Flexibility of zonation of fatty acid oxidation in rat liver

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Periportal and perivenous hepatocytes were isolated from rats subjected to different treatments that induce (starvation, cold exposure) or depress (refeeding after starvation) hepatic fatty acid oxidation. These experiments were designed to determine factors that may be involved in creating and maintaining the asymmetrical distribution of this metabolic pathway in the acinus of the liver. The uneven distribution of mitochondrial [¹⁴C]-palmitate oxidation within the acinus (i) was very flexible and changed markedly with the physiological status of the animal (periportal/perivenous ratio: 1.5, 2.0, 1.0 and 0.4 for fed, starved, re-fed and cold-exposed animals respectively), (ii) coincided with a similar zonation of carnitine palmitoyltransferase I activity in fed as well as in cold-exposed animals, (iii) was paralleled by a comparable zonation of mitochondrial 3-hydroxy-3-methyl-

glutaryl-CoA synthase activity in starved animals, and (iv) was not determined by zonal differences in any of the following parameters: sensitivity of carnitine palmitoyltransferase I to malonyl-CoA, intracellular concentration of malonyl-CoA, fatty acid synthesizing capacity, acetyl-CoA carboxylase activity, fatty acid synthase activity or relative content of the two hepatic acetyl-CoA carboxylase isoforms. Unlike mitochondrial oxidation, peroxisomal [¹⁴C]palmitate oxidation was always zoned towards the perivenous zone of the liver irrespective of the physiological status of the animal. The data presented show that changes in the acinar distribution of mitochondrial long-chain fatty acid oxidation involve specific long-term mechanisms under different physiological conditions.

INTRODUCTION

Hepatocytes express structural and functional heterogeneity depending on their periportal (PP; afferent) or perivenous (PV; efferent) location in the liver acinus [1–3]. Regarding fatty acid oxidation, this metabolic pathway has been shown to be zoned towards the PP zone of the liver acinus in normally fed rats [4,5]. However, the factors that determine this asymmetry are not well defined. It is generally agreed that hepatic fatty acid oxidation (basically ketogenesis) is primarily controlled at two steps, i.e. those catalysed by carnitine palmitoyltransferase I (CPT-I; EC 2.3.1.21) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (EC 4.1.3.5) [6–8]. CPT-I activity has been shown to be higher in the PP than in the PV zone of the livers of fed rats [4,5]. CPT-I catalyses the supposedly rate-determining step in the transport of long-chain fatty acids into the mitochondrial matrix [6–8]. This enzyme displays rather complex modes of control. CPT-I is induced in different pathophysiological conditions [6–8]. The changes in CPT-I activity are often accompanied by variations in enzyme sensitivity to malonyl-CoA, a physiological intracellular inhibitor of CPT-I [6–8]. Malonyl-CoA is the product of the reaction catalysed by acetyl-CoA carboxylase (ACC), an important rate-regulatory enzyme of fatty acid synthesis *de novo*, and a substrate for fatty acid synthase (FAS). Thus malonyl-CoA levels allow the co-ordinate control of the synthesis and oxidation of fatty acids in the liver [6–8].

In 1984, Allred and co-workers reported the identification of two isoforms of ACC [9]. This observation did not receive much attention for several years, until first Thampy [10] and later Witters and colleagues [11] rediscovered the two isoforms of

ACC, i.e. a 280 kDa isoform (ACC-280) and a 265 kDa isoform (ACC-265). The 280 kDa isoenzyme is (i) less sensitive to activation by citrate than the classical 265 kDa isoform [11], and (ii) especially abundant in tissues with a high fatty acid oxidative capacity (e.g. heart and skeletal muscle), whereas it is not expressed in rat adipose tissue, which only contains ACC-265 [11,12]. These and other observations have led to the suggestion that ACC-265 might synthesize malonyl-CoA to be channelled through FAS to lipogenesis, whereas ACC-280 could be involved in the production of malonyl-CoA for inhibition of CPT-I [11–13].

Contrary to the asymmetrical zonal distribution of CPT-I in rat liver, a homogeneous distribution within the liver acinus of fed and 24 h-starved rats has been demonstrated for mitochondrial HMG-CoA synthase [14], the putative regulatory enzyme of ketone body synthesis from acetyl-CoA [7,8]. HMG-CoA synthase activity is controlled by two mechanisms which usually operate in concert: modification of pre-existing enzyme molecules by succinylation (inactivation)/desuccinylation (activation) [15,16] and changes in the amounts of enzyme mRNA and immunoreactive protein [16–18].

Although much information has accumulated on the zonation of the hepatic enzymic equipment from experiments performed using microdissection/histochemical techniques and dual-digitonin-pulse perfusion [1–3], it is not possible to infer differences in metabolic fluxes from differences in the activities and/or amounts of enzymes that may not be rate-limiting for a particular pathway. In contrast, the use of preparations of hepatocytes isolated from the PP and PV zones of the liver by digitonin/collagenase perfusion enables the measurement of rates of metabolic pathways under defined conditions, although the

Abbreviations used: ACC, acetyl-CoA carboxylase; ACC-265, 265 kDa isoform of ACC; ACC-280, 280 kDa isoform of ACC; CPT-I, carnitine palmitoyltransferase I; FAS, fatty acid synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PP, periportal; PV, perivenous; TDGA, tetradecylglycidate.

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conditions used *ex vivo* do not necessarily reflect the situation that occurs *in vivo*. Studies on carbohydrate and amino acid metabolism using isolated PP and PV hepatocytes have shown that liver heterogeneity is flexible and may change during the development of the animal and in different pathophysiological conditions [1–3]. To date, however, no information is available as to how the zonation of hepatic fatty acid metabolism may change with the physiological status of the animal. In addition, the mechanisms underlying the acinar distribution of fatty acid oxidation, potentially connected to that of fatty acid synthesis, have not been elucidated. Therefore in the present study PP and PV hepatocytes were isolated from donor animals subjected to different treatments that induce (starvation, cold exposure) or depress (refeeding after starvation) hepatic fatty acid oxidation. The two types of hepatocytes were used to determine potential changes in the overall fluxes through the pathways of fatty acid synthesis and oxidation, as well as factors (supposedly the steps catalysed by CPT-I and/or mitochondrial HMG-CoA synthase) involved in the maintenance of the acinar distribution of hepatic fatty acid oxidation.

EXPERIMENTAL

Materials

Sodium [1-¹⁴C]acetate (56 Ci/mol), [1-¹⁴C]acetyl-CoA (54 Ci/mol), [1-¹⁴C]palmitic acid (58 Ci/mol), [1-¹⁴C]palmitoyl-CoA (47.8 mCi/mmol), L-[methyl-¹⁴C]carnitine (71 Ci/mol) and [³⁵S]streptavidin (about 1000 Ci/mmol) were supplied by Amersham International (Amersham, Bucks., U.K.). Digitonin and collagenase (type I) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). R-730 RIBI adjuvant was obtained from Immunochem Research Inc. (Hamilton, MT, U.S.A.). Tetradecylglycidate (TDGA) was kindly provided by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA, U.S.A.).

Animals

Male Wistar rats (250–300 g body weight) were used in all experiments. Animals always had free access to drinking water. They were (i) fed *ad libitum* a standard, low-fat, pelleted stock diet (fed animals), (ii) starved for 48 h (starved animals), (iii) starved for 48 h and then refed on a fat-free, high-carbohydrate, diet for 72 h (refed animals), or (iv) kept at 4 °C for 15 h prior to cell isolation (cold-exposed animals). In the last case, animals had also free access to the standard, low-fat, pelleted diet. Animals were housed individually under a 12 h light/dark cycle (light, 06:00 to 18:00 h; dark, 18:00 to 06:00 h). Hepatocytes were isolated at 09:00 h.

Isolation and incubation of hepatocytes

PP and PV hepatocytes were isolated by the digitonin/collagenase perfusion procedure of Chen and Katz [19]. The final hepatocyte preparation was suspended in Krebs/Henseleit bicarbonate buffer supplemented with 10 mM glucose. Cell viability, as determined by Trypan Blue exclusion, always exceeded 85 % in the final hepatocyte suspension. PP and PV hepatocytes were characterized according to the distribution pattern of a number of marker enzymes (glucokinase, glutamate dehydrogenase, alanine aminotransferase and lactate dehydrogenase) exactly as described before [5].

Hepatocytes were incubated in Krebs/Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 2 % (w/v) defatted and dialysed BSA. Incubations (4–6 mg of cellular protein/ml) were carried out in 25 ml Erlenmeyer flasks in a

metabolic gyratory shaker (85 oscillations per min) in a total volume of 2 ml at 37 °C, under an atmosphere of O₂/CO₂ (19:1). Since lipogenesis is markedly depressed just after the isolation procedure [20], hepatocytes were incubated for 15 min under the aforementioned conditions before experimental parameters were determined.

Rate of fatty acid oxidation

The rate of fatty acid oxidation was determined as the formation of total oxidation products from [1-¹⁴C]palmitate. Hepatocytes were incubated for 20 min either in the absence or in the presence of 5 μM TDGA, a potent and specific inhibitor of CPT-I activity [21,22] and of mitochondrial long-chain fatty acid oxidation [22,23]. Hepatocytes were subsequently incubated in the presence of 0.4 mM albumin-bound [1-¹⁴C]palmitate (0.05 Ci/mol). After 20 min, reactions were stopped by the addition of 0.5 ml of 2 M perchloric acid and total oxidation products were determined as described before [22]. Mitochondrial palmitate oxidation was thus determined as the TDGA-sensitive oxidation, whereas peroxisomal palmitate oxidation was measured as the TDGA-insensitive oxidation [22].

Rate of fatty acid synthesis *de novo*

For the determination of the rate of fatty acid synthesis *de novo*, hepatocytes were incubated with 10 mM [1-¹⁴C]acetate (0.05 Ci/mol). After 30 min, reactions were stopped with 0.2 ml of 10 M NaOH, samples were saponified and total fatty acids were extracted [24].

CPT-I assay

CPT-I activity was determined in isolated mitochondria or in digitonin-permeabilized hepatocytes. In the first case, mitochondria were isolated from intact hepatocytes and CPT-I activity was measured as the malonyl-CoA-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine [25]. Preparations of mitochondria were practically devoid of peroxisomes, as judged from measurements of recovery of catalase activity (results not shown).

When CPT-I activity was determined in a digitonin-permeabilized cell system, hepatocytes were incubated for 20 min either in the absence or in the presence of 5 μM TDGA. Samples were subsequently removed from both sets of incubations in order to monitor CPT activity exactly as described before [22]. After 40 s, reactions were stopped with 0.4 ml of 1 M HCl and [¹⁴C]palmitoylcarnitine product was extracted with n-butanol. CPT-I activity was thus determined as the TDGA-sensitive CPT activity.

Mitochondrial HMG-CoA synthase assay

Mitochondria were isolated from intact hepatocytes [26] and resuspended in a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM sucrose, 1 mM EDTA, 1 mM dithioerythritol and 1.5 % (v/v) Triton X-100. The resulting samples were dialysed for 24 h at 4 °C against 20 mM potassium phosphate/12 mM EDTA (pH 7.0) in order to achieve inactivation of HMG-CoA lyase [27]. Aliquots of the samples were subsequently incubated for 20 min at 30 °C either in desuccinylation medium (designed to desuccinylate HMG-CoA synthase, so that total enzyme activity is exposed) or in non-desuccinylation medium (designed to preserve the succinylation of HMG-CoA synthase, so that only the activity of desuccinylated synthase molecules is expressed). The compositions of these two media were exactly as described by Quant et al. [15]. Subsequent determination of HMG-CoA

synthase activity was performed according to Clinkenbeard et al. [27]. Briefly, aliquots of desuccinylated and non-desuccinylated samples were incubated at 30 °C for a further 3–5 min (depending on the source of mitochondria) in 100 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM dithioerythritol, 20 μ M acetoacetyl-CoA and 0.2 mM [1-¹⁴C]acetyl-CoA (1.2 Ci/mol). Reactions were stopped with 0.3 ml of 6 M HCl, and [¹⁴C]HMG-CoA product was determined [27].

Assays of ACC and FAS activities

ACC activity was determined in 12000 g cell supernatants as the incorporation of [1-¹⁴C]acetyl-CoA into fatty acids in a reaction coupled to the FAS reaction [24,25]. As a modification of the published procedure, the concentrations of citrate and MgCl₂ in the homogenizing buffer and in the assay mixture were 10 and 13 mM respectively. FAS used in the assay for measuring ACC activity was purified as in [24]. Residual ACC activity was removed by passing the FAS preparation over an avidin column.

FAS activity was determined in 12000 g cell supernatants by measuring the incorporation of [1-¹⁴C]acetyl-CoA into fatty acids in the presence of malonyl-CoA [24].

Antibody production

In this study a primary antiserum against rat liver ACC was utilized. Hepatic ACC for antibody production was isolated from rats starved for 72 h and then refed a fat-free, high-sucrose, diet for 48 h as described before [24]. A 1 ml sample of R-730 adjuvant suspension containing 43 μ g of purified, native ACC in physiological saline was administered subcutaneously and intracutaneously to a rabbit. At 26 and 52 days after the initial injections, the rabbits were boosted in the same way. Antiserum was collected 10 days after the second boost and stored in 1 ml aliquots at –20 °C. Relative to this antiserum, the titre at 52 days was 35 % and that at 26 days was 1 % of the final one. The following lines of evidence identified the antiserum as directed against ACC: (i) it had the ability to neutralize ACC activity in rat liver homogenates, and (ii) it recognized in immunoblots of rat liver homogenates two biotin-containing protein bands with the expected molecular masses, i.e. 265 and 280 kDa.

Western blot analysis of ACC isoforms

The distribution of the isoforms of ACC in isolated rat liver cells was determined using immunoprecipitation, SDS/PAGE, immunoblotting, autoradiography and scanning of autoradiograms.

For this purpose cells (4–6 mg of protein) were homogenized with a tight-fitting Dounce homogenizer in 1 ml of buffer containing 50 mM Hepes (pH 7.5), 0.25 M mannitol, 10 mM citrate, 4 mM MgCl₂ and 50 μ l of the following freshly prepared proteinase inhibitor mixture: PMSF (0.5 mM), Tos-Phe-CH₂Cl (15 μ g/ml), Tos-Lys-CH₂Cl (18 μ g/ml), Tos-L-arginine methyl ester (18 μ g/ml), pepstatin (5 μ g/ml), trypsin inhibitor (50 μ g/ml), leupeptin (5 μ g/ml), benzamidine (0.8 mg/ml), apro-tinin (0.4 kallikrein inhibitory units/ml) and 2-mercaptoethanol (5 μ l/ml) in 20 mM Tris, 0.5 M NaCl, pH 7.5, with 0.05 % (v/v) Tween 20 (TTBS).

A 10 μ l portion of pre-swollen Protein A-Sepharose was rotated end-over-end with 15 μ l of antiserum and 25 μ l of TTBS for at least 2 h at room temperature. The antibody bound to Protein A-Sepharose was extensively washed with TTBS and then rotated end-over-end overnight at 4 °C with up to 1.5 mg of protein from the 12000 g supernatant of a Dounce extract of

isolated hepatocytes, in the presence of 50 μ l of the aforementioned inhibitor mixture. With the Dounce homogenizing procedure, damage to mitochondria is minimal and biotin-containing proteins such as pyruvate carboxylase and propionyl-CoA carboxylase are simply removed by centrifuging at 12000 g. Using immunoprecipitates rather than cytosol for separation of isoforms further reduces interference of other biotin-containing proteins competing for streptavidin (cf. [28]) that is used for detection of ACC.

The immune complexes were extracted from the Protein A-Sepharose gels with Laemmli disintegration buffer. SDS-treated immune complexes were incubated overnight with [³⁵S]streptavidin (0.05 μ Ci/incubation). The immune precipitates were subjected to SDS/PAGE as described by Laemmli [29] using 0.75-mm thick 3.5 % (w/v) polyacrylamide gels. Stacking and resolving buffer pH values were adjusted to 6.8 and 8.8 respectively. Proteins were transferred from SDS gels to nitrocellulose in an electrophoretic transfer cell. After washing, blots were dried and visualized by autoradiography using Fuji RX film. To detect the relative content of ACC-265 and ACC-280, the intensities of the bands obtained in the autoradiograms were quantified by densitometry, using an Ultrascan XL laser densitometer (LKB, Bromma, Sweden).

Other analytical procedures

Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymic method as described in [30]. Diacylglycerol acyltransferase activity was determined in 12000 g cell supernatants by measuring the incorporation of [1-¹⁴C]palmitoyl-CoA into triacylglycerols in the presence of endogenous diacylglycerols exactly as described before [31]. Protein was determined by the method of Lowry et al. [32], with BSA as standard.

Statistical analysis

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

RESULTS

The present study was aimed at determining factors involved in creating and maintaining the acinar distribution of hepatic long-chain fatty acid oxidation. A large body of experimental evidence indicates that two factors may be *a priori* assumed to be potentially rate-controlling for hepatic long-chain fatty acid oxidation [6–8]. (i) The flux through the step catalysed by CPT-I, which is dependent on CPT-I specific activity, CPT-I sensitivity to malonyl-CoA and the intracellular concentration of malonyl-CoA. The latter may in turn be dependent on the relative activities of ACC and FAS, as well as on the relative content of ACC-265 and ACC-280 [11–13]. (ii) The flux through the step catalysed by mitochondrial HMG-CoA synthase, which is dependent on the amount of enzyme protein and on the extent of succinylation of existing enzyme molecules.

Rate of fatty acid oxidation

The rates of mitochondrial and peroxisomal [¹⁴C]palmitate oxidation were determined in PP and PV hepatocytes isolated from animals in different physiological states. Mitochondrial [¹⁴C]palmitate oxidation changed with the nutritional status as expected, i.e. it increased in starvation and decreased after

Table 1 Rate of [^{14}C]palmitate oxidation in PP and PV hepatocytes

Hepatocytes were isolated from the PP and PV zones of the liver, and the rates of mitochondrial, peroxisomal and total [^{14}C]palmitate oxidation were determined. Results are means $\pm$ S.D. for four animals from each group. Significant differences from the PP zone of the corresponding group are indicated by: * $P < 0.01$; ** $P < 0.05$.

Animal group	Rate of [^{14}C]palmitate oxidation (nmol of palmitate into product/h per mg of protein)								
	Mitochondrial oxidation			Peroxisomal oxidation			Total oxidation		
	PP	PV	PP/PV	PP	PV	PP/PV	PP	PV	PP/PV
Fed	35.9 $\pm$ 7.2	23.9 $\pm$ 3.5**	1.5	10.2 $\pm$ 2.0	15.4 $\pm$ 2.0**	0.7	46.1 $\pm$ 6.7	39.3 $\pm$ 4.1	1.2
Starved	126.2 $\pm$ 23.6	64.2 $\pm$ 22.0*	2.0	23.6 $\pm$ 7.3	38.1 $\pm$ 7.0**	0.6	149.8 $\pm$ 13.0	102.3 $\pm$ 17.5*	1.5
Refed	3.7 $\pm$ 3.6	3.5 $\pm$ 1.0	1.0	5.3 $\pm$ 0.8	6.7 $\pm$ 2.1	0.8	9.0 $\pm$ 3.3	10.2 $\pm$ 1.5	0.9
Cold-exposed	47.3 $\pm$ 8.6	120.2 $\pm$ 13.1*	0.4	18.9 $\pm$ 1.6	26.6 $\pm$ 4.3**	0.7	66.2 $\pm$ 5.0	146.8 $\pm$ 17.5*	0.5

refeeding a high-carbohydrate diet (Table 1). Cold exposure also induced [^{14}C]palmitate oxidation in liver mitochondria (Table 1). More interestingly, Table 1 shows that zonation of mitochondrial long-chain fatty acid oxidation changed with the physiological status of the animals. Thus a certain zonation of mitochondrial [^{14}C]palmitate oxidation towards the PP zone was observed in normally fed animals. The induction of mitochondrial fatty acid oxidation in starvation was greater in the PP than in the PV zone of the liver. In contrast, cold exposure induced a very specific increase of mitochondrial fatty acid oxidation in the PV zone. No zonation of mitochondrial [^{14}C]palmitate oxidation was observed in refed animals.

Peroxisomal [^{14}C]palmitate oxidation changed with the physiological status of the animals, but less markedly than mitochondrial oxidation (Table 1). The rate was always higher in PV than in PP hepatocytes, irrespective of the status of the animal (Table 1). Clearly, the contribution of the peroxisomal compartment to hepatocellular total long-chain fatty acid oxidation changed in the different animal groups and in the two zones of the liver. Except for the refed state, [^{14}C]palmitate was mostly oxidized in the mitochondrial compartment, and so total [^{14}C]palmitate oxidation mostly reflected mitochondrial oxidation (Table 1).

Changes in the rate of [^{14}C]palmitate oxidation may be an indirect reflection of opposite changes in the rate of [^{14}C]palmitate esterification to glycerolipids. The hepatic pool of glycerol 3-phosphate is markedly zoned towards the PV zone [33], and so direct comparison of rates of fatty acid esterification in PP and PV hepatocytes may be prone to error. Hence we determined the activity of diacylglycerol acyltransferase, a putative regulatory enzyme of triacylglycerol biosynthesis and the enzyme exclusively involved in this pathway [34], as a marker of triacylglycerol-synthesizing capacity. However, no zonation of diacylglycerol acyltransferase activity was evident in any of the four animal groups (PP/PV ratios: 1.0, 0.9, 1.0 and 0.9 for fed, starved, refed and cold-adapted animals respectively).

CPT-I activity

A qualitative relationship was observed between the acinar distribution of mitochondrial [^{14}C]palmitate oxidation (Table 1) and that of CPT-I activity (Table 2) in fed, refed and cold-exposed animals. Thus, in normally fed animals, CPT-I activity was slightly higher in PP than in PV hepatocytes, as shown in previous studies [4,5]. CPT-I activity decreased upon refeeding, and this was accompanied by a loss of zonation. In line with the increase in mitochondrial fatty acid oxidation in the PV zone, cold exposure specifically induced CPT-I activity in that zone;

Table 2 CPT-I activity in PP and PV hepatocytes

Hepatocytes were isolated from the PP and PV zones of the liver, and CPT-I activity was determined in a permeabilized-cell system. Results are means $\pm$ S.D. for four animals from each group. Significant differences from the PP zone of the corresponding group are indicated by: * $P < 0.01$; ** $P < 0.05$.

Animal group	CPT-I activity (nmol/min per mg of protein)		
	PP	PV	PP/PV
Fed	1.49 $\pm$ 0.13	1.13 $\pm$ 0.18**	1.3
Starved	2.44 $\pm$ 0.26	2.32 $\pm$ 0.06	1.0
Refed	1.15 $\pm$ 0.06	1.23 $\pm$ 0.43	0.9
Cold-exposed	1.54 $\pm$ 0.25	2.99 $\pm$ 0.55*	0.5

thus the PP/PV ratio was decreased to 0.5 in these animals. On the other hand, the shift of mitochondrial [^{14}C]palmitate oxidation towards the PP zone observed in the starved animals (Table 1) was not accompanied by a parallel change in CPT-I activity (Table 2). Hence CPT-I activity may contribute to the zonation of mitochondrial long-chain fatty acid oxidation in fed and cold-exposed animals, but not in the starved group.

Mitochondrial HMG-CoA synthase activity

The activity of HMG-CoA synthase was determined in extracts of mitochondria isolated from PP and PV hepatocytes. Since mitochondrial HMG-CoA synthase is subject to regulation by succinylation (inactivation)/desuccinylation (activation) [15,16], assays were performed to determine both expressed (desuccinylated) and total (desuccinylated + succinylated) synthase activity. In line with previous observations [35], expressed mitochondrial HMG-CoA synthase activity was increased by starvation and decreased upon refeeding a high-carbohydrate diet (Table 3). In addition, cold exposure did not significantly affect expressed HMG-CoA synthase activity (Table 3). Changes in synthase activity were still evident, at least in part, after treatment of mitochondrial extracts with desuccinylation medium to measure total activity (Table 3). This indicates that changes in the amount of enzyme molecules are also operative [16,18,35]. Starvation increased the active fraction (decreased the extent of succinylation) of mitochondrial HMG-CoA synthase, whereas refeeding after starvation had the opposite effect (Table 3).

With respect to zonation, no relationship was observed between the acinar distribution of mitochondrial HMG-CoA synthase activity and that of mitochondrial [^{14}C]palmitate oxidation in fed

Table 3 HMG-CoA synthase activity in mitochondria from PP and PV hepatocytes

HMG-CoA synthase activity was determined in mitochondrial extracts obtained from PP and PV hepatocytes. Prior to measuring enzyme activity, samples were incubated either in non-desuccinylation medium (to determine expressed enzyme activity) or in desuccinylation medium (to determine total enzyme activity). The fraction of active (desuccinylated) mitochondrial HMG-CoA synthase was calculated as a percentage of total activity. Results are means $\pm$ S.D. for four animals from each group. Significant differences from the PP zone of the corresponding group are indicated by: * $P < 0.01$; ** $P < 0.05$.

Animal group	HMG-CoA synthase activity (nmol/min per mg of protein)								
	Expressed activity			Total activity			Active fraction (%)		
	PP	PV	PP/PV	PP	PV	PP/PV	PP	PV	PP/PV
Fed	7.44 $\pm$ 2.38	8.34 $\pm$ 2.11	0.9	11.66 $\pm$ 2.33	12.58 $\pm$ 1.55	0.9	63.8 $\pm$ 7.3	66.3 $\pm$ 6.7	1.0
Starved	23.20 $\pm$ 1.95	12.45 $\pm$ 2.09*	1.9	25.06 $\pm$ 3.41	19.05 $\pm$ 1.88**	1.3	92.6 $\pm$ 7.2	65.4 $\pm$ 5.7*	1.4
Refed	4.13 $\pm$ 0.83	4.85 $\pm$ 1.07	0.9	7.58 $\pm$ 2.44	9.17 $\pm$ 1.15	0.8	54.5 $\pm$ 4.0	52.9 $\pm$ 6.6	1.0
Cold-exposed	10.13 $\pm$ 1.82	10.91 $\pm$ 0.84	0.9	14.90 $\pm$ 2.31	16.48 $\pm$ 3.27	0.9	68.0 $\pm$ 5.1	66.2 $\pm$ 3.1	1.0

Figure 1 Sensitivity of CPT-I to malonyl-CoA in mitochondria from PP and PV hepatocytes

Mitochondria were isolated from PP (○, □, △, ◇) and PV (●, ■, ▲, ◆) hepatocytes which had been isolated from fed (○, ●), starved (□, ■), refed (△, ▲) and cold-exposed (◇, ◆) animals. CPT-I activity was determined in the mitochondria in the presence of different concentrations of malonyl-CoA. Results were obtained using four animals from each group.

and cold-exposed animals, in which mitochondrial oxidation is predominant in the PP and the PV zone respectively (Table 1). Mitochondrial HMG-CoA synthase activity did not show such asymmetrical distribution in these two groups of animals (Table 3). Zonation of mitochondrial HMG-CoA synthase activity was also found to be absent in refed animals (Table 3). In contrast, a good relationship was observed in the starved animals between the zonation of mitochondrial [14 C]palmitate oxidation (PP/PV ratio 2.0; Table 1) and that of expressed HMG-CoA synthase activity in mitochondria (PP/PV ratio 1.9; Table 1). Although a certain zonation towards the PP zone was still evident when total synthase activity was determined, zonal differences in HMG-CoA synthase activity in the starved group seem to be mostly due to differences in the extent of succinylation of the enzyme (Table 3). Mitochondrial HMG-CoA synthase may thus contribute to the zonation of mitochondrial long-chain fatty acid oxidation observed upon starvation.

Sensitivity of CPT-I to malonyl-CoA

Figure 1 shows CPT-I inhibition by malonyl-CoA in mitochondria isolated from PP and PV hepatocytes from the four animal groups. In agreement with previous reports, the sensitivity of CPT-I to malonyl-CoA decreased in starvation and increased upon refeeding relative to the fed group (reviewed in [6–8]), whereas no significant differences were observed between fed and cold-exposed animals. More importantly, no differences in sen-

Table 4 Malonyl-CoA concentration and rate of fatty acid synthesis *de novo* in PP and PV hepatocytes

The rate of fatty acid synthesis *de novo* was determined in hepatocytes isolated from the PP and PV zones of the liver. Malonyl-CoA concentration was determined in perchloric acid extracts of the same hepatocyte suspensions as used for measuring lipogenesis. Results are means $\pm$ S.D. for four animals from each group.

Animal group	[Malonyl-CoA] (pmol/mg of protein)			Rate of fatty acid synthesis (nmol of acetyl units/min per mg of protein)		
	PP	PV	PP/PV	PP	PV	PP/PV
Fed	43 $\pm$ 6	54 $\pm$ 9	0.8	40.1 $\pm$ 9.3	49.7 $\pm$ 6.3	0.8
Starved	12 $\pm$ 4	14 $\pm$ 4	0.9	1.1 $\pm$ 0.2	1.2 $\pm$ 0.6	0.9
Refed	108 $\pm$ 26	115 $\pm$ 44	0.9	186.2 $\pm$ 42.7	169.2 $\pm$ 14.2	1.1
Cold-exposed	40 $\pm$ 10	34 $\pm$ 8	1.2	33.7 $\pm$ 8.0	27.6 $\pm$ 6.8	1.2

Table 5 Activities of ACC and FAS and the relative content of ACC in PP and PV hepatocytes

The activities of ACC and FAS, and ACC-280 content as a percentage of total ACC mass [i.e. ACC-280 mass/(ACC-265 mass + ACC-280 mass)], were determined in 12000 g supernatants of PP and PV hepatocytes. Results are means $\pm$ S.D. for four animals from each group.

Animal group	Activity (nmol/min per mg of protein)						ACC-280 (%)		
	ACC			FAS					
	PP	PV	PP/PV	PP	PV	PP/PV	PP	PV	PP/PV
Fed	0.89 $\pm$ 0.29	1.17 $\pm$ 0.43	0.8	4.35 $\pm$ 1.63	5.16 $\pm$ 2.43	0.8	29.8 $\pm$ 1.7	35.4 $\pm$ 2.0	0.8
Starved	0.12 $\pm$ 0.04	0.11 $\pm$ 0.02	1.1	1.46 $\pm$ 0.12	1.44 $\pm$ 0.58	1.0	28.9 $\pm$ 4.5	27.0 $\pm$ 2.1	1.1
Refed	1.64 $\pm$ 0.63	2.84 $\pm$ 1.03	0.6	25.64 $\pm$ 4.53	23.40 $\pm$ 5.35	1.1	39.8 $\pm$ 3.2	35.9 $\pm$ 3.5	1.1
Cold-exposed	0.61 $\pm$ 0.10	0.51 $\pm$ 0.09	1.2	3.33 $\pm$ 0.45	2.87 $\pm$ 0.52	1.2	35.3 $\pm$ 3.6	28.8 $\pm$ 3.2	1.2

sitivity to malonyl-CoA were observed between the PP and the PV zones of the liver in any of the four animal groups, indicating that the acinar distribution of mitochondrial [14 C]palmitate oxidation is not determined by the sensitivity of CPT-I to malonyl-CoA.

Intracellular concentration of malonyl-CoA, rate of fatty acid synthesis *de novo*, ACC activity and FAS activity

It is well established that changes in CPT-I activity and sensitivity to malonyl-CoA under different pathophysiological situations are also accompanied by changes in the intracellular concentration of malonyl-CoA [6–8]. In addition, malonyl-CoA levels are expected to correlate well with the rate of fatty acid synthesis *de novo* [36]. Table 4 shows the intracellular content of malonyl-CoA and the rate of fatty acid synthesis *de novo* in the different animal groups. As expected, both parameters were notably decreased in starvation and increased upon refeeding, whereas they were slightly diminished by cold exposure.

With regard to acinar distribution, both the levels of malonyl-CoA and the fatty acid-synthesizing capacity (Table 4) tended to be zoned towards the opposite zone from long-chain fatty acid oxidation (Table 1) in the fed, starved and cold-exposed animals. However, these trends were not statistically significant. In addition, no zonation was observed in the refed animals for these two parameters (Table 4). Hence malonyl-CoA levels do not determine the acinar distribution of hepatic long-chain fatty acid oxidation. Likewise, no zonation was observed in the activity of either ACC or FAS, the two enzymes involved in fatty acid synthesis from acetyl-CoA (Table 5).

ACC isoforms

The zonation of long-chain fatty acid oxidation changes quite markedly upon starvation or cold exposure, whereas fatty acid synthesis *de novo* is not zoned in either of these conditions (see above). On the basis of their different kinetic and regulatory properties and tissue distributions [11], ACC-265 and ACC-280 have been suggested to play different biological roles. In particular, ACC-265 might synthesize malonyl-CoA which would be channelled through FAS to fatty acid synthesis *de novo*, whereas ACC-280 might be involved in the synthesis of malonyl-CoA for CPT-I inhibition [11,13]. The latter hypothesis is specially supported by recent studies carried out in rat heart, which almost exclusively expresses ACC-280 [12,13,37]. If this were the case, the zonation pattern of the two isoenzymes should change at least in the starved and cold-exposed groups, i.e. there should be a selective decrease of ACC-280 relative to ACC-265 in the PP (oxidative) zone of the starved animals as well as in the PV

Figure 2 ACC isoform distribution in PP and PV hepatocytes from fed, starved, refed and cold-exposed animals

ACC was extracted by immunoprecipitation from PP and PV hepatocytes from donor rats in the indicated physiological states. The amount of cell extract incubated for immunoprecipitation was 0.74, 0.75, 1.58, 1.42, 0.69, 0.72, 0.51 and 0.51 mg of protein for fed (PV, PP), starved (PV, PP), cold-adapted (PV, PP) and refed (PV, PP) animals respectively. Following labelling with [35 S]streptavidin, SDS/PAGE and transfer to nitrocellulose, the biotin-containing isoforms of ACC were visualized by autoradiography.

(oxidative) zone of the cold-exposed animals. Figure 2 shows the separation patterns of the two ACC isoenzymes in PP and PV hepatocytes from the four different animal groups. However, the relative content of the two ACC isoenzymes did not differ, either between the two liver zones or among the different physiological states (Table 5). This observation indicates that ACC-280 from rat liver does not seem to be involved in the synthesis of a pool of malonyl-CoA that is particularly committed to inhibit CPT-I.

DISCUSSION

Previous studies on the zonation of carbohydrate and amino acid metabolism have shown that liver heterogeneity may change during the development of the animal and in certain pathophysiological conditions [1–3]. A first general conclusion which may be inferred from our studies is that the acinar distribution of mitochondrial long-chain fatty acid oxidation is also very flexible and changes with the physiological status of the animal. However, the physiological significance of these changes is not obvious. Irrespective of the various models proposed to date to explain the microstructure of the liver acinus, PP hepatocytes seem to account for the bulk of the parenchymal cells within the acinus [1,2]. Therefore the more pronounced induction of mitochondrial long-chain fatty acid oxidation in the PP zone of the liver under starvation will increase the ketogenic capacity of the liver more efficiently than in the hypothetical case of a more

prominent induction in the PV zone. PP hepatocytes seem to be better equipped than PV hepatocytes to achieve induction of oxidative processes. Compared with PV hepatocytes, PP cells have a higher density of rough endoplasmic reticulum, as well as a greater volume of mitochondria and a greater area of cristae [1,2]. Likewise, the induction of ketogenesis and gluconeogenesis by glucagon has been shown to be more efficient in PP than in PV hepatocytes maintained in long-term culture [4].

The specific induction of hepatic long-chain fatty acid oxidation in the PV zone of cold-exposed animals is more difficult to explain. The expression of some enzymes seems to be enhanced in (or even restricted to) this liver zone [1]. In a number of liver pathologies, especially upon administration of certain xenobiotics (e.g. carbon tetrachloride, ethanol, aromatic solvents), the specific expression of detoxifying enzyme systems ensues in the PV zone (cf. [1,38]). It has been suggested that the low intramitochondrial NADH/NAD⁺ ratios observed in liver mitochondria in situations where ketone body production is enhanced may be related to the stimulation of hepatic gluconeogenesis [4,7]; thus, for example, the ratio of produced 3-hydroxybutyrate/acetoacetate is lower in PP than in PV hepatocytes despite the higher oxidative capacity of the former [4]. Moreover, this ratio is decreased by treatment with glucagon *in vitro* [4] and by diabetes *in vivo* (L. Agius, personal communication). In this respect it is noteworthy that the specific PV induction of hepatic long-chain fatty acid oxidation upon cold exposure reported in the present study agrees with the specific increase in gluconeogenesis in the PV zone of the liver under similar conditions reported by Shiota et al. [39]. The physiological significance of these observations is unclear, since PV hepatocytes apparently constitute only a minor fraction of the parenchymal cells within the liver acinus [1,2]. Nevertheless, PV hepatocytes are located in the efferent side of the acinus, i.e. at the site where blood exits from the liver. Additionally, an intriguing possibility is that part of the energy produced by fatty acid oxidation in the PV zone is not coupled to oxidative phosphorylation, but enables thermogenesis upon cold exposure.

A second general conclusion of our studies is that changes in the zonation of mitochondrial long-chain fatty acid oxidation seem to involve different mechanisms under different physiological conditions. Thus the acinar heterogeneity of fatty acid oxidation in starvation and cold exposure seems to be maintained by the zonation of mitochondrial HMG-CoA synthase activity and CPT-I activity respectively. Immunohistochemical studies performed in fed and 24 h-starved rats have recently shown a homogeneous distribution within the liver acinus of mitochondrial HMG-CoA synthase [14]. Nevertheless, this observation is not completely at odds with our data, since the immunohistochemical approach only gives information about the amount of total enzyme protein and our results indicate that zonal differences in activity of the synthase in the starved animals are mostly due to differences in the extent of succinylation of existing enzyme molecules.

Although the induction of mitochondrial HMG-CoA synthase and CPT-I usually occurs in concert [7,8], rapid switches of hepatic fatty acid metabolism such as those occurring in the starved-to-fed or the suckling-to-weaning transition correlate better with changes in mitochondrial HMG-CoA synthase activity [7,8], indicating that different induction mechanisms for the synthase and the acyltransferase may occur. The 5' region of the mitochondrial HMG-CoA synthase gene contains a peroxisome-proliferator-responsive element [40] which is also present in the genes encoding other enzymes involved in hepatic fatty acid metabolism, namely fatty acid-binding protein, acyl-CoA oxidase, the bifunctional enzyme of peroxisomal β -oxidation, mito-

chondrial medium-chain acyl-CoA dehydrogenase and fatty acid ω -hydroxylase (cf. [40,41]). In contrast, no peroxisome-proliferator-responsive element has been demonstrated to date for either of the two mitochondrial CPTs.

In contrast with the dynamic zonation displayed by mitochondrial fatty acid oxidation, peroxisomal [¹⁴C]palmitate oxidation was predominant in the PV zone of the liver, irrespective of the physiological status of the animal. Likewise, peroxisome proliferation induced by hypolipidaemic agents, plasticizers and other peroxisome proliferators is especially observed in the PV zone of the liver [42]. The contribution of the peroxisomal compartment to hepatocellular total long-chain fatty acid oxidation changes in the different animal groups and in the two zones of the liver. In fed animals, peroxisomal palmitate oxidation accounted for 22 % and 39 % of total palmitate oxidation in the PP and the PV zone of the liver respectively. These values are within the range of those reported in studies where peroxisomal long-chain fatty acid oxidation in whole liver was determined by different methods such as H₂O₂ production, luminometry or CPT-I inhibition [22,23,43–45]. Nevertheless, we are aware that a recent report indicates that, on blocking CPT-I, and hence mitochondrial oxidation, with specific inhibitors (e.g. TDGA), the contribution of the peroxisomal compartment to total oxidation may be overestimated [46]. This may be the case for the following reasons: (i) peroxisomal long-chain fatty acid oxidation usually catalyses a limited number of β -oxidation cycles, whereas mitochondrial oxidation goes to completion [43,45]; the methodology used in the present paper does not take into account the fact that peroxisomes degrade fatty acids only partially, and (ii) most evidence points towards the fact that, in rat liver, mitochondrial long-chain fatty acid oxidation would be about 10 times more active than peroxisomal oxidation [43,45]: thus, in theory a 90 % inhibition of CPT-I would lead to a 10 % underestimation of mitochondrial oxidation but to a 100 % overestimation of peroxisomal fatty acid oxidation.

The acinar distribution of fatty acid synthesis *de novo* is rather constant and does not change significantly with the physiological status of the animal in spite of the profound changes in the lipogenic capacity of the whole liver under different conditions. This also applies to the relative content of the two ACC isoforms which are expressed in rat liver. Our data with supernatants of isolated PP and PV cells are in line with others in which determinations were performed with eluates obtained after selective damage of the PP and PV zones [47]. In any case, since no differences in the relative abundance of ACC-280 exist in the regions of the liver adapted to fatty acid oxidation by zonation, the possible role of ACC-280 in controlling the aggregation state of ACC, as suggested by Winz et al. [48], should be considered.

Various mechanisms have been proposed to explain liver metabolic zonation, including the differential supply of hormones, nutrients and oxygen across the portal/venous vasculature, as well as the particular innervation of the liver acinus [1,2]. However, the physiological position of the liver within the circulation does not seem to be a major determinant of zonation of gene expression [49]. In addition, experiments in which lobular fluxes were inverted by changing the direction of the flow have demonstrated that short-term regulatory mechanisms determined by the direction of the bloodstream are also operative in zone-specific gene expression [1,2]. With regard to hepatic fatty acid oxidation, the present findings indicate that at least part of the difference between the PP and PV zones involves long-term mechanisms which survive hepatocyte isolation. In contrast, the acute response of [¹⁴C]palmitate oxidation to a number of cellular modulators has been shown to be similar in isolated PP and PV hepatocytes in different situations [5,50], indicating that short-

term mechanisms which may determine the zonation of fatty acid oxidation *in vivo* do not seem to involve intrinsic differences between PP and PV hepatocytes.

It should be kept in mind that the use of isolated PP and PV hepatocytes to study the metabolic zonation of the liver may not exactly reflect the actual situation occurring *in vivo*. For example, the zonation of ACC has also been studied by microdissection methods as well as by the dual-digitonin-pulse perfusion technique. Results obtained with the former approach are similar to ours in that ACC was concentrated in the PV zone of the liver [51]. In contrast, data obtained with the dual-digitonin-pulse perfusion technique differ from ours in that a net gradient of ACC activity to the PP side was observed [52]. An advantage of the use of isolated PP and PV hepatocytes is that it enables the correlation between enzyme activities and the rate of metabolic pathways to be investigated. Nevertheless, we are aware that the time lag between the handling of the intact liver and the performance of experimental determinations might produce changes in metabolic fluxes. This problem could be important in the case of *de novo* fatty acid synthesis, which is quite dependent on the conditions of hepatocyte isolation and incubation [20]. The comprehension of liver metabolic zonation *in vivo* clearly awaits the development of new methodological approaches.

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