ferricytochrome *c*. Adding 2 μM AA to reactive system, reductive cytochrome *c* still can suppress the H_2O_2 generation (line 4) but oxidative cytochrome *c* cannot (line 5). It suggests that under normal conditions cytochrome *c* mediates the alternative electron-leak pathway in spite of the redox state because electron can be transferred to ferricytochrome *c* and reduce it. But when electron transfer is blocked by AA, ferricytochrome *c* cannot be reduced and losses the ability of being a H_2O_2 suppressor, whereas

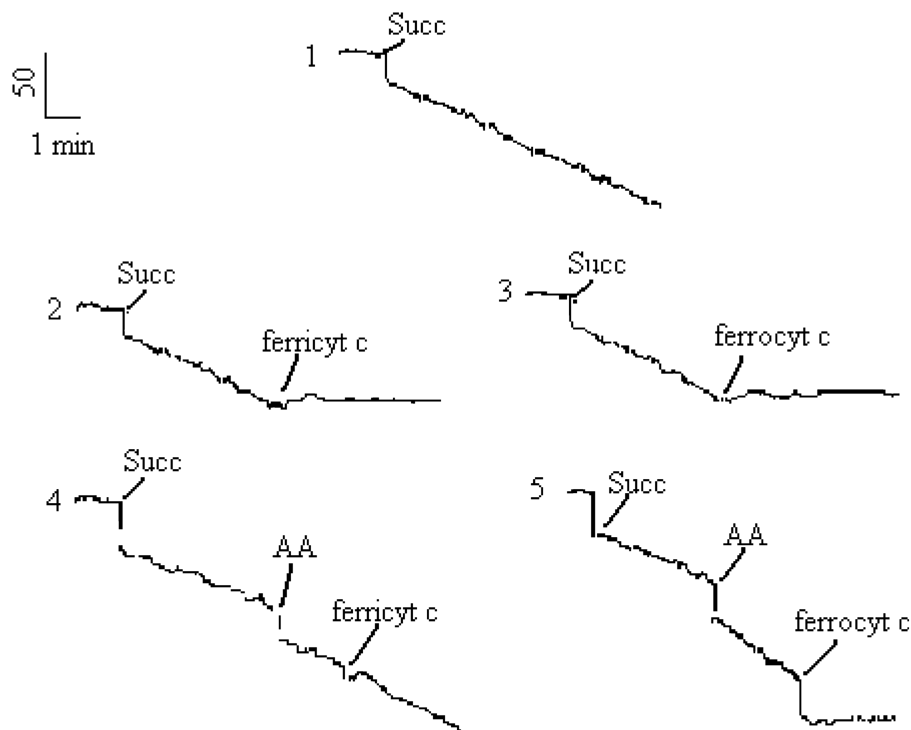

Fig. 5. The H_2O_2 suppressive ability of ferrocytochrome *c* and ferricytochrome *c* is influenced by AA in SCR system. The method is scopoletin-horseradish peroxidase spectrophotometric assay. Reaction condition: 20 mM Na-phosphate buffer, pH 7.4, 20 mM succinate, 2 mg/ml SCR, 6 U/ml HRP, 0.6 μg/ml scopoletin, no cytochrome *c* (line 1), and 5 μM cytochrome *c* (lines 2–5); 2 μM AA (lines 4 and 5).

ferrocytochrome *c* can suppress over-generated H_2O_2 directly. To ensure the operation of the alternative pathway, electron must flow to cytochrome *c* and make cytochrome *c* be reductive state.

Similar results were obtained by the elimination of pre-existed H_2O_2 by cytochrome *c*. Fig. 6 shows that cytochrome *c* has different actions on H_2O_2 elimination in the presence of different inhibitors which act at different sites of the respiratory chain. From Fig. 6A one can see a full reaction of succinate—c-dHMP system. Adding succinate to c-dHMP, H_2O_2 forms immediately. Then a balance appears. About 200 s later, a sharp H_2O_2 elimination occurs. AA and TTFA increase H_2O_2 generation but inhibit H_2O_2 elimination (Figs. 6C and D). Adding KCN to reaction system, H_2O_2 generation is abolished and the elimination occurs immediately (Fig. 6B). Because the inhibiting sites of AA and TTFA are before cytochrome *c* and that of KCN is after cytochrome *c*, we can draw a conclusion that the electron flow to cytochrome *c* is necessary for the H_2O_2 eliminating function of cytochrome *c*. Moreover, either ferrocytochrome *c* or ferricytochrome *c* can give a similar result. It means that the pre-existed H_2O_2 cannot be cleared by the alternative electron-leak pathway when electron flow to cytochrome *c* is blocked.

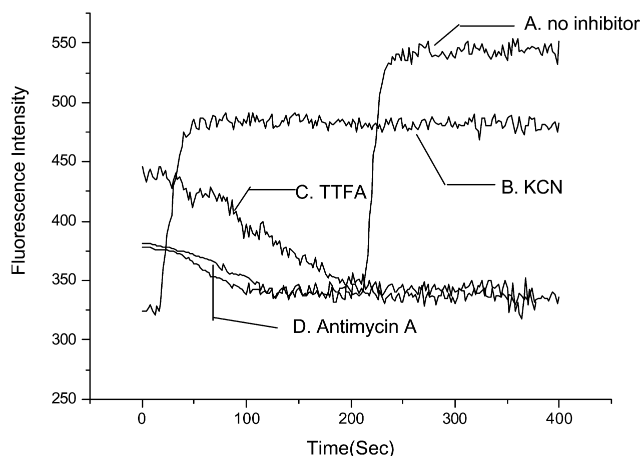

Fig. 6. Effect of cytochrome *c* on H_2O_2 elimination when different inhibitors existed. Reaction condition: 0.4 mg/ml HMP, 2.5 U HRP, 0.1 μM scopoletin, 50 mM Na-phosphate buffer, pH 7.4, 10 μM cytochrome *c*, and 50 mM succinate. (A) No inhibitor added; (B) KCN added; (C) TTFA added; and (D) AA added.

From Fig. 7 we also can see that the electron-flow to cytochrome *c* is necessary for the suppression of hydroxyl radical by the alternative electron-leak pathway. Spin trapping with DMPO was used to detect hydroxyl radical generation of SCR-succinate system. The hydroxyl radical signal is remarkable when cytochrome *c* is absent (line 1), and AA does not alter the signal in this condition (line 4). Either ferrocytochrome *c* or ferricytochrome *c* can abolish the $HO^{\cdot}$ generation (line 2 and line 3). But in the presence of AA cytochrome *c* cannot suppress the hydroxyl radical generation again, either ferricytochrome *c* (line 5) or ferrocytochrome *c* (line 6). The results infer that for the ROS suppression, the

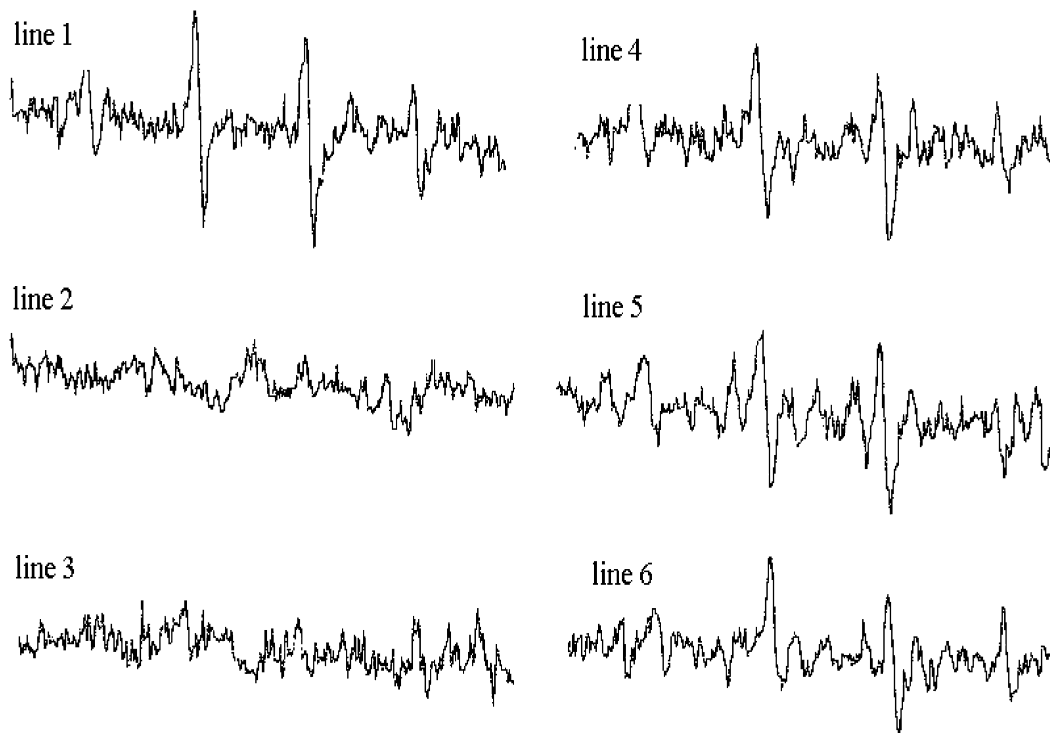

Fig. 7. AA can abolish the hydroxyl radical suppressive effect of cytochrome *c*. Line 1: no AA, no cyt c; line 2: no AA, 50 μM ferricyt c; line 3: no AA, 50 μM ferrocyt c; line 4: 2 μM AA, no cyt c; line 5: 2 μM AA, 50 μM ferricyt c; and line 6: 2 μM AA, 50 μM ferrocyt c. Reaction condition: 6.2 mg/ml SCR, 20 mM succinate, 400 mM DMPO, 100 μM AA. Measurement: CF 3385 G, SW 200 G, CT 200 mS, ST 200 S, SP 20 mW, GN 4×10^5, MA 1 G, *f* 9.47–9.48 GHz.

reductive state of cytochrome *c* is not enough and the electron-flow is necessary. If electron can transfer to cytochrome *c*, the over-generated ROS can be eliminated by the alternative electron-leak pathway. AA blocks the electron-flow before cytochrome *c*, so the cytochrome *c* cannot maintain the reductive state and the alternative electron-leak pathway cannot work to clear ROS.

Discussion

Some paper reported that one of the important functions of cytochrome *c* is antioxidation [18–21]. Several ways have been suggested for describing the antioxidant function of cytochrome *c*. First, cytochrome *c* ensures the fluency of electron flow that can reduce the electron leakage from respiratory chain. Second, soluble cytochrome *c* is well known to oxidize superoxide back to O_2. It was proposed that also in the cell, solubilized ferricytochrome *c* can react with superoxide anion, so that O_2 and ferrocytochrome *c* are formed. Apparently, to oxidize superoxide anion at a high rate in mitochondria, cytochrome *c* should be released to the intermembrane solution. During apoptosis, when cytochrome *c* released, this hemoprotein also performs the function of oxidizing superoxide. Third, cytochrome *c* inhibits H_2O_2 formation. Addition of cytochrome *c* to mitochondria in the resting state (state 4) strongly suppresses the formation of H_2O_2 and the inhibition relates to reverse electron transfer from succinate to NAD^+ [19,20]. Fourth, we have demonstrated the antioxidation of cytochrome *c* in HMP. An electron-leak model of respiratory chain was presented for explaining the antioxidative role of cytochrome *c* in mitochondria [10].

In this paper we can see that the electron-leak model of respiratory chain is also working in intact mitochondria (shown as Fig. 1). Skulachev and co-workers [19,20] indicated that the inhibition of H_2O_2 formation by cytochrome *c* relates to reverse electron transfer from succinate to NAD^+, but similar observation was also obtained in SCR system (Figs. 2 and 3) which does not contain complex I. so there must be a new pathway working at the same time with the reverse electron transfer pathway. It is the alternative electron-leak pathway mediated by cytochrome *c* that plays a role to maintain the low ROS level in cell by the elimination of pre-existed H_2O_2 (Fig. 4).

Two electron-leak pathways make cytochrome *c* work as a “bodyguard” protecting all the facilities of mitochondria from the damages of ROS. The alternative electron-leak pathway suppresses the over-generated ROS to maintain the ROS level in permissible range. The balance of the generation and elimination of ROS

makes mitochondria become a self-defensive system. The power of the cytochrome *c* as the "bodyguard" is proportional to the content of cytochrome *c*. The "bodyguard" function of cytochrome *c* is very important for the cell which is in some pathological state, for in this state some enzymes on respiratory chain are deactive and electron transfer is abnormal. The block of electron transfer brings on over-generated ROS to the damaged cell, but the alternative electron-leak pathway reduces the crisis of death.

Cytochrome *c* is a hemoprotein which contains iron atom. The presence of iron atom makes cytochrome *c* show two different states: cyt c^{3+} (ferricytochrome *c*) and cyt c^{2+} (ferrocytochrome *c*). When electron flows in respiratory chain, ferricytochrome *c* received electron to become ferrocytochrome *c* and then ferrocytochrome *c* delivers the electron to complex IV. For the alternative electron leakage, the reductive state of cytochrome *c* is needed.

From this paper, we can see that in the presence of AA the ferrocytochrome *c* can suppress H_2O_2 generation but ferricytochrome *c* cannot (Fig. 5), so the reductive state of cytochrome *c* is the necessary condition for ROS suppression. But the reductive state of cytochrome *c* is not the sufficient condition for ROS suppression. Fig. 7 shows that AA can abolish the suppression of hydroxyl radical by cytochrome *c*, either ferricytochrome *c* or ferrocytochrome *c*. So what is the necessary and sufficient condition for the normal running of the alternative electron-leak pathway? From Fig. 6 one can see that the antioxidant function disappears when the electron flow is interdicted before cytochrome *c* and the ROS elimination occurs immediately when the interdiction is appended at the down-stream of cytochrome c. It also is shown by Fig. 7 that the electron-flow to cytochrome *c* is sufficient for the suppression of hydroxyl radical by the alternative electron-leak pathway. The electron-flow to cytochrome *c* can prompt cytochrome *c* in reductive state and provide a force to drive ROS elimination. Our experiments give a new opinion: the reductive state of cytochrome *c* caused by electron-flow is necessary and sufficient for cytochrome *c* to exert antioxidant function by the alternative electron-leak pathway.

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