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Monoepoxide Production from Linoleic Acid by Cytochrome c in the Presence of Cardiolipin

Hirotaro Iwase, Takehiko Takatori, Masataka Nagao, Kimiharu Iwadate, and Makoto Nakajima

Department of Forensic Medicine, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo, Japan

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We found that cytochrome c (Cyt c) could oxidize cardiolipin (CL), and detected monoepoxides of linoleic acid (LA) in the fatty acids constituting the oxidized CL. We also found that in the presence of CL and Cyt c, free LA was oxidized and LA monoepoxides were produced. The aim of this study was to elucidate the mechanism of this lipid peroxidation. We concluded that ferric Cyt c produced some radical species from water-soluble oxygen in the presence of CL (CL-Cyt c system) and that radicals oxidized free LA or CL. The CL-Cyt c system may be another LA monoepoxide producing system in the neutrophil and may account for the lipid peroxidation observed in the ischemia-reperfusion-induced cardiac injury.

We previously reported that free LA was oxidized to LA monoepoxides (9,10-epoxy-12-octadecenoic acid (leukotoxin, LTx) and 12,13-epoxy-9-octadecenoic acid (LTx′)) by the system comprising the hydrogen peroxide (H_2O_2) generating system and Cyt c (H_2O_2-Cyt c system). The H_2O_2-Cyt c system was considered to produce hydroxyl radicals (•OH) that converted LA into monoepoxides of LA (1, 2).

As mitochondrial inner membranes contain CL and Cyt c, and mitochondrial H_2O_2 generation has been reported (3), we attempted to determine whether CL could be damaged by the H_2O_2-Cyt c system, and found that LA bound to CL was oxidized by Cyt c into many types of peroxylipid, including LA monoepoxides both in the presence and absence of H_2O_2. We also found that ferrous Cyt c was converted to ferric Cyt c in the presence of CL, and that ferric Cyt c, in turn, could produce some radical species from water-soluble oxygen in the presence of CL (CL-Cyt c system). The radicals generated by the CL-Cyt c system could oxidize not only CL itself, but also free LA added to the system. As CL seemed to catalyze the conversion of ferrous Cyt c into ferric Cyt c, CL may be a catalyst that accelerates the electron transfer in the mitochondria. However, once Cyt c and CL were separated from the electron transport system and Cyt c reduction did not occur, it might damage mitochondrial membranes. The CL-Cyt c system also may play a role in lipid peroxidation which was observed during the ischemia-reperfusion-induced cardiac injury (4).

MATERIALS AND METHODS

Materials. CL (bovine heart), LA, Cyt c (bovine heart), Cyt c reductase (porcine heart), tocopherol, Cu,Zn-superoxide dismutase (SOD, bovine erythrocytes), phospholipase-A_2 (bee venom) and L-ascorbic acid were from Sigma Chem. Co. (St. Louis, Missouri), H_2O_2 was purchased from Mitsubishi Gas Chem. Co. (Tokyo, Japan), 2, 5-dimethylfuran and 2, 2, 6, 6-tetramethylpiperidine were from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin), 3-methylthiopropionaldehyde was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). NADH was from Oriental Yeast Co., Ltd. (Tokyo, Japan), and all the other reagents used were from Wako Pure Chem (Osaka, Japan).

Chemical synthesis of LA monoepoxides and their structural analysis by gas chromatography-mass spectrometry (*GC–MS*). LA monoepoxides (LTx and LTx′) were synthesized and purified following the method of Akabane et al. (5). Fatty acid samples were methylated with diazomethane and analyzed using a mass spectrometer, TSQ70 (Finningen MAT, CA), equipped with a model 3400 gas chromatograph (Varian, Palo Alto, CA).

Extraction and analysis of fatty acids. Each reaction mixture (2 ml) was acidified with 0.3 N HCl to pH 4.5–5, and 8 ml diethylether was added, and after centrifugation (1500 g, 5 min), 6 ml organic phase was taken and evaporated under a stream of nitrogen gas. The extract was dissolved in 200 μl 100% ethanol and 20 μl resulting mixture was injected into an Inertsil-ODS column (5 μm particles, 0.46 cm × 25 cm, GL Science Inc.) mounted in a Shimadzu high performance liquid chromatography (HPLC) apparatus. The solvent system was a mixture of acetonitrile:water (73:27 v/v, pH 3), the flow rate

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was 1.0 ml/min and the column temperature was 30–40°C. Fatty acids were detected at 192 nm by an ultraviolet spectrophotometric detector (SPD-10A) and their elution patterns were traced by a recorder (CR-5A).

Preparation of micelles. In order to prepare micelles of CL, each reaction mixture was sonicated and vortex stirred for 1 min before starting the reaction by adding Cyt c.

CL oxidation by the H_2O_2–Cyt c system. To phosphate-buffered saline (50 mM phosphate, 110 mM NaCl, 5% ethanol; PBS) containing 0.2 mM CL was added 0.1 mM Cyt c, and the mixture was incubated with 0–0.5 mM H_2O_2 on a shaker for 60 min at 37°C. After the first incubation, phospholipase-A_2 (5.0 U/ml) and 1.0 mM $CaCl_2$ were added to the reaction mixture and it was incubated for 30 min at 37°C, then the fatty acids were extracted.

Free LA oxidation by the CL–Cyt c system. To PBS containing 0–0.4 mM CL and 0.1 mM LA was added 0.01 or 0.2 mM Cyt c. In another experiment, 0–0.4 mM Cyt c was added to PBS containing 0.2 mM CL and 0.1 mM LA. Each reaction mixture was incubated on a shaker for 60 min at 37°C.

CL–Cyt c system under an anaerobic condition. Just before the experiment, PBS was purged with nitrogen gas for 15 min, and during the incubation, the air in the test-tube was replaced with nitrogen gas.

The effect of Cyt c reduction on the LA monoepoxides production by the CL–Cyt c and H_2O_2–Cyt c systems. In the case of the CL-Cyt c system, PBS containing 0.2 mM Cyt c, 0.1 U/ml Cyt c reductase and 0–0.4 mM NADH was incubated for 60 min at room temperature, and then 0.1 mM LA and sonicated CL solution (final CL concentration 0.2 mM) were added to start the reaction. After incubation of 60 min at 37°C, fatty acids were extracted. In the case of the H_2O_2-Cyt c system, 0.2 mM H_2O_2 was added to start the reaction.

RESULTS

GC–MS and HPLC of Chemically Synthesized LA Monoepoxides

The results of GC-MS analysis of chemically synthesized LTx and LTx′ are shown in Fig. 1. On the HPLC with the column temperature set at 30°C, their retention time were 12.5 min and 12.0 min, respectively.

CL Oxidation by the H_2O_2–Cyt c System

CL was oxidized by Cyt c even in the absence of H_2O_2 and LA monoepoxides were detected from the fatty acids constituting the oxidized CL (Fig. 2-A). The addition of H_2O_2 had no effect on both the damage of LA and the LA monoepoxides production. This result was different from the monoepoxides production from free LA by the H_2O_2-Cyt c system, which was proportional to the H_2O_2 added (2).

Free LA Oxidation by the CL–Cyt c System

As shown in Fig. 2-B, free LA added to the CL-Cyt c system was oxidized and the LTx production was observed in the HPLC among many types of peroxylipid, indicating that some kind of free radical was generated by the CL-Cyt c system. About 90% of free LA was damaged by the system containing 0.01 mM Cyt c and 0.2 mM CL (Fig. 3-A). As shown in Fig. 3-B, when the Cyt c concentration was high (0.2 mM), the LTx production was proportional to the amount of CL added, until it reached 0.15 mM. However, when the CL concentration exceeded 0.15 mM, LTx production was saturated. It may be because radical generation was dependent on the amount of water-soluble oxygen as discussed later. When the Cyt c concentration was low (0.01 mM), LTx production was proportional to the amount of CL added, until it reached 0.2 mM. However, when the CL concentration exceeded 0.2 mM, LTx production began to decline. As shown in Fig. 3-C, when various concentration of Cyt c was added to 0.2 mM CL, the highest peak of LTx production was obtained when the concentration of Cyt c was 0.01 mM. These results suggested that radical generation was saturated when the molar ratio of CL to Cyt c exceeded 20.

Absorption Spectral Changes of Ferrous Cyt c after the Addition of CL

Ferrous Cyt c shows an absorption peak at around 550 nm, whereas ferric Cyt c does not. As shown in Fig. 4, ferrous Cyt c was converted into ferric Cyt c after the addition of CL.

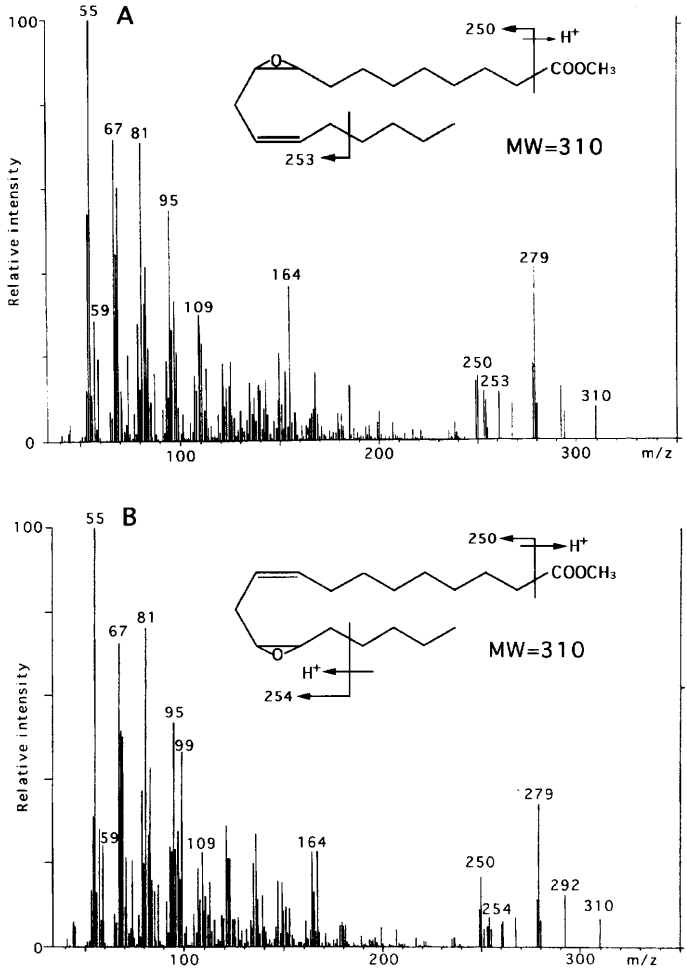

FIG. 1. GC-MS patterns of LTx (A) and LTx′ (B) which were methylated with diazomethane.

Effect of Cyt c Reduction on the LA Monoepoxides Production by the CL–Cyt c and H_2O_2–Cyt c Systems

As the NADH concentration increased and more Cyt c was reduced by Cyt c reductase, LTx production both by the H_2O_2-Cyt c and CL-Cyt c systems was decreased (Fig. 5). Ascorbic acid reduced ferric Cyt c to ferrous Cyt c and inhibited LTx production almost completely (Table 1). These results indicated that it was not ferrous Cyt c but ferric Cyt c that could oxidize the lipid.

Time Course of LTx Production

The curve of time dependent LTx production was sigmoid (Fig. 3-D). This was consistent with the finding that ferrous Cyt c does not produce radical species in the presence of CL. Cyt c used in the experiments contained 10% ferrous Cyt c, and at the start of the reaction, this ferrous Cyt c might be converted to ferric Cyt c, when LA monoepoxides production did not occur.

Radical Species Generated by the CL–Cyt c System

LTx production was inhibited almost completely under an anaerobic condition (Table 1), suggesting that the radical species was generated from water-soluble oxygen. Some scavengers of •OH

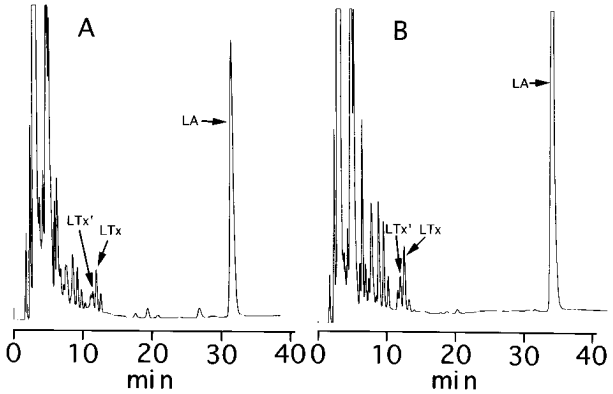

FIG. 2. A: HPLC pattern of the fatty acids constituting the CL which was oxidized by Cyt c. LTx and LTx′ were detected from the oxidized CL. B: HPLC pattern of the free LA products by the CL-Cyt c system (0.1 mM LA, 0.2 mM CL, 0.2 mM Cyt c). LA was oxidized and LTx and LTx′ was detected among the many types of peroxylipid.

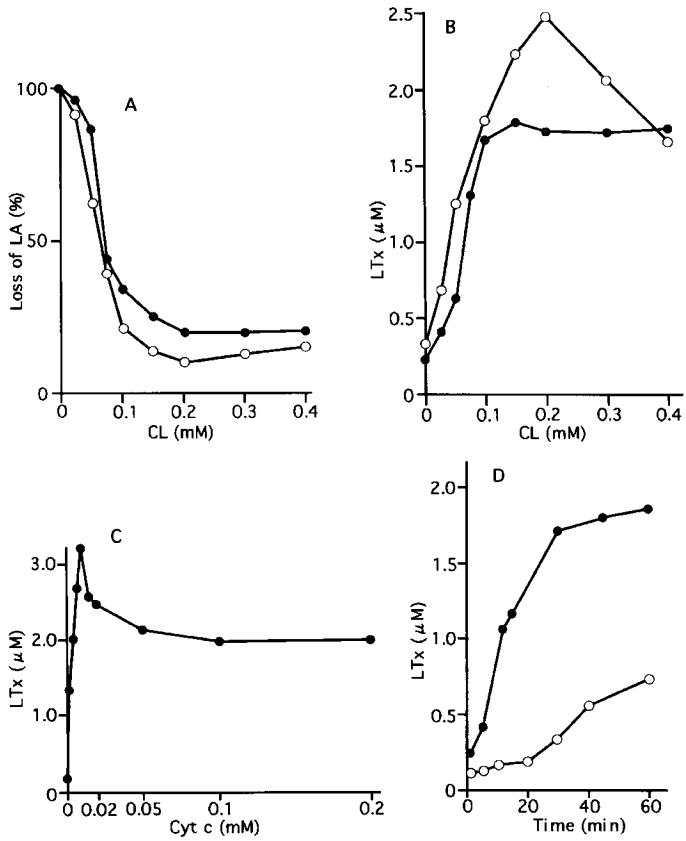

FIG. 3. A: Loss of LA in the CL-Cyt c system. ○: LA (0.1 mM) was oxidized by 0.01 mM Cyt c and 0–0.4 mM CL. ●: LA (0.1 mM) was oxidized by 0.2 mM Cyt c and 0–0.4 mM CL. B: LTx production by the CL-Cyt c system. ○: LA (0.1 mM) was oxidized by 0.01 mM Cyt c and 0–0.4 mM CL. ●: LA (0.1 mM) was oxidized by 0.2 mM Cyt c and 0–0.4 mM CL. C: LTx production by the CL-Cyt c system containing 0.1 mM LA, 0–0.2 mM Cyt c and 0.2 mM CL. D: Time course of LTx production by the CL-Cyt c system containing 0.1 mM LA, 0.2 mM Cyt c and 0.2 mM CL. ○: The reaction mixture was incubated at room temperature without shaking. ●: The reaction mixture was incubated on a shaker at 37°C. Each curve is sigmoid. The reagent Cyt c contained about 10% ferrous Cyt c, and at the start of the experiment, ferrous Cyt c may be converted to ferric Cyt c, when free radicals were not generated.

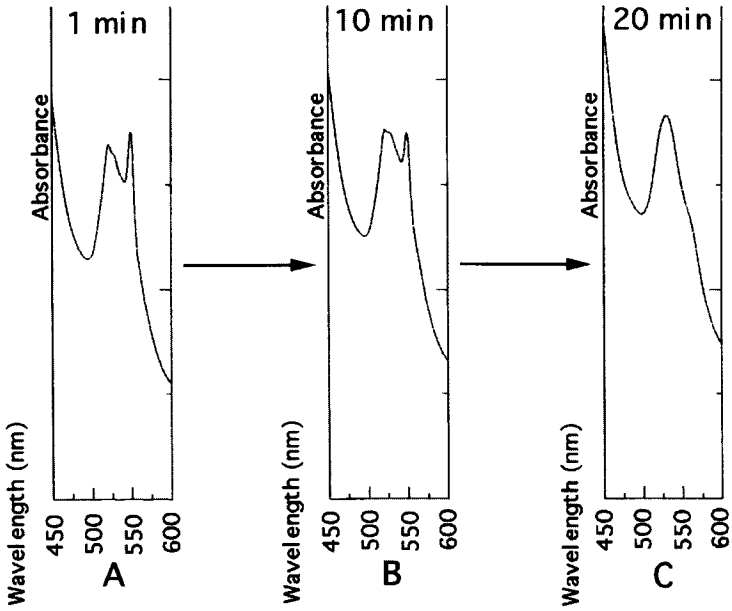

FIG. 4. Absorption spectral changes of ferrous Cyt c after the addition of CL. Cyt c (0.1 mM) was reduced by 0.02 mM ascorbic acid, the reaction was started by adding 0.2 mM CL and the absorption spectrum was recorded. The addition of CL resulted in the conversion of ferrous Cyt c to ferric Cyt c.

(N,N-dimethyl-p-nitrosoaniline, 3-methylthiopropionaldehyde) and singlet oxygen ($^1O_2$) (β-carotene, 2,5-dimethylfuran, α-tocopherol) inhibited the LTx production, whereas SOD and catalase did not, indicating that $\cdot$ OH and/or $^1O_2$ were involved. This, however, may not be the only explanation, because some scavengers of ·OH (mannitol, DMSO etc.) and $^1O_2$ (tetramethylpiperidine) did not inhibit LTx production and because all the inhibitory agents used could reduce the ferric Cyt c to ferrous Cyt c (data not shown).

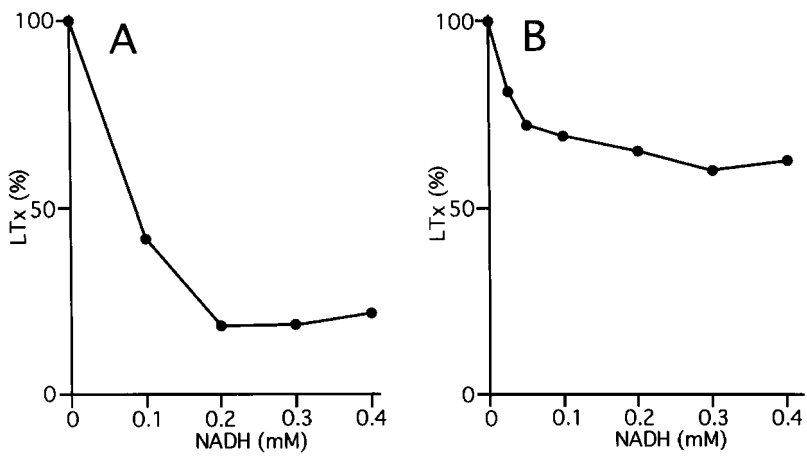

FIG. 5. A: The effect of the reduction of Cyt c on LTx production by the CL-Cyt c system. Before the experiment, Cyt c (0.2 mM) was reduced by 0.1 U/ml Cyt c reductase and 0–0.4 mM NADH for 60 min at room temperature, then 0.1 mM LA and 0.2 mM CL were added to start the reaction. B: The effect of the reduction of Cyt c on LTx production by the H_2O_2-Cyt c system. Before the experiment, Cyt c (0.2 mM) was reduced by 0.1 U/ml Cyt c reductase and 0–0.4 mM NADH for 60 min at room temperature, then 0.1 mM LA and 0.2 mM H_2O_2 were added to start the reaction.

TABLE 1A
Inhibition of LTx Production by the CL–Cyt c System under Some Conditions

Conditions	LTx production (%)
650 U/ml Catalase	101
120 U/ml SOD	103
1.0 mM 2,2,6,6-Tetramethylpiperidine	97.4
1.0 mM 1,4-Diazabicyclo-[2.2.2]octane	101
0.5 mM β-Carotene	82.9
1.0 mM 2,5-Dimethylfuran	86.6
1.0 mM D-L-α-Tocopherol	8.1
10 mM Benzoic acid	96.4
10 mM Mannitol	96.5
1.0 mM N,N-Dimethyl-p-nitrosoaniline	9.4
1.0 mM 3-Methylthiopropionaldehyde	57.5
1.0 mM Dimethyl sulfoxide (DMSO)	99.1
0.2 mM Ascorbic acid	17.6
Anaerobic	5.5

PBS containing 0.2 mM Cyt c, 0.2 mM CL and 0.1 mM LA was incubated for 60 min at 37°C under each set of conditions. LTx production is expressed as a ratio of that of the control solution, which contained only 0.2 mM Cyt c, 0.2 mM CL and 0.1 mM LA.

DISCUSSION

In the light of our results, we concluded that ferrous Cyt c was converted into ferric Cyt c in the presence of CL, and that ferric Cyt c, in turn, produced some free radical species from water-soluble oxygen in the presence CL. Although Goñi et al reported that Cyt c catalyzed autoxidation of phospholipids (6), we elucidated that some radicals produced by the CL-Cyt c system could damage not only CL itself, but also free LA added to the system. The CL-Cyt c system may be one of the LA monoepoxides producing system in the neutrophil (1, 2).

There was a report that CL was required for electron transfer in the mitochondrial respiratory

TABLE 1B
Inhibition of LTx Production by the H_2O_2–Cyt c System under Some Conditions

Conditions	LTx production (%)
120 U/ml SOD	98.8
1.0 mM 2,2,6,6-Tetramethylpiperidine	91.6
1.0 mM 1,4-Diazabicyclo-[2.2.2]octane	73.9
0.5 mM β-Carotene	74.5
1.0 mM 2,5-Dimethylfuran	87.2
1.0 mM D-L-α-Tocopherol	13.4
10 mM Benzoic acid	118
10 mM Mannitol	117
1.0 mM N,N-Dimethyl-p-nitrosoaniline	9.7
1.0 mM 3-Methylthiopropionaldehyde	27.3
1.0 mM Dimethyl sulfoxide (DMSO)	98.2

PBS containing 0.1 mM Cyt c, 0.2 mM H_2O_2 and 0.1 mM LA was incubated for 60 min at 37°C under each set of conditions. LTx production is expressed as a ratio of that of the control solution, which contained only 0.1 mM Cyt c, 0.2 mM H_2O_2 and 0.1 mM LA.

chain (7). CL may be an effective catalyst which accelerates the electron transport system in the mitochondria, because CL rapidly converted ferrous Cyt c to ferric Cyt c (Fig. 4). Cyt c must be always reduced to ferrous Cyt c in the electron transport system to protect the mitochondrial membranes from the radical generation by the CL-Cyt c system. However, once the complex of CL and Cyt c was separated from the electron transfer system, it might cause the membrane damage due to the radical generation.

CL-Cyt c system seems to be involved in some pathological disorders. In the case of diabetes mellitus, the reduced glucose intake due to the lack of insulin may result in a lack of the NADH supply to mitochondria which may increase the amount of ferric Cyt c with consequent mitochondrial membrane damage due to the promotion of radical generation by the CL-Cyt c system. This mechanism may be particularly prominent around the arteries, which receive an abundant oxygen supply, and therefore, the CL-Cyt c system may account for the atherosclerosis associated with diabetes mellitus.

Lipid oxidation has been observed in ischemia-reperfusion-induced cardiac injury (4). As Cyt c is assumed to be released from the cardiac muscle, the CL-Cyt c system may play a role in such a disorder. LTx, which has toxic effects on cardiac function (8, 9) and mitochondrial respiration (10), may be produced by the CL-Cyt c system and inhibit the cardiac contraction.

Cyt c is used as a medicine to improve the symptoms of brain infarction. However, its pharmacological mechanism of action is still unknown. LTx production from LA by the CL-Cyt c system may be involved, because LTx was found to dilate blood vessels (11).

Further researches are required on what kind of free radical is involved in the CL-Cyt c system, on whether other kinds of hemoproteins such as hemoglobin and myoglobin have the same ability as Cyt c to generate radical species or on whether these hemoproteins are involved in such disorders as ischemia-reperfusion-induced cardiac injury.

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