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PROPERTIES OF THE MITOCHONDRIAL MEMBRANE AND CARNITINE PALMITOYLTRANSFERASE I IN THE PERIPORTAL AND THE PERIVENOUS ZONE OF THE LIVER

EFFECTS OF CHRONIC ETHANOL FEEDING

JOSÉ CASTRO,* JOSEFA P. CORTÉS and MANUEL GUZMÁN
Department of Biochemistry and Molecular Biology I, Faculty of Chemistry, Complutense University, 28040-Madrid, Spain

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Abstract—Rats were fed for 35 days a high-fat diet containing either 36% of total calories as ethanol (ethanol group) or an isocaloric amount of carbohydrate (control group). Then, mitochondria were isolated from the periportal and the perivenous zone of the liver in order to study the acinar heterogeneity of the effects of prolonged ethanol administration upon the properties of carnitine palmitoyltransferase I (CPT-I) and its membrane environment. Chronic ethanol ingestion selectively decreased CPT-I activity in periportal hepatocytes but equally increased enzyme sensitivity to malonyl-CoA and enzyme energy of activation in the two zones of the liver. In control animals, mitochondrial membrane showed higher fluidity and lower degree of saturation of phospholipid fatty acyl moieties in periportal than in perivenous hepatocytes. Prolonged ethanol feeding (i) decreased mitochondrial membrane fluidity; (ii) increased the proportion of palmitic acid and decreased that of arachidonic acid in mitochondrial phosphatidylcholine and phosphatidylethanolamine, whereas it drastically reduced the content of linoleic acid and concomitantly increased that of saturated and monoenoic fatty acids in cardiolipin; (iii) suppressed the disordering effects of the addition of ethanol to mitochondrial suspensions. All these ethanol-induced alterations of membrane fluidity and fatty acyl composition were not significantly different between periportal and perivenous mitochondria. In conclusion, chronic ethanol feeding changes the activity of CPT-I in a zone-selective manner but modifies both the regulatory properties of the enzyme and the properties of its lipid environment in a non-zone-selective manner. Hence factors in addition to the properties of the mitochondrial membrane seem to be involved in the ethanol-induced alterations of the CPT-I enzyme.

Ethanol ingestion by mammals is almost invariably associated with the development of fatty liver, which may eventually lead to more severe stages of liver damage [1–3]. Among the different factors responsible for the increased accumulation of triacylglycerols in the liver, the blockade of fatty acid oxidation may make a major contribution [3–5]. Furthermore, our current line of research supports an important role for the alterations of the kinetic and regulatory properties of carnitine palmitoyltransferase I (CPT-I)† in the appearance of ethanol-induced fatty liver [5, 6]. This enzyme is believed to catalyse the rate-limiting step of the fatty acid-oxidative process in both liver [7, 8] and extrahepatic tissues [9]. Thus, under different physiopathological situations characterized by variations in the rates of hepatic fatty acid oxidation, the flux through this step changes in parallel [6–10]. In addition, CPT-I is inhibited by physiological concentrations of malonyl-CoA [7–9]. Interestingly, CPT-I sensitivity to inhibition by malonyl-CoA usually changes in concert with enzyme activity [7–9].

One of the most characteristic features of the ethanol-induced fatty liver is the predominance of perivenular lesions [11–12]. Although it is generally agreed that different processes and enzyme activities display an asymmetrical distribution within the liver acinus [13, 14], no clear explanation is available so far for this zonal heterogeneity of the ethanol effects. Nevertheless, the selective perivenular induction of the microsomal ethanol-oxidizing system upon chronic ethanol administration may be a factor involved in the generation of asymmetrical liver damage [15, 16].

It is well-known that ethanol easily diffuses into biomembranes [17–19]. Ethanol thus induces a generalized disordering of the membrane core *in vitro*, increasing the mobility of the fatty acyl side chains of phospholipids [17, 18]. On the other hand, prolonged ethanol ingestion leads to a number of physicochemical alterations in biomembranes, including the development of an adaptive resistance to the disordering effects of acute doses of ethanol, the so-called "membrane tolerance" [19]. Moreover, ethanol affects a huge number of membrane-bound enzymes, ion channels and receptors [17–19]. Since the CPT-I enzyme is particularly influenced by the

* To whom correspondence should be addressed.

† Abbreviations: CPT, carnitine palmitoyltransferase; CPT-I and CPT-II, overt and latent CPT activity respectively; DPG, cardiolipin (diphosphatidylglycerol); DPH, 1,6-diphenylhexa-1,3,5-triene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, total phospholipids; PP, periportal; PV, perivenous.

Table 1. Effect of chronic ethanol feeding on triacylglycerol accumulation and CPT-I activity and sensitivity to malonyl-CoA in the periportal and the perivenous zone of the liver

Parameter	Control group			Ethanol-fed group		
	PP	PV	PP/PV	PP	PV	PP/PV
Triacylglycerol concentration	56.7 ± 7.1	64.0 ± 6.1	0.89	106.3 ± 11.4‡	345.8 ± 29.5†‡	0.31
CPT-I activity	6.87 ± 0.93	5.22 ± 0.37*	1.32	3.32 ± 0.55‡	6.16 ± 0.74†	0.54
CPT-I sensitivity to malonyl-CoA	7.8 ± 0.9	5.0 ± 0.6†	1.55	4.6 ± 0.9‡	3.2 ± 0.3†‡	1.45

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. In addition, cellular triacylglycerols were determined in the hepatocytes from which mitochondria were isolated. Values of triacylglycerol accumulation are expressed as nmol/mg of cellular protein. Values of CPT-I activity are expressed as nmol of palmitoylcarnitine/min per mg of mitochondrial protein. Values of enzyme sensitivity to malonyl-CoA are expressed as μM. Results are the means ± SD of six animals of each type.

* $P < 0.05$ vs the periportal zone of the corresponding group.

† $P < 0.01$ vs the periportal zone of the corresponding group.

‡ $P < 0.01$ vs the corresponding zone of the control group.

properties of its membrane environment [20–22], the possibility exists that the ethanol-induced changes in the kinetic and regulatory properties of CPT-I described up to date [5, 6] are mediated, at least in part, via ethanol-induced alterations in the composition and/or physical properties of the mitochondrial membrane.

The present study was thus undertaken to investigate the effects of prolonged ethanol ingestion on the properties of the CPT-I enzyme and its membrane environment with regard to the zonal heterogeneity of the liver. Our results suggest that the ethanol-induced changes in membrane fluidity and composition of the mitochondrial membrane are not the only factors responsible for the zone-selective effects exerted by prolonged ethanol feeding upon the CPT-I enzyme.

MATERIALS AND METHODS

Animals and their treatment. Male Wistar rats (150–175 g initial body weight) were fed for 35 days the "all purpose" Lieber-DeCarli liquid diet, in which ethanol represented 36% of total calories [23]. The pair-fed control group received exactly the same diet except that carbohydrate isocalorically replaced ethanol. Animals were housed individually and kept in a constant-temperature room with a 12 hr light–dark cycle.

Isolation of mitochondria from periportal and perivenous hepatocytes. Periportal and perivenous hepatocytes were isolated by the digitonin/collagenase perfusion procedure of Chen and Katz [24] and further characterized according to the distribution pattern of several marker enzymes [25]. 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. Hepatocytes were homogenized and mitochondria were isolated as described before [5]. Preparations of mitochondria were practically devoid of peroxisomes, as judged from experiments of recovery of catalase activity (results not shown). Mitochondrial protein was determined by the method of Lowry *et al.* [26], with bovine serum albumin as a standard.

Determination of carnitine palmitoyltransferase I *activity.* Measurement of CPT-I activity was performed within 15 min of the isolation of mitochondria. Mitochondria (0.15–0.25 mg of protein) were preincubated for 4 min in 1.0 mL containing 150 mM sucrose, 60 mM KCl, 25 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM dithioerythritol, 40 μM palmitoyl-CoA and 1.3 mg/mL defatted bovine serum albumin (charcoal-treated and dialysed). Then, reactions were started by addition of 25 μL containing 1 μCi (0.4 μmol) of L-[*Me*-^{3}H]carnitine, carried out for up to 4 min and stopped with 1.0 mL ice-cold 1 M HCl. [^{3}H]Palmitoylcarnitine was extracted with butan-1-ol [5]. Blank values were determined by stopping reactions at zero time. Malonyl-CoA-insensitive CPT activity, representing the latent form of mitochondrial CPT activity (CPT-II), was always subtracted from total CPT activity experimentally determined. CPT-I activity always accounted for more than 90% of this total CPT activity (results not shown).

Determination of the order parameter. Fluorescence anisotropy was determined with 1,6-diphenylhexa-1,3,5-triene (DPH) as probe [27]. A solution of DPH (100 mM) in acetonitrile was diluted 500 times by injection into a vigorously stirred solution which contained 10 mM Tris–HCl (pH 7.4), 150 mM KCl and 1 mM EDTA. The resulting mix was stirred for at least 1 hr before use, added to 3 vol. of mitochondrial suspension (2 mg of protein/mL) and incubated for 30 min at 37°. Measurements of fluorescence anisotropy were performed with a polarization spectrophotometer (Perkin-Elmer model MPF-44E) at 37° with 366 excitation and 426 emission wavelengths, by using polarizing filters in both excitation and emission planes. Control samples containing only mitochondria or probe were tested routinely and appropriate corrections were made. Each instrument reading was the average of about 10 measurements and, in view of instrumental variation, each determination was the average of 5–7 of these readings. Values of fluorescence

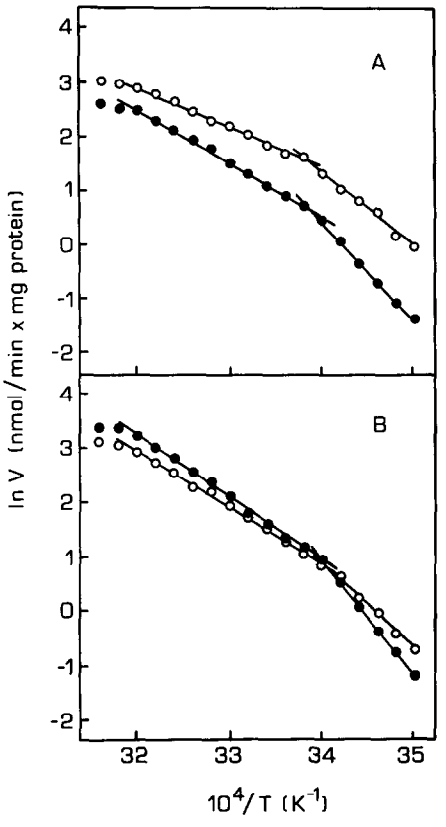

Fig. 1. Effect of chronic ethanol feeding on the energy of activation of CPT-I in periportal and perivenous mitochondria. Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control (○) or the ethanol-containing diet (●). Energies of activation (E_a) were calculated from the slope of the two segments of the Arrhenius plots. E_a for the upper segment refers to the range of *ca* 32–34 on the abcissa, whereas the values for the lower segment are those greater than 34. E_a values are given in Kcal/mol and represent the means of four animals of each type. Panel A: Periportal mitochondria. E_a for control animals: 21.5, 13.5; E_a for ethanol-fed animals: 30.5, 18.5. Panel B: Perivenous mitochondria. E_a for control animals: 26.4, 18.0; E_a for ethanol-fed animals: 34.7, 21.2. Standard deviations were always lower than 5% of the mean value.

anisotropy allowed determination of the membrane order parameter [28].

Lipid extraction and analysis. Total lipids were extracted by the method of Bligh and Dyer [29]. All the solvents contained 0.01% of butylated hydroxytoluene. Neutral lipids were separated by thin layer chromatography on silica-gel G plates with hexane/diethyl ether/formic acid (40:10:1, v/v/v) as developing system. Triacyglycerols were quantified as free glycerol after alkaline hydrolysis in a spectrophotometric assay coupled to glycerol kinase and glycerol 3-phosphate dehydrogenase [30], whereas cholesterol was determined by the cholesterol esterase/cholesterol oxidase/peroxidase method, supplied in kit form by Boehringer (Mannheim, Germany).

Individual phospholipid classes were separated by bidimensional thin layer chromatography on silica-gel G plates using chloroform/methanol/ammonia (90:54:1, v/v/v) as first developing system and chloroform/methanol/acetic acid/water (45:20:6:1, v/v/v/v) as second developing system. Phospholipids were quantified by phosphorus analysis [31].

Phospholipid fatty acids were converted into their corresponding methyl esters by the method of Morrison and Smith [32]. Fatty acid methyl esters were analysed in a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector, using a WCOT capillary column (length: 30 m, internal diameter: 0.25 mm) coated with Supelcowax 10 (Supelco Inc., Bellefonte, U.S.A.) as stationary phase. The temperature of the injector port and the detector was 250°. The oven temperature was programmed with an initial hold of 32 min at 185° to 200° at a rate of 2°/min until the end of the run. Individual methyl esters were identified by comparison with known commercial standards (supplied by Supelco) and according to previous data [33].

Statistical analysis. Statistical comparison was performed by using a two-way analysis of variance. A *post hoc* analysis was made by the Student–Neuman–Keuls test. Arrhenius plots were adjusted by linear-regression analysis.

Materials. DPH was purchased from Molecular Probes (Junction City, OR, U.S.A.). The sources

Table 2. Effects of chronic ethanol feeding on membrane order parameter in periportal and perivenous mitochondria.

Diet	Liver zone	S_{DPH}	
		No additions	+ 100 mM Ethanol
Control	Periportal	0.357 ± 0.005	0.333 ± 0.009‡
	Perivenous	0.425 ± 0.007*	0.401 ± 0.010*‡
Ethanol	Periportal	0.412 ± 0.010†	0.414 ± 0.004†
	Perivenous	0.477 ± 0.006*†	0.474 ± 0.003*†

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet and the order parameter was determined with DPH as probe (S_{DPH}). Results represent the means ± SD of four animals of each type.

* Significantly different ($P < 0.01$) vs the periportal zone of the corresponding group.

† Significantly different ($P < 0.01$) vs the corresponding zone of the control group.

‡ Significantly different ($P < 0.01$) vs assays with no additions.

Table 3. Effect of chronic ethanol feeding on membrane phospholipid composition in periportal and perivenous mitochondria

Parameter	Control group			Ethanol-fed group		
	PP	PV	PP/PV	PP	PV	PP/PV
nmol PL/mg protein	243 ± 16	251 ± 13	0.97	248 ± 23	257 ± 6	0.96
nmol PC/mg protein	116.6 ± 8.1	123.0 ± 17.5	0.95	124.7 ± 11.3	129.5 ± 9.3	0.96
nmol PE/mg protein	75.3 ± 5.2	75.0 ± 3.9	1.00	76.9 ± 10.2	76.1 ± 3.0	1.01
nmol DPG/mg protein	19.6 ± 1.7	19.3 ± 2.8	1.02	20.6 ± 2.7	21.8 ± 2.2	0.95
Molar ratio cholesterol/PL	0.05 ± 0.01	0.05 ± 0.02	1.00	0.05 ± 0.01	0.06 ± 0.01	0.83

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. Results are the means ± SD of six animals of each type.

Table 4. Effect of chronic ethanol feeding on the fatty acyl composition of total phospholipids in periportal and perivenous mitochondria

Fatty acid	Control group			Ethanol-fed group		
	PP	PV	PP/PV	PP	PV	PP/PV
16:0	13.7 ± 1.0	19.9 ± 0.6*	0.89	18.3 ± 1.5†	25.0 ± 1.1*†	0.73
16:1n-7	1.3 ± 0.2	1.6 ± 0.3	0.81	1.6 ± 0.1	1.9 ± 1.4	0.84
16:1n-9	1.7 ± 0.3	1.8 ± 0.2	0.94	1.9 ± 0.4	2.1 ± 0.2	0.90
18:0	16.8 ± 1.5	17.3 ± 0.6	0.97	20.4 ± 1.6†	20.9 ± 1.2†	0.98
18:1n-7	3.4 ± 0.3	3.7 ± 0.5	0.92	3.8 ± 0.7	4.3 ± 0.4	0.88
18:1n-9	6.6 ± 0.3	7.0 ± 0.9	0.94	6.5 ± 1.0	7.1 ± 0.5	0.92
18:2n-6	19.8 ± 0.9	16.5 ± 0.6*	1.20	15.1 ± 0.9†	11.6 ± 1.2*†	1.30
20:4n-6	26.8 ± 1.4	20.2 ± 0.8*	1.33	21.8 ± 1.3†	16.0 ± 0.7*†	1.36
22:5n-6	2.6 ± 0.8	2.3 ± 0.4	1.13	2.8 ± 0.4	2.9 ± 0.6	0.93
22:6n-3	4.7 ± 0.2	4.7 ± 0.5	1.00	4.9 ± 0.8	5.2 ± 0.3	0.94
Others	2.6 ± 0.5	2.0 ± 0.6	—	2.9 ± 0.3	3.0 ± 0.7	—

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. Results are the means ± SD of four animals of each type.

* Significantly different ($P < 0.01$) vs the periportal zone of the corresponding group.

† Significantly different ($P < 0.01$) vs the corresponding zone of the control group.

Table 5. Effect of chronic ethanol feeding on the fatty acyl composition of phosphatidylcholine in periportal and perivenous mitochondria

Fatty acid	Control group			Ethanol-fed group		
	PP	PV	PP/PV	PP	PV	PP/PV
16:0	15.3 ± 1.6	21.1 ± 0.7*	0.73	19.7 ± 1.1‡	26.2 ± 1.3*‡	0.75
16:1n-7	1.7 ± 0.4	1.6 ± 0.5	1.06	1.9 ± 0.5	2.2 ± 0.7	0.86
16:1n-9	2.0 ± 0.2	2.2 ± 0.6	0.91	2.3 ± 0.5	2.5 ± 0.4	0.92
18:0	18.6 ± 1.3	18.5 ± 0.5	1.01	20.3 ± 1.6†	21.7 ± 1.5†	0.94
18:1n-7	3.2 ± 0.5	3.8 ± 0.6	0.84	4.1 ± 0.6	4.2 ± 0.5	0.98
18:1n-9	5.8 ± 0.9	7.2 ± 0.9	0.81	7.9 ± 1.2	7.0 ± 0.9	1.13
18:2n-6	15.3 ± 1.2	14.1 ± 0.9	1.17	13.5 ± 1.1	12.2 ± 1.2	1.14
20:4n-6	29.0 ± 1.8	23.4 ± 1.3*	1.24	23.3 ± 1.0‡	17.5 ± 0.9*‡	1.33
22:5n-6	1.7 ± 0.6	1.5 ± 0.4	1.13	1.5 ± 0.3	1.3 ± 0.2	1.15
22:6n-3	3.5 ± 0.7	3.6 ± 0.9	0.97	3.1 ± 0.5	3.4 ± 0.2	0.91
Others	2.9 ± 0.4	3.0 ± 0.3	—	2.4 ± 0.6	3.4 ± 0.5	—

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. Results are the means ± SD of four animals of each type.

* Significantly different ($P < 0.01$) vs the periportal zone of the corresponding group.

† Significantly different ($P < 0.05$) vs the corresponding zone of the control group.

‡ Significantly different ($P < 0.01$) vs the corresponding zone of the control group.

Table 6. Effect of chronic ethanol feeding on the fatty acyl composition of phosphatidylethanolamine in periportal and perivenous mitochondria

	Control group			Ethanol-fed group		
Fatty acid	PP	PV	PP/PV	PP	PV	PP/PV
16:0	12.5 ± 0.7	17.7 ± 0.9*	0.71	17.0 ± 0.6‡	22.7 ± 1.5*‡	0.75
16:1n-7	0.9 ± 0.2	1.1 ± 0.3	0.82	1.2 ± 0.3	1.4 ± 0.4	0.86
16:1n-9	1.3 ± 0.2	1.6 ± 0.4	0.81	1.8 ± 0.4	1.7 ± 0.2	1.06
18:0	21.8 ± 1.1	22.9 ± 1.2	0.95	24.3 ± 0.7†	25.4 ± 1.8	0.96
18:1n-7	4.1 ± 0.6	3.9 ± 0.3	1.05	4.2 ± 0.5	4.3 ± 0.6	0.98
18:1n-9	6.9 ± 0.6	7.0 ± 1.5	0.99	6.6 ± 0.5	6.4 ± 0.8	1.03
18:2n-6	10.6 ± 1.1	9.6 ± 0.7	1.10	8.3 ± 1.4	7.5 ± 0.8	1.11
20:4n-6	26.3 ± 1.5	20.5 ± 1.1*	1.28	21.0 ± 1.3‡	15.6 ± 0.6*‡	1.35
22:5n-6	3.6 ± 0.8	4.0 ± 0.3	0.90	3.9 ± 0.5	4.3 ± 0.8	0.91
22:6n-3	7.9 ± 0.9	8.7 ± 1.6	0.91	8.0 ± 0.7	7.8 ± 1.1	1.03
Others	4.1 ± 0.6	3.0 ± 0.7	—	3.7 ± 0.5	2.9 ± 0.6	—

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. Results are the means ± SD of four animals of each type.

* Significantly different ($P < 0.01$) vs the periportal zone of the corresponding group.
† Significantly different ($P < 0.05$) vs the corresponding zone of the control group.
‡ Significantly different ($P < 0.01$) vs the corresponding zone of the control group.

Table 7. Effect of chronic ethanol feeding on the fatty acyl composition of cardiolipin in periportal and perivenous mitochondria

	Control group			Ethanol-fed group		
Fatty acid	PP	PV	PP/PV	PP	PV	PP/PV
16:0	3.1 ± 0.6	6.5 ± 0.8†	0.48	8.2 ± 0.9§	10.4 ± 1.8§	0.79
16:1n-7	0.6 ± 0.3	0.8 ± 0.3	0.75	1.3 ± 0.4‡	1.1 ± 0.4	1.18
16:1n-9	0.7 ± 0.2	1.0 ± 0.3	0.70	1.8 ± 0.6‡	1.3 ± 0.3	1.38
18:0	2.3 ± 0.6	4.0 ± 0.5*	0.58	8.2 ± 0.9§	11.6 ± 1.9§	0.71
18:1n-7	4.7 ± 0.9	6.7 ± 1.1	0.70	9.8 ± 1.3§	11.5 ± 1.4§	0.85
18:1n-9	8.8 ± 0.9	11.7 ± 1.6*	0.75	18.1 ± 2.3§	21.7 ± 2.8§	0.83
18:2n-6	75.2 ± 6.6	62.4 ± 5.1*	1.20	47.3 ± 3.8§	37.9 ± 5.6*§	1.25
20:4n-6	1.6 ± 0.5	2.3 ± 0.7	0.70	1.5 ± 0.3	1.3 ± 0.4‡	1.15
22:5n-6	0.8 ± 0.2	1.2 ± 0.4	0.67	1.1 ± 0.3	0.9 ± 0.2	1.22
22:6n-3	1.0 ± 0.4	1.5 ± 0.3	0.67	0.7 ± 0.3	0.6 ± 0.1	1.17
Others	1.2 ± 0.3	1.8 ± 0.4	—	2.0 ± 0.5	0.6 ± 0.2	—

Mitochondria were obtained from periportal and perivenous hepatocytes isolated from rats chronically fed the control or the ethanol-containing diet. Results are the means ± SD of four animals of each type.

* Significantly different ($P < 0.05$) vs the periportal zone of the corresponding group.
† Significantly different ($P < 0.01$) vs the periportal zone of the corresponding group.
‡ Significantly different ($P < 0.05$) vs the corresponding zone of the control group.
§ Significantly different ($P < 0.01$) vs the corresponding zone of the control group.

of the rest of the chemicals and solvents have been described before [6, 25].

RESULTS

Rats were chronically fed a high-fat diet containing either 36% of total calories as ethanol (ethanol group) or an isocaloric amount of carbohydrate (control group). Upon prolonged ethanol administration, a rather selective accumulation of triacylglycerols was achieved in the perivenous zone of the liver (Table 1). Since CPT-I has been suggested to play an important role in the generation of ethanol-induced fatty liver [5, 6], mitochondria were isolated from periportal and perivenous hepatocytes for studying the properties of the CPT-I enzyme and its membrane lipid environment.

Effects of ethanol feeding on CPT-I

In control animals, CPT-I was more active and less sensitive to inhibition by malonyl-CoA in periportal than in perivenous hepatocytes (Table 1). Chronic ethanol feeding uniquely reduced enzyme activity in periportal cells, whereas no significant effect was observed on perivenous enzyme activity. In addition, prolonged ethanol administration

increased enzyme sensitivity to inhibition by malonyl-CoA in the two zones of the liver (Table 1).

A first approach to study the dependence of CPT-I activity with membrane environment was to monitor the effect of changing membrane temperature on CPT-I activity. For all mitochondrial preparations, Arrhenius plots may be resolved in two-segment curves, with heat-inactivation occurring above 40° (Fig. 1). In control animals, the energy of activation of CPT-I was higher in perivenous than in periportal hepatocytes. Prolonged ethanol administration increased the energy of activation of the enzyme in the two zones of the liver, but no zonal differences were observed for this effect of ethanol feeding (Fig. 1 and its legend).

Effects of ethanol feeding on membrane fluidity

The differences in the energy of activation of CPT-I between either the periportal and the perivenous zone of the liver or the control and the ethanol-treated group are consistent with alterations in the membrane environment of the enzyme protein. To examine this further, we calculated the membrane order parameter. Membrane fluidity is reciprocally related with the order parameter and allows an indication of the physical state of membrane lipids [28]. Since CPT-I is a very hydrophobic protein [34], DPH was used as probe because it interacts with the deep, hydrophobic core of the lipid bilayer [27]. Mitochondrial membrane fluidity was higher in the periportal than in the perivenous zone of the liver (Table 2). Prolonged ethanol administration decreased membrane fluidity in a non-zone-selective manner, i.e. the decrease induced in the periportal region was similar to the reduction observed in the perivenous zone (Table 2).

Addition of a saturating dose (100 mM) of ethanol to preparations of mitochondria isolated from control animals markedly decreased the order parameter of the membrane, but this ethanol-induced increase of membrane disorder was similar in periportal than in perivenous mitochondria (Table 2). In contrast, when mitochondria isolated from ethanol-treated animals were exposed to the same concentration of ethanol, no change in the order parameter was evident (Table 2).

Effects of ethanol feeding on the phospholipid composition of mitochondria

No significant differences were observed between the two cell sub-populations or between the two animal groups with regard to mitochondrial properties such as phospholipid content (expressed as nmol phospholipid-P_i/mg protein), relative proportions of major phospholipid classes (phosphatidylcholine, phosphatidylethanolamine and cardiolipin) as well as molar ratio cholesterol/phospholipid (Table 3). In contrast, the fatty acyl composition of mitochondrial total phospholipids was different in the two liver zones and was markedly affected by prolonged ethanol administration (Table 4). In line with their higher fluidity, mitochondria from periportal hepatocytes showed a more unsaturated fatty acyl pattern, with a lower content of palmitic acid and a higher proportion of both linoleic and arachidonic acids (Table 4). Moreover, the fatty acyl composition of mitochondrial phospholipids from ethanol-treated animals presented a greater proportion of palmitic and stearic acids and a lower content of linoleic and arachidonic acids. These effects of ethanol were equally evident in both periportal and perivenous hepatocytes (Table 4).

To study these alterations in detail, we next determined the fatty acyl composition of the major mitochondrial phospholipid classes. As can be inferred from Table 5, phosphatidylcholine from periportal mitochondria was poorer in palmitic acid and richer in arachidonic acid as compared with phosphatidylcholine obtained from perivenous mitochondria. In addition, prolonged ethanol ingestion increased the proportion of palmitic acid and decreased that of arachidonic acid in mitochondrial phosphatidylcholine from the two zones of the liver (Table 5). A similar trend was observed with regard to the fatty acyl profile of phosphatidylethanolamine (Table 6).

Cardiolipin from periportal mitochondria showed a higher proportion of linoleic acid and a lower content of saturated and monoenoic fatty acyl chains as compared with cardiolipin from perivenous mitochondria (Table 7). After prolonged ethanol intake, both periportal and perivenous mitochondria suffered the same alterations upon the fatty acyl chain profile of cardiolipin, namely a strong decline in linoleic acid and a concomitant enrichment in saturated and monoenoic fatty acids (Table 7).

DISCUSSION

CPT-I seems to play a key role in the control of long-chain fatty acid oxidation [7–9]. The activity and the regulatory properties of hepatic CPT-I usually change in parallel both on the short [35–38] and on the long term [6–10] under situations in which the rate of fatty acid oxidation is altered. Thus, we have previously reported that acute [37, 38] and chronic ethanol administration [5, 6] decrease CPT-I activity and increase enzyme sensitivity to inhibition by malonyl-CoA. Surprisingly, although a rather selective accumulation of triacylglycerols ensued in the perivenous zone of the liver upon chronic ethanol ingestion, CPT-I activity was only depressed in periportal hepatocytes, remaining unchanged in perivenous hepatocytes. In contrast, enzyme sensitivity to inhibition by malonyl-CoA increased in the two liver zones. The specific accumulation of triacyglycerols in the perivenous zone of the liver upon prolonged ethanol intake may be closely related to the selective blockade of triacylglycerol secretion as very-low-density lipoproteins by this hepatocyte sub-population [39], whereas the maintenance of CPT-I activity in perivenous hepatocytes might serve as an adaptive mechanism aimed to prevent this selective perivenular accumulation of triacylglycerols. Anyway, the situation *in vivo* may be more complicated owing to the existence of gradients of hormones, substrates and oxygen [13, 14].

Chronic ethanol administration causes profound structural and functional alterations in cell and organelle membranes [17–19]. Thus, our data show that the rigidity of the mitochondrial membrane is

increased upon prolonged ethanol administration. This is in line with the increased energy of activation of CPT-I and the more saturated pattern of mitochondrial fatty acyl chains of phospholipids upon long-term ethanol ingestion (see below). Nevertheless, Waring *et al.* found no effect of chronic ethanol feeding on the order parameter of the mitochondrial membrane [40]. These authors also reported that chronic ethanol feeding induces in the mitochondrial membrane the development of a resistance to the disordering actions of ethanol *in vitro* [40]. Further, our results show that these acute and chronic effects of ethanol are symmetrical with regard to liver zone.

The increase in membrane rigidity upon long-term ethanol treatment agrees with the changes in the phospholipid fatty acyl composition towards a more saturated pattern. In this context, it should be pointed out that the mitochondrial envelope has a unique composition as compared with other cell and organelle membranes. For example, mitochondria contain very little membrane-bound cholesterol and a high membrane protein content; although the cholesterol/phospholipid and the phospholipid/protein ratios seem to be major factors determining membrane fluidity [28], this is not the case of the ethanol-induced alterations described herein. In addition, the phospholipid composition of the mitochondrial envelope is characteristic: although phosphatidylcholine and phosphatidylethanolamine are major membrane components, just like in other biomembranes, cardiolipin constitutes almost 10% of total membrane phospholipids. It is striking that the effects of chronic ethanol intake upon phospholipid fatty acyl composition are phospholipid-specific: on the one hand, phosphatidylcholine and phosphatidylethanolamine parallely suffer an enrichment in palmitic acid, declining their content in arachidonic acid; the latter may be a result of an increase in lipid peroxidation [17, 18, 41] or of a depression of the elongation–desaturation system(s) [17, 18]. On the other hand, the ethanol-induced decrease of linoleic acid content is a rather selective action on cardiolipin (see also Refs 40 and 42). This phospholipid is highly specific of mitochondria and displays an unusual abundance of C_{18}–C_{18} backbones, dilinoleoyl configuration being the major species [43]. The fact that the fatty acyl composition of cardiolipin is the most affected by long-term ethanol ingestion is of great interest since, although having lower abundance than phosphatidylcholine or phosphatidylethanolamine, cardiolipin seems to be preferentially accommodated in the solvatation shell of a number of mitochondrial membrane proteins [43]. In addition, the degree of saturation of the C_{18}–C_{18} diacylglycerol species of cardiolipin seems to be a critical structural factor determining the tight association of this phospholipid with membrane proteins [43]. Moreover, cardiolipin seems to be specifically involved in the development of membrane tolerance upon prolonged ethanol administration [44]. The idea of the pivotal role of cardiolipin for mitochondrial membrane properties is not unreasonable in view that small changes in membrane-constituent phospholipids have major effects on the membrane properties of mammalian erythrocytes [45]. It is thus very interesting that long-term ethanol intake alters both the fatty acyl composition of cardiolipin and the kinetic and regulatory properties of the CPT-I enzyme in liver mitochondria, whereas in skeletal muscle mitochondria there is no effect of chronic ethanol ingestion on the properties of either cardiolipin [44] or the CPT-I enzyme [5].

It has been reported that increased membrane fluidity is associated with increased CPT-I activity [20–22]. This is true not only for perturbations of membrane fluidity *in vitro* [21], but also for long-term alterations such as those occurring in starvation and diabetes, in which the mitochondrial membrane is less rigid [20]. More recently, Kolodziej and Zammit have observed a very close relation between increased membrane fluidity and decreased enzyme sensitivity to inhibition by malonyl-CoA [46]. Thus, in our control animals a similar relation exists when comparing the periportal with the perivenous zone of the liver: increased CPT-I activity and decreased enzyme sensitivity to inhibition by malonyl-CoA correlate well with decreased energy of activation of the enzyme, increased membrane fluidity and less saturated pattern of membrane phospholipid fatty acyl composition. Nevertheless, this correlation partially disappears upon prolonged ethanol feeding, since, with regard to liver zone, this kind of treatment symmetrically alters the properties of the membrane environment (fluidity, tolerance and composition) and the regulatory properties of CPT-I (energy of activation, sensitivity to inhibition by malonyl-CoA) but asymmetrically affects CPT-I activity. Therefore, after long-term ethanol ingestion a zone selective dissociation not only ensues between the kinetic and the regulatory properties of CPT-I (see earlier), but also between the kinetic properties of the enzyme on the one hand and the membrane environment and the regulatory properties of the enzyme on the other hand. It is thus very likely that the effects of ethanol feeding on CPT-I are not exclusively mediated via changes in the properties of the enzyme membrane environment, but also by other mechanisms such as changes in the levels of enzyme protein. In fact, a number of alterations in the nutritional and hormonal status of the animal modify the rates of synthesis and/or degradation of the hepatic CPT protein [47, 48]. Moreover, a number of drugs such as lovastatin [49] and anabolic steroids [50] increase CPT-I activity on the long-term without changing the regulatory properties of the enzyme.

In conclusion, the present data suggest that the alterations in the fluidity and composition of the mitochondrial membrane are not the only factor responsible for the zone-selective effects of prolonged ethanol ingestion upon the properties of CPT-I. Nevertheless, changes in the protein lipid microenvironment may ultimately be the more significant, and the use of a probe—such as DPH—or the determination of membrane phospholipid composition only allows the evaluation of gross changes in the membrane environment, whereas changes in microenvironments are not detected. In any case, our results raise the possibility that the control of the CPT-I enzyme (and thereby of other

proteins) is mediated via different mechanisms in the periportal and the perivenous zone of the liver.

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