Regulation of apoptosis by respiration: cytochrome *c* release by respiratory substrates

Goshi Nishimura[a], Rita J. Proske[b], Hiroko Doyama[b], Masahiro Higuchi[a,c,*]

[a] *Department of Molecular Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*
[b] *Research Center for Cardiovascular Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, The University of Texas-Houston Health Science Center, Houston, TX 77030, USA*
[c] *Department of Neurology, Baylor College of Medicine, and VA Medical Center Neurology Svc., Bldg 100, Room 2B 114, 2002 Holcombe Blvd, Houston, TX 77030, USA*

Received 21 August 2001; accepted 27 August 2001

First published online 6 September 2001

Edited by Vladimir Skulachev

Abstract Cytochrome *c* release from mitochondria is essential for apoptosis. Using human myelogenous leukemia ML-1a, its respiration-deficient and reconstituted cells, we demonstrated that respiratory function is essential for tumor necrosis factor-induced cytochrome *c* release. In a cell free system using mitochondrial fraction from ML-1a, initiation of respiration by substrates for complexes I, II, and III but not IV released cytochrome *c*, suggesting that reduction of coenzyme Q or complex III is essential for cytochrome *c* release. In the same system, disruption of mitochondrial outer membrane was neither enough nor the cause for cytochrome *c* release by succinate. These observations define an early pathway in which a change in respiration releases cytochrome *c*. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cytochrome *c*; Apoptosis; Mitochondrial respiration

1. Introduction

Changes in mitochondrial function are thought to be involved in apoptosis induction. Several reports identified molecules in mitochondria that can activate downstream apoptosis signals. Cytochrome *c* is first described as one of such molecules and released from mitochondria, which leads to the activation of caspase 3 [1]. Cytochrome *c* released from mitochondria forms a complex with Apaf-1 to catalyze the activation of caspase 9 in a dATP-dependent manner [2]. Activated caspase 9 cleaves and activates caspase 3 leading to apoptosis. It is very likely that cytochrome *c* release from mitochondria can induce apoptosis, however several other reports imply different roles of cytochrome *c* in apoptosis signaling [3,4]. Apoptosis-inducing factor [5], which can cause nuclear condensation [6], is also released from mitochondria under apoptotic conditions. Several reagents can open mitochondrial permeability transition pores, leading to a release of apoptosis-inducing factor [7]. Other reports indicate the involvement of mitochondrial caspases 2 and 9 in apoptotic signaling [8]. These reports indicate that a change in mitochondria releases certain molecules, which are responsible for apoptosis signaling.

It has been believed that caspase 8 directly activates caspase 3 [9,10]. However, several reports indicate the synergistic action of caspase 8 and mitochondria to activate caspase 3 [11], and the involvement of cytochrome *c* in caspase 8 activation [12]. In Fas-induced apoptosis signaling, activation of caspase 8 can cleave bid to associate to mitochondria, which results in cytochrome *c* release [13,14]. We and others showed that cytochrome *c* release was observed prior to caspase 3 activation in tumor necrosis factor (TNF) and UVB/staurosporine-induced apoptosis signaling [15]. Therefore, it is likely that cytochrome *c* release might be important prior to or at the same time with the activation of caspase which is the critical event in apoptosis signaling.

We have previously shown the requirements of mitochondrial respiratory function in TNF-induced apoptosis signaling by using a panel of respiration-deficient clones and their reconstituted cybrids with normal mitochondria [16]. Our study placed mitochondrial respiratory function at a step prior to the activation of a caspase 3-like protease in TNF-induced apoptosis signaling. We also showed that an antioxidant-sensitive pathway is involved prior to cytochrome *c* release in TNF-induced apoptosis signaling [17]. Since reactive oxygen species (ROS) are mainly generated dependent on mitochondrial respiration, and can induce cytochrome *c* release [18], the antioxidant-sensitive pathway might be regulated by mitochondrial respiratory function.

In this paper, we investigated the role of respiratory function in cytochrome *c* release. We demonstrated that the electron transfer through mitochondrial respiratory chain (MRC) complex II–III–IV is responsible for cytochrome *c* release by TNF. We also showed that the reduction of MRC by specific MRC substrates could release cytochrome *c* in cell free systems.

2. Materials and methods

2.1. Establishment of respiration-deficient and reconstituted clones

Respiration-deficient and reconstituted clones were established and maintained as described [16].

*Corresponding author. Fax: (1)-713-794 7786.
E-mail address: mhiguchi@bcm.tmc.edu (M. Higuchi).

Abbreviations: TNF, tumor necrosis factor; ROS, reactive oxygen species; CHX, cycloheximide; TTFA, thenoyltrifluoroacetate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; MRC, mitochondrial respiratory chain

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PII: S0014-5793(01)02859-9

2.2. DNA fragmentation assay

DNA fragmentation induced by TNF was assayed by the modified method as described [19].

2.3. Respiration measurement

Oxygen consumption was measured with a Clark oxygen electrode (model 5300; Yellow Spring Instrument Co., Yellow Spring, OH, USA) as described [20].

2.4. Cytosolic fraction

After cells were washed with phosphate-buffered saline, 20 μl of buffer (250 mM sucrose, 30 mM Tris, pH 7.7, 1 mM EDTA, 5% glycerol, 1 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) was added, and the cells were disrupted by douncing 80 times in a 0.3 ml Kontes douncer with the B pestle (Kontes Glass Company). Under these conditions, most of the cell membrane is damaged with no change in mitochondrial respiratory activity.

2.5. Mitochondrial fraction

0.5–2×10^8 ML-1a cells were suspended in 1 ml sucrose buffer (250 mM sucrose, 30 mM Tris–HCl, pH 7.7) and the cell membrane was disrupted by N_2 cavitation (250 psi for 5 min). Under these conditions, most of the cell membrane is damaged with no change in mitochondrial respiratory activity. Next, DNA and nuclear were removed by centrifugation (1500×*g*, 30 s). Supernatant fraction was centrifuged (16 000×*g*, 10 min) and the pellet was suspended in sucrose buffer and used as mitochondrial fraction. To investigate cytochrome *c* release and oxygen consumption, 2 mM KH_2PO_4, 1 mM ADP and an indicated amount of substrate for MRC complexes were added to initiate state 3 respiration.

2.6. Detection of cytochrome c release from mitochondrial fraction by Western blotting

Detection of cytochrome *c* release was done by Western blotting analysis as described previously [17].

2.7. Flow cytometry of mitochondrial fraction

Detection of outer membrane disruption by anti-cytochrome *c* oxidase was assayed by the modified method as described [21].

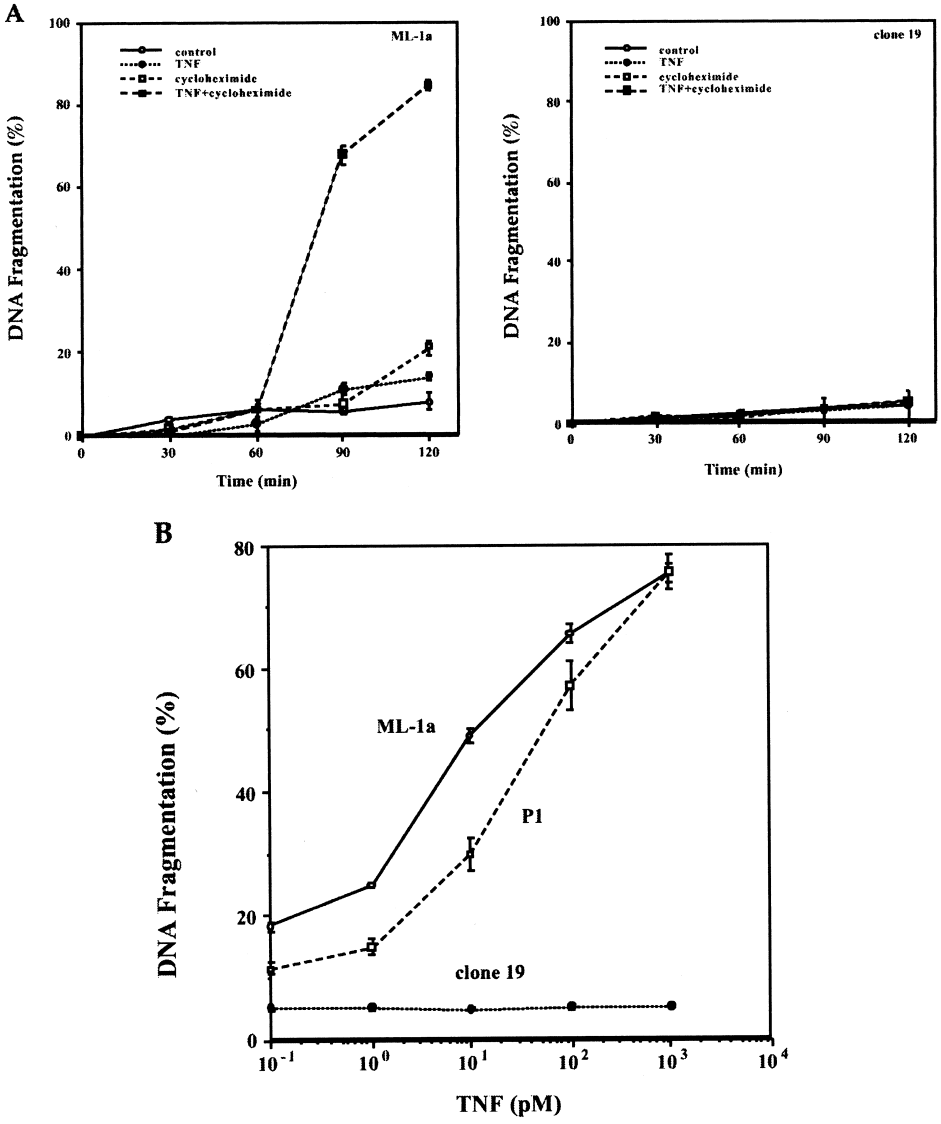

Fig. 1. A: Time-dependent induction of apoptosis by TNF and CHX in ML-1a and clone 19. B: Dose-dependent induction of apoptosis by TNF in ML-1a, clone 19 and P1 in the presence of CHX. A: [^{3}H]TdR prelabeled ML-1a (left) or clone 19 (right) were incubated with or without 1 nM TNF in the presence or absence of 1 μg/ml CHX for indicated times, and fragmented DNA was determined as described in Section 2. B: [^{3}H]TdR prelabeled ML-1a, clone 19 and P1 were incubated with an indicated amount of TNF in the presence of 1 μg/ml CHX for 90 min, and fragmented DNA was determined as described in Section 2.

3. Results and discussions

To investigate whether respiratory function is necessary for apoptosis signaling, especially cytochrome *c* release, which leads to caspase 3 activation, we used ML-1a, respiration-deficient clone 19, and the reconstituted clone P1. Respiration-dependent changes in electron transfer in the MRC might be inhibited in respiration-deficient cells and recovered in reconstituted clone. Fig. 1A shows the time-dependent effect of TNF, protein synthesis inhibitor cycloheximide (CHX) and both of TNF and CHX in ML-1a (left) and in clone 19 (right). In ML-1a, TNF in the presence of CHX showed a very strong apoptosis-inducing effect. However, CHX or TNF alone showed very little effect. In contrast, even TNF in the presence of CHX had no effect in clone 19. We also tested the dose-dependent effect of TNF in the presence of CHX on ML-1a, clone 19 and reconstituted clone P1 (Fig. 1B). The apoptosis-inducing effect of TNF was completely inhibited in clone 19 and recovered in P1.

Next, we investigated cytochrome *c* release. ML-1a, clone 19, and P1 were treated with TNF and CHX for 90 min, and tested for their effect on cytochrome *c* release from mitochondria (Fig. 2A). TNF in the presence of CHX could release cytochrome *c* in ML-1a cells. However, we could not observe an increase in TNF-induced cytochrome *c* release in clone 19. Furthermore, an increase in cytochrome *c* release by TNF was also observed in clone P1. TNF or CHX alone had little effect on releasing cytochrome *c* in ML-1a [17]. These results suggest that respiratory function is involved prior to cytochrome *c* release in TNF-induced apoptosis.

MRC is composed of five enzyme complexes: NADH: ubiquinone oxidoreductase (complex I), succinate: ubiquinone oxidoreductase (complex II), ubiquinone: ferricytochrome *c* oxidoreductase (complex III), ferrocytochrome *c*: oxygen oxidoreductase (complex IV), and F_0F_1-ATPase (complex V). Complexes I, II, III, and IV plus coenzyme Q and cytochrome *c* make up the MRC and complex V generates ATP by coupling with the membrane potential generated at complexes I, III, and IV. Several reports imply that inhibition of complexes I and III can induce apoptosis [22,23] possibly through the regulation of the redox state of mitochondria and the generation of ROS. We previously showed that inhibition of complex I by TNF can release cytochrome *c* in the presence of protein synthesis inhibitor CHX through an antioxidant-sensitive fashion [17]. Therefore, we investigated the role of electron transfer on cytochrome *c* release. To investigate whether electron transfer in the MRC might be involved in TNF-induced apoptosis, we tested the ability of inhibitors of complexes I and II (the entrances of electrons to MRC) to inhibit TNF-induced cytochrome *c* release. Thenoyltrifluoroacetate (TTFA), an inhibitor of complex II, may inhibit electron flow through complex II–III–IV, and rotenone, an inhibitor of complex I, may inhibit electron flow through complex I–III–IV. We previously showed that rotenone in the presence of CHX induced apoptosis and cytochrome *c* release, but rotenone alone showed very little effect on apoptosis induction and cytochrome *c* release [17]. Now we confirmed that rotenone alone as well as TTFA had no effect on cytochrome *c* release. TTFA inhibited cytochrome *c* release induced by TNF plus CHX in vivo, but rotenone increased cytochrome *c* release induced by TNF plus CHX, suggesting that electron flow through complex II–III–IV might be essential in cytochrome *c* release induced by TNF plus CHX (Fig. 2B). Although we used the specific inhibitors of complexes I and II, other non-specific action needs to be considered. Especially, since TTFA can work as a chelating reagent, it might work as an antioxidant.

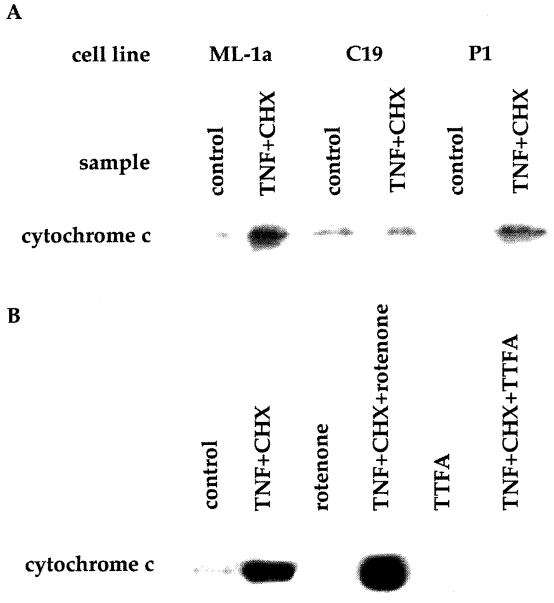

Fig. 2. Respiratory function-dependent cytochrome *c* release by TNF in vivo. A: ML-1a, clone 19, and P1 cells were incubated with 1 nM TNF in the presence of 1 μg/ml CHX for 90 min. After the cytosolic fraction was obtained, cytochrome *c* release from mitochondria was detected by Western blotting as described in Section 2. B: ML-1a was incubated with or without 1 nM TNF and 1 μg/ml CHX in the presence or absence of 10 μM rotenone or 0.25 mM TTFA for 90 min, and cytochrome *c* release from mitochondria was detected as described in Section 2.

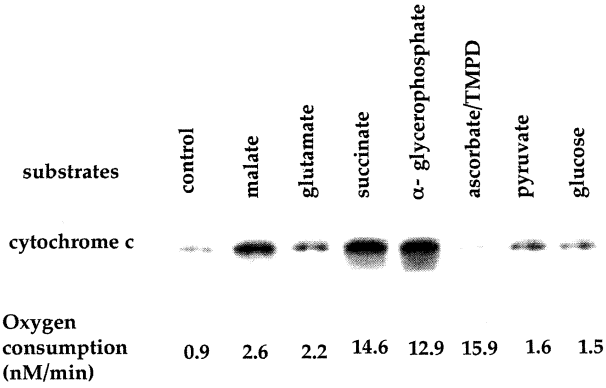

Fig. 3. Cytochrome *c* release by respiratory substrates in vitro. Mitochondrial fraction from ML-1a was incubated with substrates of each complex and the released cytochrome *c* and oxygen consumption were detected as described in Section 2.

To further investigate the roles of electron transfer in cytochrome *c* release, we tested the effect of several respiratory substrates on cytochrome *c* release in a cell free system. Mitochondrial fraction from ML-1a was incubated in the presence of malate, glutamate, pyruvate (complex I substrates), succinate (complex II substrate), α-glycerophosphate (complex III substrate), ascorbate with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) (complex IV substrate) and glucose for 15 min at 37°C. Respiratory activity of these sub-

strates was also investigated. Of the complex I substrates, malate is the most effective for cytochrome *c* release and oxygen consumption, glutamate is a weaker inducer of cytochrome *c* release and oxygen consumption, and pyruvate is a poor effector. Complex II substrate succinate and complex III substrate α-glycerophosphate were good inducers of cytochrome *c* release and oxygen consumption. Addition of ascorbate/TMPD to mitochondrial fraction increased oxygen consumption but failed to release cytochrome *c*. Glucose has a slight effect on both oxygen consumption and cytochrome *c* release (Fig. 3). Substrates for complexes I, II, and III but not IV released cytochrome *c*. Malate and glutamate reduce complex I, coenzyme Q, complex III, cytochrome *c* and complex IV; succinate reduces complex II, coenzyme Q, complex III, cytochrome *c* and complex IV; α-glycerophosphate reduces coenzyme Q, complex III, cytochrome *c* and complex IV; and ascorbate/TMPD reduces cytochrome *c* and complex IV. Therefore, reduction of coenzyme Q and/or complex III might be necessary for cytochrome *c* release. Since this is one of the most effective sites to reduce an oxygen molecule and generate ROS [24], ROS generation from this site might be followed by cytochrome *c* release. Significant release of cytochrome *c* by succinate was observed within a minute after treatment, and then cytochrome *c* release increased in a time-dependent fashion (Fig. 4A). In contrast, in the control, we could not see any cytochrome *c* release up to 10 min, and at 15 min, a small amount of cytochrome *c* release was observed (Fig. 4A). Cytochrome *c* was released in a dose-dependent fashion by succinate and a 20 mM succinate treatment for 15 min released 70% of total mitochondrial cytochrome *c* (Fig. 4B).

We investigated the mechanisms of how cytochrome *c* is released from mitochondria after succinate, a complex II substrate, treatment. One possible mechanism that releases cytochrome *c* is the disruption of mitochondrial outer membrane, which can be inhibited by overexpressing Bcl-xl [21]. We tested whether the mitochondrial outer membrane is disrupted by the ability of a much larger protein (160 kDa) to gain access to the intermembrane space by using an antibody to cytochrome *c* oxidase subunit IV after succinate and digitonin treatment. Digitonin in a low concentration is known to disrupt cellular membrane and mitochondrial outer membrane but not the inner membrane [25]. As shown in Fig. 5A, control mitochondrial fraction and mitochondrial fraction treated with succinate bound to the cytochrome *c* oxidase antibody to the same extent. The failure of these samples to show specific staining suggests that the outer membrane is intact after succinate treatment. In contrast, there is enhanced and specific staining with the cytochrome *c* oxidase antibody to digitonin-treated mitochondrial fraction.

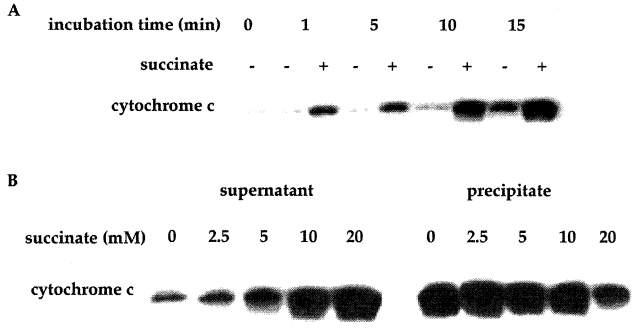

Fig. 4. A: Time course of succinate-dependent cytochrome *c* release. B: Dose response of succinate on cytochrome *c* release from and remaining in mitochondria. Mitochondrial fraction from ML-1a was incubated with 5 mM (A) or the indicated amount (B) of succinate for the indicated times (A) or 15 min (B) at 37°C and the released cytochrome *c* was detected by Western blotting as described in Section 2.

Next, we investigated whether succinate treatment generated a pore size smaller than the antibody and larger than cytochrome *c* (12 kDa). Mitochondrial fraction from ML-1a was incubated with succinate or digitonin, and oxygen consumption in the presence or absence of cytochrome *c* in the buffer was measured by using ascorbate/TMPD, which can reduce cytochrome *c* (Fig. 5B). As expected, oxygen consumption of succinate-treated mitochondria was greatly reduced, since cytochrome *c* was released by succinate treatment. The addition of cytochrome *c* reversed the inhibition of oxygen consumption, suggesting that added cytochrome *c* could penetrate mitochondrial outer membrane and reduce complex IV in the presence of ascorbate/TMPD. In contrast, inhibition of oxygen consumption by digitonin was small and the effect of cytochrome *c* addition on ascorbate/TMPD-dependent oxygen consumption in digitonin-treated mitochondria was comparable to that in control mitochondria.

We also examined the release of cytochrome *c* from mitochondria by succinate and digitonin. Digitonin could release cytochrome *c*, however, the amount of cytochrome *c* released by digitonin was extremely smaller than that by succinate (Fig. 5C). In conclusion, although disruption of the mitochondrial outer membrane can release cytochrome *c* by digitonin treatment, initiation of respiration through complex II–III–IV by succinate released considerably more cytochrome *c* without disrupting the mitochondrial outer membrane.

Several mechanisms have been considered to release cytochrome *c* from mitochondria. Several factors such as oxygen concentration, coupling/uncoupling, the state of respiration, the concentration of respiratory substrate, and antioxidants can control ROS generation from mitochondria [26]. ROS generation has been considered one of the causes of cytochrome *c* release. Therefore, the increase in ROS generation by the change of mitochondrial respiratory substrate might be one of the causes for cytochrome *c* release.

Since family members of Bcl-2 work as anti- or pro-apoptotic molecules and their structure bears striking resemblance to diphtheria toxin which forms a channel to the membrane [27], the formation of pores to the mitochondrial outer membrane has been considered one of the causes of apoptosis. Fig. 5A,C indicates that disruption of mitochondrial outer membrane alone is not sufficient and is not the cause of cytochrome *c* release by succinate. These results indicate that cytochrome *c* needs to be stabilized inside the outer membrane under non-apoptotic condition.

One of the possible mechanisms, which can stabilize cytochrome *c*, is the association of cytochrome *c* with the mitochondrial inner membrane. Cytochrome *c* is located on the mitochondrial inner membrane, this association is dependent on its interaction with acidic lipid cardiolipin and is regulated by the redox state [28]. Generation of ROS from the coenzyme Q site might also regulate oxidation of cardiolipin and affect cytochrome *c* release. Furthermore, the stimulation of oxidative metabolism induces the desorption of cytochrome *c* pool from the inner membrane into the intermembrane space

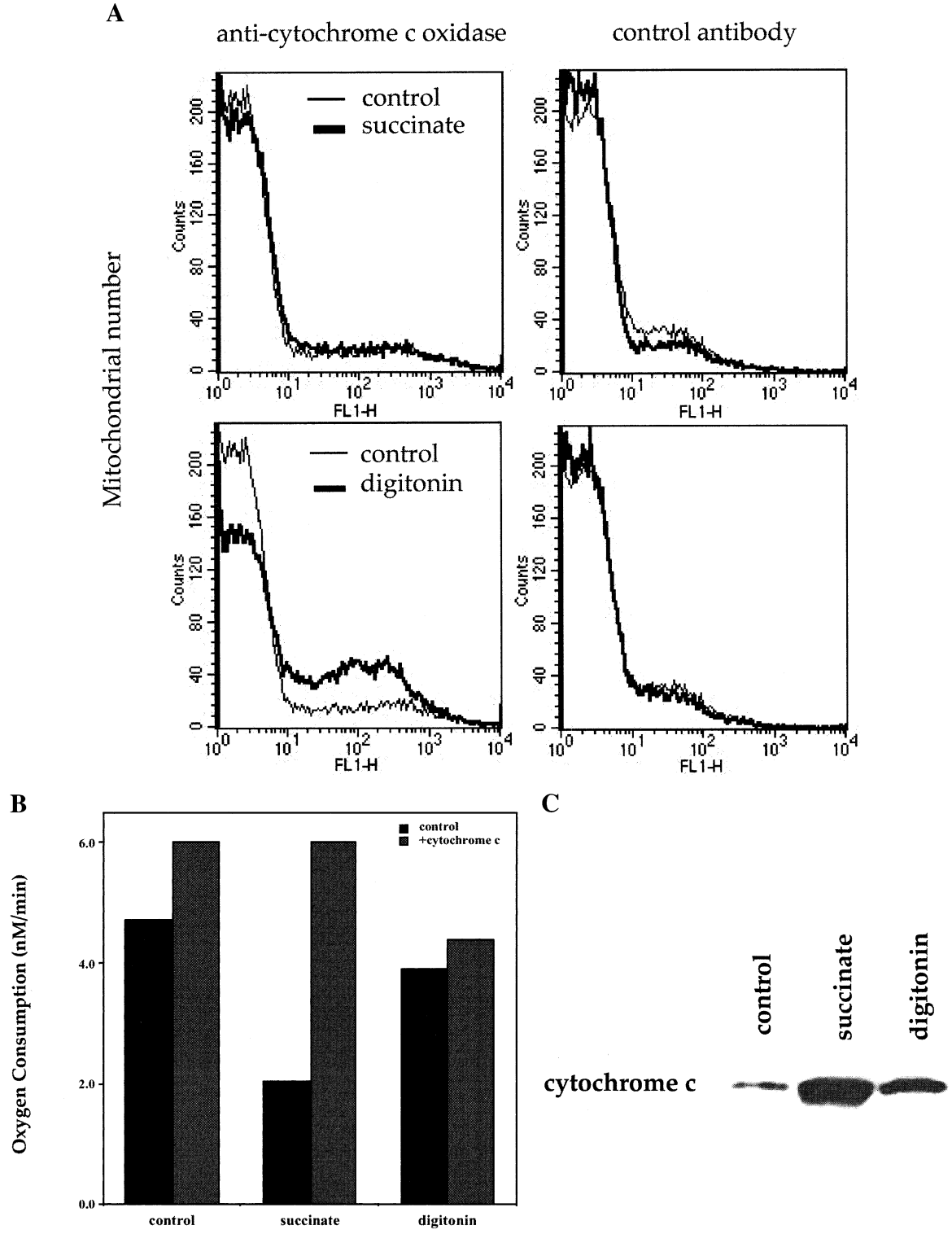

Fig. 5. The effect of succinate and digitonin on mitochondria for outer mitochondrial membrane disruption (A), for cytochrome *c* penetration (B), and for cytochrome *c* release (C). Mitochondrial fraction from ML-1a was incubated with 10 mM succinate or 12 μM digitonin for 15 min at 37°C and washed. The binding to monoclonal antibody, to cytochrome *c* oxidase subunit IV or control antibody was determined as described in Section 2 (A), oxygen consumption was determined in the absence or presence of 100 μM cytochrome *c* using ascorbate/TMPD as a substrate as described in Section 2 (B), and cytochrome *c* release from mitochondrial fraction was detected by Western blotting as described in Section 2 (C).

[29]. Therefore, the reduction of the MRC (possibly coenzyme Q or complex III) by respiratory substrates increases the amount of cytochrome *c* in the intermembrane space, overcomes the stabilizing effect located inside the outer membrane, and finally induces cytochrome *c* to penetrate the outer membrane by unknown mechanisms. Even though we could not detect the pore which enables anti-cytochrome *c* oxidase antibody to access the inner membrane from outside, cytochrome

c (molecular weight 12 kDa) which is added from outside of the mitochondrial outer membrane can access the inner membrane in succinate-treated mitochondria (Fig. 5B).

In addition, the hinge protein is very acidic, connects cytochrome *c*1 and cytochrome *c* [30], and is reported to regulate cytochrome *c* release leading to apoptosis [31]. We can speculate that the association of cytochrome *c* to the hinge protein can be regulated by respiratory substrates and may affect the desorption of cytochrome *c* pool from the inner mitochondrial membrane into the intermembrane space. In each case, cytochrome *c* needs to penetrate the outer membrane, and such a mechanism including the roles of ROS induced by respiratory substrate is now under investigation.

Acknowledgements: This research was partially supported by a Grant 98BG287 from the American Heart Association to M.H.

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