Vol. 84, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 14, 1978 Pages 202-207

CHOLESTEROL ENRICHMENT OF NORMAL MITOCHONDRIA IN VITRO: A MODEL SYSTEM WITH PROPERTIES OF HEPATOMA MITOCHONDRIA

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Received July 27,1978

SUMMARY

It is known that rat hepatoma mitochondria contain higher levels of endogenous cholesterol than do mitochondria from normal liver. In the experiments described here, normal liver mitochondria were enriched with cholesterol by a solid-state transfer process in vitro and some of their enzymic properties were compared with literature values reported for hepatoma mitochondria. Striking parallels were seen. The data indicate that normal mitochondria, enriched with cholesterol in vitro, may create an interesting model system for examining some metabolic characteristics of tumor mitochondria.

INTRODUCTION

High levels of endogenous cholesterol have been found in hepatoma mitochondria (1). These mitochondria differ from normal rat liver mitochondria in some of their metabolic properties (2). We have developed a solid-state transfer method by which mitochondria isolated from normal rat liver can be significantly enriched with exogenous cholesterol in vitro. Enrichment was accomplished by co-incubation of freshly isolated mitochondria with dextran beads to which cholesterol had been hydrophobically adducted. ADP:O and respiratory (acceptor) control ratios, using succinate as substrate, as well as ATPase activity in both the presence and absence of 2,4-dinitrophenol, were investigated. We found that mitochondria enriched with cholesterol vary markedly from normal mitochondria in some of the above parameters. Furthermore, such enriched mitochondria show remarkable enzymic similarities to mitochondria isolated from rat hepatomas.

0006-291X/78/0841-0202$01.00/0

METHODS AND MATERIALS

Isolation of Mitochondria

Mitochondria were isolated from livers of 300-400 gram male Long-Evans rats by the method of Kielley and Kielley (3), using 0.25 M sucrose and 0.001 M EDTA, pH 7.4, as the isolation and suspension medium. Final protein concentrations were determined by the Biuret method (4) employing 0.33% (w/v) deoxycholate.

Cholesterol Enrichment

20 μCi [^{3}H] -cholesterol (Spec. act., 1 mCi/mmole; Amersham, Arlington Heights, Ill.), and 38.6 mg carrier cholesterol were added to 1.0 gram benzene-washed Sephadex® G-10 beads (Pharmacia, Piscataway, N.J.) in 50 ml of benzene. The benzene was removed by rotary evaporation under vacuum resulting in uniformly coated beads. Three 25 ml erlenmeyer flasks, each containing 1.0 ml resuspended mitochondrial aliquots (40 mg protein), were incubated with either 250 mg of benzene-washed ("nude") beads, 250 mg of cholesterol-coated beads, or 500 mg of cholesterol-coated beads, respectively, for 15 minutes at 0°C with gentle shaking. The beads were extracted from the suspension by low speed centrifugation over a 60% sucrose cushion. The extent of cholesterol incorporation into the mitochondria was determined both by liquid scintillation counting and by the colorimetric assay of Webster (5). The results from both methods agreed to within 2.5%.

Mitochondrial Respiration

Oxygen consumption was measured with a YSI model 53 oxygen monitor. The incubation mixture contained 7.5 mM disodium succinate, 10 mM potassium phosphate (pH 7.4), 100 mM sucrose, 3.3 mM $MgCl_2$, 0.25 mM ADP, and approximately 2 mg of mitochondrial protein /3 ml incubation volume. ADP:O and respiratory control ratios were calculated graphically according to Estabrook (6) at 28°C.

ATPase Studies

ATP hydrolysis experiments were performed as described by Coleman (7) using 100 mM sucrose, 20 mM tris maleate (pH 7.4), 10 mM ATP and approximately 8 mg of mitochondrial protein in a final volume of 4 ml. Incubation media also contained 20 μM 2,4-dinitrophenol or 5 mM $MgCl_2$, where indicated with the Tables and Figure. Aliquots (0.5 ml) of the mitochondrial incubations were terminated with ice cold trichloroacetic acid. Orthophosphate release was determined by the method of Chen, et al.(8).

RESULTS

Table I demonstrates that the cholesterol content of mitochondria can be increased by the procedure described in Methods, which allowed for the reproducible and quantitative enrichment of mitochondria with cholesterol. Functional studies were done with mitochondria enriched approximately 6-fold and 12-fold.

The oxygen consumption studies (Table II) indicated that cholesterol

Table I EXOGENOUS CHOLESTEROL ENRICHMENT OF MITOCHONDRIA

Experiment	cholesterol content (nmole/mg protein)
Mitochondria (control) [a]	26.2 ± 0.3 (5)
Enrichment level I [b]	189.6 ± 17.8 (4)
Enrichment level II [c]	336.3 ± 9.3 (4)

Mitochondria incubated with: [a] 250 mg "nude", benzene-washed dextran beads; [b] with 250 mg cholesterol-coated beads; [c] with 500 mg cholesterol-coated beads. Cholesterol content given ± S.D. Numbers in parentheses indicate number of separate mitochondrial preparations. Each preparation was assayed in duplicate for each experimental condition.

Table II CHOLESTEROL ENRICHMENT AND OXYGEN CONSUMPTION

Experiment	ADP:O Ratio	Respiratory Control Ratio
Mitochondria (control)	1.81 ± 0.13 (16)	4.10 ± 0.18 (16)
Enrichment level I	1.80 ± 0.09 (16)	3.72 ± 0.14 (16)
Enrichment level II	1.71 ± 0.14 (16)	2.61 ± 0.13 (16)

Mitochondria were prepared, cholesterol-enriched and assayed as given in Methods and Table I. Data are given ± S.D., and values in parentheses indicate individual mitochondrial preparations, each one assayed a minimum of twice for the experimental condition.

enrichment had no effect on ADP:O ratios. Respiratory control ratios, however, were seen to decrease somewhat at high levels of cholesterol enrichment. Furthermore, the decrease in the respiratory control ratio was non-linear as a function of linear increases in the amount of cholesterol taken up by mitochondria.

Table III shows that the rate of ATP hydrolysis by mitochondria increased non-linearly with linear cholesterol incorporation. When the cholesterol level was raised to 189.6 nmole/mg mitochondrial protein, a 42% increase in ATPase activity was observed; when the cholesterol level was brought to 336.3 nmole/mg protein, the increase in ATPase activity was 137%. Furthermore, our cholesterol-enriched mitochondria retained the ability to be stimulated by low uncoupling concentrations of 2,4-dinitrophenol.

Figure I illustrates the time-course of ATP hydrolysis by control mitochondria and cholesterol-enriched mitochondria.

DISCUSSION

It has been reported that hepatoma mitochondria contain unusually high cholesterol levels. For example, Feo, et al. (1), found that different rat hepatoma lines exhibited varying mitochondrial cholesterol contents ranging from 3.9 to 6.2 times that of normal liver mitochondria.

While rat hepatoma mitochondria were found to exhibit ADP:O ratios which are not significantly different from those obtained with normal mitochondria, they did show substantially lower respiratory control ratios (1,2,9,10,11-14).

When mitochondria from several lines of rat hepatoma (which reportedly possess higher than normal levels of endogenous cholesterol) were examined, Kaschnitz, et al., (10) obtained latent ATPase activities between 2 to 3 times that of normal liver mitochondria. With regard to uncoupler stimulation of ATPase activity in hepatoma mitochondria, the literature is somewhat controversial. Early papers claimed that liver tumor mitochondria exhibited a low stimulation of ATP hydrolysis by uncouplers (15-17). After extensive study of the effects of uncouplers on hepatoma mitochondria, Kaschnitz, et al., (10), found that maximal ATPase could, in fact, be observed. In a recent paper, Pedersen (2), also demonstrated that simulation of ATPase activity by 2,4-dinitrophenol could be achieved with isolated rat hepatoma

Table III CHOLESTEROL ENRICHMENT AND MITOCHONDRIAL ATPase

Experiment	nmoles P_i /min/mg protein	
	without 2, 4-DNP[a)]	with 2,4-DNP[b)]
Mitochondria (control)	3.96 ± 0.22 (4)	42.7 ± 2.4 (3)
Enrichment level I	5.63 ± 0.27 (4)	73.1 ± 3.2 (4)
Enrichment level II	9.38 ± 0.43 (4)	125.2 ± 5.7 (3)

Data are for intact mitochondrial ATPase in the absence of added Mg^{2+}. Comparison of ATPase ± added Mg^{2+} (not shown) permitted estimation of damaged mitochondria, which was below 8%. [a)] Latent ATPase determined after 24 min; [b)] ATPase determined after 8 min in the presence of 20 μM uncoupler.

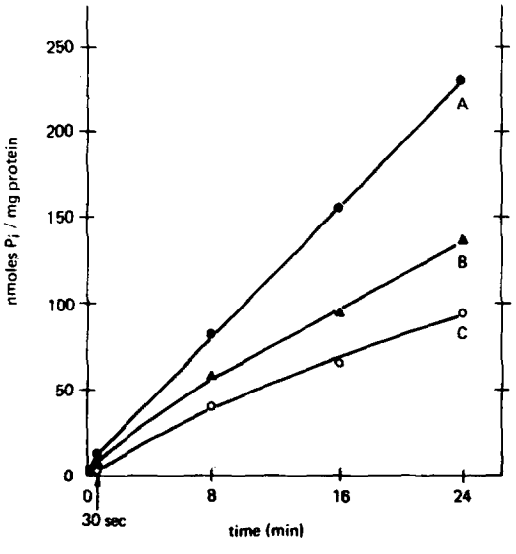

FIGURE I: Time-course of mitochondrial ATPase activity with cholesterol enrichment via the solid-state cholesterol transfer process described in Methods. (A) cholesterol enrichment 13-fold over control; (B) cholesterol enrichment 7-fold over control; (C) control incubation (employing "nude" dextran beads).

mitochondria, but only if they were incubated with ATP prior to addition of uncoupler.

In the experiments reported here, freshly isolated mitochondria from normal rat liver tissue were exogenously enriched with cholesterol to levels approximately equal to or significantly greater than those reported for hepatoma mitochondria. The mitochondria so enriched, exhibited unchanged ADP:O ratios, decreased respiratory control ratios, increased latent ATPase activity, and the retention of their capacity to manifest uncoupler-stimulated ATPase activity. These data indicate that by exogenously increasing the mitochondrial cholesterol concentration, a system is created, some of whose enzymic activities parallel those observed with mitochondria obtained from frequently studied tumor systems.

ACKNOWLEDGMENTS: Aided by grants from the American Cancer Society, (IN-14-0), and the USPHS (CA 13074). R.B. and A.W. are undergraduate honor students in the Department of Biology.

REFERENCES

1. Feo, F., Canuto, R.A., Garcea, R. and Gabriel, L. (1975) Biochim. Biophys. Acta., 413, 116-134.
2. Pedersen, P.L.(1978) Prog. Exptl. Tumor Res. 22, 190-274.
3. Kielley, W.W. and Kielley, R.K. (1951) J. Biol. Chem. 191, 485-500.
4. Cleland, K.W. and Slater, E.C. (1953) Biochem. J. 53, 547-556.
5. Webster, D. (1962) Clin. Chim. Acta. 7, 277-284.
6. Estabrook, R.W. (1967) Meth. in Enzymology 10, 41-47.
7. Coleman, P.S. (1973) Biochim. Biophys. Acta 305, 179-184.
8. Chen, P.S., Toribara, T.Y. and Warner, H. (1956) J. Analytical Chem. 19, 1756-1758.
9. Pedersen, P.L. and Morris, H.P. (1974) J. Biol. Chem. 249, 3327-3334.
10. Kaschnitz, R.M., Hatefi, Y. and Morris, H.P. (1976) Biochim. Biophys. Acta 449, 224-235.
11. Pedersen, P.L., Greenawalt, J.W., Chan, T.L. and Morris, H.P. (1970) Cancer Res. 30, 2620-2626.
12. Ohnishi, T., Hemington, J.G., La Noue, K.F., Morris, H.P. and Williamson, J.R. (1973) Biochem. Biophys. Res. Comm. 55, 372-381.
13. Floridi, A., Blotta, I. and Caputo, A.(1974) Oncology 29, 461-469.
14. Baldwin, P.E., George, D.T., Cunningham, C.C., (1975) Experientia 31, 1333-1334.
15. Delvin, T.M. and Pruss, M.P. (1962) Proc. Am. Assoc. Cancer Res. 3, 315.
16. Pedersen, P.L., Eska, T., Morris, H.P. and Catterall, W.A. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1079-1082.
17. Kolarov, J. Kuzela, S., Krempasky, V. and Ujhazy, V. (1973) Biochem. Biophys. Res. Commun. 55, 1173-1179.