The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome *c* oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles

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Abstract The effect of reactive oxygen species (ROS), produced by the mitochondrial respiratory chain, on the activity of cytochrome *c* oxidase and on the cardiolipin content in bovine heart submitochondrial particles (SMP) was studied. ROS were produced by treatment of succinate-respiring SMP with antimycin A. This treatment resulted in a large production of superoxide anion, measured by epinephrine method, which was blocked by superoxide dismutase (SOD). Exposure of SMP to mitochondrial mediated ROS generation, led to a marked loss of cytochrome *c* oxidase activity and to a parallel loss of cardiolipin content. Both these effects were completely abolished by SOD+catalase. Added cardiolipin was able to almost completely restore the ROS-induced loss of cytochrome *c* oxidase activity. No restoration was obtained with peroxidized cardiolipin. These results demonstrate that mitochondrial mediated ROS generation affects the activity of cytochrome *c* oxidase via peroxidation of cardiolipin which is needed for the optimal functioning of this enzyme complex. These results may prove useful in probing molecular mechanism of ROS-induced peroxidative damage to mitochondria which have been proposed to contribute to aging, ischemia/reperfusion and chronic degenerative diseases.

Key words: Reactive oxygen species; Cytochrome *c* oxidase; Cardiolipin; Bovine heart submitochondrial particle

1. Introduction

Oxygen free radicals are highly reactive and short-lived species which are expected to cause damage to various cellular constituents. Mitochondrial electron transport chain has been recognized as a major intracellular source of reactive oxygen species [1]. The superoxide anion radical appears to be the first oxygen reduction product generated under physiological and pathological conditions. Subsequent dismutation of superoxide anion generates H_2O_2 which, in turn, can lead to production of $^{\bullet}OH$. Peroxidation of membrane lipid components has been hypothesized to be a major mechanism of oxygen free radical attack resulting in generalized impairment of the membrane functions. The possibility that such a mechanism could cause specific damage to certain vital components of the mitochondrial membrane deserves further attention. Unsaturated fatty acids are particularly susceptible to ROS attack because of the presence of double bonds, which can undergo peroxidation through a chain of oxidative reactions. Cardiolipin is an important phospholipid constituent of the inner mitochondrial membrane, particularly rich in unsaturated fatty acids. This phospholipid plays a pivotal role in optimizing the activity of key mitochondrial proteins including several anion carriers and some electron transport complexes [2,3]. Cytochrome oxidase, the terminal enzyme complex of the respiratory chain, contains few, tightly bound cardiolipin molecules which are essential for its functioning [3]. These cardiolipin molecules are possible targets of oxygen free radicals attack, due to their high content of unsaturated fatty acids and because of their location in the inner mitochondrial membrane near to the site of ROS production.

Recent reports from this laboratory have demonstrated a decline in the cytochrome *c* oxidase activity in cardiac mitochondria isolated from animals under different physiopathological conditions such as aging [4,5] and ischemia/reperfusion [6]. This decline was attributed to a specific alteration in the mitochondrial cardiolipin content, due probably to a peroxidative attack of this phospholipid by ROS. This possibility has been confirmed by in vitro experiments [7].

In order to establish more firmly the existence of a direct correlation between ROS production and alteration of cytochrome *c* oxidase activity involving cardiolipin peroxidation, we have carried out a study on the effect of ROS, produced at the level of the respiratory chain by treatment of succinate-respiring submitochondrial particles with antimycin A [8–10], on the activity of the cytochrome *c* oxidase and on the cardiolipin content. The results obtained demonstrate that mitochondrial mediated ROS production results in a loss of cytochrome *c* oxidase activity, which can be directly ascribed to ROS-induced peroxidative damage of cardiolipin, essential for the functioning of this enzyme complex.

2. Materials and methods

All chemicals used were commercial products of highest available purity. Epinephrine, horse heart cytochrome *c*, *N*,*NNN*-tetramethyl-*p*-phenylenediamine (TMPD), ascorbic acid, 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 [11] and stored in 250 mM sucrose suspension at −20°C. Mitochondria were depleted of endogenous cytochrome *c* essentially as described in [12].

Beef-heart submitochondrial particles (SMP) were prepared essentially as described by Lee et al. [13], 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

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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 volume of 250 mM sucrose and centrifuged at $12\,000 \times g$ for 10 min. The supernatant was decanted and centrifuged at $105\,000 \times g$ for 40 min. The resulting pellet, consisting of SMP, was washed and suspended in 250 mM sucrose.

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

The generation of superoxide radical was induced by treatment of the SMP with succinate+antimycin A as follows. SMP (1 mg/ml) were incubated in a reaction medium consisting of 150 mM KCl and 25 mM Tris at pH 6.5 and 37°C. At zero time 10 mM succinate and 10 μM antimycin A were added. Aliquots for estimating the cytochrome *c* oxidase activity and the cardiolipin content were withdrawn at 60 min or at different times as specified in the legends of figures.

The production of superoxide anion was measured by the epinephrine oxidation test [14]. About 0.5 mg of SMP, dissolved in 3 ml of reaction medium as reported above, were supplemented with 1.67 mM succinate, 1.67 μM antimycin A and 1 mM epinephrine. The superoxide dependent oxidation of epinephrine to adrenochrome, was followed spectrophotometrically at 485–575 nm with an HP 8453 diode array spectrophotometer.

Cytochrome *c* oxidase activity was measured polarographically with an oxygen electrode at 25°C. The medium was 50 mM KPT (pH 7.2), 10 mM ascorbate, 0.7 mM TMPD, 0.05% *n*-dodecyl-β-D-maltoside, 10 μM cytochrome *c* and around 0.05 mg/ml of SMP protein.

Cardiolipin content was determined by the HPLC technique as previously described [15].

Cardiolipin was peroxidized in the presence of Fe^{2+}-ADP-ascorbic acid as previously described [5].

3. Results

It has been shown that the mitochondrial respiratory chain can generate superoxide anion by treatment of succinate-respiring submitochondrial particles with antimycin A [8–10]. The production of superoxide anion originates from the ubiquinone, Q cycle, of complex III where one electron from ubisemiquinone is transferred directly to molecular oxygen. Fig. 1 shows the production of superoxide anion in SMP treated with succinate+antimycin A. The rate of this production amounted to 2.7 nmol of $O_2^{\bullet -}$ per min per mg protein, in good agreement with the data reported by others [14]. The production of $O_2^{\bullet -}$ could be blocked by addition of SOD.

The activity of cytochrome *c* oxidase was measured on SMP supplemented with succinate+antimycin A, namely

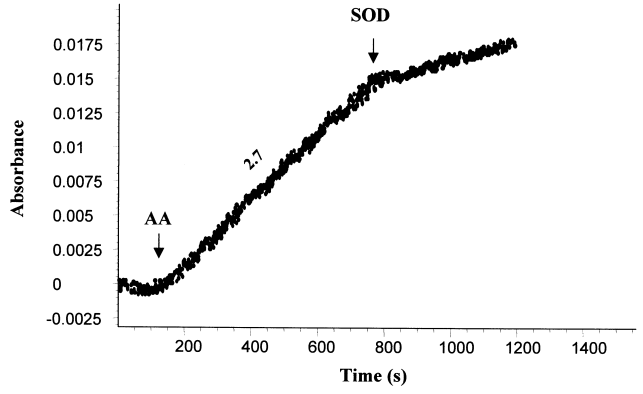

Fig. 1. Production of superoxide anion in bovine heart SMP supported by succinate+antimycin A. Superoxide anion production rate was estimated spectrophotometrically by the epinephrine method [14] as described in Section 2. Numbers on the traces indicate nmol $O_2^{\bullet -}$/min/mg protein. The experiment shown is representative of five experiments which gave similar results.

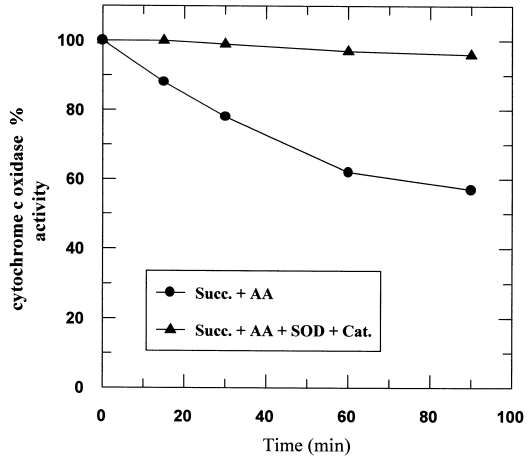

Fig. 2. Time dependence of oxygen free radical-induced loss of cytochrome *c* oxidase activity in SMP and prevention by SOD+catalase. SMP were treated with succinate+antimycin A, as described in Section 2, in the absence or presence of SOD (68 units)+catalase (94 units) and cytochrome *c* oxidase activity was measured at the times indicated in the figure. The results are expressed as percentages of the control. The experiment shown is representative of three experiments which gave similar results.

under conditions of ROS production at the level of the mitochondrial respiratory chain. As shown in Fig. 2, treatment of SMP with succinate+antimycin A resulted in a marked and time dependent loss of cytochrome *c* oxidase activity, maximal effect (more than 40% loss), being achieved within 1 h of this treatment. Addition of SOD+catalase to succinate+AA treated SMP, completely prevented the loss of cytochrome *c* oxidase activity, thus indicating a direct involvement of ROS in this effect.

The loss of cytochrome *c* oxidase activity induced by ROS production, could be due to a lowered content of cardiolipin, a phospholipid which is specifically required for the optimal functioning of this enzyme complex [3], due to ROS attack to double bonds of fatty acid constituents of the cardiolipin

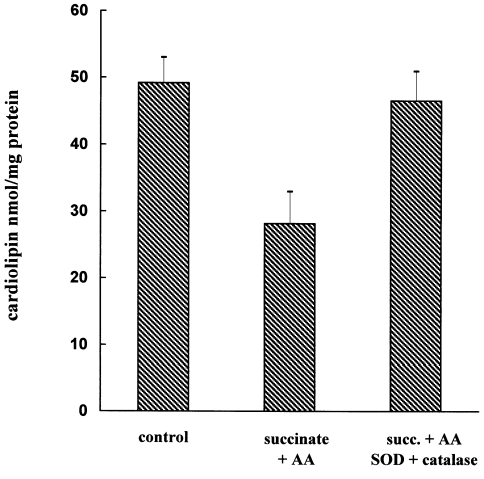

Fig. 3. Oxygen free radical-induced loss of the cardiolipin content in bovine heart SMP and prevention by SOD+catalase. SMP were incubated for 60 min in the absence (control) or presence of succinate+antimycin A, as described in Section 2 and cardiolipin content was determined by the HPLC technique as described in [15]. SOD (68 units)+catalase (94 units) were added in the incubation medium before the addition of succinate+antimycin. All values are expressed as mean ± S.E. of three independent determinations.

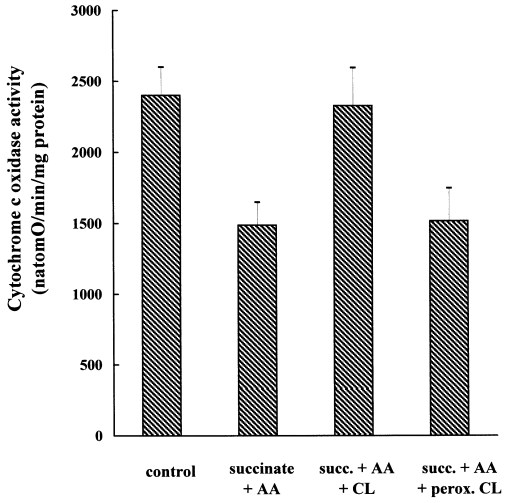

Fig. 4. Oxygen free radical-induced loss of cytochrome *c* oxidase activity in bovine heart SMP and reactivation by cardiolipin. Cytochrome *c* oxidase activity was measured on SMP incubated for 60 min in the absence (control) or presence of succinate+antimycin A. Cardiolipin (3 μM) or peroxidized cardiolipin were added to SMP treated for 60 min with succinate+antimycin A, by ethanolic injection. All values are expressed as mean ± S.E. of three independent determinations.

molecules. To assess this, the content of cardiolipin was determined in SMP treated for 1 h with succinate+antimycin A. As shown in Fig. 3, the content of cardiolipin decreased by around 40% in SMP treated with succinate+antimycin A when compared to the value obtained with untreated SMP. There exists also a quantitative correlation between this loss of cardiolipin content and that of cytochrome *c* oxidase activity (see Fig. 2). Addition of SOD+catalase to succinate+AA supplemented SMP, completely prevented the loss of cardiolipin content.

More convincing and direct evidence for a specific involvement of cardiolipin in the loss of the cytochrome *c* oxidase activity induced by ROS production in SMP, comes from the results reported in Fig. 4. It is shown here that the ROS-induced loss of cytochrome *c* oxidase activity could be completely restored to the level of untreated SMP by exogenously added cardiolipin. Interestingly enough, no restoration was afforded by peroxidized cardiolipin.

4. Discussion

The production of reactive oxygen species is considered as a major factor in oxidative cell injury via the oxidation and subsequent functional impairment of various cellular constituents. The production of ROS leads to primary reaction and damage in the immediate surrounding of where these reactive oxygen species are produced; they are trapped in their microenvironment by virtue of their high chemical reactivity. The mitochondrial electron transport chain is the major intracellular source of ROS [1], therefore the effects of these reactive species should be greatest at the level of mitochondrial membrane constituents, including the complexes of the respiratory chain [16] and lipid components such as cardiolipin, particularly rich in unsaturated fatty acids.

The present study demonstrates that ROS, produced at the level of respiratory chain by treating succinate-respiring submitochondrial particles with antimycin, induce a marked loss of the cytochrome *c* oxidase activity. This loss is completely abolished by SOD and catalase, indicating a direct involvement of ROS in this effect.

The functional integrity of the cytochrome oxidase enzyme complex depends on the presence of intact cardiolipin [17]. This phospholipid contains a large percentage of fatty acids (around 90% represented by linoleic acid) which are optimal for cardiolipin interaction with the cytochrome oxidase complex. These unsaturated fatty acids are highly susceptible to peroxidative attack by ROS. Our results demonstrate that ROS generation leads to a marked loss of the mitochondrial cardiolipin content. This loss of cardiolipin content is quantitatively associated to a parallel loss of the cytochrome *c* oxidase activity. Both these effects are completely abolished by SOD+catalase, which prevents ROS production. In addition, the ROS-induced loss of cytochrome *c* oxidase activity was almost completely restored to the level of untreated SMP by exogenously added cardiolipin, while no restoration was afforded by peroxidized cardiolipin. Taken together, these results demonstrate that mitochondrial mediated ROS generation affects the activity of cytochrome *c* oxidase via peroxidation of cardiolipin, due to oxy-radical attack to double bonds of its fatty acid constituents.

In addition to cytochrome oxidase enzyme, cardiolipin serves as cofactor for a number of other inner mitochondrial membrane proteins including anion transporters and respiratory complexes [3]. Therefore, it is possible that ROS-induced peroxidative damage to mitochondrial cardiolipin may affect the activity of other mitochondrial membrane-bound proteins, the activity of which is cardiolipin dependent. Experiments are underway to support this possibility.

The impairment of cytochrome *c* oxidase activity may increase the electron 'leak' from the electron transport chain, generating more superoxide radicals and perpetuating a cycle of oxygen radical-induced damage. The pattern of results presented here may prove useful in probing the molecular mechanism of ROS-induced peroxidative damage to mitochondrial membrane constituents which have been proposed to contribute to aging [18], apoptosis [19,20] and to chronic degenerative diseases including ischemia/reperfusion [5,21,22], cancer [23], Alzheimer [24], and chronic inflammation [23], and in development of effective antioxidant strategies.

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