Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations

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Abstract Rat heart mitochondrial membranes exposed to the free radicals generating system *tert*-butylhydroperoxide/Cu^{2+} undergo lipid peroxidation as evidenced by the accumulation of thyobarbituric acid reactive substances. Mitochondrial lipid peroxidation resulted in a marked loss of both cytochrome *c* oxidase activity and cardiolipin content. The alterations in the properties of cytochrome *c* oxidase were confined to a decrease in the maximal activity (V_{max}) with no change in the affinity (K_m) with respect to the substrate cytochrome *c*. Various lipid soluble antioxidants could prevent the lipid peroxidation reaction and the associated loss of cytochrome *c* oxidase activity. External added cardiolipin but no other phospholipids, nor peroxidized cardiolipin was able to prevent the loss of cytochrome oxidase activity induced by lipid peroxidation. These results establish a close correlation between oxidative damage to cardiolipin and alterations in the cytochrome oxidase activity and may prove useful in probing molecular mechanism of free radicals induced peroxidative damage of mitochondria which has been proposed to contribute to aging and to chronic degenerative diseases.

Key words: Lipid peroxidation; Cytochrome oxidase; Cardiolipin; Rat heart mitochondria

1. Introduction

Considerable attention is being addressed to the problem of the mechanism of cell injury by an acute oxidative stress. Mitochondrial electron transport chain has been recognized as a major intracellular source of oxygen radicals [1]. These oxygen radicals attack cellular macromolecules, oxidizing membranous phospholipids and damaging proteins and DNA (for review see [2]). Lipid peroxidation has been hypothesized to be a major mechanism of oxygen free radicals toxicity. Unsaturated fatty acids are particularly susceptible to oxygen radical attack because of the presence of double bonds, which can undergo peroxidation through a chain of oxidative reactions. Cardiolipin, a phospholipid of unusual structure, localized almost exclusively within the inner mitochondrial membrane where it is biosynthesized [3], is particularly rich in unsaturated fatty acid. Because cardiolipin plays a pivotal role in facilitating the activities of key mitochondrial inner membrane proteins including several anion carrier systems [4,5] and some of the electron transport complexes [6,7], it would be expected that changes that increase its susceptibility to oxidative damage would be deleterious to normal mitochondrial function. The interaction of cardiolipin with cytochrome *c* oxidase, the terminal enzyme complex of the electron transport chain, has been well established. A large number of studies indicate a specific and tight association between cytochrome oxidase and cardiolipin that is functionally important for maximal activity of this enzyme complex (for review see [8]).

Recently we have reported an aged linked decline in the cytochrome oxidase activity in rat heart mitochondria [9,10]. This decline was attributed to a specific decrease in the cardiolipin content of the inner mitochondrial membrane, due probably to a peroxidative attack of this phospholipid by oxy-radicals which are produced during aging process [11]. In order to get an in depth view of the molecular mechanism of peroxidative damage to cardiocytes, we have carried out a study on the effect of in vitro induced lipid peroxidation of rat heart mitochondria on the activity of cytochrome oxidase and on the cardiolipin content.

2. Materials and methods

t-BuOOH, $CuCl_2$, butylated hydroxytoluene (BHT), *n*-propylgallate, 2-thiobarbituric acid, vitamin E, horse heart cytochrome *c*, *NNN′N*-tetramethyl *p*-phenylenediamine (TMPD), ascorbic acid and *n*-dodecyl β-D-maltoside were obtained from Sigma Chemical Co.

Male Fisher rats were used throughout these studies. Rat heart mitochondria were prepared by differential centrifugation of heart homogenates essentially as described in [12].

Mitochondrial protein concentration was measured by the usual biuret method.

Lipid peroxidation in isolated mitochondria was induced by the addition of 25 μM $CuCl_2$ and t-BuOOH as indicated. Reaction tubes contained 1.5 mg protein/ml of mitochondria suspended in well oxygenated incubation buffer (150 mM NaCl, 20 mM HEPES, pH 7.4), at 37°C. After 30 min of incubation, the reaction was stopped by the addition of 1 mM EDTA and mitochondria were centrifuged and resuspended in 0.22 M mannitol, 70 mM sucrose and 2 mM HEPES.

Lipid peroxidation was estimated by the appearance of thiobarbituric acid reactive substances (TBARS) and of conjugated dienes according to Buege and Aust [13]. TBARS concentration is expressed as nmol of malondialdehyde (MDA)/mg protein.

Liposomes and peroxidized liposomes were prepared as previously described [14].

The fusion of liposomes with mitochondria was carried out essentially as described by Hackenbrock et al. [15] and in [14].

Cytochrome *c* oxidase activity was measured polarographically with an oxygen electrode at 25°C. The medium was 100 mM KPT (pH 7.2), 10 mM ascorbate, 1 mM TMPD, 0.05% *n*-dodecyl β-D-maltoside, 30 μM cytochrome *c* and 0.05–0.1 mg of mitochondrial protein.

Cardiolipin content was determined by the HPLC technique as previously described [16].

3. Results

Results on the effects of treatment of heart mitochondria with t-BuOOH/Cu^{2+} on the lipid peroxidation (MDA produc-

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Abbreviations: t-BuOOH, *tert*-butylhydroperoxide; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Lipos, liposomes

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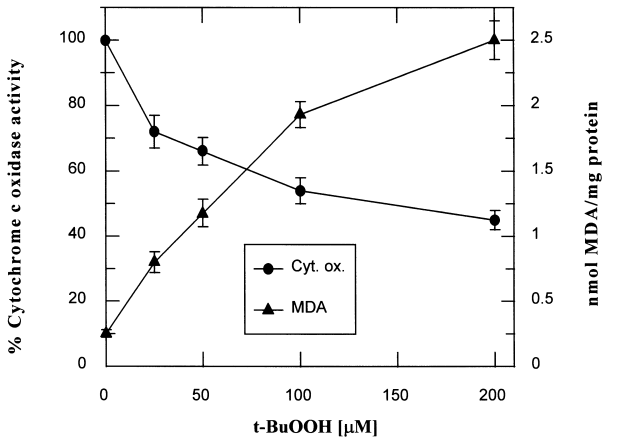

Fig. 1. Correlation between cytochrome *c* oxidase activity and lipid peroxidation in rat heart mitochondria. Rat heart mitochondria were subjected to peroxidation as described in Section 2 with 25 μM $CuCl_2$ and increasing amounts of t-BuOOH as indicated in the figure. Cytochrome *c* oxidase activity was assayed polarographically as described in the experimental section and the results are expressed as percentage of the control. The appearance of TBARS was assayed and expressed as nmol malondialdehyde (MDA)/mg mitochondrial protein. All values are expressed as means ± S.E. of three independent determinations.

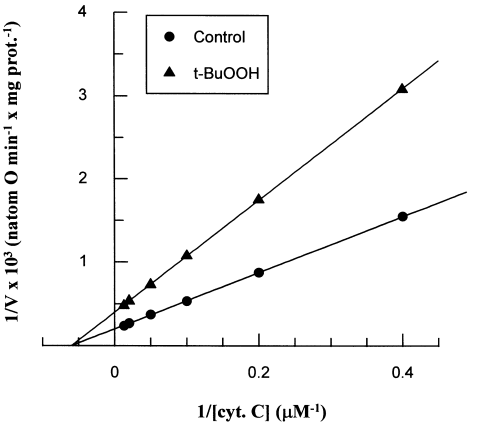

Fig. 2. The effect of lipid peroxidation on the kinetic behavior of cytochrome oxidase in rat heart mitochondria. Rat heart mitochondria were subjected to peroxidation as described in Section 2 with 25 μM $CuCl_2$ and 200 μM t-BuOOH. The kinetic parameters of cytochrome *c* oxidase were determined with various concentrations of cytochrome *c* (2.5–75 μM) using double reciprocal plots. The experiment shown is representative of three different experiments which gave similar results.

tion) and on cytochrome oxidase activity are reported in Fig. 1. Incubation of isolated rat heart mitochondria with t-BuOOH/Cu^{2+} resulted in lipid peroxidation as measured by the appearance of TBARS. Lipid peroxidation could be also demonstrated by the generation of lipid conjugated dienes (results not shown). Both t-BuOOH and $CuCl_2$ needed to be present for this reaction to occur. The activity of cytochrome oxidase was measured in these t-BuOOH/Cu^{2+} treated mitochondria. On increasing of the level of lipid peroxidation there was a parallel loss of cytochrome oxidase activity. It should be noted that neither t-BuOOH nor MDA and other products of lipid peroxidation had appreciable effect on the cytochrome oxidase activity when added directly to mitochondria during the incubation phase with ascorbate+TMPD, this excluding any direct interaction of these oxidant agents with the cytochrome oxidase enzyme complex (results not reported).

The alterations in the properties of cytochrome oxidase following treatment of heart mitochondria with t-BuOOH/Cu^{2+} was confined to a decrease in the maximal rate of oxygen consumption (V_{max}) with no change in the affinity (K_m) with respect to the substrate cytochrome *c* (Fig. 2).

The close correlation between the level of TBARS production and the loss of the cytochrome *c* oxidase activity suggests that lipid peroxidation was responsible for this effect. To test this possibility, we measured the oxidase activity and lipid peroxidation in the presence and absence of lipid soluble antioxidants such as BHT, *n*-propylgallate, vitamin E. As reported in Table 1, all these lipid soluble antioxidants provided protection against lipid peroxidation of heart mitochondria induced by t-BuOOH/Cu^{2+}. By inhibiting lipid peroxidation, these lipid soluble antioxidants provided protection of cytochrome oxidase activity. Thus, lipid peroxidation appears to account for the inactivation of cytochrome oxidase.

The decrease of cytochrome *c* oxidase activity induced by lipid peroxidation might be due to loss of cardiolipin, a phospholipid which has been shown to be specifically required for full activity of this enzyme complex [8]. The content of cardiolipin was determined in the mitochondria treated with different concentrations of t-BuOOH. As shown in Fig. 3, the content of mitochondrial cardiolipin decreased on increasing the concentration of the oxidant t-BuOOH. There is also a good correlation between the loss of cardiolipin content and the inactivation of cytochrome oxidase (Fig. 1), this suggesting an involvement of cardiolipin in this effect.

More direct evidence for a specific involvement of cardiolipin in the inactivation of cytochrome oxidase due to the lipoperoxidation of mitochondria, comes from experiments in which the effect of exogenous added cardiolipin was tested on its capability to prevent this inactivation. As cardiolipin is poorly permeable to mitochondrial membranes, the procedure of the fusion of mixed phospholipid liposomes with mitochon-

Table 1
Effects of antioxidants on the lipid peroxidation reaction and on the cytochrome oxidase activity in rat heart mitochondria

Treatment/addition	Cytochrome *c* oxidase activity (natom O/min/mg protein)	TBARS (nmol MDA/mg protein)
Control	2703 ± 220	0.25 ± 0.02
t-BuOOH	1138 ± 183	2.5 ± 0.15
t-BuOOH/BHT	2520 ± 250	0.45 ± 0.03
t-BuOOH/*n*-propyl gallate	2259 ± 210	0.50 ± 0.04
t-BuOOH/vitamin E	1904 ± 180	1.12 ± 0.07

Rat heart mitochondria were subjected to lipid peroxidation as described in Section 2 with 25 μM $CuCl_2$ and 200 μM t-BuOOH. Cytochrome oxidase activity and TBARS level were determined as described under Section 2. *n*-propylgallate (5 mM), BHT (20 μM) and vitamin E (20 μM) were dissolved in dimethyl sulfoxide. All values are means ± S.E. of three independent determinations.

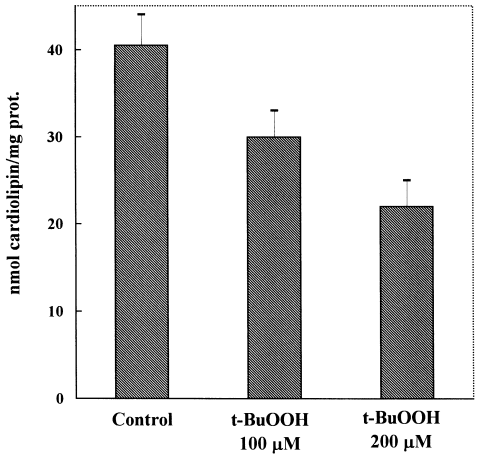

Fig. 3. The effect of lipid peroxidation on the mitochondrial content of cardiolipin. Rat heart mitochondria were subjected to lipid peroxidation as described in the legend of Fig. 1. Cardiolipin content of mitochondria was determined by the HPLC technique as described in Section 2. All values are expressed as means ± S.E. of three independent determinations.

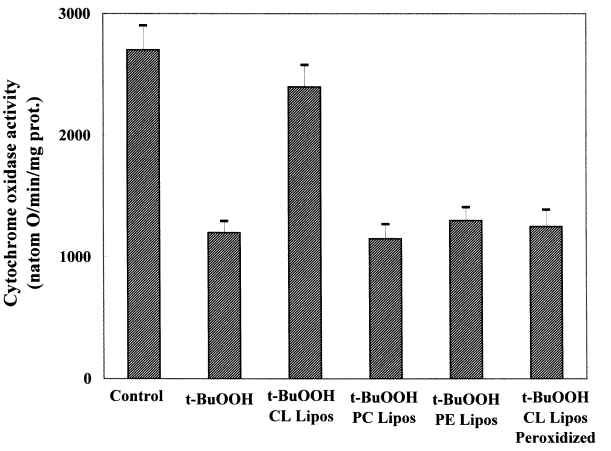

Fig. 4. Protection by exogenous cardiolipin of the cytochrome *c* oxidase inactivation induced by lipid peroxidation. Rat heart mitochondria were subjected to lipid peroxidation as described in Section 2 with 25 μM $CuCl_2$ and 200 μM t-BuOOH. Enrichment of mitochondria with exogenous cardiolipin was obtained by the procedure of the fusion of mitochondrial membranes with liposomes as previously described [11]. The different types of liposomes used were: PC, PC/CL (4:1 molar ratio) and PC/PE (1:1 molar ratio). Peroxidized PC/CL liposomes were obtained as described in [11]. Liposomes were added to mitochondria 10 min before starting the lipid peroxidation reaction. Cytochrome oxidase activity was measured as described above. All values are expressed as means ± S.E. of three different determinations.

dria was used in order to incorporate cardiolipin into these organelles [11]. As shown in Fig. 4, exogenously added cardiolipin almost completely protected cytochrome oxidase by inactivation induced by lipid peroxidation. No protection was afforded by peroxidized cardiolipin nor by other phospholipids such as PC and PE.

4. Discussion

The present study demonstrates that lipid peroxidation of cardiac mitochondria, following treatment with the free radicals generating system t-BuOOH/Cu^{2+}, leads to a marked reduction of cytochrome oxidase activity. Inhibition of lipid peroxidation by antioxidants protected against enzyme inactivation. Thus, the inactivation of mitochondrial cytochrome oxidase by these oxidant agents appears to depend upon a peroxidation of membrane lipids.

The functional integrity of cytochrome oxidase depends on the presence of intact cardiolipin [8]. This phospholipid, in contrast to other major phospholipids of the mitochondrial membrane (PC and PE), contains a large percentage of polinsaturated fatty acids (around 90% represented by linoleic acid) which are optimal for cardiolipin interaction with the cytochrome oxidase complex [17]. These unsaturated fatty acids are known to be highly susceptible to peroxidative injury. Our data show that lipid peroxidation is associated to a marked loss of mitochondrial cardiolipin content. Thus, it is reasonable to assume that the loss of cardiolipin, due to the t-BuOOH/Cu^{2+} induced peroxidative damage of its polin-saturated fatty acids, is mainly responsible for the observed inactivation of cytochrome *c* oxidase. This assumption is supported by several lines of experimental evidences presented here. In fact, there is a strong correlation between the inactivation of cytochrome oxidase and the loss of cardiolipin content in mitochondria treated with free radicals generating system. More important, exogenously added cardiolipin afforded almost full protection of cytochrome oxidase activity against inactivation induced by lipid peroxidation. This protection was specific for cardiolipin in that neither PC nor PE may replace cardiolipin in this effect of protection. Moreover, peroxidized cardiolipin was ineffective in protecting cytochrome oxidase by inactivation induced by lipid peroxidation, suggesting that peroxidized cardiolipin is unable to interact with this enzyme complex, as previously reported by Robinson et al. [17].

In conclusion, our results demonstrate that in vitro lipid peroxidation of mitochondrial membranes results in a marked inactivation of cytochrome oxidase. The basis of this inactivation appears to be a specific oxidative damage of cardiolipin which is required for optimal activity of this enzyme complex. The pattern of results presented here may prove useful in probing molecular mechanism of free radicals induced peroxidative damage of mitochondrial membranes which has been proposed to contribute to aging [18] and to chronic degenerative diseases, including ischemia [19,20], cancer and chronic inflammation [21] and in development of effective antioxidant strategies.

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