Activity of carnitine palmitoyltransferase in mitochondrial outer membranes and peroxisomes in digitonin-permeabilized hepatocytes

Selective modulation of mitochondrial enzyme activity by okadaic acid

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A procedure is described for the rapid measurement of the activity of mitochondrial-outer-membrane carnitine palmitoyltransferase (CPT_o) and peroxisomal carnitine palmitoyltransferase (CPT_p) in digitonin-permeabilized hepatocytes. CPT_o activity was determined as the tetradecylglycidate (TDGA)-sensitive malonyl-CoA-sensitive CPT activity, whereas CPT_p activity was monitored as the TDGA-insensitive malonyl-CoA-sensitive CPT activity. Under these experimental conditions, the respective contributions of CPT_o and CPT_p to total hepatocellular malonyl-CoA-sensitive CPT activity were 74.6 and 25.4%, which correlated well with the values of 76.9 and 23.1% for the respective contributions of the mitochondrial and the peroxisomal compartment to total hepatocellular palmitate oxidation. The sensitivity of CPT_o to inhibition by malonyl-CoA was very similar to that of CPT_p; thus 50% inhibition of CPT_o and CPT_p activities was achieved with malonyl-CoA concentrations of 2.6 ± 0.5 and 3.0 ± 0.4 μ M respectively. Short-term incubation of hepatocytes with the phosphatase inhibitor okadaic acid (i) increased the activity of CPT_o and the rate of mitochondrial palmitate oxidation, (ii) decreased the affinity of CPT_o for palmitoyl-CoA substrate, and (iii) decreased the sensitivity of CPT_o to inhibition by malonyl-CoA. By contrast, neither the properties of CPT_p nor the rate of peroxisomal palmitate oxidation were changed upon incubation of cells with okadaic acid. Results indicate therefore that CPT_o, but not CPT_p, may be regulated by a mechanism of phosphorylation/dephosphorylation. The physiological relevance of these findings is discussed.

INTRODUCTION

Translocation of long-chain fatty acids into the mitochondrial matrix is a rather complex process which involves the co-ordinate action of a long-chain acyl-CoA synthetase, the mitochondrial-outer-membrane carnitine palmitoyltransferase (CPT_o), a carnitine/acylcarnitine translocase and the mitochondrial-inner-membrane carnitine palmitoyltransferase (CPT_p) [1–3]. Diet- and hormone-induced changes in the activity and sensitivity to malonyl-CoA of rat liver CPT_o are usually accompanied by parallel variations in the flux through the fatty-acid-oxidative pathway [1–3]. It is thus generally accepted that CPT_o plays a pivotal role in the control of long-chain fatty acid oxidation by liver mitochondria [1–3].

Similarly to mitochondria, peroxisomes possess the full enzymic equipment required for the β -oxidation of long-chain fatty acids [4,5]. Although the mechanism of fatty acid entry into peroxisomes remains unclear [5], it has been suggested that the transport of long-chain fatty acyl substrates into the peroxisomal matrix may occur by a carnitine-dependent mechanism [6]. In fact, peroxisomes have a carnitine palmitoyltransferase (CPT_p) enzyme which may make an important contribution to the hepatic synthesis of long-chain acylcarnitines [2,7]. Previous observations indicate that CPT_p and CPT_o share a number of common kinetic and regulatory properties, e.g. chain-length specificity for acyl-CoA substrate [7], pattern of sensitivity to inhibition by malonyl-CoA [7] and long-term modulation by diet and drugs [8].

Although most of the studies on rat liver CPT enzymes have focused on the long-term regulation of the kinetic and regulatory properties of CPT_o [2,3,9,10], the molecular mechanisms involved in these alterations are poorly understood. However, long-term changes are probably preceded by short-term control events. Short-term adaptive changes of hepatic CPT_o activity have so far not received as much attention as long-term adaptive changes. This may be due to the fact that short-term modulation of CPT_o activity is difficult to preserve during the procedure of cell disruption and subsequent isolation of mitochondria for enzyme assay. This problem may be circumvented by assaying CPT_o activity in a permeabilized-cell system [11]. The use of this procedure has shown that hepatic CPT is controlled in the short term by different types of agonists, including some which are potential modulators of the phosphorylation state of the enzyme [11–13]. In those studies, however, CPT activity was determined as the hepatocellular malonyl-CoA-sensitive CPT activity [11–13], and thus CPT_o and CPT_p activities were determined together.

In the present paper we describe a procedure for the rapid and separate determination of CPT_o and CPT_p activities in digitonin-permeabilized hepatocytes. For this purpose we made use of tetradecylglycidate (TDGA), a potent and specific inhibitor of CPT_o [14], which has no significant effect on CPT_p [6]. This procedure was employed to study the short-term regulation of CPT_o and CPT_p activities. The selective effects of the phosphatase inhibitor okadaic acid on CPT_o and mitochondrial palmitate oxidation suggest that a mechanism of phosphorylation/

Abbreviations used: CPH, 2-chloro-6-phenylhexanoate; CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CPT_i, CPT_o and CPT_p, CPT activity located in the mitochondrial inner membrane, mitochondrial outer membrane and peroxisomes respectively; TDGA, tetradecylglycidate.

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dephosphorylation may be involved in the control of CPT_o, but not of CPT_p.

MATERIALS AND METHODS

Materials

[1-¹⁴C]Palmitic acid and L-[Me-¹⁴C]carnitine were from Amersham International (Amersham, Bucks., U.K.). The latter was kindly given by Dr. H. R. Scholte, Erasmus University (Rotterdam, The Netherlands). Collagenase (type I), digitonin and okadaic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TDGA and 2-chloro-6-phenylhexanoate (CPH) were given by Dr. J. M. Lowenstein, Brandeis University (Waltham, MA, U.S.A.).

Isolation and incubation of hepatocytes

Male Wistar rats (250–300 g) which had free access to food and water were used all through this study. Hepatocytes were isolated as described previously [15] and incubated in Krebs–Henseleit bicarbonate buffer supplemented with 10 mM-glucose and 1% (w/v) defatted and dialysed BSA. Incubations (4–6 mg of cellular protein/ml) were carried out in a total volume of 2 ml at 37 °C under an atmosphere of O₂/CO₂ (19:1). Since stock solutions of TDGA and okadaic acid were prepared in dimethyl sulphoxide, control incubations had the corresponding dimethyl sulphoxide content. No significant effect on CPT activity and on the rate of fatty acid oxidation was observed at the concentrations of dimethyl sulphoxide used (0.1–0.2%, v/v).

Determination of CPT_o and CPT_p activities

Hepatocytes were incubated for 20 min in the absence or in the presence of 5 μ M-TDGA. This concentration of TDGA allows maximal inhibition of CPT_o activity (see the Results and discussion section). Samples were removed from both sets of incubations in order to monitor malonyl-CoA-sensitive CPT activity. CPT_o activity was thus determined as the TDGA-sensitive malonyl-CoA-sensitive CPT activity, whereas CPT_p activity was monitored as the TDGA-insensitive malonyl-CoA-sensitive CPT activity.

Measurement of malonyl-CoA-sensitive CPT activity was performed as described previously [11]. Standard determinations of CPT activity were carried out with 50 μ M-palmitoyl-CoA. CPT activity which was insensitive to 100 μ M-malonyl-CoA, representing CPT_i activity, was always subtracted from the CPT activity experimentally determined. CPT_i activity always accounted for less than 15% of the total CPT activity determined under our assay conditions and was not affected by either TDGA or okadaic acid. Furthermore, the degree of inhibition of hepatocellular CPT activity by 100 μ M-malonyl-CoA was similar at all the palmitoyl-CoA concentrations employed in the present study, i.e. 20–400 μ M (results not shown).

Determination of mitochondrial and peroxisomal palmitate oxidation

Hepatocytes were incubated for 20 min in the absence or in the presence of 5 μ M-TDGA (see the Results and discussion section). Samples were removed from both sets of incubations in order to monitor the rate of [1-¹⁴C]palmitate oxidation. Mitochondrial palmitate oxidation was thus determined as the TDGA-sensitive oxidation, whereas peroxisomal palmitate oxidation was measured as the TDGA-insensitive oxidation. For determination of palmitate oxidation, hepatocytes were incubated for 20 min with 0.4 mM-[1-¹⁴C]palmitate (0.05 Ci/mol) bound to BSA. In-

cubations were carried out for 20 min and oxidation products were quantified as described previously [16].

Other analytical methods

For assay of marker enzyme activities, samples of the cell suspensions were added to the CPT assay mixture and then the extracellular medium was separated from the 'ghosts' as described in [17]. Catalase, glutamate dehydrogenase, lactate dehydrogenase and monoamine oxidase were assayed by standard spectrophotometric methods [18].

Highly purified mitochondria and peroxisomes were isolated from hepatocyte suspensions as described in [7].

Intracellular levels of malonyl-CoA were measured by a radioenzymic method as described previously [15].

Protein was determined by the method of Lowry *et al.* [19] with BSA as a standard.

Statistical analysis

Results shown represent the means $\pm$ S.D. of the number of experiments indicated in every case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t* test.

RESULTS AND DISCUSSION

Recovery of marker enzyme activities in the extracellular medium after hepatocyte permeabilization with digitonin

Permeabilization of hepatocytes with digitonin allows the study of cellular CPT activity in a more or less natural environment and alleviates the necessity of preparing mitochondria for enzyme assay [11]. Previous experiments had shown that the optimal concentration of digitonin for monitoring hepatocellular malonyl-CoA-sensitive CPT activity was approx. 40 μ g/mg of cellular protein [11]. Exposure of the cells to this concentration of digitonin liberated more than 99% of total lactate dehydrogenase (a cytosolic marker enzyme) within 15 s. The integrity of the mitochondrial outer and inner membranes was proved by the fact that less than 1% of total monoamine oxidase (a mitochondrial-outer-membrane marker enzyme) and of total glutamate dehydrogenase (a mitochondrial-matrix marker enzyme) was released from the permeabilized cells after 1 min of exposure to digitonin. Under these conditions, the permeability of the peroxisomal membrane was not affected, since non-detectable amounts of catalase (a peroxisomal-matrix marker enzyme) was released. Moreover, no CPT activity was recovered outside the permeabilized cells. All these data indicate that the membrane environment of CPT_o, CPT_i and CPT_p is preserved throughout the 1 min procedure of cell permeabilization and enzyme assay.

Determination of CPT_o and CPT_p activities in digitonin-permeabilized hepatocytes

TDGA was used to determine separately CPT_o and CPT_p activities in a digitonin-permeabilized hepatocyte system. Increasing doses of TDGA decreased in parallel the malonyl-CoA-sensitive CPT activity in permeabilized hepatocytes and the rate of palmitate oxidation in intact hepatocytes (Fig. 1). TDGA interacts specifically and irreversibly with CPT_o, rendering the enzyme inactive [14], whereas CPT_p has been reported to be either unaffected [6] or significantly affected [20] by TDGA. As a control, we preincubated hepatocytes for 20 min with or without 5 μ M-TDGA, and highly purified mitochondria and peroxisomes were subsequently isolated [7] in order to determine CPT_o and CPT_p activities respectively. Under these conditions, CPT_o activity was observed to be completely inhibited by TDGA,

Fig. 1. Effect of TDGA on hepatocellular malonyl-CoA-sensitive CPT activity and on the rate of palmitate oxidation

Hepatocytes were incubated for 20 min in the presence of increasing doses of TDGA. Then samples were removed to determine both malonyl-CoA-sensitive CPT activity (○) and the rate of palmitate oxidation (●). Results show the means ± s.d. of four different cell preparations.

Table 1. Effect of preincubation of hepatocytes with TDGA on the activity of CPT_o and CPT_p

Hepatocytes were preincubated for 20 min with or without 5 μM-TDGA. Then cells were separated from the incubation medium and mitochondria and peroxisomes were subsequently isolated. Catalase and glutamate dehydrogenase activities are expressed as μmol/min per mg of protein, whereas malonyl-CoA-sensitive CPT activity is expressed as nmol/min per mg of protein. Results are means ± s.d. of three different cell preparations. Values in parentheses represent percentage inhibition by TDGA.

Fraction	TDGA in pre-incubation	Malonyl-CoA-sensitive CPT	Catalase	Glutamate dehydrogenase
Mitochondria	—	4.13 ± 0.56	36.2 ± 6.7	3.62 ± 0.25
	+	0 (> 99)	34.3 ± 5.9	3.84 ± 0.58
Peroxisomes	—	5.06 ± 0.38	7465 ± 1367	0.17 ± 0.04
	+	4.66 ± 0.20 (8)	7837 ± 1095	0.19 ± 0.02

whereas CPT_p activity was practically unaffected by this inhibitor (Table 1). Therefore the TDGA-sensitive malonyl-CoA-sensitive CPT activity was assumed to be CPT_o activity, whereas the TDGA-insensitive malonyl-CoA-sensitive CPT activity should represent CPT_p activity. Likewise, TDGA-sensitive palmitate oxidation should reflect the contribution of the mitochondrial compartment to total hepatic palmitate oxidation, whereas TDGA-insensitive palmitate oxidation represents peroxisomal palmitate oxidation (see also [21]). The reasons for the different results reported by Skopin *et al.* [20] as compared with those obtained by Buechler & Lowenstein [6] or by us (Table 1) are not apparent. It might be argued that rat liver also contains a microsomal medium-chain carnitine acyltransferase which is sensitive to inhibition by malonyl-CoA [22]. Although at low concentration palmitoyl-CoA is a substrate for microsomal medium-chain carnitine palmitoyltransferase, the activity of this enzyme is strongly inhibited at the concentrations of palmitoyl-CoA used in our assay [22]. Therefore, we believe that this

Table 2. Effect of okadaic acid on the activity of CPT_o and CPT_p and on the rate of mitochondrial and peroxisomal palmitate oxidation

Hepatocytes were incubated for 20 min with or without 5 μM-TDGA. Incubations were continued for an additional 20 min period with or without 1 μM-okadaic acid. Subsequently, samples were removed to determine the activity of CPT_o and CPT_p (expressed as nmol of palmitoylcarnitine formed/min per mg of cellular protein) as well as the rate of mitochondrial and peroxisomal palmitate oxidation (expressed as nmol of palmitate converted into product/h per mg of cellular protein). The rate of mitochondrial and peroxisomal palmitate oxidation was also monitored in parallel incubations carried out in the presence or the absence of 0.1 mM-CPH. Results show the means ± s.d. of ten different cell preparations for TDGA experiments and four different cell preparations for CPH experiments. Values in parentheses represent the contributions either to total enzyme activity or to total palmitate oxidation. *Significantly different (*P* < 0.01) from incubations with no additions.

Parameter	Addition to the incubations	
	None	1 μM-okadaic acid
Malonyl-CoA-sensitive CPT activity		
Total	4.05 ± 0.37	5.83 ± 0.66*
Mitochondrial	3.02 ± 0.31 (74.6)	4.85 ± 0.54*
Peroxisomal	1.03 ± 0.08 (25.4)	0.98 ± 0.15
Palmitate oxidation		
Total	43.7 ± 5.9	61.2 ± 8.8*
Mitochondrial		
TDGA-sensitive	33.6 ± 4.2 (76.9)	51.0 ± 7.1*
CPH-insensitive	31.9 ± 5.1 (73.0)	47.3 ± 4.5*
Peroxisomal		
TDGA-insensitive	10.1 ± 1.8 (23.1)	10.2 ± 2.3
CPH-sensitive	11.8 ± 2.4 (27.0)	13.9 ± 3.1

enzyme activity does not make an important contribution to the total malonyl-CoA-sensitive CPT pool under the experimental conditions employed in this study.

As shown in Table 2 (column 1), CPT_o and CPT_p accounted for 74.6 and 25.4 % of total malonyl-CoA-sensitive CPT activity respectively, which correlates well with the values of 76.9 and 23.1 % for the respective contributions of the mitochondrial and the peroxisomal compartment to total hepatocellular palmitate oxidation. Similar values were obtained for mitochondrial and peroxisomal palmitate oxidation by use of CPH (Table 2). This compound is a rather selective inhibitor of peroxisomal and cytosolic 3-ketothiolase (EC 2.3.1.16) IB, whereas mitochondrial 3-ketothiolases are not significantly affected [23]. Therefore CPH-insensitive palmitate oxidation represents mitochondrial oxidation, whereas CPH-sensitive palmitate oxidation represents peroxisomal oxidation. On the basis of marker-enzyme recovery, Derrick & Ramsay [7] estimated that CPT_p could constitute about 20 % of the hepatic CPT_p plus CPT_o pool in fed rats. By using tritiated substrates, Rognstad has recently found that peroxisomally initiated palmitate oxidation represents 18–26 % of total hepatic palmitate oxidation [21]. Our results are thus in line with these other experimental approaches.

As in mitochondria and peroxisomes isolated from rat liver [1–3,7], both CPT_o and CPT_p in our permeabilized-cell assay were sensitive to inhibition by physiological (micromolar) concentrations of malonyl-CoA (Fig. 2). The sensitivity of CPT_o to malonyl-CoA was very similar to that of CPT_p; thus 50 % inhibition of CPT_o and CPT_p activities was achieved with malonyl-CoA concentrations of 2.6 ± 0.5 and 3.0 ± 0.4 μM respectively. Therefore, the very similar features displayed by CPT_o and CPT_p in isolated peroxisomes or mitochondria as compared

Fig. 2. Effect of okadaic acid on the sensitivity of CPT_o and CPT_p to inhibition by malonyl-CoA

Hepatocytes were incubated for 20 min with or without 5 μ M-TDGA. Incubations were continued for an additional 20 min period with (●, ■) or without (○, □) 1 μ M-okadaic acid. Subsequently, samples were removed to determine the activity of CPT_o (○, ●) and CPT_p (□, ■) in the presence of increasing concentrations of malonyl-CoA. Results show the means $\pm$ S.D. of six different cell preparations.

Fig. 3. Effect of okadaic acid on the activity of CPT_o and CPT_p at different doses of palmitoyl-CoA

Hepatocytes were incubated for 20 min with or without 5 μ M-TDGA. Incubations were continued for an additional 20 min period with (●, ■) or without (○, □) 1 μ M-okadaic acid. Subsequently, samples were removed to determine the activity of CPT_o (○, ●) and CPT_p (□, ■). Results show the means $\pm$ S.D. of four different cell preparations.

with these enzymes in digitonin-permeabilized hepatocytes indicate that cell permeabilization with digitonin is a suitable technique for the study of these two enzyme activities *in situ*.

Short-term effects of okadaic acid on CPT_o and CPT_p activities and on the rate of mitochondrial and peroxisomal palmitate oxidation

By using a permeabilized-hepatocyte system we have previously shown that the activity of the total malonyl-CoA-sensitive CPT pool was controlled in the short term by different types of cell agonists [11–13]. An extremely useful probe for the study of

protein phosphorylation in intact cells has recently become available. This is okadaic acid, a powerful and specific inhibitor of protein phosphatases 1 and 2A [24,25], which are the major protein phosphatases acting on many phosphoproteins *in vivo* [24–26]. Enzymes whose phosphorylation state is increased in hepatocytes after cell incubation with okadaic acid include the rate-limiting enzymes of glycogen synthesis and degradation, glycolysis, gluconeogenesis and fatty acid synthesis [24,25]. We have recently reported that okadaic acid increases the activity of the total malonyl-CoA-sensitive CPT pool and the rate of palmitate oxidation in isolated rat hepatocytes [13]. Hence we investigated in the present study whether CPT_o and/or CPT_p are the targets for okadaic acid action.

Incubation of hepatocytes with okadaic acid had no significant effect on either CPT_p activity or the rate of peroxisomal palmitate oxidation (Table 2). By contrast, okadaic acid markedly increased CPT_o activity and the rate of mitochondrial palmitate oxidation (Table 2). It should be pointed out that these effects of okadaic acid were not due to changes in sensitivity to inhibition by TDGA; thus the TDGA concentration causing 50% of maximal inhibition of either CPT_o activity or mitochondrial palmitate oxidation was 0.10 ± 0.01 μ M ($n = 3$) both in the presence and in the absence of 1 μ M-okadaic acid in the hepatocyte incubations. Likewise, addition of okadaic acid to the hepatocyte incubations significantly stimulated CPH-insensitive (= mitochondrial) palmitate oxidation, whereas it had no effect on CPH-sensitive (= peroxisomal) palmitate oxidation (Table 2).

Okadaic acid rendered CPT_o not only more active but also less sensitive to inhibition by malonyl-CoA (Fig. 2); 50% inhibition of CPT_o activity was obtained with malonyl-CoA concentrations of 2.6 ± 0.5 and 4.5 ± 0.7 μ M when hepatocyte incubations were carried out in the absence or in the presence of 1 μ M-okadaic acid respectively ($n = 6$, $P < 0.01$ by the Student *t* test). Okadaic acid, on the other hand, did not affect the sensitivity of CPT_p to inhibition by malonyl-CoA (Fig. 2). As stated above, CPT activity that was insensitive to malonyl-CoA was always subtracted from the CPT activity experimentally determined and was not affected by either TDGA or okadaic acid. Therefore, CPT_o and CPT_p activities in Fig. 2 fall to zero at 100 μ M-malonyl-CoA, and IC₅₀ values may be directly inferred from these plots. However, at first glance the data in Fig. 2 may also suggest that a fraction of CPT_o becomes refractory to inhibition by malonyl-CoA after cell exposure to okadaic acid. Thus when values of 1/percentage of inhibition are plotted against 1/[malonyl-CoA] for CPT_o in okadaic acid-treated cells, the plot obtained intersects the ordinate at a point equivalent to 71% inhibition. Decreased sensitivity of CPT_o to inhibition by malonyl-CoA is a major factor involved in the stimulation of hepatic long-chain fatty acid oxidation under different ketotic states [1–3,10,27]. Hence this could also be an important mechanism underlying the promotion of mitochondrial palmitate oxidation by okadaic acid, irrespective of whether okadaic acid decreases the sensitivity of the CPT_o enzyme molecules to inhibition by malonyl-CoA or makes a sub-population of enzyme molecules refractory to inhibition by malonyl-CoA.

Harano *et al.* [28] have observed that glucagon increases both the activity and the phosphorylation state of CPT in short-term hepatocyte incubations. Ketotic states are characterized by an increased supply of fatty acids to the liver and a decrease in the affinity of CPT_o for acyl-CoA, which will allow the enzyme to handle high substrate concentrations [1–3,27]. Harano *et al.* [28] claimed, however, that enhanced phosphorylation of CPT was accompanied by an increase in enzyme affinity for palmitoyl-CoA. However, these authors only determined the activity of the total CPT system. In addition, their 69 kDa immunoprecipitated CPT protein is now believed to be the CPT_i enzyme

Fig. 4. Effect of okadaic acid on the affinity of CPT_o and CPT_p for palmitoyl-CoA

Data from Fig. 3 are represented as percentage of the enzyme activity determined in the presence of 400 μ M-palmitoyl-CoA.

(e.g. [29–31]). Thus we were interested in studying whether okadaic acid changes the affinity of CPT_o for palmitoyl-CoA.

Fig. 3 shows the plots for velocity versus palmitoyl-CoA concentrations for CPT_o and CPT_p in digitonin-permeabilized hepatocytes. Within the whole range of palmitoyl-CoA concentrations employed, incubation of hepatocytes with okadaic acid increased the activity of CPT_o, but had no effect on the maximal activity of CPT_p. Values for apparent $K_{0.5}$ and V_{max} could not be inferred from double-reciprocal plots, since these were rather curvilinear (see also [27,32]). However, an okadaic acid-induced change in the affinity of CPT_o for palmitoyl-CoA was evident when data of velocity from Fig. 3 were plotted as the percentage of enzyme activity as determined with a near-to-maximal concentration of palmitoyl-CoA (400 μ M). Thus incubation of hepatocytes with okadaic acid increased by 2-fold the apparent $K_{0.5}$ of CPT_o for palmitoyl-CoA (Fig. 4). Therefore, okadaic acid stimulated CPT_o activity and mitochondrial palmitate oxidation, despite decreasing enzyme affinity for palmitoyl-CoA substrate. In line with data obtained with isolated rat liver organelles [7], CPT_o and CPT_p showed identical affinity for palmitoyl-CoA substrate when assayed in our permeabilized-hepatocyte system (Fig. 4). Nevertheless, the affinity of CPT_p for palmitoyl-CoA was not changed upon incubation of cells with okadaic acid (Fig. 4).

We are aware that at least two limitations are inherent to the experimental procedure employed herein. (i) In order to measure CPT_o and CPT_p activities, we permeabilize the plasma membrane, and this causes the cytosol to leak out of the cell, leading to dilution of cytosolic components, including malonyl-CoA. Therefore the inhibition exerted by malonyl-CoA on the two enzymes in isolated hepatocytes does not take place when cells are permeabilized with digitonin. Moreover, the malonyl-CoA content of our isolated hepatocytes was $12.7 \pm 2.1 \mu$ M in control incubations and $0.8 \pm 0.3 \mu$ M in incubations with 1 μ M-okadaic acid ($n = 4$, $P < 0.01$ by the Student t test), in line with the observation that acetyl-CoA carboxylase activity is strongly inhibited by okadaic acid [24]. Thus the degree of inhibition exerted by malonyl-CoA on palmitate oxidation must be different in control and okadaic acid-treated hepatocytes. (ii) CPT_o and CPT_p activities in permeabilized hepatocytes are several-fold higher than the respective rates of mitochondrial and peroxisomal palmitate oxidation in intact hepatocytes (Table 2), and this cannot be accounted for (at least in okadaic acid-treated cells) by

the inhibition exerted by malonyl-CoA on the two enzymes *in situ*. Unfortunately, we do not know the actual reasons for this discrepancy. Because of these two limitations, any quantitative correlation between CPT_o or CPT_p activity and the rate of palmitate oxidation may only hold for selected assay conditions of CPT_o or CPT_p activity. We believe, however, that our permeabilized-hepatocyte system allows us to establish qualitative relationships between changes in CPT_o or CPT_p activity and changes in mitochondrial or peroxisomal palmitate oxidation respectively.

Physiological considerations

The use of the present permeabilized-hepatocyte system shows that CPT_p shares with CPT_o very similar features with regard to malonyl-CoA sensitivity and palmitoyl-CoA affinity. Although the physiological role of CPT_p remains unclear [2], the similar contributions of CPT_p and peroxisomal palmitate oxidation to the total malonyl-CoA-sensitive CPT pool and the total palmitate-oxidative system respectively might indicate a possible limiting effect of the palmitoyltransferase on peroxisomal palmitate oxidation. It has been suggested that the long-chain acylcarnitines oxidized by peroxisomes are firstly synthesized by CPT_o in the mitochondrial outer membrane and then converted into their corresponding CoA esters by CPT_p to undergo β -oxidation in the peroxisomal matrix [6,33]. If that was the case, increased CPT_o activity upon treatment with okadaic acid will most likely be accompanied by a stimulation of peroxisomal palmitate oxidation. However, this is not what we observe in our experimental system. Our results thus indicate that CPT_p itself might be involved in the formation of long-chain acylcarnitines for peroxisomal oxidative metabolism. The physiological importance of CPT_p in shuttling chain-shortened products out of the peroxisomes for oxidation by mitochondria is still unknown [2,5].

CPT_p activity is determined by the nutritional status of the animal. Thus CPT_p activity has been shown to be induced upon high-fat feeding [8] and starvation [8,34], although glucagon has no effect on the levels of CPT_p mRNA [8]. In addition, the sensitivity of CPT_p to inhibition by malonyl-CoA decreases in starvation [34]. Our results suggest that these alterations in the properties of CPT_p may be controlled by a mechanism that does not involve a change in the phosphorylation state of the enzyme.

Under the experimental conditions employed in the present work, a qualitative relationship was observed between changes in CPT_o activity and changes in the rate of mitochondrial palmitate oxidation, indicating that CPT_o may be a key regulatory enzyme in the control of long-chain fatty acid oxidation by liver mitochondria. The okadaic acid-induced stimulation of hepatic mitochondrial palmitate oxidation seems to be due (at least in part) to profound changes in the kinetic and regulatory properties of CPT_o, namely increased specific activity, decreased sensitivity to inhibition by malonyl-CoA and decreased affinity for palmitoyl-CoA substrate. Although the exhaustive molecular characterization of CPT_o has allowed the study of the mechanisms involved in the long-term regulation of this enzyme protein [8,29,35], the molecular processes underlying the alterations in the kinetic and regulatory properties of hepatic CPT_o are still unknown [2,3,9,10]. Very recently a polyclonal antibody against rat liver CPT_o has been obtained, and the levels of immunoreactive CPT_o protein have been shown to increase several-fold under different ketotic states [36]. However, the question of how the regulatory properties of the enzyme change under these situations remains unsolved. It has been suggested that post-transcriptional modifications of the existing enzyme molecules could be involved in the alterations induced by fasting and diabetes on the regulatory properties of CPT_o [10,37]. It is

noteworthy that the modifications induced by okadaic acid (presumably by increased enzyme phosphorylation) on hepatic CPT₀ are precisely those appearing in ketotic states such as fasting, diabetes and hyperthyroidism [2,3,9], in which the phosphorylation degree of target regulatory enzymes should be enhanced. Hence it is tempting to speculate that increased phosphorylation of CPT₀ may be a key mechanism to render the enzyme more active, less sensitive to inhibition by malonyl-CoA and less saturable by acyl-CoA substrate. Direct evidence of CPT₀ phosphorylation remains to be obtained.

These investigations were supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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Received 16 December 1991/23 April 1992; accepted 8 May 1992