THE EFFECT OF (−)CARNITINE ON THE METABOLISM OF PALMITATE IN LIVER CELLS ISOLATED FROM FASTED AND REFED RATS

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1. Introduction

In studies on fatty acid metabolizing enzymes in fasted and fed rats it has been observed that the activity of palmityl-CoA: carnitine palmityltransferase (EC 2.3.1.21) and acyl-CoA: glycerophosphate acyltransferase (EC 2.3.1.15) in the liver vary in opposite directions depending on the nutritional state of the animal [1]. In fasting the activity of carnitine palmityltransferase increases and the glycerophosphate acyltransferase decreases. Fasting also increases the content of carnitine in the liver [2,3], and in perfusion studies it has been observed that addition of carnitine to the perfusion fluid accelerates ketogenesis in livers from fed rats [3].

In studies on isolated mitochondria Borrebaek [4] found that the acylation of glycerophosphate has a certain preference over the acylation of carnitine when a low concentration of palmitate was added to the incubation system. Both reactions were easily saturated with high palmitate concentrations.

These observations seen together suggest that both the relative activities of carnitine palmityltransferase and glycerophosphate acyltransferase and variations in the concentration of carnitine can have directing effects on the metabolic fate of fatty acids in the liver.

Recent studies on carnitine in isolated liver cells have shown that these cells have lost 3/4 to 4/5 of their normal carnitine content during the preparation procedure. The cells again take up carnitine when it is added to the medium (Christiansen and Bremer, unpublished results). Glycerol or fructose are known to increase the concentration of glycerophosphate in the liver [5].

To find out whether both the changes in enzyme activities and the changes in the concentrations of the acyl acceptors (carnitine and glycerophosphate) may regulate the metabolic fate of fatty acids in the liver, we have tested the effects of carnitine, glycerol, and fructose on fatty acid metabolism in cells from fasted and fasted/carbohydrate refed rats. The experiments have shown that the rate of fatty acid oxidation and ketogenesis increase when carnitine is added to the incubation medium, especially in cells from animals refed carbohydrate. However, cells from carbohydrate refed animals esterify much more and oxidize less than cells from fasted animals, even when the cells contain 2 mM carnitine (normal content 0.1–0.3 mM). Glycerol and fructose inhibit fatty acid oxidation and some times even esterification, most likely because they lead to a drop in ATP and fatty acid activation in the cells.

2. Materials and methods

[1-^{14}C]Palmitate was obtained from the Radiochemical Center, Amersham, England. [CH_3-^{3}H](−)Carnitine was prepared according to Stokke and Bremer [6]. Essentially fatty acid free bovine albumin, *N*-2 hydroxyethylpiperazin-*N'*-2-ethanesulfonic acid (HEPES), hexokinase, glucose 6-dehydrogenase, and β-hydroxybutyrate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Parenchymal liver cells were prepared and purified according to Seglen [7] except that only 0.03 M HEPES was used in the suspension medium. Cell suspensions were always examined microscopically for trypan blue exclusion. A high percentage (90–95%) of unstained cells was routinely observed. The cellular

respiration was not stimulated by succinate, indicating that the plasma membrane was not damaged. The liver cells removed extracellular palmitate from the albumin-bound form and metabolized it with a high rate of 44–52 nmol × mg protein^{-1} × 30 min^{-1}.

Since the uptake of carnitine in the liver cells is time dependent, incubations were performed as follows: 5 ml of the cell suspension (approx. 50 mg protein) were preincubated with or without 4 mM (−)carnitine for 30 min at 37°C. At this time the carnitine content of the cells incubated with carnitine had increased from approximately 0.1 mM to about 2 mM. Samples of 0.5 ml of the preincubated cell suspension was then added to 1.5 ml oxygenated suspension buffer containing substrates and other additions to give the following final concentrations: Albumin, 0.3 mM; [1-^{14}C]palmitate, 0.25 mM and where indicated: Glycerol, 5 mM; fructose, 7.5 mM; (−)carnitine, 1 mM. The bovine albumin was dialyzed before use. All incubations were done in duplicate. Flasks where the formation of radioactive CO_2 was to be measured had a center well which contained 0.2 ml 10% NaOH and a folded filter paper, and a side arm which contained 0.2 ml 5 M $HClO_4$. At the end of the incubation the $HClO_4$ was tipped in and the shaking was continued for 60 min to trap all $^{14}CO_2$. Analyses on lipids and acid soluble products were performed on an identical pair of incubation flasks. At the end of the incubation samples of 0.5 ml of the incubation mixture were pipetted into 10 ml of chloroform–methanol (2:1) and into 0.25 ml 2 N $HClO_4$.

After about 2 h the chloroform–methanol mixture was filtered and treated according to Folch et al. [8]. Total lipid radioactivity was measured in 1 ml of the washed chloroform phase. The remaining chloroform phase was evaporated to dryness and the lipid residue dissolved in 100 μl hexane. A sample of 25 μl was chromatographed with hexane–diethylether–glacial acetic acid (80:20:1) on silicic acid thin-layer plates (Stahl H). The lipid spots were made visible in a iodine vapour chamber. The spots corresponding to phospholipids, diglyceride, free fatty acids and triglyceride were scraped from the plates for determination of distribution of radioactivity.

The acid soluble radioactivity was measured in the $HClO_4$ extracts. The recovery of radioactivity in the different fractions was better than 95%.

Ketone bodies and ATP were measured in the $HClO_4$ extracts after neutralization with KOH. Acetoacetate and β-hydroxybutyrate were measured with β-hydroxybutyrate dehydrogenase and ATP by measuring the appearance of NADPH in the presence of hexokinase, glucose 6-phosphate dehydrogenase, NADP, and glucose.

Long-chain acyl-CoA was determined as acid insoluble CoA after alkaline hydrolysis [9] by an exchange assay with palmitylcarnitine transferase [10]. Long-chain acylcarnitine was measured as butanol-extractable radioactivity in incubations with nonradioactive palmitate and [CH_3-^{3}H](−)carnitine.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer Model 3375. An external standard method was used for quenching corrections.

3. Results and discussion

Fig.1 shows the time course of [1-^{14}C]conversion to oxidized products (acid-soluble products + CO_2) and complex lipids (phospholipids, diglyceride and triglyceride) in liver cells from a normally fed rat incubated with and without carnitine in the medium. The addition of carnitine decreased the formation of triglyceride with about 45% and increased the formation of acid soluble products correspondingly (mainly ketone bodies). The formation of radioactive CO_2 decreased as the rate of fatty acid oxidation

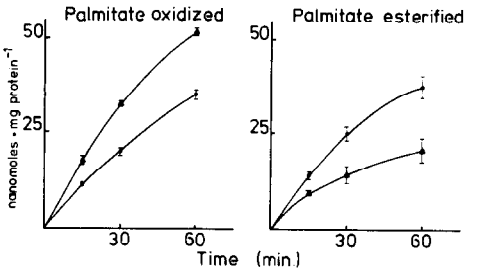

Fig.1. Effect of (−)carnitine on the metabolism of palmitate in liver cells isolated from normally fed rats. The incubation conditions were as described in Materials and methods. Values from two different experiments are indicated by the bars at each point. Palmitate oxidized includes acid soluble products + CO_2. Palmitate esterifies includes phospholipids, diglyceride and triglyceride. (●) Cells preincubated without carnitine. (▲) Cells preincubated with 4 mM carnitine.

increased (not shown). The incorporation of palmitate into phospholipids was much less affected than triglyceride formation. The rate of fatty acid activation (the sum of reaction products) was not affected by the addition of carnitine in this experiment, but in cells from carbohydrate-refed rats, where the effect of carnitine was more pronounced, it induced some increase in fatty acid activation (table 2).

Table 1
The effects of (−)carnitine, glycerol, and fructose on the metabolism of palmitate in liver cells isolated from a rat fasted for 24 h.

Additions	Palmitate metabolized	Palmitate esterified	Palmitate oxidized	Ratio esterified / oxidized	Long-chain acyl-CoA	Long-chain acylcarnitine	ATP
None	45.8	12.6	33.2	0.38	0.36	–	7.1
Glycerol	37.9	15.9	22.0	0.72	0.16	–	2.3
Fructose	36.3	15.4	20.9	0.74	0.16	–	2.8
Glycerol + fructose	31.7	13.3	18.4	0.72	0.11	–	1.3
Carnitine	44.7	9.0	35.7	0.25	0.30	4.7	7.1
Carnitine + glycerol	37.7	10.4	27.3	0.38	0.17	2.8	2.7
Carnitine + fructose	35.2	8.0	27.2	0.29	0.14	1.1	2.0
Carnitine + glycerol + fructose	31.7	7.0	24.7	0.28	0.13	1.2	1.1

Cells corresponding to 5.4 mg protein were incubated as described in Materials and methods. The results are expressed as nmoles × mg protein^{-1} × 30 min^{-1}.

Table 2
The effects of (−)carnitine, glycerol, and fructose on the metabolism of palmitate in liver cells isolated from a rat fasted for 48 h and then refed carbohydrate for 48 h

Additions	Palmitate metabolized	Palmitate esterified	Palmitate oxidized	Ratio esterified / oxidized	Long-chain acyl-CoA	Long-chain acylcarnitine	ATP
None	52.1	45.9	6.2	7.4	0.31	–	7.5
Glycerol	48.2	44.0	4.2	10.5	0.14	–	5.5
Fructose	44.9	40.6	4.3	9.4	0.17	–	5.1
Glycerol + fructose	41.4	36.7	4.7	7.8	0.13	–	2.8
Carnitine + Carnitine	56.7	37.4	19.3	1.9	0.27	2.1	7.9
Carnitine + glycerol	52.4	36.7	15.7	2.3	0.16	0.9	5.0
Carnitine + fructose	48.0	30.2	17.8	1.7	0.19	1.1	4.0
Carnitine + glycerol + fructose	43.2	26.3	16.9	1.6	0.17	1.0	2.3

Cells corresponding to 4.6 mg protein were incubated as described in Materials and methods. The results are expressed as nmoles × mg protein^{-1} × 30 min^{-1}.

Tables 1 and 2 show the metabolism of palmitate in liver cells isolated from a fasted and a fasted-carbohydrate-refed rat, and the effects of different additions on this metabolism. Comparison with fig.1 shows that the cells from the fasted rat oxidized more and esterified less palmitate than did the cells from the normally fed rat, while the cells from the carbohydrate-refed rat oxidized less and esterified more. This is in agreement with previous observations [11].

Carnitine had the strongest effect in the cells from the carbohydrate-refed rat (table 2) where the formation of acid soluble products was increased about three times (mainly ketone bodies) while there was only a small effect in the cells from the fasted rat. Carnitine reduced the formation of triglyceride and radioactive CO_2. However, in spite of the stronger effect of carnitine in the 'refed' cells, they were not converted to 'fasted' cells. Even in the presence of carnitine the rate of oxidation in the cells from the refed rat was only about half of the rate in the cells from the fasted rat, and the rate of esterification was still about four times higher. Thus, the esterification/oxidation ratio in the presence of carnitine was 1.9 in the cells from the refed rat compared to 0.25 in the cells from the fasted rat. These ratios were obtained with cells containing about 2 mM (−)carnitine which is well above the physiological range of 0.1–0.3 mM [2,3]. It is clear therefore that an increased carnitine concentration alone does not explain the altered fatty acid metabolism in the liver of fasted (or diabetic) animals. It seems likely that the distribution of fatty acids between oxidation and esterification is influenced also by the relative activities of the enzymes carnitine palmityltransferase and glycerophosphate acyltransferase which change their activities in the liver in opposit directions depending on the nutritional state of the animal [1].

Tables 1 and 2 show that both glycerol and fructose always inhibited fatty acid oxidation in our experiments. Esterification was increased only in cells from fasted rats while in cells from refed rats both oxidation and esterification was inhibited, probably because fatty acid activation was inhibited by the drop in ATP in the cells [5]. In our experiments ATP dropped with 30–85%, and there were similar decreases in the levels of long-chain acyl-CoA and long-chain acylcarnitine. It has been suggested that glycerol and fructose primarily inhibit fatty acid oxidation by being converted to products which compete with the fatty acids as oxidizable substrates in the mitochondria, rather than by increasing glycerophosphate concentration which might act as a trap for acyl-CoA. The theory about competing oxidizable substrates is made less likely by our results. Such a mechanism should give increased levels of acyl-CoA and acylcarnitine. The opposit effect was observed. Also, fructose decreased the β-hydroxybutyrate/acetoacetate ratio (not shown) at the same time as the rate of fatty acid oxidation and ketogenesis decreased. This indicates that the NADH/NAD ratio decreased in the mitochondria.

The esterification rate was only moderately increased by fructose and/or glycerol in cells from fasted rats. This effect nearly disappeared in the presence of carnitine, and it changed to inhibition under all conditions in cells from refed rats. It seems doubtful therefore that glycerophosphate concentration limited esterification to any great extent, even in fasted cells.

The level of acyl-CoA was very similar in the two types of cells. Still the rates of esterification were very different. Again this suggests that the activity of the glycerophosphate acyltransferase (more than the level of its substrates) regulates the rate of esterification.

The rate of fatty acid β-oxidation presumably is determined by the intramitochondrial level of long-chain acyl-CoA. Nothing is known about the difference between the extra- and the intra-mitochondrial long-chain acyl-CoA/CoA ratios in the intact cell. However, the function of carnitine and the carnitine palmityltransferase may be visualized as equalizing these ratios. Such an equalization then may be obtained by increasing the carnitine palmityltransferase activity and/or by increasing the carnitine concentration. (The carnitine palmityltransferase's K_M for carnitine is relatively high, especially in the presence of high acyl-CoA concentrations [12]). Thus, while there is apparently no major effect of carnitine on the total acyl-CoA in the cells, there may still be a drop in extra-mitochondrial and a pronounced increase in intra-mitochondrial acyl-CoA. Such a redistributing effect of carnitine on the acyl-CoA within the cell may explain both the increased rate of oxidation and a certain drop in esterification, especially in the cells from the carbohydrate refed rats.

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