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Age-dependent decline in the cytochrome *c* oxidase activity in rat heart mitochondria: role of cardiolipin

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Abstract Cardiolipin is a major mitochondrial membrane lipid and plays a pivotal role in mitochondrial function. We have recently suggested a possible involvement of this phospholipid in the age-linked decline of cytochrome *c* oxidase activity in rat heart mitochondria [G. Paradies et al. (1993) *Arch. Gerontol. Geriatr.* 16, 263–272]. The aim of this work was to test our earlier proposal. We have investigated whether addition of exogenous cardiolipin to mitochondria is able to reverse, in situ, the age-linked decrease in the cytochrome oxidase activity. The method of fusion of liposomes with mitochondria developed by Hackenbrock [Hackenbrock and Chazotte (1986) *Methods Enzymol.* 125, 35–45] was employed in order to enrich the mitochondria cardiolipin content. We demonstrate that the lower cytochrome *c* oxidase activity in heart mitochondria from aged rats can be fully restored to the level of young control rats by exogenously added cardiolipin. No restoration was obtained with other phospholipids or with peroxidized cardiolipin. Our data support a key role for cardiolipin in the age-linked decline of rat heart mitochondrial cytochrome *c* oxidase activity.

Key words: Cytochrome oxidase; Cardiolipin; Aging; Rat heart mitochondria

1. Introduction

Aging is known to cause a decline in cardiac functional competence. Mitochondria are considered a likely subcellular locus of this decline due to the central role played by these organelles in cardiac cell bioenergetics (for review see [1]). The age-dependent decline in heart performance may be related to age-linked changes in the mitochondrial membranes lipids which influence the activity of diverse membrane bound proteins including certain anion carrier proteins [2–6] and cytochrome oxidase [7].

Cardiolipin is a phospholipid of unusual structure, localised almost exclusively within the inner mitochondrial membrane where it is biosynthesised [8]. It is known that cardiolipin interacts with a number of the inner mitochondrial membrane proteins including several anionic carrier systems [9,10] and some of the electron transport complexes [11,12]. Among these, the interaction of cardiolipin with cytochrome oxidase, the terminal enzyme complex of the electron transport chain, has been best characterised (for review see [13]). In this case, 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.

We have recently reported an age-dependent decrease in the cytochrome oxidase activity in rat heart mitochondria [7]. Treatment of aged rats with the pharmacological agent acetyl-L-carnitine completely restored the normal activity of cytochrome oxidase [14]. These changes in the cytochrome oxidase activity were correlated with parallel changes in the mitochondrial cardiolipin content. In view of this, we proposed that the reduced mitochondrial cytochrome oxidase activity in aged animals is primarily due to a specific decrease in the cardiolipin content.

The aim of this work was to test the idea that lowered cardiolipin content in aged animals is the cause for the lowered cytochrome *c* oxidase activity. Evidence will be presented that the lower cytochrome oxidase activity in heart mitochondria from aged rats can be fully restored to the level of young control rats by exogenously added cardiolipin.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine and phosphatidylethanolamine, bovine heart cardiolipin, horse heart cytochrome *c*, (TMPD), *n*-dodecyl β-D-maltoside, ADP, ferrous sulfate and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals

Male Fisher rats aged 5 months (young) and 26 months (aged) were used for these studies. Rat heart mitochondria were prepared by differential centrifugation of heart homogenates essentially as described in [15]. Mitochondrial protein concentration was measured by the Bradford method using serum albumin as standard [16].

2.3. Preparation of liposomes

Liposomes (small unilamellar vesicles) were prepared by sonicating 1.7 mg of phospholipids in 1 ml of incubation medium of 25 mM phosphate buffer (pH 6.7) with the microtip probe of a Branson sonifier (mod. 250) at 40 W for 6 cycles of 2.5 min in an ice bath under N_2 stream.

2.4. Preparation of peroxidized liposomes

Liposomes were peroxidized using the Fe^{2-}-ADP-ascorbic acid method. Briefly aliquots of liposomes (≈1.7 mg of phospholipids) were dispersed in 1 ml of oxygenated buffer containing 20 μM Fe^{2+} and 120 μM ADP. The peroxidation reaction was initiated by adding 200 μM of ascorbic acid. Incubation was carried out at 37°C in a shaking water bath for 6 min. The extent of lipid peroxidation was monitored by conjugated dienes formation as described in [17].

2.5. Fusion of liposomes with mitochondrial membranes

The liposomes–mitochondrial membranes fusion was carried out essentially as described by Hackenbrock et al. [18]. Briefly, 1 ml of freshly sonicated liposomes was added to 1 mg of mitochondrial protein at 30°C with constant stirring. After 40 min of incubation, mitochondria were centrifuged, the mitochondrial pellet was washed and resuspended in 0.25 M sucrose.

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Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Lipo, liposome(s)

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2.6. Cytochrome c oxidase assay

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.

2.7. Cardiolipin determination

Mitochondrial cardiolipin content was determined by the HPLC technique previously described [19].

3. Results

Phospholipids are poorly permeable to mitochondrial membranes. Hackenbrock et al. [18] presented a procedure for the fusion of mixed phospholipid liposomes with mitochondrial membranes, which enriches the mitochondrial energy transducing membrane with bulk exogenous phospholipids. The method requires the manipulation of pH and utilises a class of liposomes designated 'small unilamellar vesicles'. Using this experimental procedure, we have studied the effect of fusion of mitochondria from young and aged rats, with liposomes composed of various phospholipids (PC, PC/CL and PC/PE) on the activity of cytochrome oxidase. The results of these experiments are reported in Table 1. Heart mitochondria from aged rats exhibited a 35% lower cytochrome oxidase activity as compared with young control rats (see also [7]). This lower activity was almost completely restored to the level of young control rats following fusion of mitochondria from aged rats with PC/CL liposomes. No restoration was obtained with peroxidized PC/CL liposomes nor with PC or PC/PE liposomes, thus suggesting a specific role of cardiolipin in this effect. Fusion of mitochondria from young control rats with PC/CL liposomes had practically no effect on the cytochrome oxidase activity, indicating that the liposomes–mitochondrial membranes fusion procedure used in our experiments did not affect the functioning of this enzyme complex in normal mitochondria.

Results on the changes in the cardiolipin content in mitochondria following fusion of these organelles with different types of liposomes are reported in Fig. 1. As previously reported, the heart mitochondrial cardiolipin content was significantly lower (40%) in aged animals than in young control animals [7]. Fusion of mitochondria from aged rats with PC/CL liposomes resulted in a considerable enrichment in the mitochondrial cardiolipin content, the level of which increased from 10.6 ± 1.4 to 25 ± 2.0 mol %. No change in the cardiolipin content was observed following fusion of mitochondria from aged rats with PC or PC/PE liposomes.

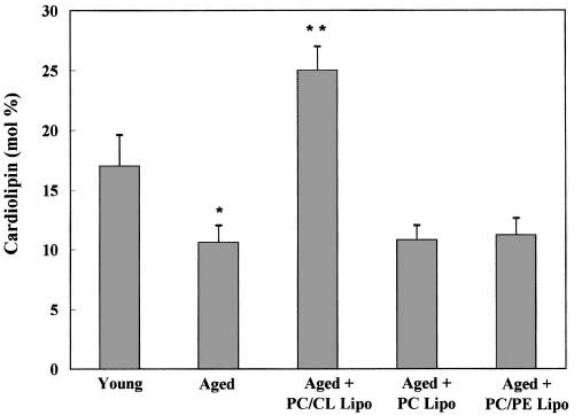

Fig. 1. Cardiolipin content in heart mitochondria from young and aged rats and the effect of fusion with liposomes. Cardiolipin content of mitochondria was determined by the HPLC technique described in Section 2. The fusion of mitochondria with liposomes was carried out as described in the legend of Table 1 and in Section 2. Each value represents the mean ± SE obtained for four experiments, with two rats for each group. $^{*}P < 0.05$ vs. young. $^{**}P < 0.01$ vs. aged.

4. Discussion

Several studies have shown a dependence of cytochrome *c* oxidase on intact cardiolipin [13,20–22]. This has been demonstrated essentially by reconstitution experiments of isolated cytochrome oxidase activity in artificial membranes such as liposomes. In these studies it has been shown that removal of cardiolipin from isolated cytochrome oxidase enzyme decreases the electron transport 60–70% of its original activity and recovery of full activity is dependent upon addition of exogenous cardiolipin. This effect of cardiolipin cannot be accomplished by other phospholipids. On this basis it has been concluded that selective association of cardiolipin with cytochrome oxidase modifies the immediate lipid environment of the protein thus facilitating its enzymatic function.

Previous studies from this laboratory have demonstrated an age-linked decrease in the cytochrome oxidase activity in rat heart mitochondria [7,14]. As this decrease was associated with a parallel decrease in the mitochondrial content of car-

Table 1
Decreased cytochrome *c* oxidase activity in heart mitochondria from aged rats and specific reactivation by cardiolipin-liposomes

Mitochondria	Cytochrome *c* oxidase (natoms O/min/mg prot.)	Change (%)
Young	2740 ± 194	
Aged	1775 ± 182*	−35
Young+PC/CL liposomes	2788 ± 277	+2
Aged+PC/CL liposomes	2723 ± 214**	−1
Aged+PC liposomes	1752 ± 192	−36
Aged+PC/PE liposomes	1806 ± 163	−34
Aged+peroxidized PC/CL liposomes	1729 ± 185	−37

The fusion of mitochondria with liposomes, composed of various phospholipids, was carried out as described in Section 2. PC/CL liposomes (4:1 molar ratio) and PC/PE liposomes (1:1 molar ratio). Young and aged control mitochondria were treated in the same manner as the liposomes-treated mitochondria, but in the absence of liposomes. Peroxidized PC/CL liposomes were obtained as described in Section 2. Each value represents the mean ± SE obtained for four separate experiments, with four rats for each group.
$^{*}P < 0.01$ vs. young control; $^{**}P < 0.05$ vs. aged.

diolipin and because of the reported dependence of cytochrome oxidase on this phospholipid we have suggested a possible involvement of cardiolipin in the age-linked decline of cytochrome oxidase activity. The results reported in this paper provide experimental support for this argument. In fact, it is shown here that the lower activity of cytochrome oxidase as well as the lower cardiolipin content in heart mitochondria from aged rats can be fully restored to the level of young control rats by exogenously added cardiolipin. It is conceivable that exogenous cardiolipin is incorporated into the mitochondrial membranes of aged rats thus restoring the normal cardiolipin content needed for full activity of this enzyme complex. Further support for such mechanism comes from experimental studies in submitochondrial particles where it was demonstrated that initiation of lipid peroxidation caused inhibition of cytochrome *c* oxidase and that this inhibition was reversible upon addition of phospholipids [23].

Neither PC nor PE may replace cardiolipin in this effect of restoration. These results are consistent with previous data from Robinson et al. [21] who found that only exogenously added cardiolipin and its derivatives, but not other phospholipids, were effective in restoring the maximal activity of cytochrome oxidase in CL-depleted enzyme.

In addition, as reported in Table 1, peroxidized cardiolipin was ineffective in restoring the lower activity of cytochrome oxidase in heart mitochondria from aged rats. This suggests that peroxidized cardiolipin is unable to interact with cytochrome *c* oxidase. This result may have important physiological implications. In fact, cardiolipin, due to its unusually high content of unsaturated bonds ($\approx$90% represented by linoleic acid) is a likely target for peroxidative attack by oxygen radicals which are produced during the aging process [24]. Therefore, it is possible that the dramatic decrease in the cardiolipin content found in mitochondria from aged rats may be a consequence of oxidative damage of this phospholipid by oxy radicals produced during the aging process. However, the possibility that the lower content of cardiolipin in mitochondria from aged rats may be due to alteration of cardiolipin biosynthesis, as reported in certain physiophatological conditions [25–27] should also be considered.

Whatever the mechanism responsible for the age-dependent decrease in the cardiolipin content in rat heart mitochondria, it remains the fact that this decrease may affect the activity of cytochrome oxidase, due to its critical dependence on this phospholipid. On the other hand, a reduced capacity of this enzyme with aging will also increase the risk of an incomplete reduction of oxygen and thus further oxygen radical production.

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