Redox State of Cytochrome *c* Regulates Cellular ROS and Caspase Cascade in Permeablized Cell Model

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Abstract: In a permeablized cell system, oxidized cyt *c* is able to induce caspase cascade whereas reduced cyt *c* cannot. In *in vitro* experiments, oxidized cyt *c* can promote H_2O_2 generation. It is suggested that the redox state of cyt *c* might regulate the initiation of apoptosis via regulation of cellular ROS level.

Keywords: Redox state, cytochrome *c*, apoptosis, caspase.

INTRODUCTION

It has been suggested that a critical event leading to cell apoptosis is the release of cyt *c* from mitochondria into cytosol [1]. Once released into cytosol, it binds to Apaf-1 forming a complex with caspase-9 [2]. This complex then initiates activation of other effector caspases, which cleave cellular proteins and finally commit the death progress[2]. Emerging evidence suggests that the apoptotic execution process is highly regulated even after cyt *c* is released. Some studies suggested that release of cyt *c* does not inevitably lead to caspase activation and cell death, at least in short-term assays [3,4]. These invoke a model that an important level of regulation of apoptosis occurs within the cytosol, preventing immediate irreversible engagement of apoptosis as soon as cyt *c* leakage from mitochondria. It was found that ionizing radiation (IR) and etoposide induce the release of cyt *c* from mitochondria in two distinct stages [5]. The early release of low level of cyt *c* into cytosol precedes the activation of caspase-9 and -3, but has no effect on ATP levels or the mitochondrial membrane potential ($\Delta\psi m$). In contrast, the late great loss of cyt *c* is dependent of caspase activation and associated with reduction of $\Delta\psi m$ and ATP, furthermore contributing to cell apoptosis. The distinct stages of cyt *c* release are not limited to IR, and in case of the v-AbI oncoprotein-suppressed apoptosis induced by withdrawal of IL-3, providing cells with survival factors stopped further cyt *c* released and conferred clonogenic survival to the remainning cell population [6]. Thereby, it is indicated that the ability of cyt *c*-induced caspases activation creates opportunities for a feedforward amplification loop, suggesting that the early released cyt *c* into cytosol may act as a regulator in the initiation of apoptosis at appropriate time in case of some apoptotic inducers. It was found that cells are resistant to apoptotic stimuli when are in a high redox potential with abundant thiol compounds present, and the anti-apoptotic function of Bcl-2 may partly be mediated by increasing the level of the reduced form of gluthathione, GSH [7-10]. It was also testified that the cellular redox potential can regulate the redox state of cytosolic cyt *c* [11]. Therefore, the redox state of cyt *c* might be one of the regulators in the cytosol to regulate apoptosis.

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Although the relationship between redox state and pro-apoptotic function of cyt *c* is of interest, discrepant data have been reported on this issue [12-15]. However, the mechanism through which the redox state of cytosol cyt *c* regulates apoptosis is still dearth of sound explanation. The aim of the current study was to determine how the redox state of cyt *c* is involved in regulating the activation of caspase cascade. The results indicated that oxidized cyt *c*, but not reduced cyt *c*, induces a time-dependent activation of caspases and a rapid cellular ROS burst (within 5 min treatment). In addition, the initial ROS accumulation (about 5-30 min) is prior to the activation of caspase, loss of $\Delta\psi m$ and the release of cyt *c* from mitochondria, suggesting that ROS is pivotal for activation of downstream caspases cascade. *In vitro* experiments, the oxidized cyt *c* can act as a catalyst promoting H_2O_2 generation with concurrent O_2 consumption. Therefore, it suggested the potential regulating function of the redox state of cytosol cyt *c* in the initiation of apoptosis and the oxidized cyt *c* exhibiting its cell toxicity partially through increment of cellular ROS.

MATERIALS AND METHODS

Cell Culture and Digitonin-Permeabilized Cells

HepG2 cells was cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified air/CO_2 (19:1) atmosphere containing at 37°C. To prepare permeabilized cells, HepG2 cells were washed in PBS, resuspended in 100 ml of buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH_2PO_4, 5 mM $MgSO_4$, 5 mM succinate, 1 mM EGTA, 5 mM Tris, pH 7.4).Cells were permeabilized with 5 mg of digitonin, and 5 mM rotenone was added to maintain pyridine nucleotides in a reduced form.

Preparation of Oxidized and Reduced of Cytc

Equine heart cyt *c* in solution of 0.5% NaCl was reduced or oxidized with sodium dithionite or potassium ferricyanide and desalted by passing the a-5 ml High-Trap size exclusion column (Phamacia) using the method reported [16].

Western Blot

Total protein extracted from treated and control cells was resolved on 12.5% SDS–PAGE gels and transferred electro-

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phoretically onto nitrocellulose membrane (Hybond-C, Amersham, UK). Filters were blocked in PBS-T for 1 h and then exposed to primary antibodies for 2 h. Filters were washed and exposed to a suitable secondary antibodies in the same buffer for 2 h. Filters were developed using enhanced chemiluminescence (ECL, Amersham, UK) and exposed to autoradiography film.

Measurements of Δψm

Δψm (mitochondrial membrane potential) was determined using the method previously reported.[17] In briefly, the retention of the dye 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$) in mitochondrial matrix was measured as the indicator of Δψm.

ROS Detection

The measurement of ROS was performed on the Hitachi F-4500 FL Spectrophotometer and the instrument parameters were as previously reported (EM of 530 nm, EX of 485 nm). When $DCFH_2$ was used, it was prepared from $DCFH_2$-DA following the reported method.

Measurement of O_2 Consumption

Oxygen consumption was measured with a Strathkelvin 1302 oxygen electrode (Strathkelvin Instruments Oxymeter 928) at 25 °C. The assay system contained in a final volume of 2 ml: 50 mM Tris-HCl buffer, pH 7.4, 10 μM DCFH2, and the presence or absence of 0.25 μM cyt *c*. The concentrations of SOD and catalase, when used, were 150 μg/ml and 100 μg/ml, respectively.

High-performance Liquid Chromatography (HPLC) Analysis of Cyt c

Chromatography of cyt.*c* was performed using an analytical ZORBAX OLIGO column (6.2×80 mm, Agilent). A gradient of 20% acetonitrile in 0.02M phosphate buffer (pH 7.0) to 2 M NaCl with 20% acetonitrile in 0.02M phosphate buffer (pH 7.0) over 40 min at a flow rate of 1.0 ml/min was used.

RESULTS

1. Redox State of Cyt c Regulates Caspase Cascade

HepG2 cells (10^6) were permeablized with digitonin and incubated with 0.5 μg of oxidized or reduced cyt *c* at 37°C, respectively. Conversions into active forms of caspases were confirmed by western analyses at various intervals. The decreasing procaspase-9 and -3 in (Fig. **1A**) suggested that the procaspases were cleaved to their active form in the cell extract treated with the oxidized cyt *c*. Both processes were time-dependent and involved a lag-phase for procaspases activation. No cleavage of procaspase-3 could be detected prior to 15 min. At the time of 90 min, the conversions were almost complete. The result is consistent with the view that once initiated, the activation of caspase-3 is a very rapid process [18]. However, reduced cyt *c* totally abolished the activation of caspases with up to 90 minutes-incubation (Fig. **1A**), indicating that only oxidized form of cyt *c* serves as a proapoptotic molecule in this permeablized cell model.

In respect of apoptosis, ROS is in general associated with induction and regulation of cell death [19]. Therefore, it was of interest to determine whether oxidized cyt *c* would affect the cellular ROS level. As seen in (Fig. **1B**), when cells were incubated with oxidized cyt *c*, a rapid (within 5 min) and sustained (maintained for at least 2 hr) increase in cellular ROS level was detected by measurement of the fluorescent increase of DCF. The total pool of accumulated ROS was partially (~60%) decreased by the pan-caspases inhibitor zVAD-fmk, but there was no detectable effect of caspases inhibitor on the initial rapid ROS burst (5~30 min). Combined with the results, it was suggested that the initial ROS burst (5~30 min) might be induced through a caspase-independent pathway. On the other hand, antioxidant was usually used to determine the involvement of ROS in the caspase cascade. N-acetylcysteine (NAC) can protect cells from the effects of ROS. [20], therefore preincubation with NAC (2.5 mM) could almost prevent the accumulation of cellular ROS as depicted in (Fig. **1B**). Furthermore, the results of western blot showed that the activation of caspases was also totally abolished by NAC pretreatment (Fig. **1A**).

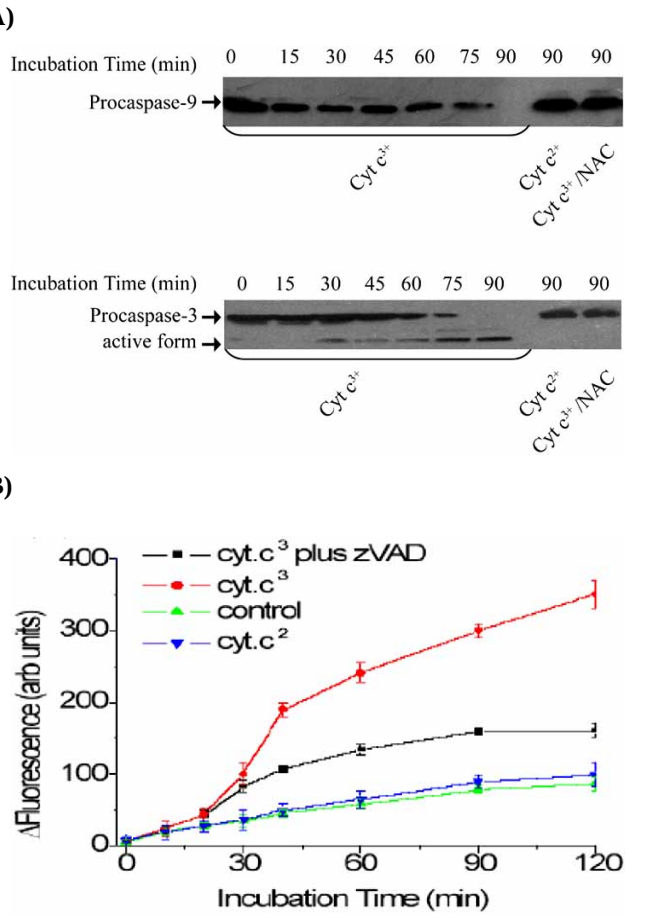

Figure 1. Effects of reduced and oxidized cyt *c* on activation of caspases and cellular ROS.
Preincubation of permeabilized cells with oxidized or reduced cyt *c* for a different time, (A) an immunoblot analysis was performed for the caspase assay mixture containing either oxidized or reduced cyt *c* using specific antibodies against caspase-3 and caspase-9. (B) Cellular ROS accumulation was monitored fluorimetrically.

Therefore, it was indicated that the initial ROS burst was indeed contributed to the activation of caspases, and the activation of caspases feedforward amplify the ROS generation. Finally, as expected, the reduced cyt *c* had no effect on cellular ROS level (Fig. **1B**), further illustrating that the oxidized cyt *c* exhibits its cell toxicity most if not all through up-regulating cellular ROS level.

2. Mitochondrial Impairment by Incubation with Oxidized Cyt *c*

In view of the fact that the cellular ROS accumulation caused by oxidized cyt *c* was so prominent, experiments were performed to determine the impairment of mitochondria. To characterize this process, the cationic lypophilic fluorochrome 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6$], was used to evaluate whether the up-regulation of cellular ROS was associated with the disruption of Δψm.[17] Results showed that $DiOC_6$ dye uptake remained essentially unaltered during the first 30 min after oxidized cyt *c* treatment, a time at which cellular ROS had been significantly accumulated. A moderate reduction in Δψm was detected 30 min after incubation with oxidized cyt *c*, which seemed at a time coinciding with a dramatic increase in caspase activity (Fig. **2A**). Once initiated, the depolarization of mitochondrial membrane potential was a rapid process, taking 60 min or less to reach completion. After 15 min-incubation with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 μM), the entire cell population exhibited a dramatic decrease in fluorescence, confirming that the $DiOC_6$ dye was sensitive to mitochondrial transmembrane depolarization (data not shown). When cells were left pretreated with zVAD-fmk, the loss of Δψm was markedly reduced by the pancaspase inhibitor (Fig. **2A**).These data suggested that activated caspases impinge on the mitochondria to induce mitochondrial permeability transition (PT) and a subsequent loss in Δψm during apoptosis provoked by exogenous oxidized cyt *c*. Similar findings have been reported in UVB irradiation-induced apoptosis, in which caspase inhibitor were able to block PT-induced Δψm loss.[21] The open of PT pore would facilitate the release of proapoptotic proteins to cytosol, therefore the time-dependent loss of cyt *c* in mitochondria was detected by immunoblot analysis. Mitochondrial cyt *c* did not decrease until 30 minutes-incubation with oxidized cyt *c* (Fig. **2B**), which seemed to occur at the same time or prior to the time of a decrease of Δψm.

Since the Δψm loss was effectively blocked by the caspase inhibitor zVAD-fmk, we then tested whether the redistribution cyt *c* from mitochondria into cytosol would also relay on caspases activation. Cells were left either untreated or pre-incubated with zVAD-fmk and then induced to undergo apoptosis by exposure to oxidized cyt *c* for 60 min. As shown in (Fig. **2C**), pre-treatment of cell with zVAD-fmk could partially inhibit the mitochondrial loss of cyt *c*. Therefore, it was suggested that the decrease in Δψm was not critical for the release of cyt *c* into cytosol in this cases, and the release of cyt. *c* was through caspase-dependent and –independent pathway.

It has been known that the proapoptotic Bcl-2 family protein Bax can directly release cyt *c* from mitochondria, and ROS can facilitate this procedure.[22, 23] There-

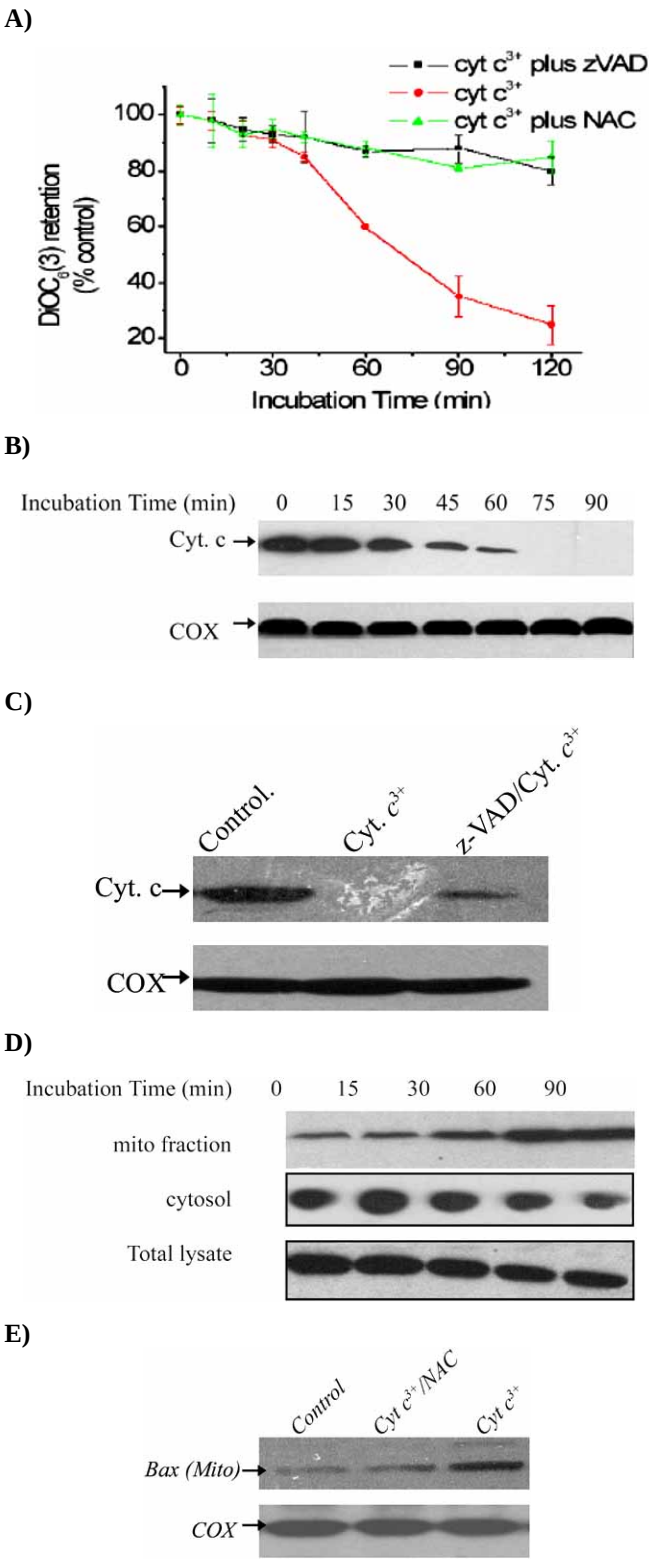

Figure 2. Impairment of mitochondrial function by oxidized cyt *c*. After pre-incubation with oxidized cyt *c* for a different time, (A) mitochondrial membrane potential was monitored fluorimetrically; (B) The retention of cyt *c* in mitochondria was detected by immunoblot analysis; (C) The effect of zVAD-fmk on the cyt *c* release was detected by immunoblot analysis; (D) The translocation of Bax to OMM was monitored by immunoblot analysis; (E) The inhibitor effects of NAC on the translocation of Bax was detected by immunoblot analysis.

fore, it was interested in whether Bax translocation was involved in the cyt *c* release. As shown in (Fig. **2D**), oxidized cyt *c* trigger a slight increase of Bax in OMM at the incubation time about 15 min. The mitochondria from untreated cells contained some detectable Bax (shown in Fig. **2D**) might due to the cytosol contamination. Therefore, the translocation of Bax occurred at the time of or proceeding the release of cyt *c*, suggesting its translocation might be involved in release of cyt *c* to cytosol through caspase-independent way. Pre-treatment cell with NAC could completely suppress the accumulation of Bax in OMM induced by oxidized cyt *c* as shown in (Fig. **2E**), suggesting that ROS might be involved in the Bax translocation and the release of cyt *c*. Combined, the data suggested that dedication of ROS accumulation, especially the initial caspase-independent ROS increase, to the loss of $\Delta\psi m$ and Bax translocation plays a pivotal role in the oxidized cyt *c*-induced mitochondrial dysfunction. As expected, reduced cyt *c* totally abolished the deteriorated effect on mitochondria (data not shown).

3. Oxidized Cyt c Promotes H_2O_2 Generation

To address the mechanism of the oxidized cyt *c*-induced ROS increase, we examined whether oxidized cyt *c* could induce the ROS generation *in vitro* experiments. In the absence of other oxidants, the transformation of the nonfluorescent $DCFH_2$ to the fluorescent DCF was significantly promoted by addition of oxidized cyt *c* (Fig. **3A**) with the concurrent increase of O_2 consumption (Fig. **3B**). In contrast, the reduced cyt *c* had no effect on $DCFH_2$ oxidation and O_2 consumption. Moreover, reduced cyt *c* suppressed the auto-oxidation of $DCFH_2$ and consequently decreased the consumption of O_2 (Fig. **3A, B**). Interdicted of O_2 consumption, fluorescent DCF increase was totally blocked in oxidized cyt *c* system (Fig. **3A**). Furthermore, preincubation of catalase could completely abolish the ability of oxidized cyt *c* to promoting $DCFH_2$ oxidation (Fig. **3A**). As catalase is a special enzyme to H_2O_2, it was suggested that endogenous H_2O_2 could be formed in the oxidized cyt *c* system to promoting the oxidation of $DCFH_2$.

An HPLC method was developed to gain further evidences. All elution buffers were bubbled with N_2 for 30 min to minimize the O_2 effect. The elution of standard H_2O_2 gave a peak at a retention time of 2.1 min (Fig. **4A**). Then elution of oxidized cyt *c* solution (preincubated with O_2) resulted in two elution peaks (Fig. **4B** (I)). The retention time of the second elution peak was 5.5 min. Fig. **4B** (II) showed the optical absorption spectrum of the collection of second elution peak. The Soret maximum of 409 nm indicated that the eluted protein was cyt *c* and stayed oxidized. The first elution peak was with the same retention time of H_2O_2, and this elute peak collection can quickly blank the KMnO4/H+ solution and decrease the fluorescence of scopoletin/horseradish peroxidase (data not shown), giving a sound evidence that H_2O_2 is indeed formed in oxidized cyt *c* solution. Injection of the reduced cyt *c* resulted in only one peak with the retention time of 6.6 min (Fig. **4C** (I)), which was later than that of oxidized cyt *c*. Fig. **4C** (II) showed the recorded spectrum of the peak collection. Spectrum exhibited a red shift of the Soret maximum from 409 nm to 413 nm with a concomitant increase in the extinction coefficient together with appearance of Q bands at 520 and 550 nm, suggesting the protein was the reduced cyt *c*. Therefore, it was evident that oxidized cyt *c* can promote H_2O_2 generation with the O_2 consumption.

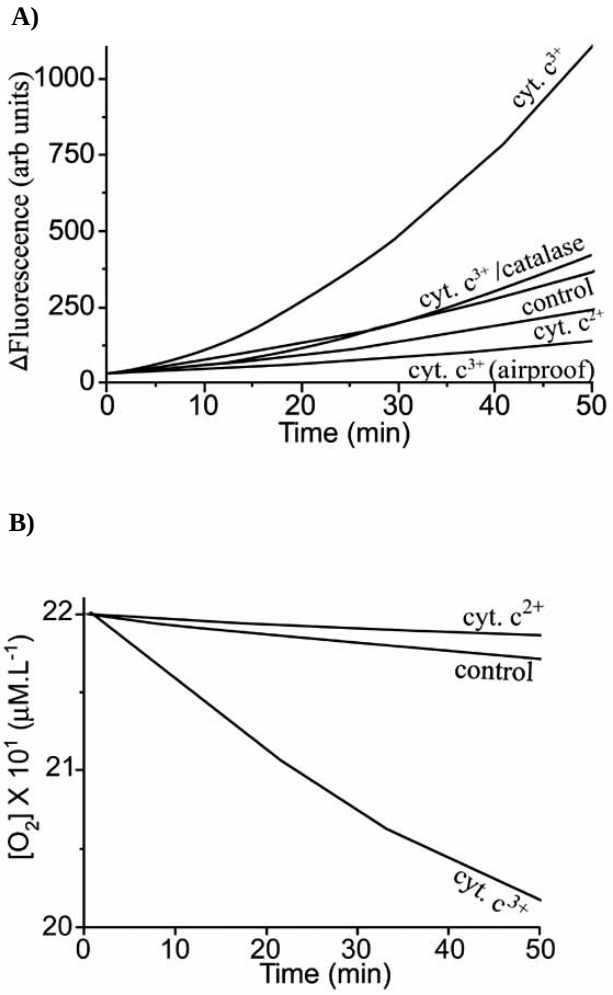

Figure 3. Promotion of H_2O_2 generation by oxidized cyt c.
$DCFH_2$ (10 μM) was incubated at 25°C in 50 mM Tris-HCl (pH 7.4) buffer containing 50 μM DTPA, with in the absence of any additional reagents or in the presence of 0.25 μM cyt c^{3+}, 0.25 μM cyt c^{3+} plus 100 μg/ml catalase, or 0.25 μM cyt c^{2+}. DCF generation was monitored fluorimetrically (A) and O_2 consumption was detected oxymetrically (B).

DISCUSSION

Despite considerable studies focusing on whether the redox state of cyt c has the ability to regulate cell apoptosis in cytosol [12-14], assigning an emergent conclusion to this issue has been still difficult. Recent findings presented evidence indicating the oxidized form of cyt *c* is important for the engagement of the caspase cascade in the absence of any other apoptotic inducer. In the current study, we used the permeabilized cell system to further investigate the mechanism of cyt *c* redox state on the regulation of cell apoptosis. Our observations confirm that oxidized cyt *c*, but not reduced cyt *c*, contribute to the caspases activation and the mitochondrial dysfunction in a time-dependent manner, which is accordant with the previous reports [14,15]. Furthermore, the ability of oxidized cyt *c* to promote caspases

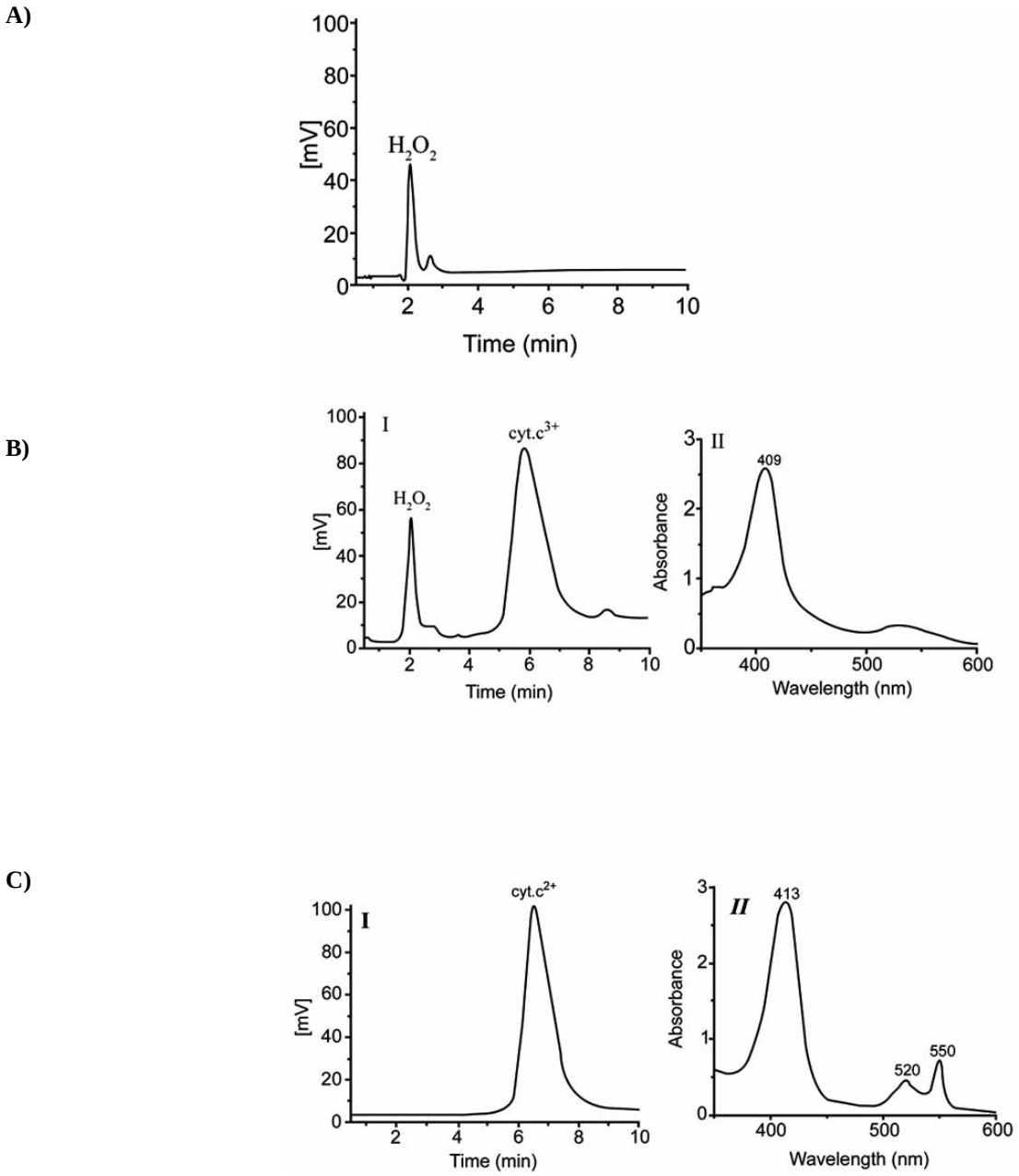

Figure 4. HPLC analysis of cyt *c* and H_2O_2.
Chromatography was performed using an analytical ZORBAX OLIGO column. (A) Chromatograms of standard H_2O_2; (B) Chromatograms of oxidized cyt *c* incubated with O2 for 4 h (I) and the optical absorption spectrum of the second elute collection (II); (C) Chromatograms of reduced cyt *c* (I) and the optical absorption spectrum of the elute collection (II).

activation mainly depends on the initial rapid accumulation of cellular ROS. The ATP synthesis was not apparent deteriorated until 40 min treatment with oxidized cyt *c* (data not shown). In addition, pre-incubation of CsA can almost prevent the loss in $\Delta\psi m$, but has no effect on the initial ROS burst (data not shown), suggesting that mitochondria were not the main source of the initial ROS burst. *In vitro* system, the likelihood of oxidized cyt *c* being a potent catalyst promoting H_2O_2 generation was testified, and there seem a little reason to suspect that this aerobic reaction could not proceed *in vivo*. This can account for oxidized cyt *c*-induced rapid ROS increase in the cell system when no detectable activation of caspases and dysfunction of mitochondria. These results indicate that cell with cytosolic cyt *c* is not destined to death, and the redox state of cyt *c* might be a key regulator of apoptosis in some conditions. It is consistent with the finding that cells are resistent to apoptotic stimuli when are in a high redox potential with abundant thiol compounds present [7,8]. In the high redox potential, the cytosol cyt *c* staying in reduced state might have no ability to promote ROS accumulation and consequently cannot promote the caspase cascade. The PT pore opening is also strongly promoted by an oxidized state of PN and of critical dithiols at discrete sites, so the reduced cyt *c* has no effect on the impairment of mitochondria. Therefore, the ability of oxidized cyt *c*-induced caspase activation to induce PT pore opening, which in turn

can induce further caspase activation (by release of cyt *c* and other IMS proteins), creates opportunities for a feedforward amplification loop, suggesting that the early released cyt *c* may act as a regulator in the initiation of apoptosis at appropriate time.

In summary, the redox state of cytosolic cyt *c* might be the regulatory step leading to an elusive commitment point for the engagement of apoptosis in some conditions. Clearly, additional studies are required to investigate the biochemical mechanisms by which oxidized cyt *c* regulates H_2O_2 generation at the level of cell.

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