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Rapid report

Cardiolipin-dependent decrease of cytochrome *c* oxidase activity in heart mitochondria from hypothyroid rats

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Abstract

Cardiolipin plays an important role in mitochondrial membrane structure and function. We have recently reported a decrease in the cytochrome *c* oxidase activity in heart mitochondria from hypothyroid rats (G. Paradies et al. (1993) Arch. Biochem Biophys. 307, 91–95). A possible involvement of cardiolipin in such a decrease has been proposed. The aim of this work was to test our earlier proposal. We have investigated whether addition of exogenous cardiolipin to hypothyroid mitochondria is able to reverse, in situ, their decreased 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 mitochondrial cardiolipin content. We demonstrate that the decreased activity of this enzyme complex in heart mitochondria from hypothyroid rats can be completely restored to the level of control rats by exogenously added cardiolipin but not by other phospholipids. These data provide strong evidence for the involvement of cardiolipin in the thyroid hormone induced changes of mitochondrial cytochrome oxidase activity.

Keywords: Cytochrome oxidase; Cardiolipin; Hypothyroidism; Heart mitochondria; (Rat)

The thyroid hormones are known as one of the major factors involved in the regulation of cardiac function. Mitochondria are considered to be likely subcellular targets of thyroid hormone action in view of their crucial role in the energy metabolism. Thyroid hormones regulate mitochondrial respiration with increased rates in hyperthyroidism and decreased rates in hypothyroidism. Changes in membrane lipid content, lipid composition and lipid-proteins interactions do occur in mitochondria isolated from hypo- and hyperthyroid animals (for a review, see [1]). These changes have been considered at least in part, responsible for the changes in the activity of certain mitochondrial anion carrier proteins [2–7] and electron transport systems [8,9].

Cardiolipin is a phospholipid of unusual structure, found almost exclusively at the level of the inner mitochondrial membrane where it is biosynthesized [10]. The biosynthesis of this phospholipid in hepatic and cardiac mitochondria has been shown to be under thyroid hormone regulation [11,12]. Cardiolipin plays an important role in mitochondrial membrane structure and function (for a review, see Hoch [13]). This

Abbreviations: CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TMPD, *N,N,N′,N*-tetrametyl-*p*-phenylenediamine; Lipo, liposomes

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phospholipid interacts with various proteins of the inner mitochondrial membrane including several anion carrier systems [14,15] and certain electron transport complexes [16,17]. Among these, the interaction of cardiolipin with cytochrome oxidase, the terminal enzyme complex of the electron transport chain, has been best characterised. 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 (for a review, see Robinson [18]).

We have recently reported a reduced cytochrome oxidase activity in heart mitochondria from hypothyroid rats [8]. Treatment of hypothyroid rats with thyroid hormone completely restored the activity of this enzyme complex to the level of control rats. These changes in the cytochrome oxidase activity were associated with parallel changes in the mitochondrial cardiolipin content. On this basis we proposed a possible involvement of cardiolipin in the decreased activity of this enzyme complex in hypothyroid rats.

The purpose of this study is to test the idea that lowered cardiolipin content in hypothyroid animals is the cause for the lowered mitochondrial cytochrome *c* oxidase activity. We demonstrate that the reduced cytochrome oxidase activity in heart mitochondria from hypothyroid rats can be in situ fully restored to the level of control rats by exogenously added cardiolipin.

Male Wistar rats (200–250 g), housed at a temperature of 22°C, were used throughout these studies. Animals were made hypothyroid by adding 6-*n*-propyl-2-thiouracil (PTU; 0.05%, w/v) to their drinking water for 3–4 weeks [8]. Control animals received only the solvent for the same period. Animals were killed 24 h after final administration.

Rat heart mitochondria were prepared by differential centrifugation of heart homogenates essentially as described previously [19].

Mitochondrial protein concentration was measured by the Bradford method using serum albumin as standard [20].

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 six cycles of 2.5 min in an ice bath under N_2 stream.

The fusion of liposomes with mitochondria was carried out essentially as described by Hackenbrock et al. [21]. 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.

Cytochrome *c* oxidase activity was measured polarographycally 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, varying concentration of cytochrome *c* (2.5–75 μM) and 0.05–0.1 mg of mitochondrial protein in a final volume of 1 ml.

Mitochondrial cardiolipin content was determined by the HPLC technique previously described [22].

The method of fusion of vesicular lipids with mitochondrial membranes developed by Hackenbrok

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

Mitochondria	K_m (μM)	V_{max} (natoms O/min/mg protein)	Change (%)
Control	16.6 ± 0.9	4333 ± 218	
Hypothyroid	15.4 ± 0.8	2707 ± 175 *	−38
Control + PC/CL liposomes	17.5 ± 1.2	4351 ± 374	+1
Hypothyroid + PC/CL liposomes	18.0 ± 1.1	4232 ± 316 **	−2
Hypothyroid + PC liposomes	16.8 ± 1.0	2607 ± 198	−40
Hypothyroid + PE/PC liposomes	17.6 ± 1.1	2805 ± 214	−35

The fusion of mitochondria with liposomes, composed of various phospholipids, was carried out as described in the experimental section. PC/CL liposomes (4:1 molar ratio) and PC/PE liposomes (1:1 molar ratio). Control and hypothyroid mitochondria were treated in the same manner as the liposomes-treated mitochondria, but in the absence of liposomes. Each value represents the mean ± S.E. obtained for four separate experiments, with four rats for each group. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. hypothyroid.

[21] has been frequently employed in order to enrich the phospholipid fraction of the mitochondrial inner membrane. The method requires the manipulation of pH and utilises a class of liposomes designated 'small unilamellar vesicles'. Using this procedure, we have studied the effect of fusion of mitochondria from control and hypothyroid rats with liposomes composed of various phospholipids (PC, PC/CL and PC/PE) on the cytochrome oxidase activity. The results of these experiments are reported in Table 1. Heart mitochondria from hypothyroid rats exhibited a 38% ($P < 0.05$) lower V_{max} of the cytochrome *c* oxidase activity as compared with control rats, with no changes in the K_m values (see also [8]). This reduced activity was completely restored to the level of control rats following fusion of hypothyroid mitochondria with PC/CL liposomes. This restoration required an 4-fold excess of added cardiolipin as referred to the endogenous content of this phospholipid. No restoration was obtained with PC nor PC/PE liposomes. These results are consistent with previous data from Robinson et al. [23] 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 cardiolipin depleted enzyme reconstituted in liposomal membranes. It should be noted that fusion of mitochondria from control rats with PC/CL liposomes had practically no effect on the cytochrome oxidase activity, indicating that the liposome-mitochondrial membranes fusion procedure used in our experiments did not affect the functioning activity of this enzyme complex in control mitochondria. It should also be noted that the affinity of this enzyme complex toward its substrate cytochrome *c* remained unchanged in all the liposome-mitochondrial membrane preparations.

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. Heart mitochondrial cardiolipin content was significantly lower (around 40%) in hypothyroid rats than in control animals (see also [8]). Fusion of hypothyroid mitochondria with PC/CL liposomes resulted in a marked enrichment in the mitochondrial cardiolipin content, the level of which increased from 24.6 ± 1.9 to 59.5 ± 4.4 nmol/mg protein. No change in the cardiolipin content was observed following fusion of hypothyroid mitochondria with PC nor PC/PE liposomes.

The data here reported provide strong evidence for a direct involvement of cardiolipin in the thyroid

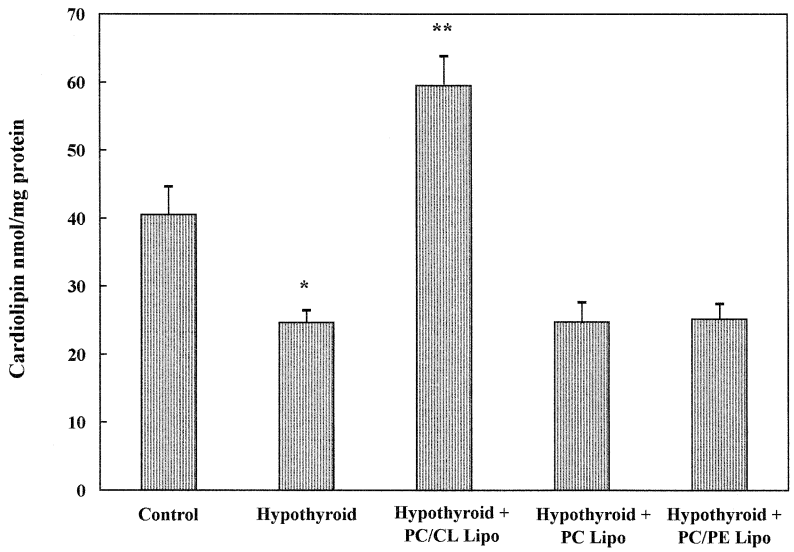

Fig. 1. Cardiolipin content in heart mitochondria from control and hypothyroid rats and the effect of fusion with liposomes. Cardiolipin content of mitochondria was determined by the HPLC technique as described in [22]. The fusion of mitochondria with liposomes was carried out as described in the legend of Table 1 and in the experimental section. Each value represents the mean ± S.E. obtained for four separate experiments, with two rats for each group. Lipo = liposomes. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. hypothyroid.

hormone induced changes of cytochrome oxidase activity in rat heart mitochondria. In fact, it is shown that the decreased cytochrome oxidase activity as well as the lower cardiolipin content in heart mitochondria from hypothyroid rats can be in situ fully restored to the level of control rats by exogenously added cardiolipin. Similar results have been obtained in vivo following treatment of hypothyroid rats with thyroid hormones, as previously reported [8]. It is conceivable that exogenously added cardiolipin is incorporated into the mitochondrial inner membrane of hypothyroid rats thus restoring the normal cardiolipin content needed for full activity of this enzyme complex. 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 [24].

Our results are in general agreement with previous study from Robinson et al. [23] on the activating properties of cardiolipin on isolated cytochrome oxidase activity reconstituted in artificial membranes such as liposomes. In this study it was shown that removal of cardiolipin from the isolated cytochrome oxidase enzyme decreases the electron transport 60–70% of its original activity and recovery of full activity was dependent upon addition of exogenous cardiolipin.

It has been recently reported that the mitochondrial cardiolipin biosynthesis is under thyroid hormone regulation [11,12]. Thus, thyroid hormone induced alterations in the mitochondrial cardiolipin levels may be considered directly responsible for the observed changes in heart mitochondrial cytochrome oxidase activity in hypo- and hyperthyroid animals.

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References

[1] Hoch, L.F. (1988) Prog. Lipid Res. 27, 199–270.
[2] Chen, Y.D. and Hoch, F.L. (1976) Arch. Biochem. Biophys. 172, 741–744.
[3] Paradies, G. and Ruggiero, F.M. (1988) Biochim. Biophys. Acta 935, 79–86.
[4] Paradies, G. and Ruggiero, F.M. (1989) Arch. Biochem. Biophys. 269, 595–602.
[5] Paradies, G. and Ruggiero, F.M. (1990) Arch. Biochem. Biophys. 278, 424–430.
[6] Paradies, G. and Ruggiero, F.M. (1990) Biochim. Biophys. Acta 1016, 207–121.
[7] Paradies, G. and Ruggiero, F.M. (1991) Biochim. Biophys. Acta 1070, 180–186.
[8] Paradies, G., Ruggiero, F.M., Dinoi, P., Petrosillo, G. and Quagliariello, E. (1993) Arch. Biochem. Biophys. 307, 91–95.
[9] Paradies, G., Ruggiero, F.M., Petrosillo, G. and Quagliariello, E. (1994) Biochim. Biophys. Acta 1225, 165–170.
[10] Hostetler, K.Y. and Van den Bosh, H. (1972) Biochim. Biophys. Acta 260, 380–386.
[11] Hostetler, K.Y. (1991) Biochim. Biophys. Acta 1086, 139–140.
[12] Cao, S.G., Cheng, F., Angel, A. and Hatch, G.M. (1995) Biochim. Biophys. Acta 1256, 241–244.
[13] Hoch, F.L. (1992) Biochim. Biophys. Acta 1113, 71–133.
[14] Noel, H. and Pande, S.V. (1986) Eur. J. Biochem. 155, 99–102.
[15] Kadenbach, B., Mende, P., Kolbe, H.V.J., Stipani, I. and Palmieri, F. (1982) FEBS Lett. 139, 109–112.
[16] Fry, M. and Green, D.E. (1981) J. Biol. Chem. 256, 1874–1880.
[17] Nicolay, K. and deKruijff, B. (1987) Biochim. Biophys. Acta 892, 320–330.
[18] Robinson, N.C. (1993) J. Bioenerg. Biomembr. 25, 153–163.
[19] Paradies, G. (1984) Biochim. Biophys. Acta 766, 446–450.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Hackenbrock, C.R. and Chazotte, B. (1986) Methods Enzymol. 125, 35–45.
[22] Ruggiero, F.M., Landriscina, C., Gnoni, G.V. and Quagliariello, E. (1984) Lipids 19, 171–178.
[23] Robinson, N.C., Zborowski, J. and Talbert, L.H. (1990) Biochemistry 29, 8962–8969.
[24] Wiesner, R., Ludwig, P., Schewe, T. and Rapoport, S.M. (1981) FEBS Lett. 123, 123–126.