IN VITRO STIMULATION OF LECITHIN SYNTHESIS IN RAT LIVER MITOCHONDRIA AND MICROSOMES AFTER TREATMENT WITH PHOSPHOLIPASE C

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SUMMARY

Rat liver mitochondria in which diglycerides were generated by phospholipase C treatment were shown to incorporate labeled choline from cytidine-5'diphospho-[Me-^{14}C]choline into lecithin to an extent which could not be ascribed to microsomal contamination. The response of this enzymatic activity to the extent of phospholipase C degradation was qualitatively different in microsomes and mitochondria, suggesting clearly different properties of this enzyme in the two subcellular fractions.

INTRODUCTION

It is generally assumed that the major mitochondrial phospholipid constituents phosphatidylcholine (lecithin) and phosphatidylethanolamine are synthesized on the endoplasmic reticulum (1-6). Of the enzymes involved in the complete biosynthesis of these lipids it is particularly the CDP-choline: 1,2 diglyceride choline phosphotransferase (E.C. 2.7.8.2.)*) and its ethanolamine-linked equivalent which have been recovered in the microsomal fraction of tissue homogenates (3,5,7). Addition of diglycerides has been found to stimulate the incorporation of ^{14}C-labeled choline from CDP-[Me-^{14}C]choline into lecithin when microsomal preparations were used as the enzyme source (3,5). Any incorporation observed with mitochondrial preparations has usually been ascribed to microsomal contamination (1-5). There is only one recent report in which appreciable cholinephosphotransferase activity is observed in a mitochondrial fraction (8). The addition of diglycerides as choline acceptors suffers from the poor dispersability of these compounds, which neccessitates the use of detergents. We have attempted to circumvent this obvious dis

*) CDP: cytidine-5'diphospho-

advantage by generating the diglycerides *in situ* by means of phospholipase C (E.C. 3.1.4.3)**). In the figures shown, experiments are presented in which the capacity was measured of isolated rat liver mitochondria and microsomes to incorporate the radioactivity from CDP-[Me-^{14}C]choline into the phosphatidylcholine.

METHODS AND MATERIALS

Mitochondria from the livers of male wistar rats (160 - 190 g) were isolated essentially according to the method of Loewenstein *et al.* (9) with the exception that the homogenization medium was 0.25 M sucrose, 3.0 mM Tris-HCl, 1.0 mM EDTA, pH 7.4 and the washes were performed in the medium without EDTA. Microsomes were prepared from a 10 min 20.000 g supernatant by centrifuging for 1 h at 105.000 g. After cellfractionation protein was determined by a Lowry procedure (10). For PLC treatment mitochondria or microsomes (2 ml, 40 mg of protein) were incubated at 37° C with the indicated amounts of PLC in presence of 5.0 mM $CaCl_2$. The reaction was stopped by addition of 2.0 ml 15 mM phenanthroliniumchloride pH 5.5, followed by one or two washes. Phospholipid hydrolysis by PLC was calculated by measuring chloroform soluble phosphorus (11). Treated or untreated fractions (3.0 mg of protein) were incubated for the time indicated at 37° C with the indicated amounts of CDP-[Me-^{14}C]choline in 1.0 ml medium consisting of 0.187 M sucrose, 11.0 mM cysteine HCl, 22.0 mM $MgCl_2$, 41.25 mM Tris-HCl pH 7.4. The reaction was stopped by addition of chloroform-methanol followed by extraction according to Bligh and Dyer (12). From the specific radioactivity of the CDP-choline, the amount of radioactivity in the chloroform extract and the extent of phospholipid degradation by PLC the amount of lecithin formed per µmole of phospholipid could be calculated. Since in preliminary experiments we found the enzyme activity to be membrane-bound we considered it meaningful to express the specific enzyme activity on the basis of amount of membrane (i.e. phospholipid) rather than amount of protein. Invariably more than 95 % of the chloroform soluble radioactivity was found to

**) Phospholipase C will be abbreviated as PLC.

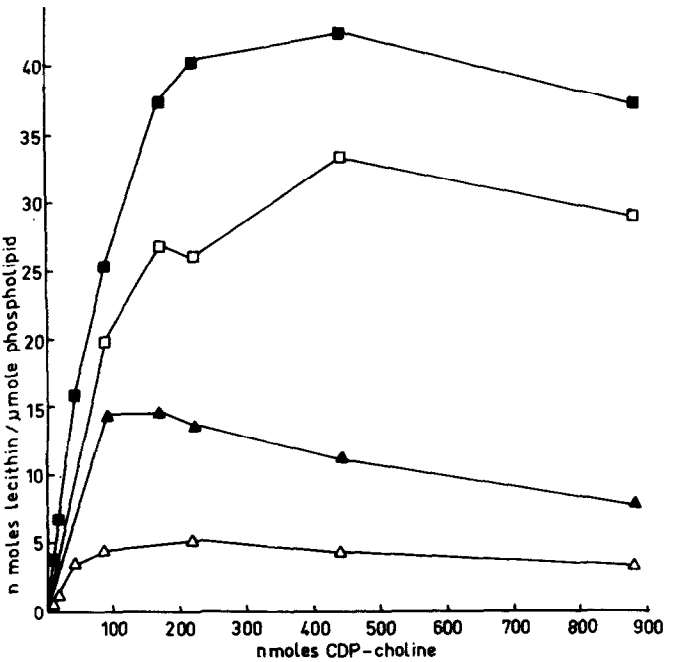

Fig. 1. For PLC treatment mitochondria or microsomes were incubated for 3 min. with 1.0 unit of PLC. Percentage of phospholipid degraded was 4.8 % for mitochondria and 21.4 % for microsomes. Incubation time: 30 min. Δ——Δ mitochondria; ▲——▲ PLC-treated mitochondria; □——□ microsomes; ■——■ PLC-treated microsomes.

be associated with lecithin after thin-layer chromatography. The extent of mitochondrial contamination with microsomes was judged by assay of NADPH-cytochrome *c* reductase (13) and varied from 2-10 % (also on phospholipid rather than on protein basis). CDP-[Me-^{14}C]choline (New England Nuclear) was diluted with unlabeled CDP-choline (Sigma) to a specific radioactivity of 32.000 dpm/220 nmoles. PLC, purified from *Bacillus cereus* was a gift from Dr. R.F.A. Zwaal from the Laboratory of Biochemistry, University of Utrecht.

RESULTS AND DISCUSSION

Fig. 1 shows the dependence on CDP-choline concentration of lecithin formation by untreated or PLC-treated mitochondria or microsomes. It is obvious that the microsomal contamination (2-10 %) cannot account for the extent of mitochondrial lecithin synthesis observed, particularly after PLC degradation. In presence of approximately 150 nmoles of CDP-choline the activity in PLC-

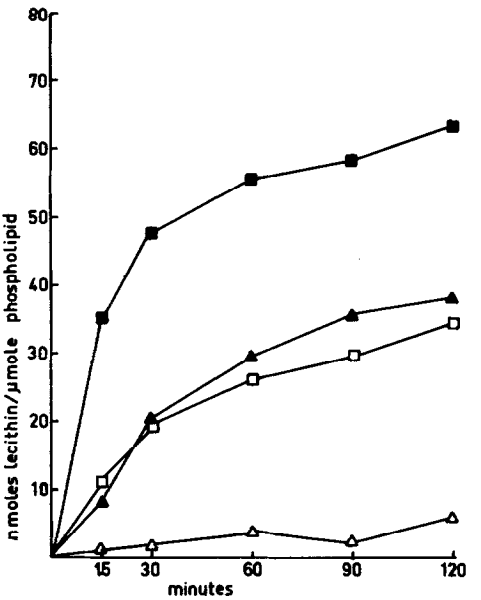

Fig. 2. Untreated or PLC-treated mitochondria (5.0 min., 0.18 units PLC) or microsomes (2.0 min. 0.78 units PLC) were incubated for various periods of time with 220 nmoles of CDP-[Me-^{14}C]choline. The amount of phospholipid degraded after PLC treatment was 50.9 % for mitochondria and 51.3 % for microsomes. Δ——Δ mitochondria; ▲——▲ PLC-treated mitochondria; □ —— □ microsomes; ■ —— ■ PLC-treated microsomes.

treated mitochondrial is more than one third the activity of similarly treated microsomes.

In fig. 2 which presents a time course experiment in the presence of 220 nmoles CDP-choline mitochondrial activity after PLC treatment even exceeds 50 % of the microsomal activity. The rate of incorporation is approximately linear for 30 minutes.

An additional indication that mitochondrial activity is not due to the presence of microsomes is found in fig. 3. From this figure it is clear that the microsomal and mitochondrial cholinephosphotransferases are separate enzyme systems because of their quite different response to PLC treatment. In microsomes lecithin synthesis continues to increase with increasing amounts of microsomal phospholipid being degraded by the preceeding PLC treatment, whereas with mitochondria a maximum is observed at approximately 20 % hydrolysis of mitochondrial phospholipids. It is emphasized that at this extent of phospholipid hydrolysis mitochondrial and microsomal activities are similar !

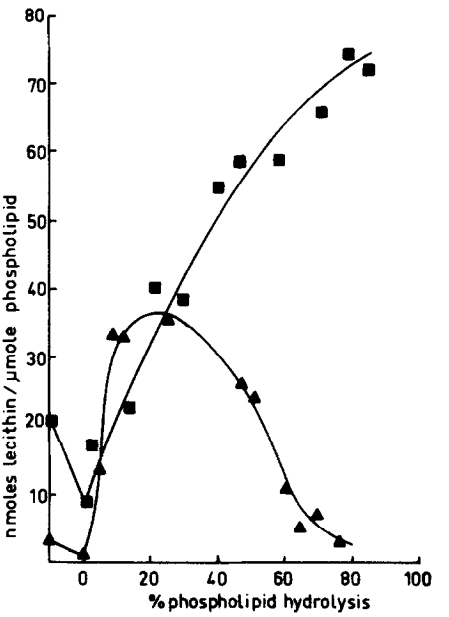

Fig. 3. Cellfractions treated with PLC (0-6 min. with 0,5-4.0 units PLC) were incubated for 30 min. with 220 nmoles CDP-[Me-^{14}C]choline as described in Materials and Methods. Δ— mitochondria; ▲——▲ PLC-treated mitochondria;□ — microsomes; ■ ——■ PLC-treated microsomes.

In conclusion, our experiments show that lecithin formation from diglycerides and CDP-choline can be catalysed by isolated mitochondria provided the diglyceride is present in suitable form. It is not likely that *in vivo* diglycerides will ever be available in mitochondria in amounts as were required in our experiments to demonstrate appreciable lecithin synthesis. However, lower rates of synthesis are probably sufficient to meet with the need of the relatively slowly turning over rat-liver mitochondria for net phospholipid synthesis. Cardiolipin synthesis by mitochondria also has been found to be of moderate magnitude (14) and the relatively high turnover rates of certain mitochondrial phospholipid constituents (15) might be merely a reflection of the rapid exchange of phospholipids which can take place between mitochondria and microsomes (16). It is by no means certain as yet that this exchange process bears any relation to the phenomenon of mitochondrial biogenesis.

The origin *in vivo* of the diglyceride acceptor remains to be established. Although the formerly established capacity of mitochondria to fully acylate

glycero-3-phosphate has recently been disputed (17), the activity of mitochondria to synthesize phosphatidic acid should not be disregarded. To what extent other cell fractions such as the cytoplasm (18) may contribute to the subsequent dephosphorylation is not yet clear.

ACKNOWLEDGEMENT

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