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Peroxisomal fatty acid oxidation and inhibitors of the mitochondrial carnitine palmitoyltransferase I in isolated rat hepatocytes

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Fatty acid oxidation was studied in the presence of inhibitors of carnitine palmitoyltransferase I (CPT I), in normal and in peroxisome-proliferated rat hepatocytes. The oxidation decreased in mitochondria, as expected, but in peroxisomes it increased. These two effects were seen, in variable proportions, with (+)-decanoylcarnitine, 2-tetradecylglycidic acid (TDGA) and etomoxir. The decrease in mitochondrial oxidation (ketogenesis) affected saturated fatty acids with 12 or more carbon atoms, whereas the increase in peroxisomal oxidation (H_2O_2 production) affected saturated fatty acids with 8 or more carbon atoms. The peroxisomal increase was sensitive to chlorpromazine, a peroxisomal inhibitor. To study possible mechanisms, palmitoyl-, octanoyl- and acetyl-carnitine acyltransferase activities were measured, in homogenates and in subcellular fractions from control and TDGA-treated cells. The palmitoylcarnitine acyltransferase was inhibited, as expected, but the octanoyltransferase activity also decreased. The CoA derivative of TDGA was synthesized and tentatively identified as being responsible for inhibition of the octanoylcarnitine acyltransferase. These results show that inhibitors of the mitochondrial CPT I may also inhibit the peroxisomal octanoyl transferase; they also support the hypothesis that the octanoyltransferase has the capacity to control or regulate peroxisomal fatty acid oxidation.

INTRODUCTION

Fatty acid β-oxidation in liver cells occurs in mitochondria and in peroxisomes. The two oxidative systems exhibit considerable overlap in substrate specificity (Lazarow & de Duve, 1976; Tolbert, 1981) a fact that has hindered efforts to differentiate the role of each. Observations on inherited peroxisomal disorders have allowed identification of several substrates, very-long-chain fatty acids and others, which require peroxisomes for their oxidation (Wilson *et al.*, 1988; Lazarow & Moser, 1989). In mitochondria, fatty acid oxidation is regulated by a mechanism in which CPT I, a mitochondrial enzyme (Bremer, 1983), plays a central role (McGarry & Foster, 1980). Several CPT I inhibitors, which characteristically inhibit ketogenesis, are available and allow study of the role of mitochondria *in situ*, in cells or in organisms (McGarry & Foster, 1973; McGarry *et al.*, 1973; Tutwiler *et al.*, 1981; Declercq *et al.*, 1987). In contrast, short-term regulatory mechanisms other than substrate availability have not been characterized in peroxisomes at a molecular level. These organelles contain carnitine acyltransferases for medium- and short-chain fatty acids (COT and CAT) (Markwell *et al.*, 1973; Leighton *et al.*, 1982; Miyazawa *et al.*, 1983), enzymes for which no physiological role has been firmly established (Bieber, 1988). Pharmacologically, it is possible to inhibit selectively the oxidation of fatty acids in peroxisomes, but the mechanism of this effect is not known (Leighton *et al.*, 1984).

To evaluate the role of peroxisomes in whole cells, under conditions in which the mitochondrial oxidation of fatty acids is depressed, the activity of both systems was measured simultaneously, in normal and in peroxisome-proliferated rat hepatocytes treated with CPT I inhibitors.

EXPERIMENTAL

Hepatocytes and incubations

The isolated hepatocytes were prepared by established procedures, as previously described (Leighton *et al.*, 1984), from male Sprague–Dawley rats (200–250 g), fed *ad libitum* with either standard pelleted diet, or for 1–2 weeks with pellets containing 50 mg of ciprofibrate/kg or 750 mg of bezafibrate/kg. The rats were fasted overnight before preparation of hepatocytes. The viability of the hepatocytes used was $94.4 \pm 1.5\%$, estimated from Trypan Blue exclusion. For the incubations, run in duplicate or in triplicate, peroxisome-proliferated hepatocytes equivalent to approx. 1.5 mg of protein, or normal hepatocytes (approx. 4.5 mg of protein) were incubated in 1 ml for 20 min in plastic vials at 37 °C under O_2/CO_2 (19:1). The medium contained 120 mM-NaCl, 4.8 mM-KCl, 2.0 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 25 mM-$NaHCO_3$, 50 mM-methanol, 10 mM-semicarbazide, 0.17 mM fatty-acid-free BSA and 0.5 mM fatty acid. The inhibitors were added in methanol. When preincubation of the cells was required, the reaction was started by addition of the substrate, otherwise by addition of cells. The reaction was stopped with 0.4 ml of cold 30% (w/v) trichloroacetic acid. Blanks, without exogenous substrate but with fatty-acid-free BSA, were routinely incubated for 0 and 20 min to correct for endogenous activity. For H_2O_2 measurements, 0.5 ml of the acid supernatant was mixed with 0.5 ml of Nash (1953) reagent, and the A_{412} was determined after

Abbreviations used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21), total activity, assayed in the presence of detergent; CPT I, mitochondrial external CPT, the enzyme inactivated by TDGA and etomoxir and the site of ketogenesis regulation by malonyl-CoA; CPT II, CPT functional in the mitochondrial matrix; COT, carnitine octanoyltransferase, enzyme present in peroxisomes, not in mitochondria; CAT, carnitine acetyltransferase (EC 2.3.1.7), enzyme present in various subcellular fractions; TDGA, 2-tetradecylglycidic acid (sodium 2-tetradecyloxiranecarboxylate dihydrate); CPZ, chlorpromazine; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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90 min at 37 °C for colour development. Acetoacetate and β-hydroxybutyrate were measured in 0.75 ml of the neutralized supernatant (Williamson *et al.*, 1962). Controls were performed to evaluate the effect on ketogenesis of methanol and semicarbazide, at the concentrations employed for the assays: methanol does not affect it; semicarbazide, with or without methanol, exerts a minor inhibition, a 10–20 % decrease in total ketogenesis which preferentially affects the detection of acetoacetate.

Subcellular fractionation

For the fractionation experiments, hepatocytes from ciprofibrate-treated rats were employed. For each gradient a large-scale incubation was done in 25 ml, with palmitate as substrate, under the same conditions as used for the 1 ml standard incubations. The controls for the experiments in which the effect of a preincubation with TDGA was studied were performed with the same batch of hepatocytes. At the end of the large-scale incubations, a 1 ml sample was removed for measurements to validate the comparison of these observations with those with the standard 1 ml incubation volume. The remaining cells were washed with a solution of 0.25 M-sucrose/1 mM-EDTA/3 mM-imidazole, pH 7.4, and were resuspended in 1 ml of the same solution. After homogenization with a Dounce glass homogenizer, an extract or postnuclear supernatant was prepared (de Duve *et al.*, 1955), of which 0.5 ml was layered on top of a continuous gradient of Nycodenz, ranging from 1.05 to 1.28 g/cm^3. Centrifugation was done for 50 min at 40000 rev./min at 8 °C in a VTi-65 Beckman rotor. The results are presented in accordance with standard procedures (Leighton *et al.*, 1968). Purified mitochondria were prepared as described by de Duve *et al.* (1955). Purified peroxisomes were prepared as described previously (Bronfman *et al.*, 1979), by using Nycodenz instead of metrizamide.

Enzyme assays

Carnitine acyltransferases were assayed radioactively or spectrophotometrically, as follows. The spectrophotometric assay was used in subcellular-fractionation and whole-homogenate measurements, as previously described (Leighton *et al.*, 1982) in the direction of acylcarnitine formation. For the assay, the fractions were diluted with 10 mM-sodium pyrophosphate, pH 8.1, containing 0.1 % sodium deoxycholate, and CoA release was monitored at 412 nm in the presence of DTNB. The final assay mixture, at pH 8.0 and 25 °C, contained 2.5 mM-(−)-carnitine, 0.1 mM-acyl-CoA, 58 mM-Tris/HCl, 5 mM-sodium pyrophosphate, 0.05 % deoxycholate and 0.25 mM-DTNB. CPT I in isolated mitochondria was assayed radioactively, in the absence of detergent to prevent the contribution of CPT II, by using butan-1-ol extraction as described by Bremer (1981). The incubations were done at pH 7.4 and 20 °C in 1 ml containing 15 mM-Tris/HCl, 0.15 M-KCl, 1 % fatty-acid-free BSA, 50 μM-palmitoyl-CoA, 0.2 mM-[^{3}H]carnitine (3000 c.p.m./nmol) and 3 mg of mitochondrial protein. This method cannot be used with octanoyl-CoA, since it is soluble in acid. To study the direct effect of TDGA-CoA on isolated organelles, preincubations were done for 20 min at 20 °C as described by Declercq *et al.* (1985) with 20 μM-TDGA-CoA. CPT I in mitochondria was measured radioactively, and COT in peroxisomes was measured spectrophotometrically, with or without addition of deoxycholate to evaluate structure-linked latency.

Glutamate dehydrogenase (marker for mitochondria), NADPH–cytochrome *c* reductase (marker for endoplasmic reticulum), catalase (marker for peroxisomes) and protein were measured as previously described (Bronfman *et al.*, 1984).

Materials

Several drugs were given by their manufacturers: bezafibrate {2-[4-(chlorobenzamidoethyl)phenoxy]-2-methylpropanoic acid} from Boehringer Mannheim, Mannheim, Germany; ciprofibrate {2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid} from Sterling–Winthrop Research Institute, Rensselaer, NY, U.S.A.; nafenopin {2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid} from Ciba–Geigy, Basle, Switzerland; TDGA (McN 3802-21-98) from McNeil Pharmaceutical, Spring House, PA, U.S.A.; etomoxir {sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate dihydrate} from Byk Gulden Pharamzeutika, Konstanz, Germany; and acylcarnitines from Otsuka Pharmaceutical Factory, Tokushima, Japan. Nycodenz was purchased from Nyegaard and Co., Oslo, Norway. Biochemicals in general were from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[^{3}H]Carnitine (71 Ci/mmol) was from Amersham. TDGA-CoA was synthesized as described by Kawaguchi *et al.* (1981), forming an acylimidazole in the first step. The TDGA-CoA was washed with diethyl ether, after acidification, and was recovered from the interphase where it precipitates.

Statistics

Measurements are presented as individual values, means, or as means ± S.D.

RESULTS

Effect of CPT I inhibitors on the palmitate-dependent production of H_2O_2 and ketones by hepatocytes

The inhibition of CPT I should decrease ketogenesis from long-chain fatty acids. As shown in Table 1, over 90 % of the ketogenesis induced by palmitate was suppressed by TDGA and etomoxir, two inactivating drugs. The inhibition by (+)-decanoylcarnitine was moderate, probably because of the decreased sensitivity of CPT I to competitive inhibition in mitochondria from peroxisome-proliferated liver cells (Christiansen, 1978; Mannaerts *et al.*, 1979). In contrast, a large increase in palmitate-dependent peroxisomal H_2O_2 generation was seen with the three inhibitors employed. TDGA and etomoxir appeared equally active, and TDGA was selected for subsequent observations. Similar results (not shown) were obtained with hepatocytes from either ciprofibrate- or nafenopin-treated rats or from untreated rats. As shown in Fig. 1, the effect of TDGA

Table 1. Effect of CPT inhibitors on palmitate-induced H_2O_2 and ketone-body production by peroxisome-proliferated hepatocytes

The cells were preincubated for 15 min with the drugs mentioned or without additions, before starting the reaction with palmitate/BSA, as described in the Experimental section. The values are means ± S.D. from measurements in triplicate, with hepatocytes from a bezafibrate-treated rat. Relative values (percentages) are shown in parenthesis.

Additions	Production (nmol/min per mg of protein)	
	H_2O_2	Ketone bodies
None	1.77 ± 0.19 (100)	8.87 ± 0.80 (100)
(+)-Decanoylcarnitine (5 mM)	3.26 ± 0.23 (184)	5.71 ± 0.88 (64)
TDGA (20 μM)	5.14 ± 0.23 (290)	0.62 ± 0.21 (7)
Etomoxir (20 μM)	3.74 ± 0.07 (211)	0.54 ± 0.17 (6)

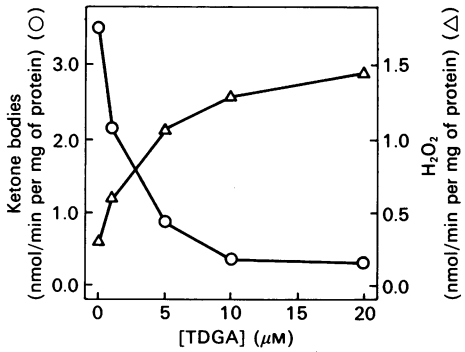

Fig. 1. Dose–response effect of TDGA on palmitate-dependent ketogenesis and on H_2O_2 generation

Hepatocytes from a ciprofibrate-treated rat were preincubated for 15 min with TDGA, before addition of 0.5 mM-palmitate in 0.17 mM-BSA and incubation for 20 min. The generation of ketone bodies (○) and H_2O_2 (△) allows monitoring of the mitochondrial and peroxisomal fatty-acid-oxidation activities respectively.

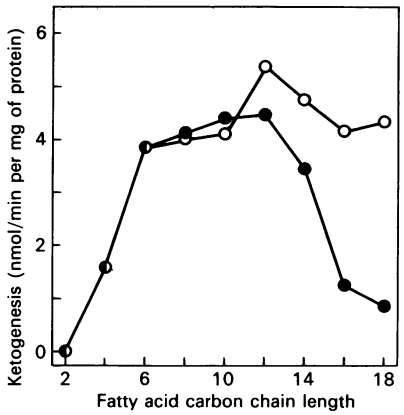

Fig. 2. Ketogenesis from saturated fatty acids in peroxisome-proliferated hepatocytes: effect of TDGA

Hepatocytes were from a ciprofibrate-treated rat. The cells were incubated with 0.5 mM saturated fatty acid, of the chain length specified in the abscissa, for 20 min, after preincubation for 15 min in the absence (○) or in the presence (●) of 10 μM-TDGA. β-Hydroxybutyrate and acetoacetate net productions were measured as described in the Experimental section. Each point is the average of two separate incubations. The endogenous ketogenesis, in the absence of added substrate, was 0.35 and 0 nmol/min per mg of protein for control and TDGA-treated cells respectively.

was dose-dependent in hepatocytes preincubated 15 min with the drug. A 50% decrease in ketogenesis was obtained with 1.2 μM-TDGA, whereas the increase in H_2O_2 production reached 50% of the maximum at 3.2 μM-TDGA. Both phenomena have different sensitivities to the drug. A similar conclusion is derived from the data in Table 1 when different drugs are compared: the increase in peroxisomal activity and the inhibition of ketogenesis vary independently.

Effect of TDGA on the peroxisomal and mitochondrial oxidation of different saturated fatty acids in normal and peroxisome-proliferated hepatocytes

The oxidation of long-chain fatty acids is regulated at the level of CPT I by malonyl-CoA (McGarry & Foster, 1980), whereas medium-chain fatty acids apparently by-pass this regulatory step (Aas & Bremer, 1968; Aas, 1971). To evaluate further the relationship between CPT I function and peroxisomal fatty acid

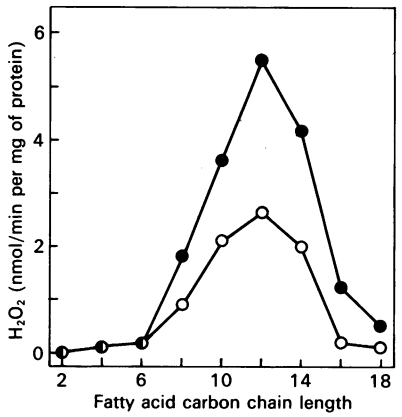

Fig. 3. H_2O_2 Generation induced by saturated fatty acids, in peroxisome-proliferated hepatocytes: effect of TDGA

Measurements were made in the same incubations used for Fig. 2. The endogenous rates of H_2O_2 generation, in the absence of added substrate, were 0.07 and 0.22 nmol/min per mg of protein, without (○) or with (●) preincubation with 10 μM-TDGA.

oxidation, observations were made with fatty acids of different chain length and CPT I inhibitors. The same response pattern was observed with normal and peroxisome-proliferated hepatocytes, even though the rate of peroxisomal oxidation in peroxisome-proliferated cells was 10–20-fold higher; ketogenesis also increased, approx. 2-fold.

In Figs. 2 and 3 the effects of TDGA on ketogenesis and H_2O_2 production are shown for hepatocytes from a ciprofibrate-treated rat. These results were very reproducible and were also observed with other peroxisome proliferators. For $C_{10:0}$ and shorter fatty acids, ketogenesis was not decreased by TDGA. With $C_{12:0}$ and $C_{14:0}$, and particularly with long-chain fatty acids, $C_{16:0}$ and $C_{18:0}$, a marked inhibition of ketogenesis was observed (Fig. 2). In contrast, the production of H_2O_2 was enhanced 2-fold or more with $C_{8:0}$ and with longer fatty acids (Fig. 3). The rate of H_2O_2 generation reflects that of acetyl-CoA generation in peroxisomes. With $C_{12:0}$, for example, it approaches the rate of mitochondrial ketogenesis. Yet a substrate–product relationship for the acetyl-CoA generated in peroxisomes and ketogenesis in mitochondria has not been found: free acetate and not ketones is the main product (Leighton *et al.*, 1989). In normal rat hepatocytes (Figs. 4 and 5) TDGA also inhibited ketogenesis, particularly from $C_{12:0}$ and longer fatty acids, and increased the rate of H_2O_2 generation with $C_{8:0}$ and longer fatty acids. As expected, the rates of H_2O_2 production are much lower than in peroxisome-proliferated cells.

To check if the enhancement of H_2O_2 generation induced by TDGA was sensitive to the peroxisomal inhibitor CPZ (Leighton *et al.*, 1984), observations were made with $C_{12:0}$ and $C_{16:0}$ as substrates (Table 2). The $C_{12:0}$-dependent H_2O_2 generation was inhibited by almost 50% in both the absence and presence of TDGA. With $C_{16:0}$, the effect was more pronounced.

Effect of TDGA on the activity of CPT, COT and CAT

The independent behaviour observed in the effects of TDGA and other drugs upon mitochondrial and peroxisomal activities raised the possibility that, in addition to the irreversible inhibition of CPT I by TDGA-CoA (Kiorpes *et al.*, 1984), other mechanisms might be involved. Peroxisomes contain other carnitine acyltransferases: COT, which is present in peroxisomes and not in mitochondria, and CAT, present both in peroxisomes and in mitochondria. They would participate in the shuttling to the cytosol of shortened acyl-CoA residues and acetyl-CoA, as

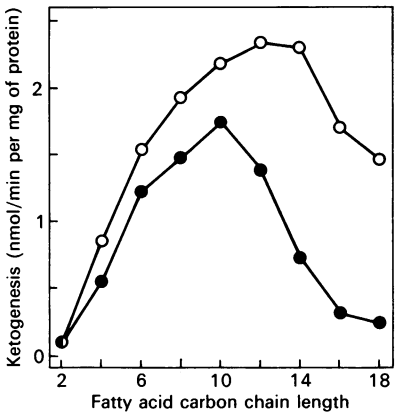

Fig. 4. Ketogenesis from saturated fatty acids in normal hepatocytes: effect of TDGA

Hepatocytes were from a control untreated rat. The general procedure was as described in the legend for Fig. 2. The results shown in Fig. 5 were obtained from the same incubations. The endogenous ketogenesis, in the absence of added substrate, was 0.23 and 0.09 nmol/min per mg of protein, without (○) or with (●) preincubation with 10 μM-TDGA.

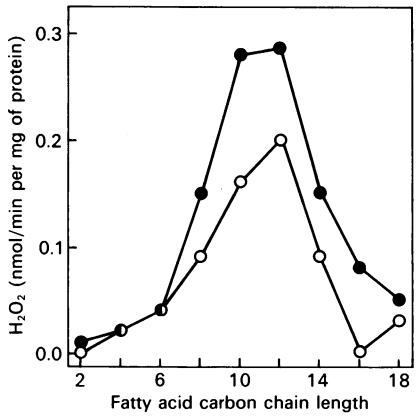

Fig. 5. H_2O_2 generation induced by saturated fatty acids in normal hepatocytes: effect of TDGA

Measurements were made in the same incubations used for Fig. 4. The endogenous rates of H_2O_2 generation, in the absence of added substrate, were 0.09 and 0.13 nmol/min per mg of protein, without (○) or with (●) preincubation with 10 μM-TDGA.

carnitine esters (Tolbert, 1981; Leighton *et al.*, 1982; Farrell & Bieber, 1983). Inhibitors of CPT I might also act on COT or CAT in the cell, a possibility that has not been evaluated.

The activities of CPT, COT and CAT were measured in postnuclear supernatants or extracts from hepatocytes preincubated for 15 min with TDGA and from control cells, which were then incubated for 20 min with palmitate, to mimic the conditions for the fatty-acid-oxidation measurements. The extracts were subfractionated to correlate the activity of the transferases with their subcellular localization. The incubation with TDGA decreased total CPT activity by approx. 30%. The insensitive fraction could correspond in part to activity of CPT II, located in the mitochondrial matrix and insensitive to TDGA. In addition, it was found that the activity of COT was also inhibited by 50% with TDGA. In contrast, the activity of CAT was stimulated by approx. 40%. Since free TDGA would be removed during washing of the cells, the effects described apparently correspond to an irreversible inhibition, just as shown for CPT I (Declercq *et al.*, 1985). Since the substrate-specificity patterns for mitochondrial CPT and peroxisomal COT overlap, measurements were made in purified organelles to check if the inhibition detected with the substrate employed for COT, octanoyl-CoA, was due to COT inhibition or was the result of a partly inhibited CPT I acting on octanoyl-CoA. The results of these experiments are shown in Figs. 6 and 7. The subfractionation of an extract from peroxisome-proliferated hepatocytes incubated as indicated above (Fig. 6) showed a large fraction of catalase not bound to particles, and a small peak at around 1.20 g/cm^3 that corresponds to peroxisomes. Adequate resolution was obtained among particle-bound catalase, the peroxisomal marker, and glutamate dehydrogenase, the mitochondrial marker. The endoplasmic-reticulum marker, NADPH–cytochrome *c* reductase, revealed a distribution that overlapped with cytosolic catalase and the mitochondrial peak. COT, in contrast with CAT and CPT, showed a bimodal distribution, with the larger fraction of the enzyme in the low-density peak, a distribution pattern common to peroxisomal enzymes in which only part of the enzyme is retained in the organelle, and the rest appears in the cytosol (Leighton *et al.*, 1975). After TDGA treatment, the activity of the transferases,

Table 2. Inhibition of hepatocyte H_2O_2 production by CPZ

The values correspond to means ± S.D. of four different experiments with hepatocytes from ciprofibrate-treated rats. The cells were preincubated for 15 min with or without 10 μM-TDGA, before being added to the incubation mixture, with CPZ when required. TDGA, in the absence of CPZ, decreased ketogenesis from lauric acid by 51±9% and from palmitic acid by 90±14%. With CPZ, in the absence of TDGA, ketogenesis was 107±17% and 67±20% of the basal values for lauric acid and palmitic acids respectively.

	H_2O_2 production (nmol/min per mg of protein)		Inhibition by CPZ (%)
	Without CPZ	CPZ (0.5 mM)	
Lauric acid	2.22±0.08	1.30±0.07	42
+TDGA	4.28±0.79	2.30±0.71	46
Palmitic acid	0.44±0.19	0.13±0.03	71
+TDGA	1.57±0.60	0.23±0.11	85

modified as mentioned before, showed the same subcellular distribution pattern, with the characteristic bimodality of COT (Fig. 7). If the 50% inhibition of COT after TDGA had been due to inhibition of CPT I acting non-specifically on octanoyl-CoA, a selective decrease in the COT peak overlapping with mitochondria should have occurred. This was not seen in the experiment illustrated, nor in a duplicate set of experiments which gave the same inhibition with TDGA and the same pattern of subcellular distribution. When comparing the proportion of the transferases equilibrating at densities higher than the main mitochondrial peak, it can also be seen that the fraction of the activity present in the peroxisomal region is smaller for CPT and larger for COT and CAT, in agreement with previous data (Markwell *et al.*, 1973; Bieber *et al.*, 1981; Leighton *et al.*, 1982).

Effect of TDGA-CoA on mitochondrial CPT and peroxisomal COT

To evaluate directly the evidence that TDGA inhibited COT, in addition to its well-established effect on CPT I, studies were

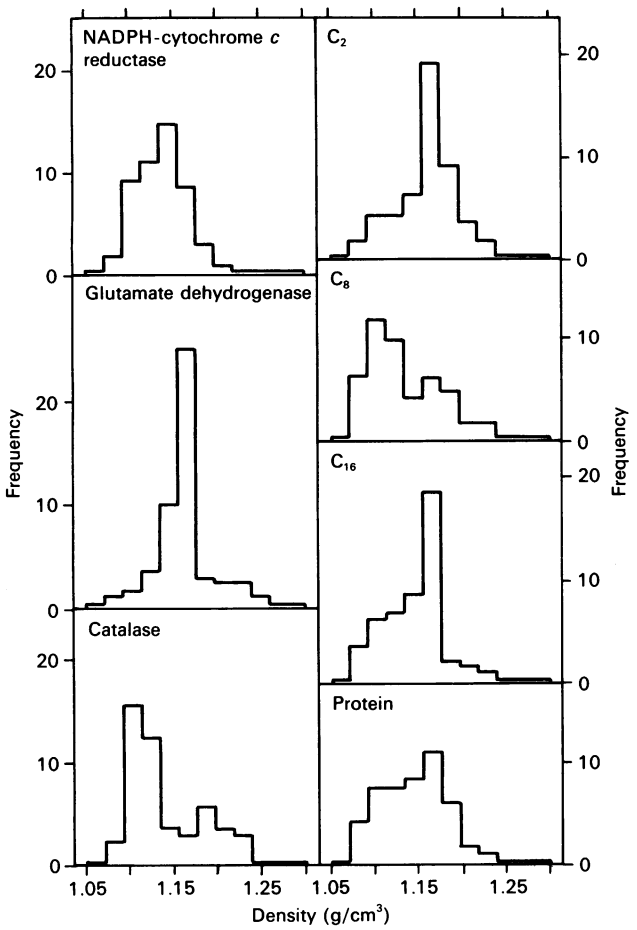

Fig. 6. Subcellular distribution of carnitine acyltransferases (CAT, COT and CPT): isopycnic Nycodenz-density-gradient fractionation of an extract prepared from hepatocytes

Hepatocytes from a ciprofibrate-treated rat were incubated and fractionated as described in the Experimental section. Standardized representation of the results in which the ordinate represents the frequency of the components for each fraction, $Q/\Sigma Q \cdot \Delta\rho$, where Q represents the activity found in the fraction, ΣQ the total activity recovered in the fractions and $\Delta\rho$ the increment in density of the gradient for each fraction. The abscissa illustrates the standardized density limits for each fraction. The Figure corresponds to one of two essentially equal gradients. The recoveries for CAT (C_2), COT (C_8) and CPT (C_{16}) were 126%, 99% and 101% respectively.

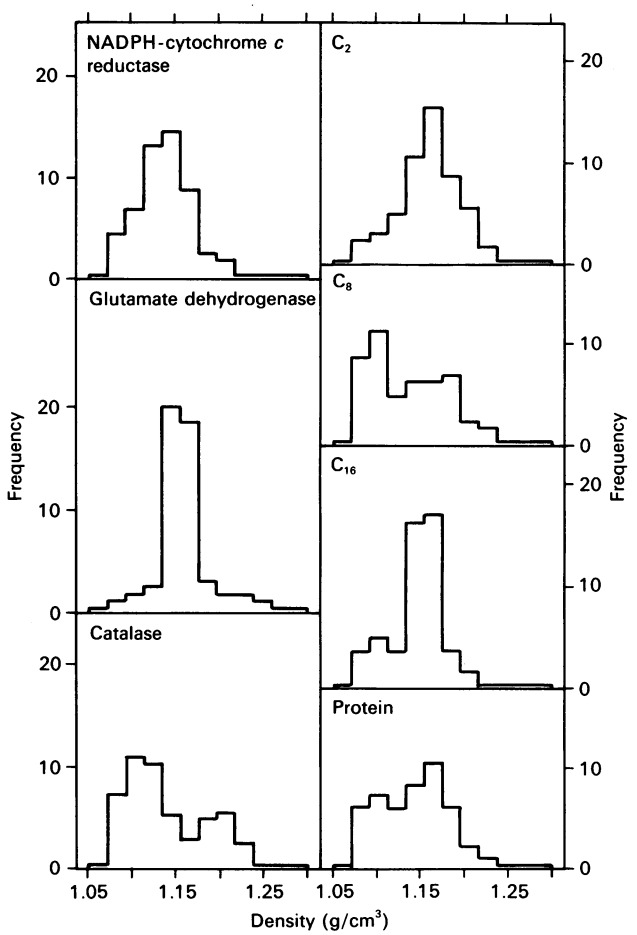

Fig. 7. Subcellular distribution of carnitine acyltransferases (CAT, COT and CPT), after TDGA treatment: isopycnic Nycodenz-density-gradient fractionation of an extract prepared from hepatocytes

Hepatocytes from a ciprofibrate-treated rat were preincubated with TDGA and processed as described in the Experimental section. The recoveries for CAT (C_2), COT (C_8) and CPT (C_{16}) were 89%, 110% and 90% respectively. The Figure corresponds to one of two essentially equal gradients. The hepatocytes treated with TDGA are from the same batch of cells employed for the experiment illustrated in Fig. 6.

made with isolated organelles. CPT and COT were measured in isolated mitochondria and peroxisomes, respectively, in the absence or presence of the inhibitor. Since the active inhibitor of CPT I is the CoA derivative of TDGA (Kiorpes *et al.*, 1984), TDGA-CoA was synthesized and inhibition assays were performed as described in the Experimental section. It was observed that incubation of mitochondria for 20 min with 20 μM-TDGA-CoA led to a 90% inhibition of the CPT I activity. For COT, after incubation of isolated peroxisomes for 20 min with 20 μM-TDGA-CoA, the inhibition was 80%, and it was 88% when the preincubation was done in the presence of 0.1% deoxycholate. Incubation of peroxisomes with free TDGA gave only 31% inhibition, and 17% when the incubation was done in the presence of deoxycholate. Therefore TDGA inhibits COT and, for this enzyme also, TDGA-CoA appears to be the active form of the inhibitor.

DISCUSSION

Detection of peroxisomal and mitochondrial β-oxidation activity in isolated hepatocytes

The measurement of H_2O_2 production, detected as formaldehyde released from catalase-mediated methanol peroxidation, allows estimation of the rate of peroxisomal fatty acid oxidation in intact cells (Inestrosa *et al.*, 1979; Mannaerts *et al.*, 1979). The method is indirect, yet its results correlate well with measurements by organ differential spectrophotometry of catalase intermediates (Foerster *et al.*, 1981) and with measurements in subcellular fractions (Inestrosa *et al.*, 1979; Mannaerts *et al.*, 1979). The validity of this measurement has received further support in studies on the production of free acetate, the product of acetyl-CoA generated in peroxisomes (Leighton *et al.*, 1989).

The activity of mitochondria and peroxisomes *in situ* can be monitored by measuring ketogenesis and H_2O_2 generation. Since both organelles exhibit a considerable overlap in substrate specificity, and because peroxisomes *in situ* express only a minor

fraction of the optimum activity detected in cell-free systems (Leighton *et al.*, 1982), whole-cell studies are necessary to establish the metabolic contribution of each organelle.

The present results illustrate the peroxisomal and mitochondrial substrate specificity with saturated fatty acids in hepatocytes. Peroxisomes oxidize preferentially medium-chain-length fatty acids ($C_{10:0}$, $C_{12:0}$ and $C_{14:0}$), whereas mitochondria are very active on $C_{6:0}$ to $C_{18:0}$ acids. Major physiological substrates such as $C_{16:0}$ or $C_{18:0}$ are oxidized mainly in mitochondria, both in the absence and in the presence of peroxisome proliferation.

Opposite effects of CPT I inhibitors on mitochondria and peroxisomes

The increase in peroxisomal activity concomitant with mitochondrial inhibition could result in part from increased substrate availability. However, medium-chain fatty acids would not be expected to accumulate after inhibition of the carnitine-dependent mitochondrial transport system employed by long-chain fatty acids. A spill-over mechanism would not be expected, and in fact ketogenesis from medium-chain fatty acids was only minimally affected. Since two oxiranecarboxylic fatty acid analogues that inactivate CPT I, and the (+)-carnitine derivatives, all gave the same response pattern, the inhibition of ketogenesis from long-chain fatty acids would be the consequence of CPT I inhibition. But the stimulation of peroxisomes, particularly with medium-chain fatty acids as substrate, is a novel phenomenon, apparently unrelated to CPT I.

Since CPT I and COT exhibit overlap in substrate specificity (Bieber, 1988) the possible inhibition of COT by CPT I inhibitors cannot be rejected *a priori*. Accordingly, we explored the hypothesis that the inhibition of COT, in addition to CPT I, constitutes the basis of the opposite effects exerted by these drugs on peroxisomal and mitochondrial fatty acid oxidation.

COT inhibition and peroxisomal activity

COT and CAT in the peroxisomes would participate in the transport of the products of fatty acid oxidation. This proposal is based on their substrate specificity and kinetic constants (Tolbert, 1981; Leighton *et al.*, 1982; Farrell & Bieber, 1983; Farrell *et al.*, 1984). The properties of COT and its differentiation from those of CPT have been under experimental re-evaluation. Healy *et al.* (1988) studied the relationship between COT and the easily solubilized fraction of CPT, and Ramsay (1988) concluded that both activities correspond to different enzymes in bovine liver. In human liver, in addition to its presence in peroxisomes and mitochondria, COT is the only transferase detected in the endoplasmic reticulum (Bronfman & Leighton, 1984), another localization for which a functional explanation is lacking. As stressed by Ramsay (1988) and Bieber (1988), the overlap in enzyme specificity for the various transferases is partly responsible for the present ambiguity in this field.

To explain the increase in peroxisomal activity seen with CPT inhibitors, we propose that they also inhibit COT, a hypothesis based on two assumptions: that COT is indeed required to shuttle medium-chain acyl-CoAs from the peroxisome, and that an impairment of this process will favour further β-oxidation of the acyl-CoA retained in peroxisomes. A selective COT inhibitor, suitable for studies with hepatocytes, would allow a direct evaluation of this hypothesis, but apparently none is available. The inhibition reported with 2-bromopalmitate would not be specific for COT (Healy *et al.*, 1988). Malonyl-CoA apparently inhibits COT, in addition to CPT I (Saggerson & Carpenter, 1981; Lund & Bremer, 1983), a finding that raises intriguing regulatory possibilities, yet the sensitivity of COT to malonyl-CoA has been examined by others, with different results (Healy *et al.*, 1988).

The inhibition of peroxisomal fatty acid oxidation in hepatocytes by CPZ has been confirmed by Vamecq (1987). He attributes the effect to COT inhibition. Our alternative explanation for the CPZ effect in whole cells is that the drug acts as an uncoupler of the mitochondrial oxidative phosphorylation and decreases the cell ATP concentration, a condition to which peroxisomes are more sensitive than are mitochondria (Leighton *et al.*, 1987); yet we do not know at what level ATP is affecting peroxisomes selectively. In fact, with FCCP and other uncouplers, the selective inhibition of peroxisomes is readily seen (Leighton *et al.*, 1987). However, COT and CPT, which we have confirmed are sensitive to CPZ *in vitro*, are not affected by FCCP under the same conditions, (C. Necochea, C. Skorin & F. Leighton, unpublished work). Therefore the hypothesis that the partial inhibition of COT would cause peroxisomal inhibition, and not stimulation, is not supported by the results mentioned above, nor by the present results.

The finding that peroxisomes contain a membrane ATPase (del Valle *et al.*, 1988), and the proposition that peroxisomes *in situ* exhibit an ATP-dependent structure-linked latency (Wolvetang *et al.*, 1990), in contrast with the well-characterized high permeability of the peroxisomal membrane *in vitro* (van Veldhoven *et al.*, 1983; Labarca *et al.*, 1986), are elements that might be related to the proposed role of carnitine acyltransferases in transport through the peroxisomal membrane.

Selective COT inhibitors, suitable for use with whole cells or animals, would allow exploration of the regulatory role proposed for this enzyme. In addition, they would help to establish the mechanism through which CPT I inhibitors counteract the characteristic induction of peroxisomal β-oxidation enzymes by clofibrate-like drugs (Hertz & Bar-Tana, 1987).

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