Expression of liver carnitine palmitoyltransferase I and II genes during development in the rat

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The enzyme activity and the expression (protein and mRNA concentrations) of genes encoding for hepatic carnitine palmitoyltransferases (CPT) I and II were studied during neonatal development, in response to nutritional state at weaning and during the fed–starved transition in adult rats. The activity, the protein concentration and the level of mRNA encoding CPT I are low in foetal-rat liver and increase 5-fold during the first day of extra-uterine life. The activity and gene expression of CPT I are high during the entire suckling period, in the liver of 30-day-old

rats weaned at 20 days on to a high-fat diet and in the liver of 48 h-starved adult rats. The activity and CPT I gene expression are markedly decreased in the liver of rats weaned on to a high-carbohydrate diet. By contrast, the activity, the protein concentration and the level of mRNA encoding CPT II are already high in the liver of term foetuses and remain at this level throughout the suckling period, irrespective of the nutritional state of the animals either at weaning or in the adult.

INTRODUCTION

At birth the diet of the neonatal rat has a high fat and low carbohydrate content (see Girard et al., 1992). To meet the energy needs of the newborn, fatty acid oxidation develops rapidly after birth in many peripheral tissues and in the liver, where it results in a high rate of ketone-body production (see Girard et al., 1992).

The postnatal development of hepatic long-chain fatty acid (LCFA) oxidation seems to be closely related to the emergence of the carnitine palmitoyltransferase (CPT) system, which controls the entry of LCFA into the mitochondria [reviewed by McGarry and Foster (1980) and McGarry et al. (1989)]. The CPT system is composed of three distinct activities: overt CPT (CPT I), carnitine–acylcarnitine translocase and latent CPT (CPT II). It is generally accepted that CPT I represents the main locus of control for LCFA oxidation (reviewed by McGarry et al., 1989). Three mechanisms of regulation have been described: (1) changes in maximal activity of the enzyme; (2) variations in the concentration of malonyl-CoA, a lipogenic intermediate and a potent inhibitor of CPT I; (3) modifications in the sensitivity of CPT I to malonyl-CoA inhibition [reviewed by Zammit (1984) and McGarry et al. (1989)]. It has been shown that during the foetal–neonatal transition the activity of hepatic CPT I is increased, whereas the malonyl-CoA concentration and the sensitivity of CPT I to malonyl-CoA inhibition are markedly decreased (Girard et al., 1992). Conversely, at the time of weaning on to the high-carbohydrate diet, all of these parameters are reversed, in keeping with an overall decrease in the rate of hepatic LCFA oxidation (Girard et al., 1992). Although the mechanisms that govern lipogenic activity and malonyl-CoA concentration have been documented (see Iritani, 1992), those controlling the changes in CPT I activity and sensitivity to malonyl-CoA inhibition remain unknown. The recent cloning of

a cDNA encoding CPT I (Esser et al., 1993b) and the availability of antibodies directed against CPT I (Kolodziej et al., 1992; Esser et al., 1993a) allow these questions to be addressed.

The data concerning the expression of CPT II are more controversial. It has been reported that CPT II gene expression is increased by 3–4-fold in the liver of starved and diabetic rats (Brady and Brady, 1987, 1989; Brady et al., 1988) or in cyclic-AMP-treated hepatoma cells (Wang et al., 1989). However, neither the activity of CPT II nor the rate of LCFA oxidation was determined in those experiments. Other studies suggest that the increase in CPT II expression in the liver of starved or diabetic rats is much smaller (about 40%) than that observed for CPT I (Kolodziej et al., 1992). Moreover, the addition of cyclic AMP to hepatoma cells does not increase CPT II activity and protein level (Prip-Buus et al., 1992).

The aim of the present work was to investigate the changes in the expression of CPT I and CPT II (mRNA and protein levels) in the rat liver during the perinatal period and the suckling–weaning transition.

MATERIALS AND METHODS

Animals

Female Wistar rats bred in our laboratory were housed at 24 °C in individual plastic cages with light from 15:00 to 03:00 h in order to sample the liver between 09:00 and 10:00 h, i.e. during the absorptive period (fed state).

Immediately after birth, the litter size was standardized to 10 newborn rats that were allowed to suckle. The suckling newborns were weaned at 20 days to a semi-synthetic high-carbohydrate low-fat diet (HC: 72% carbohydrate, 1% fat, 27% protein, in terms of energy) or a high-fat carbohydrate-free diet (HF: < 1% carbohydrate, 72% fat, 28% protein, in terms of energy) as described previously (Coupé et al., 1990).

The studies were performed in 19- and 21-day foetal rats, in 1- and 14-day-old suckling rats and in 30-day-old weaned rats. Adult rats fed on laboratory chow pellets (65% carbohydrate, 11% fat, 24% protein, in terms of energy) or starved for 48 h were used as controls. The livers were used immediately for the isolation of mitochondria, or were frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

Isolation of mitochondria and Western-blot analysis of CPT I and CPT II

Liver mitochondria were isolated by the differential-centrifugation technique previously described (Herbin et al., 1987), followed by a purification on a Percoll gradient as described by Reinhart et al. (1982). Mitochondria were used for the assay of CPT I and CPT II activities and for quantification of the immunoreactive proteins by Western-blot analysis. Before immunoblotting, mitochondrial proteins were solubilized (5 mM Tris/HCl, 150 mM KCl, 1% octyl glucoside) as described by Woeltje et al. (1987). After centrifugation (1 h at 100 000 g), the solubilized proteins were submitted to SDS/PAGE (8.5% acrylamide) as described by Laemmli (1970). The separated proteins were transferred on to a reinforced nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and the blots were incubated with the anti-CPT I antiserum (1/100, v/v; Kolodziej et al., 1992; Esser et al., 1993a). After revelation of immune complexes (see below), the nitrocellulose membranes were dehybridized (5 min in 0.2% SDS/50 mM glycine/24 mM HCl, pH 2.6). Then the membranes were incubated with the anti-CPT II antiserum (1/100, v/v; Woeltje et al., 1987). Immune complexes were quantified by using ^{125}I -Protein A (0.5 $\mu\text{Ci}/\text{ml}$ for 1 h). Quantifications were performed by scanning densitometry of the autoradiographs.

Extraction and Northern-blot analysis of total RNA

Total RNA was extracted with guanidinium thiocyanate and purified by pelleting through a CsCl cushion as described by Chirgwin et al. (1979). The concentration of RNA was measured by the A_{260} . Northern-blot analyses of total RNA (20 μg) were performed after 1%-agarose-gel electrophoresis in 2.2 M formaldehyde as previously described (Pégorier et al., 1992). After transfer to nylon membranes (Hybond N; Amersham International, Amersham, Bucks., U.K.), the filters were hybridized overnight at 65°C in the absence of formamide with the CPT I (Esser et al., 1993b) and CPT II (Woeltje et al., 1990) cDNA probes labelled with [^{32}P]dATP by using the Multiprime labelling system kit (Amersham). For Northern blotting, the following fragments were used: for CPT I an *Eco*R1–*Eco*R1 fragment from a subclone of p61a (Esser et al., 1993b), obtained by engineering an *Eco*R1 site through PCR 5' to the first ATG codon (Met-1) and for CPT II a *Sma*I–*Bam*HI fragment from the pBKS-CPT II.4 construct (Woeltje et al., 1990). Quantifications were performed by scanning densitometry of the autoradiographs. The transferred RNAs were hybridized with an oligonucleotide specific for 18 S RNA (Chan et al., 1984) labelled with [^{32}P]ATP to monitor the amount of total RNA.

Measurements of mitochondrial CPT I and CPT II activities

CPT I activity was assayed as described previously (Herbin et al., 1987). In all the experiments ($n = 35$), the formation of palmitoyl[^3H]carnitine from palmitoyl-CoA (80 μM) and L-[methyl-

^3H]carnitine (1 mM, 1.6 Ci/mol) was almost completely suppressed ($98 \pm 1\%$) by a high concentration of malonyl-CoA (100 μM), suggesting that only CPT I activity was measured without any significant contribution of CPT II.

CPT II activity was assayed in solubilized mitochondrial extracts (1% octyl glucoside) as described for CPT I, except that the concentration of L-[methyl- ^3H]carnitine was 2.5 mM (0.8 Ci/mol). The treatment of mitochondria with 1% octyl glucoside leads to a total inactivation of CPT I activity (results not shown), as previously reported by Declercq et al. (1987).

Analytical methods

Proteins were determined by the method of Lowry et al. (1951), with BSA as standard.

Chemicals

Fatty-acid-free BSA, L-carnitine, malonyl-CoA, palmitoyl-CoA and reagents for Northern- and Western-blot analysis were purchased from Sigma (St. Louis, MO, U.S.A.). L-[methyl- ^3H]Carnitine was obtained from Amersham International.

Statistics

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed by the Wilcoxon (1947) rank-order test.

RESULTS

Developmental changes in liver CPT I and CPT II activities

The activity of CPT I is low in foetal-rat liver (Table 1). It increases 5-fold during the first day of extra-uterine life and remains at a high level throughout the suckling period (Table 1). After weaning, the CPT I activity is 60–70% lower in the liver of rats weaned on to a HC diet than in rats weaned on to the HF diet (Table 1). As previously reported, the activity of CPT I is 2.5-fold higher in 48 h-starved adult-rat liver than in fed ones (Table 1). By contrast, the activity of CPT II is not significantly modified either during neonatal development or in response to nutritional state of the animals (Table 1).

Table 1 Changes in hepatic CPT I and CPT II activities during development and in response to nutritional environment

The activity of CPT I was measured in freshly isolated liver mitochondria. The activity of CPT II was assayed on mitochondrial proteins solubilized with 1% octyl glucoside. Rats were weaned at 20 days on to a high-carbohydrate diet (HC) or a high-fat diet (HF) and were studied at 30 days. Results are means $\pm$ S.E.M. of 4–6 different animals: * $P < 0.01$ compared with 21-day foetuses; † $P < 0.01$ compared with rats weaned on to the HC diet; ‡ $P < 0.01$ compared with fed adult rats.

Liver source	CPT activity (nmol/min/mg mitochondrial prot.)	
	CPT I	CPT II
21-day foetuses	0.75 $\pm$ 0.17	4.2 $\pm$ 0.3
1-day-old suckling newborns	3.4 $\pm$ 0.4*	4.5 $\pm$ 0.5
14-day-old suckling newborns	3.0 $\pm$ 0.2*	4.6 $\pm$ 0.5
30-day-old HC-weaned rats	1.7 $\pm$ 0.1	5.1 $\pm$ 0.6
30-day-old HF-weaned rats	2.4 $\pm$ 0.2†	6.3 $\pm$ 0.7
Fed adult rats	1.2 $\pm$ 0.4	4.8 $\pm$ 0.5
48 h-starved adult rats	2.7 $\pm$ 0.6‡	6.7 $\pm$ 0.7

Figure 1 Developmental changes in the concentration of liver immunoreactive CPT I and CPT II proteins

Solubilized mitochondrial proteins (70 μ g) from rat liver were size fractionated and blotted with an anti-CPT I antiserum (a) as described in the Materials and methods section. After de-hybridization of the nitrocellulose membrane, it was blotted with the anti-CPT II antiserum (b). Rats were weaned at 20 days on to a high-carbohydrate (HC) or a high-fat (HF) diets and were studied at 30 days. Adult rats were either fed (F) or starved (S) for 48 h. This blot is representative of the five different experiments quantified in Table 2.

To determine whether this developmental pattern is associated with corresponding changes in the expression of the genes encoding for these enzymes, the amount of immunoreactive proteins and the level of related mRNA were determined in each of these experimental conditions.

Developmental changes in liver CPT I and CPT II gene expression

Western blots were probed with the anti-CPT I antiserum produced either by Kolodziej et al. (1992) or by Esser et al. (1993a). Irrespective of the anti-CPT I antiserum used, the developmental pattern was the same (results not shown). As shown on Figure 1, the CPT I immunoreactive protein is undetectable in the liver of 19-day foetuses, whereas the level of CPT II is already high. The changes in the concentration of immunoreactive CPT I and CPT II are similar to those observed for maximal enzyme activities. Densitometric quantification of Western blots (Table 2) shows that the concentration of CPT I protein is increased 9-fold at birth and remains elevated in situations associated with high rates of LCFA oxidation, i.e.

Figure 2 Developmental changes in mitochondrial CPT I and CPT II mRNA levels in rat liver

Total RNA was extracted from the liver and analysed by the Northern-blot technique as described in the Materials and methods section. For details see the legend of Figure 1. This blot is representative of the five different experiments quantified in Table 2.

suckling period, weaning on to HF diet and 48 h starvation. A 60 % decrease in the concentration of CPT I protein occurs in the liver of rats weaned on to the HC diet as compared with rats weaned on to the HF diet (Table 2). Nevertheless, the level of hepatic mitochondrial CPT I of rats weaned on to the HC diet remains 2-fold higher than in liver mitochondria of fed adult rats (Table 2). In agreement with the data on CPT II activity, the level of immunoreactive CPT II protein was not significantly modified either during development or in response to nutritional state (Figure 1, Table 2), although in the latter instance CPT II activities tended to be higher by about 40 %.

The developmental pattern of CPT I and CPT II mRNA concentrations was closely related to the changes in the concentration of immunoreactive proteins. CPT I mRNA is

Table 2 Changes in the expression of hepatic CPT I and CPT II genes during development and in response to nutritional environment

The levels of hepatic CPT I and CPT II mRNA and the concentration of immunoreactive proteins were determined from densitometric analysis of five different Northern and Western blots. For the experimental design see the legend of Figure 1. Results are expressed as arbitrary units, the reference value (1) being the level of mRNA and the concentration of protein found in the liver of fed adult rats. Rats were weaned at 20 days on to either a high-carbohydrate diet (HC) or a high-fat diet (HF). Results are means $\pm$ S.E.M. of 4–6 different animals: * P < 0.01 compared with 21-day foetuses; † P < 0.01 compared with rats weaned on to the HC diet; ‡ P < 0.01 compared with fed adult rats.

Liver source	Relative level of CPT I (arbitrary units)		Relative level of CPT II (arbitrary units)	
	mRNA	Protein	mRNA	Protein
21-day foetuses	0.8 $\pm$ 0.2	0.5 $\pm$ 0.1	1.2 $\pm$ 0.1	0.9 $\pm$ 0.1
1-day-old suckling newborns	5.6 $\pm$ 0.5*	4.5 $\pm$ 0.5*	1.4 $\pm$ 0.2	1.3 $\pm$ 0.1
14-day-old suckling newborns	3.2 $\pm$ 0.5*	3.9 $\pm$ 0.4*	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2
30-day-old HC-weaned rats	1.4 $\pm$ 0.1	1.9 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
30-day-old HF-weaned rats	2.9 $\pm$ 0.3†	3.5 $\pm$ 0.4†	1.2 $\pm$ 0.1	1.4 $\pm$ 0.2
Fed adult rats	1	1	1	1
48 h-starved adult rats	6.3 $\pm$ 0.7‡	3.7 $\pm$ 0.5‡	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1

undetectable in the liver of 19-day fetuses, whereas the level of CPT II mRNA is already high (Figure 2). The concentration of CPT I mRNA increased 7-fold at birth, and remained elevated during the suckling period. Subsequently it fell by 60–70 % in the liver of rats weaned on to the HC diet (Figure 2, Table 2). The levels of CPT I mRNA were 2-fold higher in the liver of rats weaned on to the HF diet compared with that in liver of rats weaned on to the HC diet (Figure 2, Table 2). In the liver of 48 h-starved adult rats the induction of CPT I mRNA was 6-fold with respect to that in animals fed *ad libitum* (Figure 2, Table 2).

The concentration of CPT II mRNA remained remarkably constant whatever the stage of development or the nutritional status of the animals (Figure 2, Table 2).

DISCUSSION

The present work provides the first evidence that the postnatal rise in CPT I activity is due to the induction of the gene encoding for this enzyme, as suggested by the rapid accumulation of CPT I mRNA after birth. The fact that hepatic CPT I mRNA can be maintained at a high level in rats weaned on to a HF diet, whereas it decreases when rats are weaned on to a HC diet, suggests that these changes are dependent on hormonal and/or nutritional factors rather than on the precise stage of development. The high plasma glucagon and the low plasma insulin levels that prevail immediately after birth (Girard et al., 1973), during the suckling period (Girard et al., 1977), in rats weaned on to a HF diet (Decaux et al., 1986) or in starved adult rats (Balks and Jungermann, 1984) could be responsible for the induction and/or the maintenance of a high hepatic CPT I activity and mRNA levels. Although the role of pancreatic hormones in the regulation of CPT I gene expression remains to be elucidated, they appear to exert long-term effects on CPT I activity. Hence, neither insulin nor glucagon is able to exert short-term control on CPT I activity either *in vivo* (Peñas and Benito, 1986; Grantham and Zammit, 1988; Penicaud et al., 1991) or *in vitro* (Boon and Zammit, 1988; Guzman and Geelen, 1988). Although activation of CPT I by agents that elevate cyclic AMP and/or inhibit protein phosphatases 1 and 2A has been reported (Guzman and Geelen, 1992), this has now been shown to have been due to the way in which cells were permeabilized and CPT activity was assayed (M. Guzman and V. A. Zammit, unpublished work). By contrast, long-term treatment with insulin is able to restore a normal CPT I activity in the liver of starved (Penicaud et al., 1991) or diabetic (Grantham and Zammit, 1988) adult rats. Whether this long-term effect of insulin results from changes in CPT I gene expression remains to be elucidated. However, indirect evidence for an effect of pancreatic hormones on liver CPT I activity has been obtained from experiments in post-mature fetuses. This experimental condition induces *in utero* a fall in the insulin/glucagon molar ratio (Portha et al., 1976), to a level very close to that observed immediately after birth (Girard et al., 1973), and a marked rise in the activity of CPT I in the liver of post-mature fetuses (Chalk et al., 1983; Herbin et al., 1988).

It is possible that CPT I gene expression could be regulated by circulating substrates such as glucose and/or non-esterified fatty acids. It is noteworthy that in situations where CPT I gene expression is markedly stimulated (suckling period, weaning on to HF diet, starvation) the concentration of plasma non-esterified fatty acids is high, whereas the glucose availability is decreased. Conversely, when rats are weaned on to a HC diet CPT I expression is markedly depressed and remains so in the fed adult rat liver. Thus glucose or an intermediate arising from its

metabolism could decrease CPT I gene expression and/or enhance the rate of mRNA degradation. Such a down-regulation of mRNA levels by glucose concentration has been previously reported for GLUT-1 (Wertheimer et al., 1991) and the glucose-regulated protein family (Shiu et al., 1977). Conversely, during the suckling period or after weaning on to a HF diet, the absorption of large amounts of fat could promote the transcription and/or the stabilization of hepatic CPT I mRNA. Indeed, fatty acids have been shown to stimulate the expression of hepatic fatty acid-binding protein (L-FABP; Dempsey et al., 1985), adipose-cell FABP (aP2; Amri et al., 1991) and peroxisomal acyl-CoA oxidase (Keller et al., 1993).

Finally, this work also demonstrates that CPT II gene expression is not markedly affected during development and in response to changes in the nutritional state of adults or weaned animals. These results are not consistent with previously published observations that have reported a 3–4-fold increase in CPT II gene expression during starvation, diabetes (Brady and Brady 1989; Brady et al., 1988) and after cyclic AMP treatment of hepatoma cells (Wang et al., 1989). The possible explanation for such discrepancies has been given by McGarry and co-workers (Woeltje et al., 1990; Brown et al., 1991, 1993; Weis et al., 1993), where the authenticity of the CPT II clones used by Brady's group has been questioned. Using the CPT II cDNA cloned by Woeltje et al. (1990), we find changes in mRNA concentration that are consistent with previous observations concerning CPT II protein level and activity. Firstly, it has been shown that the concentration of immunoreactive CPT II was similar in term-foetal and adult rat liver (Kolodziej et al., 1992). Secondly, the activity of CPT II is not modified during the foetal-neonatal transition (Saggerson and Carpenter, 1982) and only moderately during the fed-starved transition in the adult-rat liver (Saggerson et al., 1984; Grantham and Zammit, 1986). Moreover, there is no change in the concentration of CPT II protein [measured by using an anti-(human CPT II) antiserum] in cyclic-AMP-treated hepatoma cells (Prip-Buus et al., 1992).

In conclusion, the marked influence of developmental stage and nutritional status on CPT I gene expression reported here provides additional evidence in support of the key regulatory role of this enzyme in the control of hepatic LCFA oxidation.

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