Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation

Inter-tissue and inter-species expression of CPT I and CPT II enzymes

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The outer mitochondrial membrane enzyme carnitine palmitoyltransferase I (CPT I) represents the initial and regulated step in the β-oxidation of fatty acids. It exists in at least two isoforms, denoted L (liver) and M (muscle) types, with very different kinetic properties and sensitivities to malonyl-CoA. Here we have examined the relative expression of the CPT I isoforms in two different models of adipocyte differentiation and in a number of rat tissues. Adipocytes from mice, hamsters and humans were also evaluated. Primary monolayer cultures of undifferentiated rat preadipocytes expressed solely L-CPT I, but significant levels of M-CPT I emerged after only 3 days of differentiation *in vitro*; in the mature cell M-CPT I predominated. In sharp contrast, the murine 3T3-L1 preadipocyte expressed essentially exclusively L-CPT I, both in the undifferentiated state and throughout the differentiation process *in vitro*. This was also true of the mature mouse white fat cell. Fully developed adipocytes from the hamster and human behaved similarly to those of the rat. Thus the mouse white fat cell differs fundamentally from those of the other species examined in terms of its choice of a key regulatory enzyme in fatty acid metabolism. In contrast, brown adipose tissue from all three rodents displayed the same isoform profiles, each expressing overwhelmingly M-CPT I. Northern blot analysis of other rat tissues established L-CPT I as the dominant isoform not only in liver but also in kidney, lung, ovary, spleen, brain, intestine and pancreatic islets. In addition to its primacy in skeletal muscle, heart and fat, M-CPT I was also found to dominate in the testis. The same inter-tissue isoform pattern (with the exception of white fat) was found in the mouse. Taken together, the data bring to light an intriguing divergence between white adipocytes of the mouse and other mammalian species. They also raise a cautionary note that should be considered in the choice of animal model used in further studies of fat cell physiology.

INTRODUCTION

The carnitine palmitoyltransferase (CPT) enzyme system operates body-wide to permit the entry of long-chain fatty acids into the mitochondrial matrix, where they can undergo β-oxidation as a source of energy or, in the liver, provide substrates for ketone body synthesis [1]. The first component of the system, CPT I, located on the mitochondrial outer membrane, effects the transesterification of a fatty acyl group from CoA to carnitine. The acylcarnitine product is able to traverse the inner mitochondrial membrane by means of a specific carnitine/acylcarnitine translocase. The transesterification is then reversed to regenerate fatty acyl-CoA by the action of a distinct enzyme denoted CPT II, loosely associated with the matrix face of the inner membrane.

The regulation of fatty acid flux through the CPT system is believed to be exerted at the level of CPT I. This enzyme is uniquely inhibited by malonyl-CoA, the product of acetyl-CoA carboxylase and the first committed intermediate in *de novo* fatty acid synthesis. Hence, in the liver, during periods where the insulin-to-glucagon ratio is high and fatty acid synthesis is active, malonyl-CoA levels rise and inhibit CPT I, thereby blocking β-oxidation and avoiding a futile cycle [1–3]. Conversely, in the fasted state, the malonyl-CoA concentration decreases, inhibition of CPT I is relieved and fatty acid oxidation and ketogenesis can proceed. It is now clear that this regulatory mechanism also operates in non-hepatic tissues. Even in tissues that do not perform significant fatty acid synthesis, malonyl-CoA is present and its concentration is seen to change with physiological state [4–6], as in the rat liver. Production of malonyl-CoA by acetyl-CoA carboxylase in these cells seems therefore to fulfil a purely regulatory function.

Although in broad operational terms the CPT system has long been recognized as a key element in fuel homoeostasis, only recently has detailed understanding of its structure/function/regulatory aspects begun to emerge. Current understanding can be summarized as follows. CPT II is expressed as a single gene product body-wide [7,8] and cDNA species have been isolated for the rat [9] and human [10] enzymes. In contrast, CPT I is known to exist in at least two isoforms. CPT I in rat liver mitochondria (L-CPT I) has a $K_m^{carnitine}$ of approx. 30 μM compared with approx. 500 μM for the skeletal muscle enzyme

Abbreviations used: CPT I, CPT II, outer- and inner- membrane forms of mitochondrial carnitine palmitoyltransferase respectively; I_{max}, maximum percentage inhibition achieved; L-CPT I and M-CPT I, liver-type and muscle-type isoforms of CPT I respectively.

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(M-CPT I), whereas the IC_{50} for inhibition by malonyl-CoA is approx. 3 μM for the liver and approx. 0.03 μM for the muscle variant [4]. The sensitivity of hepatic CPT I to malonyl-CoA varies somewhat with physiological state, whereas this phenomenon is not observed with the muscle-type enzyme [11]. Rat and human cDNA species encoding L- and M-CPT I have also been isolated [12–17], and expression of the rat CPT I proteins in COS cells has confirmed that the markedly different kinetic properties of the two enzymes are intrinsic characteristics and not a consequence of the tissue-specific environment [15].

It is now clear that L- and M-CPT I can each be found in a variety of tissue and cell types. L-CPT I is the sole isoform not only in liver, but also in human fibroblasts [13]; it is expressed in intestine [18] and at minor levels in rat heart [19] and white adipocytes [15]. M-CPT I, which is essentially the sole isoform in skeletal muscle, is also the predominant species in rat heart [20] and, interestingly, in both brown and white adipocytes [15]. In addition, the relative expression of CPT I isoforms has been shown to change in the heart during development of the rat [21], with carnitine feeding of a carnitine-deficient mouse [22] or after electrical stimulation of cultured neonatal rat cardiac myocytes [23].

The apparent plasticity of the pattern of isoform expression in the heart raised the possibility that other tissues might also display altered CPT I expression patterns in different physiological or developmental states. In particular, we suspected that a switching of isoforms might occur during the differentiation of fibroblastoid preadipocytes (presumably expressing L-CPT I) to mature adipocytes (which express M-CPT I in the rat). Accordingly, we have examined the expression of L- and M-CPT I isoforms during the differentiation of mouse 3T3-L1 cells and of primary rat preadipocytes, as well as in mature adipocytes from several species including, critically, human. In addition, we have performed a detailed analysis of CPT mRNA expression in a variety of rat and mouse organs. The results indicate that there is indeed a marked change in the expression of L- and M-CPT I during differentiation of the rat white preadipocyte. However, they also show that the balance between the two isoforms in any given tissue is highly unpredictable. Most importantly, it turns out that the mouse white adipocyte differs fundamentally from those of the other species examined. This has implications for the choice of animal model where fatty acid metabolism and its relationship to adiposity are to be studied.

EXPERIMENTAL

Materials

Liposyn III was a gift from Abbott Laboratories (North Chicago, IL, U.S.A.). Nuserum was from Collaborative Biomedical (Bedford, MA, U.S.A.). Other tissue culture reagents and TRIzol Reagent were from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.). 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

Animals

Tissues for RNA preparation were taken from: Sprague–Dawley rats at 6 and 12 weeks of age (approx. 125 and 350 g respectively); NIH Swiss mice at approx. 8 weeks of age (approx. 30 g) or 9-month-old 'retired breeders'; and golden Syrian hamsters at approx. 8 weeks of age (approx. 140 g). All of the above animals were male (except the donors of ovaries and periovarian fat) and were maintained on a 4 % fat chow diet (Teklad) with a 12 h/12 h light/dark cycle. They were killed during the first 4 h of the light period.

Fischer 344 rats (approx. 12 weeks of age, 220–250 g) were used for the preparation of primary preadipocytes. These animals were barrier-reared and fed with a sterile NIH 31 diet and sterile water.

Animals were from Harlan Sprague-Dawley, Indianapolis, IN, U.S.A.

Human tissues

Cardiac tissue was obtained from a 70-year-old male undergoing a heart transplant. A sample from the right ventricular wall was dissected largely free of non-muscle tissue, rapidly frozen in liquid nitrogen and stored at -70 °C until the preparation of RNA. Human subcutaneous fat was harvested from two individuals undergoing elective liposuction. The 'slurry', obtained through large-bore tubing, was kept cool and subjected to collagenase digestion within 45 min of collection. Adipocytes were purified as for the rodent cells. The example shown in Figure 5 was taken from the hip region of a 56-year-old female. The study of human tissue (otherwise to be discarded) was conducted in accordance with the declaration of Helsinki of the World Medical Association and with the approval of the University of Texas Southwestern Institutional Review Board. Informed consent was obtained.

RNA preparation

Animals were anaesthetized with Nembutal and cervically dislocated before the rapid removal of organs. Selected tissues were pooled from 2–16 animals depending on the species and RNA yield (e.g. rat liver and mouse adipocytes respectively). They were either coarsely chopped and homogenized immediately in guanidine thiocyanate or frozen and crushed in liquid nitrogen before homogenization. Whole organs were used for liver, lung, ovary, spleen, brown adipose tissue and heart. Separation of rat kidney cortex from medulla was achieved after bisection of the organ longitudinally. Approx. 5 cm of intestine closest to the stomach (small intestine) or the rectum (large intestine) were rinsed with ice-cold saline before homogenization. Testes were used after the outer membrane had been discarded. The cerebellum and midbrain were removed from the brain before homogenization of the remaining tissue. Skeletal muscle was harvested from the hindlimb. Mature white adipocytes were prepared by the method of Rodbell [24] from the epididymal fat pad (or, where stated, the periovarian or retroperitoneal fat pads). Rat pancreatic islets were isolated as described [25]. For RNA preparation from 3T3-L1 cells, the monolayers from 1–8 plates (150 mm) were washed and collected as described below, followed by immediate homogenization in guanidine thiocyanate. Preparation of RNA from each organ/cell type was then achieved by centrifugation through $CsCl_2$ [26].

Primary monolayer cultures of rat preadipocytes (before and after differentiaton *in vitro*) were lysed with a phenol/guanidine isothiocyanate reagent (TRIzol). RNA was isolated by extraction with chloroform, precipitation with isopropyl alcohol, washing with ethanol and resuspension in water [27].

Rat preadipocyte culture

Fischer 344 rats were decapitated under CO_2 analgesia before removal of the epididymal fat pad. The fat was minced and digested with collagenase (1 mg/ml in Hanks balanced salt solution). Stromal–vascular cells were isolated by centrifugation at 800 ***g*** for 10 min and were plated in 100 mm culture dishes at a density of approx. 4×10^4 cells/cm^2 in alpha minimal essential Eagle's medium supplemented with 10 % (v/v) fetal calf serum,

100 i.u./ml penicillin and 100 μg/ml streptomycin (basal medium). After 12 h, a period during which no cell replication occurs [28], the adherent cells were washed three times with Hanks balanced salt solution, treated with trypsin and counted. Cells were plated for 12 h before replating to permit separation of the preadipocytes from other cell types. We have previously shown that 95% of cells isolated by this procedure are preadipocytes [29,30]. Cells were replated in basal medium at the same density in 25 cm^2 flasks. After 2 days, the basal medium was replaced and after a further 2 days the cells were confluent. At this point they were cultured either in basal medium (undifferentiated cultures) or in an enriched medium containing alpha minimal essential Eagle's medium, 10 μg/ml insulin, 10 mM glucose, 5 μl/ml 10% (v/v) Liposyn III, 20% (v/v) Nuserum and antibiotics [30]. These high doses of insulin and glucose elicit a maximum differentiation response. After 2 days the media were replaced and after a further 1 day the cells were lysed for RNA extraction. Hence the cells were in culture for 7.5 days and had achieved confluence 3 days before harvesting.

3T3-L1 cell culture

Stock cultures of confluent 3T3-L1 cells were trypsin-treated and reseeded on 150 mm tissue culture dishes at a dilution of 1:15 in Dulbecco's modified Eagle's medium (containing 4.5 mg/l D-glucose, 584 mg/l L-glutamine and 110 mg/l sodium pyruvate) additionally supplemented with 8 μg/ml (+)-biotin and 8 μg/ml calcium pantothenate [31]. Cells were grown to 2 days post-confluence (usually 4–5 days) with fresh medium every 2–3 days. At this point the medium was changed with the addition of 10 μg/ml insulin, 0.5 mM methylisobutylxanthine and 0.25 μM dexamethasone (differentiation medium [32]). After a further 48 h (day 0 post-differentiation) the dexamethasone and methylisobutylxanthine were withdrawn and culture continued in the presence of insulin. The medium was changed every 2 days for up to 50 days after differentiation. For cell harvest, the monolayers were washed with 10 ml of ice-cold PBS and scraped with a rubber 'policeman' into 5 ml of PBS per plate. The suspension was centrifuged for 2 min at 1200 ***g*** and 4 °C, at which point the cells either sedimented or floated depending on the stage of development and hence triacylglycerol accumulation. After removal of the liquid phase, cells were used directly for RNA isolation (see above) or for CPT assay. For the latter purpose they were resuspended in 150 mM KCl/5 mM Tris/HCl (pH 7.2) at 0.5–2 ml per plate and were broken by using 10 cycles of a glass homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 8000 ***g*** in a Microfuge for 5 min at 4 °C. The floating fat (when present) and the supernatant were aspirated and the pellet was resuspended in KCl/Tris to give a suitable concentration for CPT assay (usually 1–3 mg of protein/ml). Direct assay of this preparation provides a measure of CPT I because the mitochondria are largely intact. For assay of CPT II, an aliquot of the sample was made 1% (w/v) with regard to octylglucoside and kept on ice for 30 min. Under these conditions the mitochondria are solubilized, inactivating CPT I and exposing matrix CPT II in active form [33]. The CPT assay was as described [15]. Protein was measured by the Lowry method [34].

Northern blot analysis

RNA samples (20 μg) were subjected to electrophoresis on 1% (w/v) agarose gels and transferred to nylon membranes as described [35]. Cross-linking was achieved with a UV Stratalinker (Stratagene). Single-stranded ^{32}P-labelled probes were prepared [36]. Membranes were probed sequentially for M-CPT I, L-CPT I, CPT II and 28 S rRNA. For analysis of rat, mouse and hamster RNA species we used rat cDNA templates located within the coding regions as follows: L-CPT I, b.p. 1154–1720 [12]; M-CPT I, b.p. 1–549 [14]; CPT II, b.p. 368–1725 [9]. For human RNA species we used human cDNA templates: L-CPT I, b.p. 464–1058 [13]; M-CPT I, b.p. 55–950 [16]; CPT II, b.p. 1461–1974 [10]. In all cases a 1.6 kb human 28 S rRNA probe (denoted pA_{BE} in [37]) was used. (For each of the Northern blots presented, the 28 S probe confirmed the approximately equivalent loading of all lanes. For simplicity the 28 S probe data are shown only in Figure 5.) Membranes were hybridized [12] at 44 °C with 2×10^6 c.p.m. of probe/ml overnight (mRNA species) or 10^4 c.p.m./ml for 1 h (28 S rRNA), then washed with 1 × SSC [38]/1% SDS at 60 °C. For mRNA detection, membranes were exposed at −70 °C to X-OMAT AR film (Kodak) with an intensifying screen for 48 h in most cases. In some instances exposure was performed for 7 days (see the Results section). For 28 S rRNA, exposure was for 16 h at −20 °C. Where necessary, membranes were stripped between probes by washing with 0.1 × SSC/0.5% SDS for 30 min at 80 °C.

RESULTS

At the outset of this study we knew that purified adipocytes from the rat epididymal fat pad (and also from retroperitoneal and periovarian fat depots) express primarily M-CPT I, with a small contribution from the L-type enzyme [15]. We had also established that human fibroblasts express only L-CPT I [13], and that in both cell types the CPT I isoform make-up is reflected in the shape of the malonyl-CoA inhibition curve.

To explore the temporal pattern of CPT I isoform expression during adipocyte differentiation, we began by studying the malonyl-CoA sensitivity of CPT I in the 3T3-L1 cell line at confluence (preadipocytes) and at 6 and 12 days after differentiation, expecting to see a shift from an L-type to an M-type profile during this time. As predicted, the preadipocytes displayed an IC_{50} of 3–5 μM, characteristic of L-CPT I; however, surprisingly, no discernible shift in the malonyl-CoA sensitivity curve was observed at 6 or 12 days after differentiation (Figure 1). Absolute values for CPT I activity in the absence of malonyl-CoA also did

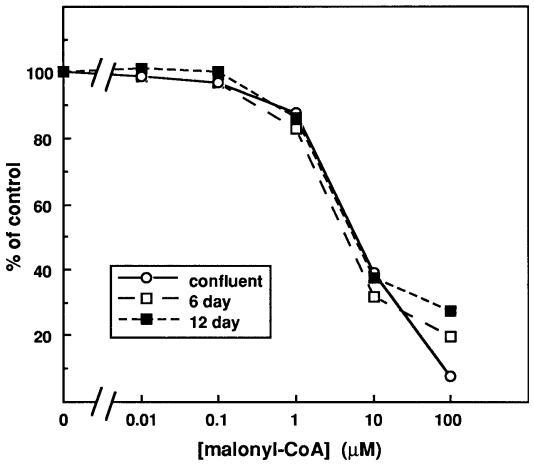

Figure 1 Effect of malonyl-CoA on CPT I activity in 3T3-L1 cells

Cells were harvested and assayed at the stage of preadipocyte confluence and at 6 or 12 days after differentiation. Results are expressed relative to the activity in the absence of malonyl-CoA. Values are the means of three independent determinations at each time point.

Table 1 CPT activities in 3T3-L1 cells

3T3-L1 cells were cultured, harvested and assayed as described in the Experimental section. Results are expressed as means ± S.D. for three independent determinations.

Cell type	Specific activity (nmol/min per mg of protein) CPT I	CPT II	I_{max} (%)	CPT II/CPT I
Confluent	1.06 ± 0.20	1.19 ± 0.44	92.1 ± 2.3	1.12
6 days after differentiation	1.3 ± 0.16	6.24 ± 0.52	80.1 ± 2.9	4.8
12 days after differentiation	1.14 ± 0.18	5.8 ± 1.78	72.7 ± 0.3	5.09

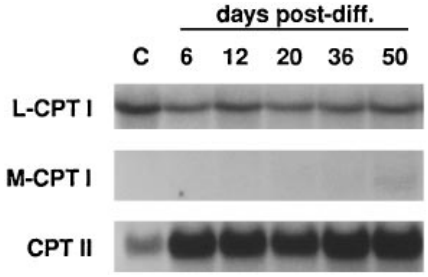

Figure 2 Northern blot of CPT mRNA species in 3T3-L1 cells

Total RNA was prepared from cells at 2 days after confluence (C) and at 6, 12, 20, 36 and 50 days after differentiation. Autoradiographs were exposed for 2 days (L-CPT I and CPT II) or 7 days (M-CPT I). Equal loading of each lane was confirmed by the use of a 28 S rRNA probe (results not shown).

not change significantly (Table 1). However, the maximum inhibition (I_{max}) achieved at 100 μM malonyl-CoA was seen to decrease (Figure 1 and Table 1). This was probably due to the 4–5-fold elevation in the CPT II level that accompanied differentiation (Table 1). Although the assay designed to measure CPT I is performed under circumstances where the mitochondria remain mostly intact, a small amount of damage inevitably occurs, with concomitant exposure of the malonyl-CoA-insensitive CPT II. Thus, as mitochondrial matrix CPT II activity increases, the apparent I_{max} for CPT I would be predicted to decrease. This increase in CPT II activity with adipocyte differentiation is consistent with previous observations on mitochondria from purified rat epididymal fat pad adipocytes, which showed that the CPT II-to-CPT I ratio is higher in mature adipocytes than in any other cell or tissue type examined so far [15]. However, the phenomenon complicates the kinetic analysis of a putative isoform switch.

As an alternative approach, we turned to Northern blot analysis of the 3T3-L1 cells with rat cDNA probes that in preliminary experiments had been shown to recognize the mouse CPT mRNA species. As seen in Figure 2, the signal for CPT II mRNA had clearly increased by 6 days after differentiation and remained essentially unchanged thereafter, consistent with the kinetic analysis. In contrast, the amount of L-CPT I mRNA did not change noticeably, even up to 50 days after differentiation. Importantly, no signal for M-CPT I was detected until 20 days; even after 50 days its intensity was trivial compared with that for L-CPT I. (It should be noted that the panel for M-CPT I was exposed for 7 days as opposed to 2 days for the other probes.) Again, these results were consistent with the kinetic analysis of

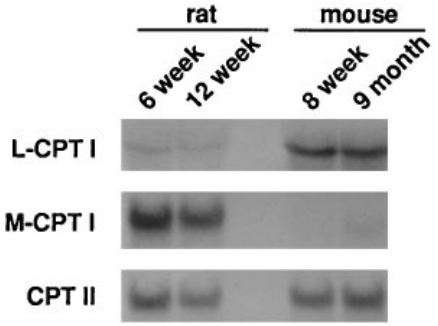

Figure 3 Northern blot of CPT mRNA species in rat and mouse adipocytes

Autoradiographs were exposed for 2 days (L-CPT I and CPT II) or 7 days (M-CPT I). Equal loading of each lane was confirmed by the use of a 28 S rRNA probe (results not shown).

the 3T3-L1 cells, but seemed to be in conflict with the observations described above for rat adipocytes. This inconsistency between the model *in vitro* and the results obtained with purified mature rat adipocytes raised several possibilities. One was that the long-term cell line is inherently incapable of developing the full pattern of CPT gene expression seen *in vivo*. A second was that the time limit imposed by the culture system prevents the adipocytes from maturing fully. A third was that the phenomenon reflected the murine origin of the 3T3-L1 cell line.

To distinguish between these possibilities, epididymal fat pad adipocyte RNA was prepared from rats and mice of different ages. The resulting Northern blot is shown in Figure 3. No significant difference in CPT expression pattern is apparent between samples from the 6-week-old and 12-week-old rats. By 6 weeks, therefore, the adult pattern of expression has been achieved, M-CPT I mRNA predominating. The use of even younger animals was impracticable because the epididymal fat pad is insufficiently developed. In stark contrast, in the mouse, the signal for M-CPT I was absent at 8 weeks of age, and even in 9-month-old animals it was barely detectable. Yet the L-CPT I signal was strong and of equal intensity in both age groups. (In Figure 3 also, the panel for M-CPT I was exposed for 7 days to make the signal apparent.) The amount of CPT II mRNA did not change significantly in either species over the time frames examined. It can be concluded that the 3T3-L1 cells display a time course of CPT I isoform expression that is remarkably similar to that of the mouse adipocyte *in vivo*, i.e. that no detectable induction of the muscle form of CPT I occurs on differentiation of the cells but that a very low level of M-CPT I gene expression does arise after long-term adipocyte maturation. However, this rendered the 3T3-L1 cells unhelpful in the study of the predicted isoform switch in the rat adipocyte.

Because an equivalent of the 3T3-L1 cell line of rat origin is not available, we examined rat preadipocytes in primary culture. As described in the Experimental section, these cells can be maintained for several days in tissue culture and caused to differentiate. A Northern blot with RNA from cultured primary rat (epididymal fat pad) preadipocytes before and after differentiation *in vitro* and from mature rat adipocytes is shown in Figure 4. After 3 days of culture in the enriched differentiation medium, more than 95% of these cells contain triacylglycerol droplets and adopt an adipocyte morphology [29,30]. L-CPT I mRNA was the only CPT I signal detected in the undifferentiated preadipocytes, albeit of low intensity. At 3 days after differentiation this was seen to intensify to a level similar to that in the mature cells. At the same time an M-CPT I signal appeared but

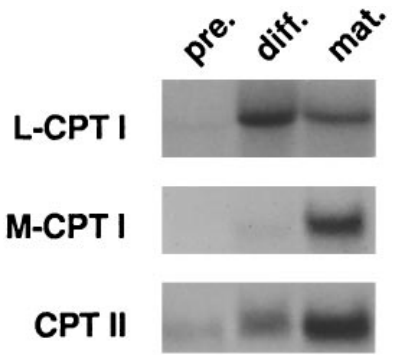

Figure 4 Northern blot of CPT mRNA species during differentiation of primary rat preadipocytes

Cells were cultured as described in the Experimental section. Total RNA was prepared from undifferentiated preadipocytes (pre.), cells differentiated *in vitro* (diff.) and mature adipocytes *in vivo* (mat.). Equal loading of each lane was confirmed by the use of a 28 S rRNA probe (results not shown).

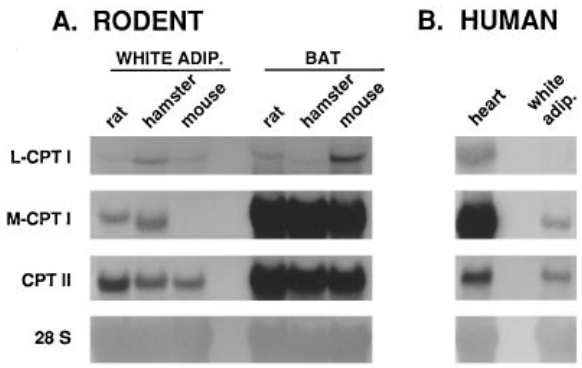

Figure 5 Northern blot of CPT mRNA species in adipocytes from various species

(A) Total RNA from white adipocytes (white adip.) and whole brown adipose tissue (BAT) from rat, hamster and mouse. (B) Total RNA from human heart and white adipocytes. Abbreviation: 28 S, ribosomal 28 S RNA.

this was substantially weaker than that found in mature adipocytes. The CPT II message also increased on differentiation and was stronger still in mature cells. This model *in vitro* therefore indicates that M-CPT I mRNA emerges in the rat adipocyte at a relatively early stage in cell development.

The above findings uncovered a clear and surprising difference between CPT I expression in adipocytes of the rat and the mouse. Two immediate questions were raised. Which animal model more closely represents the situation in human adipocytes? Is there also a difference in CPT I isoform expression between the brown adipocytes of the two rodents? Figure 5 shows an analysis of CPT mRNA expression in adipocytes from three rodent species and humans. Figure 5(A) establishes that the adult hamster adipocyte contains predominantly the message for M-CPT I, similar to that in the rat. As usual, no M-CPT I signal was seen in the mRNA from mouse white adipocytes (8-week-old animals) although the L-CPT I message was clearly detectable. In contrast, whole brown adipose tissue from all three rodent species expressed only a minor amount of L-CPT I mRNA compared with that for the muscle isoform. The results obtained with human white adipocyte RNA are shown in Figure 5(B). (The following technical consideration should be noted in the interpretation of Figure 5: the human L-CPT I mRNA migrates with the 28 S rRNA and, consequently, when total RNA is used for Northern blot analysis, the mRNA mingles with the ribosomal material, causing the radioactive band to be unusually broad, in contrast with the much narrower signals for rat L-CPT I. To demonstrate that the human L-CPT I signals shown in Figure 5(B) are not the result of background interaction with the ribosomal species, the control analysis with a specific 28 S probe is shown for this figure, confirming the approximately equal loading of the heart and adipocyte lanes.) As a control, we also analysed RNA from human heart. In keeping with previous findings [1], this tissue was found to express both isoforms, M-CPT I predominating, as with the rat. In the human white adipocyte, the L-CPT I RNA was barely detectable, whereas a robust M-CPT I signal was seen. (A second human sample gave identical results.) A CPT II mRNA was found in both human tissues, as expected. Thus the human white fat cell seems to behave like its counterpart from rat and hamster in terms of CPT I isoform expression.

To expand our undersanding of the expression pattern of the CPT I enzymes in organs other than fat, we prepared RNA from a number of rat tissues and probed for the presence of L-CPT I, M-CPT I and CPT II (Figure 6). L-CPT I mRNA was found to be the sole or predominant CPT I isoform in liver, kidney cortex, kidney medulla, lung, ovary, spleen, brain, pancreatic islets and both the large and small intestine. Small amounts of M-CPT I mRNA were seen in the lung, ovary and both regions of the kidney. As expected, the M-CPT I signal dominated over that for L-CPT I in rat heart. The faint signals for L-CPT I seen in

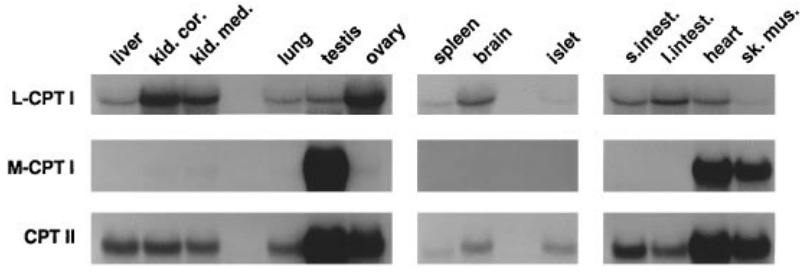

Figure 6 Northern blot of CPT mRNA species in rat tissues

Equal loading of each lane was confirmed by the use of a 28 S rRNA probe (results not shown). Abbreviations: kid. cor., kidney cortex; kid. med., kidney medulla; s.intest., small intestine; l.intest, large intestine; sk. mus., skeletal muscle.

skeletal muscle RNA have not been observed before and might be the result of contaminaton with non-myocyte material. Interestingly, M-CPT I mRNA was also present at high levels in testis. CPT II mRNA was expressed ubiquitously but at enormously variable levels. The strongest signals were in heart, skeletal muscle and gonadal tissues. In view of the discrepancy between the rat and mouse at the level of the white adipocyte, we also analysed a series of non-fat tissues from the latter species (liver, kidney, lung, testis, spleen, brain, intestine, heart and skeletal muscle). The results were essentially identical with those found in the rat.

DISCUSSION

For historical reasons the two isoforms of mitochondrial CPT I that have been characterized so far are referred to as L-CPT I and M-CPT I, in accordance with their expression in rat liver and skeletal muscle respectively [1]. However, it is becoming increasingly clear that each of these entities is expressed in tissues other than that for which it is named (see the Introduction section). The present work extends the list of rat tissues expressing predominantly L-CPT I to include kidney (both cortex and medulla), intestine (both large and small), lung, spleen, brain, pancreatic islets and ovary. In sharp contrast, M-CPT I dominates in the testis, although we have not yet defined the cell type(s) involved. This same tissue distribution seems to apply to the mouse (with the exception of white fat cells, as elaborated below).

It should be noted that each of the organs studied contains more than one cell type and this probably explains why several of the Northern blot lanes in Figure 6 show the presence of some message for each CPT I isoform. One should therefore interpret such data carefully when drawing conclusions about a particular cell type. As an instructive example, Northern blot analysis of RNA from whole rat epididymal fat pad showed a dominant L-CPT I message, but when mature adipocytes were purified away from the stromal–vascular material M-CPT I emerged as the primary isoform [15]. We now know that the removal of preadipocytes was a contributory factor (see below).

The radically different malonyl-CoA sensitivities and $K_m^{carnitine}$ values displayed by the the two CPT I isoforms suggest important differences in the physiological regulation of β-oxidation at different body sites and might also reflect local (perhaps even subcellular) variations in the concentrations of substrates and the natural inhibitor. Furthermore the regulation of fatty acid entry into the mitochondria takes on a different significance depending on the tissue in question. For example, in liver it is a pivotal element in the control of ketone body production [1–3]; in heart and skeletal muscle the primary objective is to control energy production for contraction [39]; in the pancreatic β-cell the inhibition of CPT I by malonyl-CoA might well have a crucial role in the regulation of insulin secretion [6,40]. To what extent the malonyl-CoA/CPT I interaction operates in other tissues remains to be established.

We demonstrated earlier that the ratio of L-CPT I/M-CPT I expressed in rat heart decreases during development at the same time as the accumulation of carnitine in that organ [21]. A teleological explanation might lie in the much lower $K_m^{carnitine}$ of the liver isoform, which could be expected to support the majority of fatty acid flux into mitochondria in the neonatal, relatively carnitine-deficient, rat heart; as carnitine stores build up during suckling, M-CPT I becomes the dominant isoform [21]. However, a more general theory to explain the choice of CPT I isoform in other tissues is not yet possible.

If CPT I isoform expression can change in a given cell type in response to metabolic circumstances, we asked whether the same might be true during the more drastic process of cell differentiation, focusing initially on the developing white fat cell. We began with the 3T3-L1 preadipocyte, which was derived from the mouse fibroblast 3T3 line [41] and has been broadly accepted as a paradigm for the study of fat cell differentiation [42]. The results were surprising.

Both kinetic analysis and Northern blots revealed that the undifferentiated 3T3-L1 preadipocytes do indeed express L-CPT I, and exclusively so. However, no change in malonyl-CoA sensitivity was observed on differentiation. Although kinetic analysis has proved to be extremely useful in the characterization of CPT I isoforms and provided key evidence suggesting the existence of more than one form of the enzyme long before a molecular biological approach was available to confirm it [4], the approach does have certain drawbacks, some of which are exaggerated in studies with adipocytes. First, the level of CPT I activity in the adipocyte mitochondrion is low when compared with liver or muscle tissue [15]. Secondly, the ratio of CPT II to CPT I is unusually high, which impacts on the apparent I_{max} of CPT I for malonyl-CoA and complicates direct comparisons between sensitivity curves. Although one can attempt to correct for this by considering uninhibitable activity to represent leakage of CPT II [15], such manipulation of the data is undesirable when small changes in sensitivity are in question. Thirdly, the degree to which the malonyl-CoA sensitivity curve will shift with a change in isoform composition is dependent on the carnitine concentration in the assay. Another technique, the use of [^{3}H]etomoxir labelling to distinguish CPT I isoforms by fluorography of SDS/polyacrylamide gels [19], is also difficult to apply with adipocytes because the low level of labelling achieved often requires exposure of the autoradiograph for several weeks (N. F. Brown and J. D. McGarry, unpublished work). However, now that cDNA species are available for both L-CPT I and M-CPT I, it is possible to detect the expression of both enzymes at the level of mRNA. Although an important caveat with this approach is that relative levels of mRNA might not be a precise reflection of steady-state levels of enzyme protein, for the technical reasons described we believe it is the most appropriate method of study in this case. Using the Northern blot technique we confirmed that the message for M-CPT I did not appear until the 3T3-L1 cells had been maintained in a differentiated state for 20 days, and even at 50 days the signal was slight when compared with that for L-CPT I. Further, in mouse white adipocytes *in vivo*, M-CPT I mRNA was not observed until a very advanced age of the animals, and even then it was at trivial levels. This represents a clear difference from the rat cells, where by a few weeks of age the muscle isoform is already predominant and was initially detected after only 3 days of differentiation of primary preadipocytes *in vitro*. (We have shown previously [43], and in unpublished work during this study, that neither preadipocytes nor mature adipocytes prepared from the rat epididymal fat pad as described contain detectable mRNA for the uncoupling protein, precluding brown adipocyte contamination as the source of the M-CPT I signal.)

Assuming that the function of the epididymal fat pad adipocyte is essentially the same in rats and mice, it is not clear why the two species should require different CPT I enzymes. For both differentiated 3T3-L1 cells [44] and freshly isolated rat adipocytes [45], the rate of oxidation of exogenous fatty acids is believed to represent a minor fate relative to esterification. Furthermore, at least with respect to CPT II, both the murine 3T3-L1 cells and the primary rat adipocyte cultures behave similarly, showing a marked increase in mRNA very rapidly on differentiation. At present we are unable to provide a teleological explanation for

this phenomenon of divergent isoform expression in the white adipocyte. Conceivably it might be related to the relatively small size of the mouse and its higher metabolic rate, which could be associated with accelerated fatty acid oxidation even in white adipose tissue. Theoretically, for any given level of malonyl-CoA, fatty acid flux through CPT I would be favoured if the operative isoform were the liver-type enzyme. We are not aware of any comparative measurements of malonyl-CoA (or carnitine) concentrations in rat and mouse adipocytes. Indeed, reliable data would be difficult to obtain because the long (approx. 2 h) adipocyte preparation renders meaningless the assay of metabolites that turn over rapidly. Moreover, the availability of such information would still leave unsolved the additional conundrum of why brown adipose tissue, which is characterized by a high capacity for fatty acid oxidation and thermogenesis, utilizes M-CPT I in all three species examined (rat, mouse and hamster). Clearly, additional experiments, possibly including mammals of similar or smaller sizes compared with the mouse, will be needed to answer these questions. Also deserving of further study is the observation that, whereas the appearance of M-CPT I message in the rat adipocyte occurs much more rapidly after differentiation than in the mouse cells, the level seen in the rat preadipocyte differentiation model *in vitro* was still only a fraction of that found in mature rat adipocytes. It seems that the expression of the M-CPT I gene is not merely dependent on the induction of adipogenesis but also requires some secondary, as yet undefined, factor/s associated with adipocyte maturation.

Finally, the finding that human white fat cells display a CPT I profile similar to that of the rat (and hamster) could have important implications for the choice of animal models in future studies of adipocyte metabolism. The rat has been the primary model employed in the field of CPT research and it now seems that, at least in terms of the adipocyte, it probably reflects the situation in humans more closely than does the mouse. In this regard, despite its popularity, the 3T3-L1 cell might not be the most appropriate system for elucidating the regulation of fatty acid metabolism in the human fat cell. By extension, although the use of the transgenic mouse has become increasingly important as a tool in biochemical research, it would seem prudent, from the standpoint of whole body fatty acid metabolism, to extrapolate results obtained with this model to humans with caution.

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