Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome *c* dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis

Giuseppe Petrosillo, Francesca Maria Ruggiero, Marilva Pistolese, Giuseppe Paradies*

Department of Biochemistry and Molecular Biology and C.N.R Unit for the Study of Mitochondria and Bioenergetics, University of Bari, Via E, Orabona 4, 70126, Bari, Italy

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Abstract Cytochrome *c* release from mitochondria is a critical event in the apoptosis induction. Dissociation of cytochrome *c* from the mitochondrial inner membrane (IMM) is a necessary first step for cytochrome *c* release. In the present study, the effect of reactive oxygen species (ROS) on the dissociation of cytochrome *c* from beef-heart submitochondrial particles (SMP) and on the cardiolipin content was investigated. Exposure of SMP to mitochondrial-mediated ROS generation resulted in a large dissociation of cytochrome *c* from SMP and in a parallel loss of cardiolipin. Both these effects were directly and significantly correlated and also abolished by superoxide dismutase+catalase. These results demonstrate that ROS generation induces the dissociation of cytochrome *c* from IMM via cardiolipin peroxidation. The data may prove useful in clarifying the molecular mechanism underlying the release of cytochrome *c* from the mitochondria to the cytosol. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Reactive oxygen species; Cytochrome *c*; Cardiolipin; Apoptosis

1. Introduction

There is a growing evidence for a role of mitochondria in the apoptosis induction. Release of cytochrome *c* from mitochondria appears to be a central event since it activates the caspases cascade for execution phase of apoptotic process [1,2]. The molecular mechanism responsible for this release is still not well understood. It has been shown that cytochrome *c* release from mitochondria is preceded by its dissociation from the inner mitochondrial membrane (IMM) [3]. Thus, an early event in the release of cytochrome *c* from mitochondria is its dissociation from the IMM where it is bound to cardiolipin. The binding of cytochrome *c* to cardiolipin has been extensively studied by NMR and EPR spectroscopy, mainly at the level of artificial membranes such as liposomes, and some molecular aspects of this interaction have been also defined [4–7]. Cardiolipin is a phospholipid particularly rich in unsaturated fatty acids and thus easily susceptible to reactive oxygen species (ROS) attack. ROS, which are produced primarily at the level of the mitochondrial respiratory chain, have been implicated in the regulation of apoptosis [8–10]. It would be therefore expected that oxidative damage to cardiolipin by ROS may alter the interaction of cytochrome *c* with this phospholipid at the level of the IMM and this, in turn, would induce the dissociation of cytochrome *c* from the mitochondrial membrane. According to this, it has been reported, by in vitro studies with liposomes, a loss of molecular interaction between cytochrome *c* and cardiolipin due to lipid peroxidation [11]. In addition, it has been also reported that changes in the cardiolipin content due to oxidative damage [12] or to alteration in its biosynthetic pathway [13] trigger the release of cytochrome *c* from mitochondria in the apoptotic process. Further support to the possible involvement of cardiolipin in the apoptotic process comes from very recent results showing that this phospholipid provides specificity for targeting of tBid to mitochondria [14]. tBid has been shown to induce cytochrome *c* release from mitochondria and liposomes [15,16].

In order to establish more firmly the involvement of ROS and cardiolipin in the release of cytochrome *c* from mitochondria, we have carried out a study on the effect of ROS, produced at the level of mitochondrial respiratory chain, on the dissociation of cytochrome *c* from beef-heart submitochondrial particles (SMP) and on the cardiolipin content. The results obtained demonstrate that mitochondrial-mediated ROS production induces the dissociation of cytochrome *c* from SMP, which can be directly ascribed to ROS-induced peroxidative damage of cardiolipin.

2. Materials and methods

All chemicals used were commercial products of highest available purity. Antimycin A (AA), bovine-heart cardiolipin, bovine erythrocytes superoxide dismutase (SOD) and bovine-liver catalase, were purchased from Sigma Chemical Company.

Beef-heart mitochondria were prepared according to Löw and Vallin [17] and stored in 250 mM sucrose suspension at −20°C.

Beef-heart SMP were prepared essentially as described by Lee et al. [18], except that EDTA was omitted from the sonication medium. Briefly, frozen mitochondrial suspension was thawed and diluted with 250 mM sucrose to a concentration of about 20–30 mg/ml. The mitochondria were then subjected to sonication for 2 min at the maximal output with a Branson (mod. 250) sonifier in an ice bath under N_2 stream. The suspension was diluted with an equal

*Corresponding author. Fax: (39)-80-5443317.
E-mail address: g.paradies@biologia.uniba.it (G. Paradies).

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volume of 250 mM sucrose and centrifuged at 12 000 × *g* for 10 min. The supernatant was decanted and centrifuged at 105 000 × *g* for 50 min. The resulting pellet, consisting of SMP, was washed, suspended in 250 mM sucrose and subsequently frozen.

Proteins were determined by the usual biuret method using bovine serum albumin as standard.

Before treatment, SMP were washed in 50 mM phosphate buffer, pH 7.2, centrifuged and resuspended in the same medium.

The generation of superoxide radical was induced by treatment of the SMP with succinate+antimycin A (AA) [19]. SMP (0.5 mg/ml) were incubated in a reaction medium consisting of 50 mM phosphate buffer, pH 7.2, at 37°C for 1 h in the presence of 10 mM succinate and 10 μM AA. After incubation, SMP were centrifuged at 105 000 × *g* for 50 min. and the supernatant was withdrawn for the determination of the cytochrome *c* content.

Cytochrome *c* content in the supernatant was determined spectrophotometrically at $\Delta_{550-540}$ nm of reduced minus oxidized difference spectra [20] with an HP 8453 diode array spectrophotometer. Extinction coefficient value of 19 $mM^{-1} \times cm^{-1}$ was used. Total cytochrome *c* associated to SMP was determined with the same technique.

Cardiolipin content was determined by the high-performance liquid chromatography technique as previously described [21].

Cardiolipin was peroxidized in the presence of Fe^{2+}–ADP–ascorbic acid as previously described [22].

3. Results

The cytochrome *c* content of SMP was quantified by spectrophotometric analysis. 0.75 ± 0.06 nmol/mg protein of cytochrome *c* was found associated to these particles, a value which is very similar to that reported by others [23,24]. The effect of ROS, produced at the level of the mitochondrial respiratory chain, on the cytochrome *c* dissociation from beef-heart SMP was investigated. ROS were generated by treating SMP with succinate+AA. We have previously reported that this treatment results in a large production of ROS [19]. Preincubation of SMP for 60 min in the presence of succinate+AA, namely under condition of ROS production at the level of mitochondrial respiratory chain, resulted in a large cytochrome *c* dissociation (around 30%) from SMP (Fig. 1). There was no detectable cytochrome *c* dissociation in the control untreated SMP. Addition of SOD+catalase to succinate+AA-treated SMP totally prevented the cytochrome *c* dissociation, this indicating a direct involvement of ROS in this effect. Similar cytochrome *c* dissociation was obtained by treating SMP with Fe–ADP–ascorbate which are known to induce mitochondrial membrane lipid peroxidation.

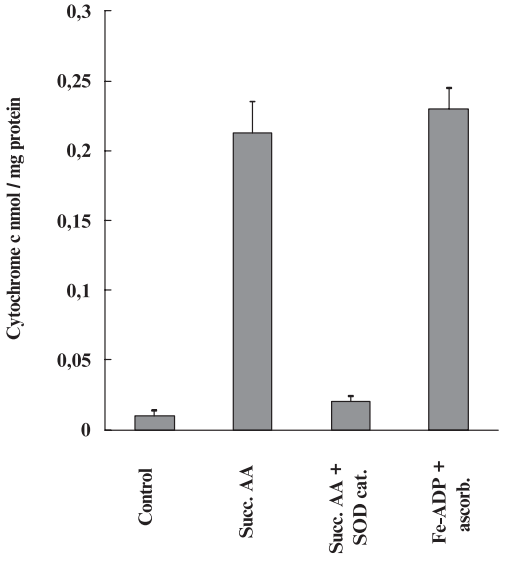

Fig. 1. Oxygen-free radical-induced cytochrome *c* dissociation from SMP and prevention by SOD+catalase. SMP were incubated at 37°C for 60 min in the reaction medium described in the Section 2 in the absence (control) or presence of succinate+AA. After incubation, SMP were centrifuged and the supernatant was withdrawn for the determination of cytochrome *c* content. SOD (68 units) and catalase (94 units) were added to the incubation medium before the addition of succinate+AA. Where indicated, 5 μM Fe^{2+}, 120 μM ADP and 200 μM ascorbate were added in the incubation phase. All values are expressed as mean ± S.E.M. of four independent determinations.

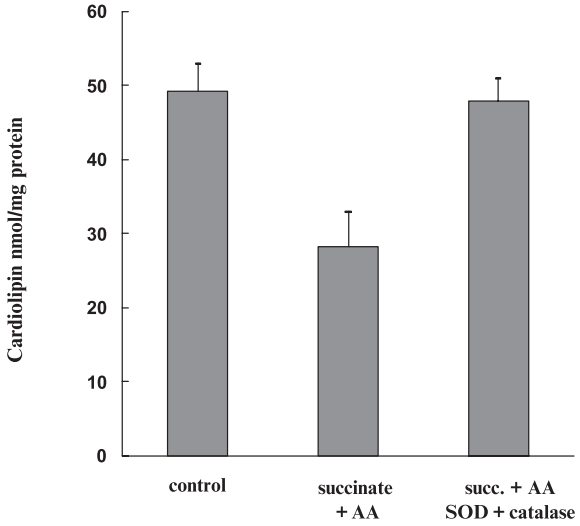

Fig. 2. Oxygen-free radical-induced loss of cardiolipin in SMP and prevention by SOD+catalase. SMP were incubated for 60 min in the absence (control) or presence of succinate+AA. Cardiolipin content of SMP was determined as described in Section 2. SOD (68 units) and catalase (94 units) were added to the incubation medium before the addition of succinate+AA. All values are expressed as mean ± S.E.M. of four independent determinations.

Since cytochrome *c* associates strongly with cardiolipin to the IMM, the ROS-induced cytochrome *c* dissociation from SMP could be due to cardiolipin peroxidation induced by ROS attack. To assess this, the content of cardiolipin was determined in SMP treated with succinate+antimycin. As shown in Fig. 2, the content of cardiolipin decreased in succinate+AA-treated SMP as compared to the value obtained with untreated SMP. Addition of SOD+catalase to succinate+AA-supplemented SMP completely prevented the loss of cardiolipin content.

When a comparison was made between levels of cardiolipin and dissociation of cytochrome *c* in succinate+AA-treated particles at different times of incubation, a strong linear correlation was observed (see Fig. 3). These data provide strong evidence that cardiolipin levels may directly influence cytochrome *c* dissociation from SMP.

More firm and direct evidence for the involvement of cardiolipin peroxidation in the dissociation of cytochrome *c* from SMP comes from the results reported in Fig. 4. It is shown here that exogenous-added cardiolipin to succinate+AA-treated SMP almost completely prevented the cytochrome *c* dissociation from these particles, whilst peroxidized cardiolipin and other phospholipid components of the IMM such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were unable to prevent this release.

4. Discussion

The generation of ROS is considered a major factor in oxidative cell injury via oxidation and subsequent functional impairment of various cellular constituents. The mitochondrial electron transport chain is a major intracellular source of ROS [25], therefore the effects of these reactive species should be greatest at the level of mitochondrial membrane constituents, given that they are highly reactive and short-lived species. Among the mitochondrial constituents, cardiolipin molecules appear particularly susceptible to ROS attack because of their high content of unsaturated fatty acids (90% represented by linoleic acid) and their location in the IMM near to the site of ROS generation. In this regard, recent studies from this laboratory have demonstrated that mitochondrial-mediated ROS generation affects the activity of complexes III and IV of the respiratory chain via cardiolipin peroxidation [19,26]. These results have been useful to explain the molecular basis of the decline in the cytochrome *c* oxidase activity observed in mitochondria isolated from animals in certain physiopathological conditions such as aging [27] and ischemia/reperfusion [28], which are characterized by an increase in the basal rate of ROS production.

Besides its specific interaction with integral membrane proteins including anion carriers and complexes of the respiratory chain, cardiolipin plays an important role in the association of cytochrome *c* to the IMM [11]. It is reasonable to expect that alterations in the structure and/or in the content of cardiolipin may disturb its interaction with cytochrome *c*, leading to dissociation of this protein from IMM, an early event in the release of cytochrome *c* from mitochondria and subsequent apoptosis.

Studies carried out with liposomes have shown that peroxidized cardiolipin failed to bind cytochrome *c* [11]. In the present study, we have studied more directly the relationship

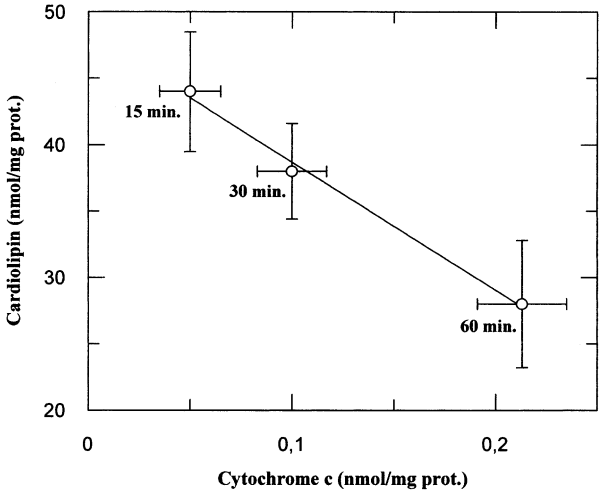

Fig. 3. Dissociation of cytochrome *c* vs. cardiolipin levels in SMP supplemented with succinate+AA at different times of incubation. SMP were incubated at 37°C in the presence of succinate+AA. At the indicated times, the incubation was interrupted by centrifugation of SMP. Measurements of cardiolipin level and cytochrome *c* dissociation were determined as described in Section 2 and in the legends of Figs. 1 and 2. Levels of cardiolipin were compared to values of cytochrome *c* dissociated from SMP. Each value represents the mean ± S.E.M. of both the cytochrome *c* and the cardiolipin measurements of four independent determinations; $r^2 = 0.99$.

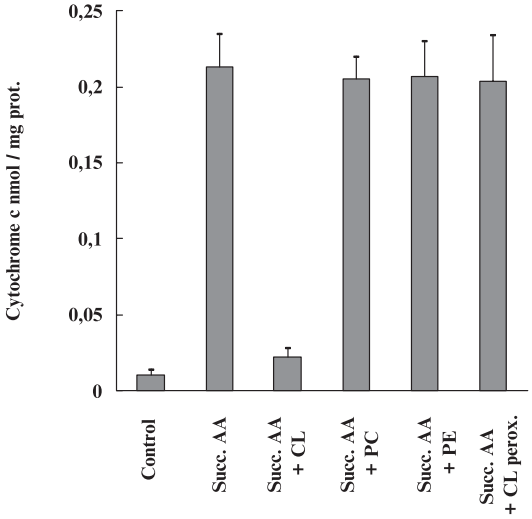

Fig. 4. Oxygen-free radical-induced cytochrome *c* dissociation from SMP and prevention by cardiolipin. Experimental conditions are similar to those described in the legend of Fig. 1. Cardiolipin (CL), PC, PE, and peroxidized cardiolipin were added to SMP at concentration of 3 μM, by ethanolic injection. All values are expressed as mean ± S.E.M. of four independent determinations.

between ROS-induced cardiolipin peroxidation and dissociation of cytochrome *c* from IMM. We demonstrate that ROS, produced at the level of the respiratory chain by treating succinate-respiring SMP with AA, induce the dissociation of cytochrome *c* from these particles. This dissociation is totally blocked by SOD+catalase, indicating a direct involvement of ROS in this effect. We found also that ROS generation leads to a marked loss of mitochondrial cardiolipin content which is similarly abolished by SOD+catalase. Moreover, there exists a quantitative correlation between the dissociation of cytochrome *c* from SMP and loss of cardiolipin levels in these particles. In addition, the ROS-induced dissociation of cytochrome *c* from SMP is totally prevented by exogenous cardiolipin, whilst peroxidized cardiolipin and other major phospholipids components of the mitochondrial membrane such as PC and PE have no effect in this respect. Taken together, these results demonstrate that mitochondrial-mediated ROS generation promotes the dissociation of cytochrome *c* from the IMM via cardiolipin peroxidation, due to oxyradical attack on double bonds of its unsaturated fatty acids. The molecular mechanism responsible for the ROS-induced disruption of the interaction between cytochrome *c* and cardiolipin at the level of the IMM is not clear. However, unmodified acyl chains of cardiolipin appear to be essential for its interaction with cytochrome *c* to anchor the protein to the membrane [6]. Peroxidative damage of these acyl chains by ROS could be responsible for the alteration of the interaction between cytochrome *c* and cardiolipin at the level of the IMM.

The release of cytochrome *c* from mitochondria is a critical event in the apoptotic pathway of cell death [1,2,29]. The molecular mechanism responsible for this release is still unknown. ROS generation is considered one of the causes of cytochrome *c* release and subsequent apoptosis [9]. The release of cytochrome *c* from mitochondria is preceded by its dissociation from the IMM [3,30]. Both ROS and cardiolipin seem to be involved in this process [10]. Our results are strongly supportive of this hypothesis and give further support to the view that an early event in the release of cyto-

chrome *c* from mitochondria is the ROS-mediated dissociation of cytochrome *c* from the IMM due to ROS-induced oxidative damage of cardiolipin. The data may prove useful in elucidating the mechanism underlying the release of cytochrome *c* from the mitochondria to the cytosol, which is considered an early event in the apoptosis.

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