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Effect of Cytochrome *c* on the Generation and Elimination of $O_2^{\cdot-}$ and H_2O_2 in Mitochondria*

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The primary recognized function of cytochrome *c* is to act as an electron carrier transferring electrons from complex III to complex IV in the respiratory chain of mitochondria. Recent studies on cell apoptosis reveal that cytochrome *c* is responsible for the programmed cell death when it is released from mitochondria to cytoplasm. In this study we present evidence showing that cytochrome *c* plays an antioxidative role by acting on the generation and elimination of $O_2^{\cdot-}$ and H_2O_2 in mitochondria. The $O_2^{\cdot-}$ and H_2O_2 generation in cytochrome *c*-depleted Keilin-Hartree heart muscle preparation (HMP) is 7–8 times higher than that in normal HMP. The reconstitution of cytochrome *c* to the cytochrome *c*-depleted HMP causes the $O_2^{\cdot-}$ and H_2O_2 generation to exponentially decrease. An alternative electron-leak pathway of the respiratory chain is suggested to explain how cytochrome *c* affects on the generation and elimination of $O_2^{\cdot-}$ and H_2O_2 in mitochondria. Enough cytochrome *c* in the respiratory chain is needed for keeping $O_2^{\cdot-}$ and H_2O_2 at a lower physiological level. A dramatic increase of $O_2^{\cdot-}$ and H_2O_2 generation occurs when cytochrome *c* is released from the respiratory chain. The burst of $O_2^{\cdot-}$ and H_2O_2, which happens at the same time as cytochrome *c* release from the respiratory chain, should have some role in the early stage of cell apoptosis.

Mitochondria represent a primary source of ROS[1] in most aerobic mammalian cells (1–3). The H_2O_2 concentration in liver cells has been estimated to be 10^{-7}–10^{-9}mol/liter and that of superoxide anion to be about 10^{-11} mol/liter (1, 3–6). The precursor of H_2O_2 has been shown to be the superoxide anion formed through a single electron reduction of O_2 by the electrons leaked from the substrate side of the respiratory chain (5). The rate of H_2O_2 production in isolated mitochondria under state 4 respiration is 0.6–1.0 nmol·mg^{-1}min^{-1}, which is about 2% of the total oxygen uptake under physiological conditions (1, 4). Two sites in the respiratory chain have been found to be responsible for the generation of $O_2^{\cdot-}$. One of these sites is located in complex I and the other in complex III (2, 7). $O_2^{\cdot-}$ production is probably via autooxidation of the reduced flavin mononucleotide of NADH dehydrogenase in complex I (2). Two components, the ubisemiquinone (8) and the reduced cytochrome *b* (9) have been suggested to be the autooxidizable factors causing $O_2^{\cdot-}$ production in complex III. Using the purified succinate-cytochrome *c* reductase, Zhang *et al.* (10) confirmed that the exact site leaking electrons to generate $O_2^{\cdot-}$ in complex III is in the Q-cycle. $O_2^{\cdot-}$ is a short-lived ion that can rapidly evolve to H_2O_2 along three pathways: 1) the catalysis of superoxide dismutase; 2) chemical dismutation; and 3) $HOO^{\cdot}$, in equilibrium with $O_2^{\cdot-}$, reacting with membrane polyunsaturated fatty acids to produce heat and H_2O_2 (11). Both $O_2^{\cdot-}$ and H_2O_2 are potentially dangerous if they are not removed in time. Therefore, a mechanism to protect respiration enzymes from the damage of the generated ROS is needed around the respiratory chain.

In our early studies on the purified succinate-cytochrome *c* reductase, we found that the electrons transferred from succinate to cytochrome *c* can be further delivered to the extra added H_2O_2 (12). An alternative electron leak pathway of respiratory chain was later suggested (13, 14). In this study, using the cytochrome *c*-depleted Keilin-Hartree heart muscle preparation (c-dHMP), we show that the alternative electron leak pathway may acted on the scavenging of $O_2^{\cdot-}$ and H_2O_2 in the mitochondrial respiratory chain.

EXPERIMENTAL PROCEDURES

Chemicals—Lucigenin (bis-*N*-methylacridinium nitrate), catalase, horseradish peroxidase, superoxide dismutase from bovine erythrocytes, bovine serum albumin, phenylmethylsulfonyl fluoride, mannitol, and EDTA were purchased from the Sigma. Luminol (3-aminophthalhydrazide) was obtained from Acros Organics. Cytochrome *c* was from Koch-Light Laboratories Ltd. 1,10-Phenanthroline anhydrous and ADP were from ICN Biomedical. Succinic acid disodium salt was from the Aldrich Chemical Company. HEPES was from Boehringer. NADH was from Amresco. Aprotinin was from Roche Molecular Biochemicals. Percoll was from Amersham Biosciences. All other reagents were of analytical grade.

Keilin-Hartree Heart Muscle Preparation and Succinate-Cytochrome c Reductase (SCR) Preparation—HMP was prepared according to the method of Keilin and Hartree (15). Succinate-cytochrome *c* reductase was purified from HMP according to the method of King (16). c-dHMP was prepared according to the method of Tsou (17). Protein concentration was determined by the Bradford method.

Measurement of the Activity of Succinate Oxidase of HMP—Activity was measured by the rate of oxygen consumption. A system containing 2 ml of pH 7.4 phosphate buffer and 8 mM succinate was warmed at 30° C, 300 mg of HMP was added, and the oxygen consumption was measured. The mitochondria respiratory control ratio was determined as the ratio of oxygen uptake in state 3/state 4 (18). The oxygen consumption was measured with a Clark oxygen electrode.

Assay for H_2O_2 Generation and Elimination—H_2O_2 generation were detected using luminol plus horseradish peroxidase-derived chemiluminescence with the BPCL Ultra-weak luminescence analyzer at 37° C. The reaction mixtures contained 500 μM luminol, 2.5 units of horseradish peroxidase, 50 mM Na-phosphate buffer pH 7.4, 4 mg/ml c-dHMP, and different cytochrome *c* concentrations in a total volume of 1 ml. The luminol plus horseradish peroxidase-derived chemiluminescence was initiated by adding 300 μM NADH as substrate. The integral of the

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[1] The abbreviations used are: ROS, reactive oxygen species; HMP, heart-muscle preparation; c-dHMP, cytochrome *c*-depleted HMP; SCR, succinate-cytochrome *c* reductase; LDCL, lucigenin-derived chemiluminescence.

signal peak reflects the formation of H_2O_2. The relation between cytochrome *c* concentration and H_2O_2 formation is plotted as the integrated area of the peak on the ordinate with the cytochrome *c* concentration on the abscissa.

Assay for O_2^- Generation—O_2^- generation was detected via lucigenin-derived chemiluminescence (LDCL) using a BPCL Ultra-weak luminescence analyzer at 37° C. For enzymatic systems, the reaction mixtures contained 3 μM lucigenin, 20 mM Na-phosphate buffer pH 7.4, 3 mg/ml c-dHMP, and different concentrations of cytochrome *c* in a total volume of 1 ml. The reaction was started by adding as substrate 200 μM NADH. When 20 mM succinate was used as substrate, the lucigenin concentration was 5 μM, and the protein concentration of HMP was 1 mg/ml. The integral of the chemiluminescence peak reflects the formation of O_2^-. The relation between cytochrome *c* concentration and the formation of the superoxide anion is plotted as of integrated area of the peak on the ordinate and the cytochrome *c* concentration on the abscissa.

RESULTS

Two Generative Sites of O_2^- and H_2O_2 in HMP—Keilin-Hartree HMP is the first preparation used in the study of cytochromes in the early stage. This preparation is a suspension of physically disintegrated mitochondrial membrane, which contains all the components of respiratory chain. Succinate-cytochrome *c* reductase is purified from HMP, which is part of respiratory chain transferring electrons from succinate to cytochrome *c*. With these two preparations we studied the generation and elimination of O_2^- and H_2O_2.

Lucigenin was used as a chemiluminescent probe to monitor the O_2^- generation. The concentration of lucigenin used in the experiments was 3 μM. It has been shown that at this concentration of lucigenin, the generation of photons is proportional to the amount of O_2^-. Although the validity of lucigenin as a chemiluminescent probe for detecting biological O_2^- has been questioned based on the observation that the lucigenin may itself act as a source of O_2^- via autooxidation of the lucigenin cation radical in several *in vitro* enzymatic systems (19, 20), 3 micromoles per liter lucigenin do not undergo redox cycling and are useful for HMP (21, 22).

As shown in Fig. 1*A*, two chemiluminescence peaks can be observed when the NADH concentration is more than 0.7 mM, and only peak I appears when NADH is less than 0.7 mM. With succinate as the substrate, only peak II can be observed, as shown in Fig. 1*B*. With purified succinate-cytochrome *c* reductase, only peak II can be observed in the presence of succinate, as shown in Fig. 1*B*. This observation indicates that peak I is formed by the O_2^- generation of complex I and peak II is formed by complex III.

O_2^- is a short-lived ion that can rapidly evolve to H_2O_2. Both O_2^- and H_2O_2 are potentially dangerous if they are not removed in time. We addressed whether the respiratory chain has a mechanism to protect itself from damage cause by the generated ROS.

Scavenging of O_2^- and H_2O_2 by a Cytochrome c-mediated Electron Leak Pathway—Forman found that O_2^- can be oxidized by ferricytochrome *c* rapidly (23), and this reaction has been used to measure the superoxide anion. It is reasonable to consider that the direct oxidization of O_2^- by ferricytochrome *c* of the respiratory chain is one of the protective mechanisms. In yeast, there is an enzyme named cytochrome *c* peroxidase that catalyzes the reduction of H_2O_2 by ferrocytochrome *c* (24). This reaction implies that the H_2O_2 could be scavenged by the electron delivery from cytochrome *c* to H_2O_2. The problem is that the cytochrome *c* peroxidase is found only in yeast. Can H_2O_2 be reduced by ferrocytochrome *c* in the absence of peroxidase? The following observation, as shown in Fig. 2 may support this possibility.

A system containing mainly cytochrome *c* and a catalytic amount of purified succinate-cytochrome *c* reductase is employed to observe the electron delivery from cytochrome *c* to

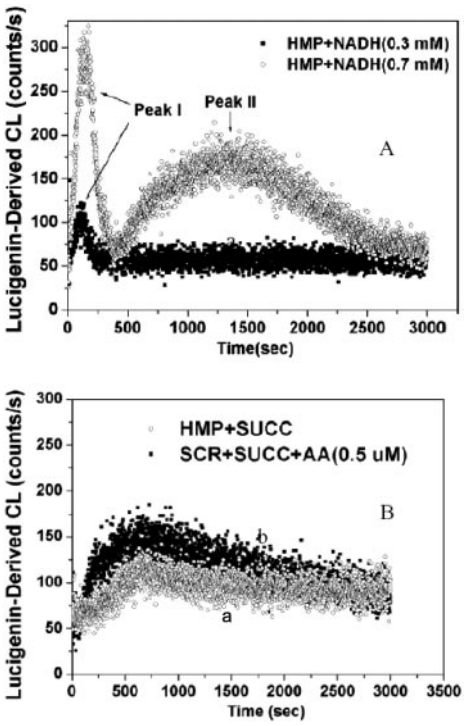

FIG. 1. **Profiles of lucigenin-derived chemiluminescence (*CL*) elicited with HMP.** *A*, NADH-supported chemiluminescence. The CL response was initiated by 1 mg/ml HMP and continuously monitored at 37° C for 3000 s. *B*, *a*, succinate-supported HMP. *b*, succinate-supported SCR. The assay mixture (1 ml) contained 50 mM phosphate buffer, pH 7.4, 3 μM lucigenin, and 20 mM sodium succinate. The CL response was initiated by 1 mg/ml HMP or SCR continuously monitored at 37° C for 3000 s.

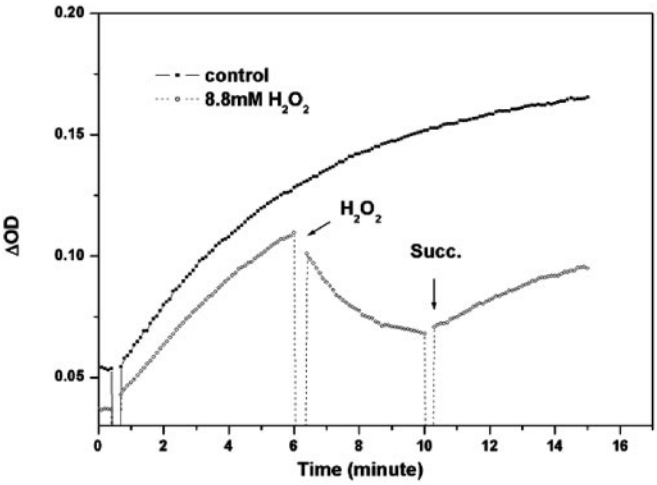

FIG. 2. **Re-reduction of H_2O_2-oxdized cytochrome *c*.** Reaction medium: 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA, 5 μg SCR, and 20 μM succinate with 8.8 mM H_2O_2 added.

H_2O_2. In this system, the absorption change at 550 nm mainly reflects the redox change of cytochrome *c*; the disturbance of other cytochromes in SCR was decreased to a limited value. As shown in Fig. 2, the process of electron transfer from succinate to cytochrome *c* can be observed by the increased absorption at 550 nm when adding succinate to the system. The addition of H_2O_2 during the reduction of cytochrome *c* causes a decreased absorption at 550 nm. The decrease of 550 nm indicates that the reduced cytochrome *c* is oxidized by H_2O_2. With further addition of succinate, the H_2O_2-oxidized cytochrome *c* can be re-reduced. This result implies that the electrons transported to the cytochrome *c* can be further delivered to H_2O_2 even in the

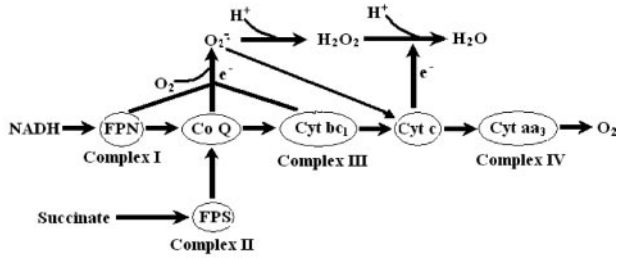

FIG. 3. **A proposal of cytochrome *c*-mediated electron-leak pathway.**

absence of cytochrome *c* peroxidase. This experiment can be performed when H_2O_2 concentrations is as low as 10^{-7}mol/liter, a value near the physiological concentration. Thus, the electron delivery from cytochrome *c* to H_2O_2 can happen in the absence of peroxidase. The cytochrome *c* of the respiratory chain not only transfers electrons to O_2 through the cytochrome *c* oxidase but also delivers electrons to H_2O_2 through an electron leak path (13, 14).

Based on the above observation, an electron-leak pathway mediated by cytochrome *c* can be suggested in mitochondrial respiratory chain as shown in Fig. 3.

To confirm the mechanism shown in Fig. 3, the cytochrome *c*-depleted HMP was prepared according to the method developed by Tsou (17). The effect of cytochrome *c* on the generation and elimination of O_2^- and H_2O_2 was observed by the reconstitution of cytochrome *c* to the c-dHMP.

Down-regulative Effect of Cytochrome c on the Generation of O_2^-—The precursor of H_2O_2 was shown to be the O_2^- formed through a single electron reduction of O_2 by the leaked electrons from the substrate side of the respiratory chain. We found that O_2^- generation in c-dHMP is about 7 times higher than that in normal HMP. The reconstitution of cytochrome *c* causes the O_2^- generation to decrease exponentially.

Fig. 4*A* shows the decay curve of O_2^- generation when cytochrome *c* is reconstituted to c-dHMP. The NADH concentration used in this experiment was 200 μM. The O_2^- generation decreased sharply when the reconstructed cytochrome *c* concentration was less then 0.8 μM and became constant at about 6 μM, a value equivalent to the content of cytochrome *c* in normal HMP. Least squares analysis showed that the curve can be represented by LDCL ($\times 10^4$) = $0.82 + 11.41e^{-19.67x} + 4.18e^{-0.73x}$. Fig. 4*B* shows the comparison of the restored activity and the O_2^- generation by the reconstitution of cytochrome *c* to the c-dHMP. It can be found that enough cytochrome *c* in the respiratory chain is necessary for both maintaining the lower O_2^- generation and high activity of the respiratory chain.

The generation of O_2^- in complex III was detected using succinate as the substrate. The O_2^- formation was about 8 times higher in c-dHMP then that in normal HMP. When cytochrome *c* was reconstituted, the value of O_2^- formation decreased to the level of normal HMP. Fig. 5*A* shows that the O_2^- formation also decayed exponentially with the reconstituted cytochrome *c*. Least squares analysis of the results showed that the curve could be represented by LDCL ($\times 10^4$) = $2.02 + 12.68e^{-8.90x} + 8.55e^{-1.19x}$. Fig. 5*B* shows the comparison of the restored activity and the O_2^- generation during the reconstitution of cytochrome *c* to the c-dHMP.

The above result implies that the content of cytochrome *c* in the respiratory chain strongly affects the level of O_2^- generation in the substrate side of the chain. The O_2^- generation was markedly increased, whereas cytochrome *c* was removed from the HMP. The reconstitution of cytochrome *c* to c-dHMP caused the O_2^- generation to decrease exponentially.

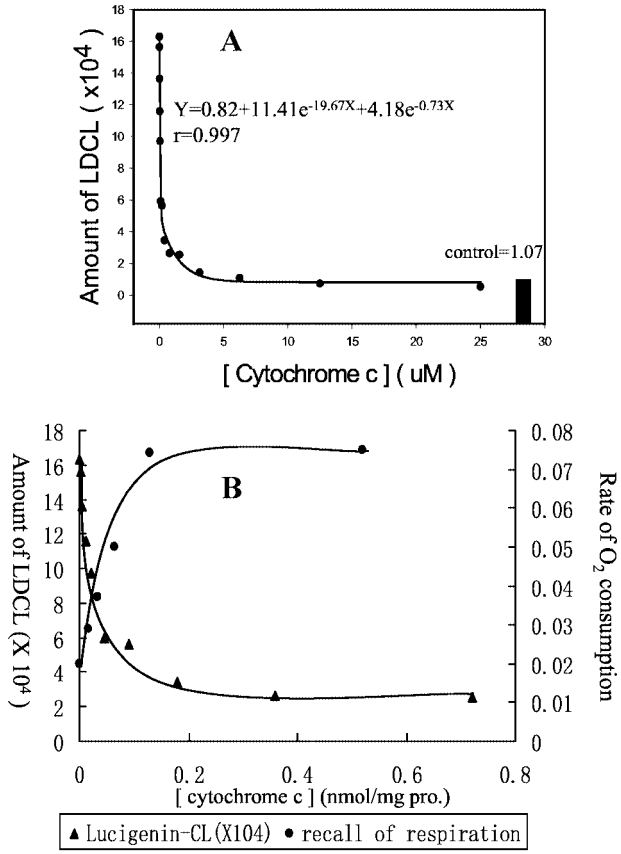

FIG. 4. **Decreased generation of superoxide anion with concentration of cytochrome *c* reconstituted to c-dHMP for NADH at a concentration of 200 μM.** *A*, total O_2^- generation for various cytochrome *c* concentrations. Ordinate is the integral peak area. The exponential decay is described by LDCL ($\times 10^4$) = $0.82 + 11.41e^{-19.67x} + 4.19e^{-0.73x}$ ($r = 0.997$). *B*, the decrease of superoxide anion generation occurs with the increase of succinate oxidase activity when cytochrome *c* is added to the reaction system. The control is the normal HMP. The protein concentration of HMP in the system was 2.17 mg/ml and the lucigenin was 3 micromole per liter.

Down-regulative Effect of Cytochrome c on the Generation of H_2O_2—Luminol plus horseradish peroxidase-derived chemiluminescence was used to detect H_2O_2 generation when cytochrome *c* was reconstituted to c-dHMP. The results are shown in Fig. 6. H_2O_2 generation in c-dHMP is about 7 times greater than that in the untreated HMP. The H_2O_2 generation obviously decreased, whereas cytochrome *c* was reconstructed to the c-dHMP. When the reconstructed cytochrome *c* concentration reached 5 μM, H_2O_2 generation decreased to the same value as that in normal HMP. Least squares analysis of the decay curve result in an formula of LDCL ($\times 10^3$) = $0.20 + 49.72e^{-0.94x} + 60.35e^{-0.21x}$ ($r = 0.999$).

The above result suggests that the amount of cytochrome *c* in the respiratory chain strongly affects the generation of H_2O_2. Enough cytochrome *c* is necessary to keep a lower physiological H_2O_2 concentration in mitochondria. When cytochrome *c* was released from the respiratory chain, H_2O_2 generation increased markedly.

Comparison of the Cytochrome c-depleted HMP and the KCN-inhibited HMP on the Generation of O_2^- and H_2O_2—The depletion of cytochrome *c* stopped the electron transfer of the respiratory chain at complex III, whereas KCN inhibition blocked the electron transfer at the terminal oxidase. Both of the treatments increased the electron leak in the substrate side of the respiratory chain. It was found that the increasing level is very different in these two treated HMP. As shown in the Fig. 7*A*,

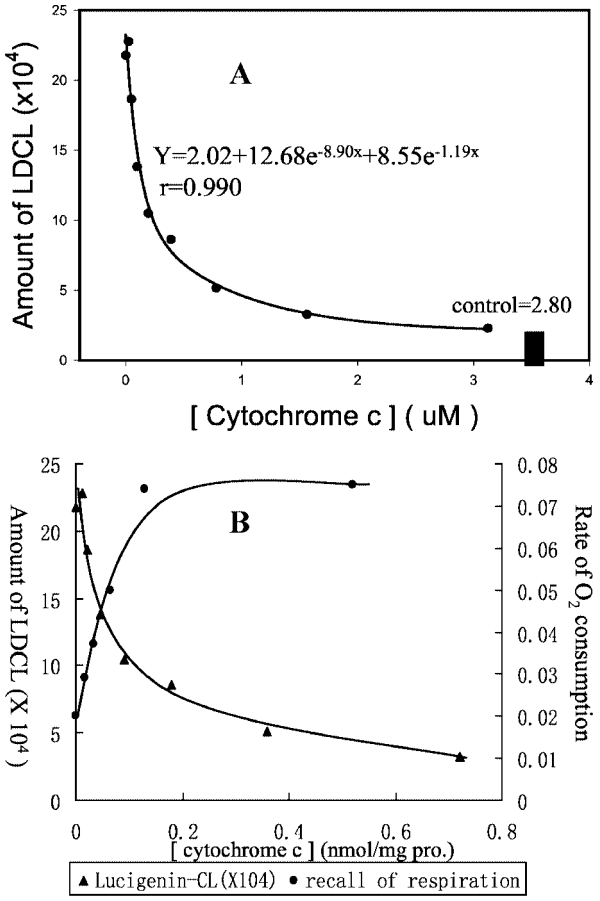

FIG. 5. **Decreased generation of superoxide anion with concentration of cytochrome *c* reconstituted to c-dHMP with succinate as substrate.** *A*, total O_2^- generation for various cytochrome *c* concentrations. Ordinate is the integral peak area. The exponential decay is described by LDCL ($\times 10^4$) = $2.02 + 12.68e^{-8.90x} + 8.55e^{-1.19x}$ ($r = 0.990$). *B*, the decrease of superoxide anion generation occurs with the increase of succinate oxidase activity when cytochrome *c* is added to the reaction system. The control was untreated HMP. The reaction system contained 20 mM Na-phosphate, 5 μM lucigenin, 2.17 mg c-dHMP and different concentrations of cytochrome *c* reconstituted. The reaction was started by adding 20 mM succinate.

the enhancement of H_2O_2 caused by KCN inhibition is only 60% of the enhancement caused by the depletion of cytochrome *c* from HMP. The enhancement of O_2^- occurred in c-dHMP but not in KCN-inhibited HMP (Fig. 7*B*). The generative levels of O_2^- and H_2O_2 are essentially different in the preparations with or without cytochrome *c*.

DISCUSSION

It is well established that mitochondria are the main source of ROS generation in cell. An antioxidative role of cytochrome *c in vivo* has been suggested (25, 27). In the living cell, a compound generated in a metabolic step is always eliminated in the next step of the metabolic reaction chain. The oxygen radicals also obey the rule of metabolism. It has been well accepted that the generation of O_2^- and H_2O_2 in the substrate side of respiratory chain is a result of electron leakage of the chain. In this study, an alternative electron-leak pathway is suggested to explain the elimination of the generated O_2^- and H_2O_2. Based on the scheme shown in Fig. 3, the level of O_2^- and H_2O_2 is in a balanced state between generation in the substrate side of respiratory chain and elimination in the oxygen side cytochrome *c*. The lower content of cytochrome *c* in the respiratory chain causes the higher level of O_2^- and H_2O_2 accumulated.

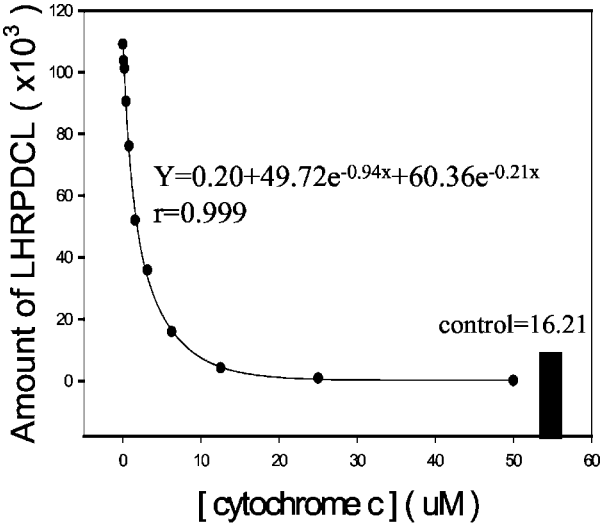

FIG. 6. **H_2O_2 generation for various concentrations of cytochrome *c* reconstituted to c-dHMP with NADH as substrate.** Ordinate is the integral peak area. The exponential decay is described by LDCL ($\times 10^3$) = $0.20 + 49.72e^{-0.94x} + 60.36e^{-0.21x}$ ($r = 0.999$). The control is untreated HMP. The c-dHMP protein concentration was 4 mg/ml.

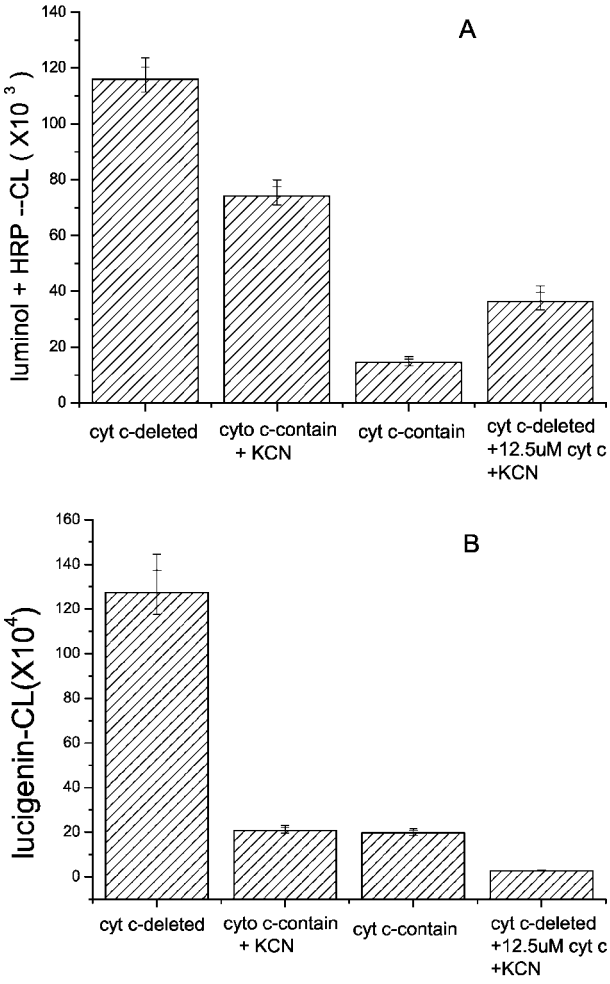

FIG. 7. **The influence of KCN to ROS generation in HMP system.** *A*, reaction of H_2O_2 generation. *B*, reaction of superoxide anion generation.

The experimental results demonstrate that cytochrome *c* strongly affects the generation and elimination of O_2^- and H_2O_2 in the mitochondrial respiratory chain. The amount of O_2^- and

H_2O_2 generated in the c-dHMP is 7–8 times higher than that in normal preparations. The reconstitution of cytochrome *c* to the c-dHMP causes O_2^- and H_2O_2 generation to decrease exponentially. This result implies that sufficient cytochrome *c* in the respiratory chain is needed for both the high activity of respiration and the low generation of O_2^- and H_2O_2. This property of cytochrome *c* is facilitated to the mitochondria for keeping reactive oxygen species at a normal physiological level. The release of cytochrome *c* from the respiratory chain will cause a dramatic increase of O_2^- and H_2O_2 generation.

The exponential decay curves of the O_2^- and H_2O_2 generation were all simulated as an equation having one constant term and two exponential terms. The following three mechanisms might be involved in the effect of cytochrome *c* on the generation and elimination of O_2^- and H_2O_2. First, the reconstitution of cytochrome *c* facilitates the electron transfer of the respiratory chain, thus the leakage of electrons reduced and the decreased generation of O_2^- and H_2O_2. This mechanism may correspond to the constant term of the simulation formula. Second, the O_2^- generated by the electron leakage can be oxidized by ferricytochrome *c* directly based on the reaction mentioned by Forman and Fridovich (23). Third, the H_2O_2 can be scavenged by the cytochrome *c*-mediated electron delivery shown in Fig. 3. The second two mechanisms may correspond to the two exponential terms of the simulation formula. The cytochrome *c* not only affects the generation of O_2^- and H_2O_2 by making the electron transfer of the respiratory chain more fluent, but also eliminating the generated O_2^- and H_2O_2 through a cytochrome *c*-mediated electron-leak pathway.

The O_2^- and H_2O_2 generation in cytochrome *c*-depleted HMP and KCN-inhibited HMP are quite difference. The level of H_2O_2 in KCN-HMP is only 60% of that in c-dHMP. This result reflects that the cytochrome *c* in KCN-HMP scavenges about 40% H_2O_2 through the cytochrome *c*-mediated electron-leak pathway. Once the O_2^- is generated in the substrate side of the respiratory chain, one portion is dismutated to H_2O_2 and another portion is rapidly scavenged by ferricytochrome *c* directly. This could be the reason that O_2^- is not accumulated in preparations containing cytochrome *c*. The more cytochrome *c* that is reconstituted to the c-dHMP, the less O_2^- and H_2O_2 are accumulated. These results support that the alternative electron-leak pathway mediated by cytochrome *c* is working in mitochondria.

Cytochrome *c* is strongly involved in cell apoptosis (26). Two groups of cytochrome *c* in mitochondria are suggested: the bound cytochrome *c* in the outer face of the inner membrane of mitochondria and the free cytochrome *c* in the space of inner and outer membrane (27, 28). In our experiments, the reconstituted cytochrome *c* is in the loosely bound state with the HMP membrane, therefore the loosely bound cytochrome *c* may have an antioxidative effect in mitochondria. It is revealed that a dramatic increase of O_2^- and H_2O_2 in mitochondria appears at the same time that cytochrome *c* leaves the respiratory chain. Therefore, the involvement of electron leakage in cell apoptosis is an interesting project. The burst of O_2^- and H_2O_2 happens earlier than that of cytochrome *c* arriving at the precaspase-stimulating position, suggesting that ROS may act as a kind of apoptogen in the very early stage of apoptosis.

Cytochrome *c* is a small, very stable hemoprotein containing covalently bound heme c as a prosthetic group. The primary recognized function of cytochrome *c* is to transfer electrons from complex III (QH_2-cytochrome *c* reductase) to complex IV (cytochrome *c* oxidase) in the respiratory chain. The finding of cytochrome *c* starting cell apoptosis implies that the cytochrome *c* has a different function in different locations in the cell. The observations in this study lead us to propose that cytochrome *c* may act as an antioxidative factor to regulate the O_2^- and H_2O_2 level of mitochondria, and this is involved in programmed cell death.

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