Immunoquantitative Analysis of Human Carnitine Palmitoyltransferase I and II Defects

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ABSTRACT. Carnitine palmitoyltransferase deficiency realizes two distinct clinical forms. We previously showed and confirmed in the present work that CPTII (identified as the carnitine palmitoyltransferase activity assayable in detergent conditions) is decreased in the muscular form whereas it is unaffected and CPTI is decreased in the hepatic form. The antibody previously prepared against human liver mitochondrial CPTII recognizes the same enzyme in muscle, liver, and fibroblasts. Immunoprecipitation experiments were performed in fibroblasts from patients with the muscular and hepatic forms of the defect. As compared with controls, cell lines from two patients with the hepatic form of the defect did not exhibit any qualitative nor quantitative abnormality of cross-reacting material, whereas cell lines from two patients with the muscular form of the defect exhibited a decreased amount of cross-reacting material. These data suggest that CPTII deficiency could result from a decreased production of protein. The amount of cross-reacting material in the two sets of patients only correlates with CPTII activity, which is decreased in the muscular presentation and unaffected in the hepatic form. These results strengthen the hypothesis of distinct proteins supporting CPTI and CPTII activities. (*Pediatr Res* 27: 497–500, 1990)

Abbreviations

CPT, carnitine palmitoyltransferase
CPTI, carnitine palmitoyltransferase I
CPTII, carnitine palmitoyltransferase II
CRM, cross-reacting material

CPT is involved in the mitochondrial oxidation of long-chain fatty acids. Their transport across mitochondrial membranes is thought to be accomplished through the sequential action of CPTI and CPTII (1, 2). *In situ*, both enzymes display very different kinetic and regulatory properties (3–5). Moreover, it was reported that CPTI activity, unlike CPTII activity, was rapidly lost in the solubilized form with detergents (6, 7). The basis of these differences is not clear. One possibility is that CPTI and CPTII are distinct proteins (8–10). Others have proposed that the two enzymes are identical (11–13). Their different behavior while membrane-associated would be ascribed to differences in their physical environments.

In human pathology, CPT deficiency realizes two different

clinical presentations: one with muscular (14), the other with hepatic (15) symptoms. The analysis of CPT activities of fibroblasts from patients with muscular and hepatic forms of the deficiency showed that CPTI deficiency was involved in the hepatic form, whereas CPTII defect was evidenced in the muscular form (16).

CPT from human liver was purified from mitochondrial membranes solubilized with Triton X100 according to procedures (17) that are thought to select (6–9) the protein that supports CPTII activity, and antibodies against this protein were raised in rabbits (Demaugre F, Cepanec C, Leroux JP, unpublished data).

In our study, we show that immune serum recognized a similar protein in liver, skeletal muscle, and fibroblasts. In fibroblasts of patients with CPTII defect (muscular form), a decreased amount of protein was recognized by the immune serum, whereas no qualitative nor quantitative abnormality was observed in fibroblasts of patients with CPTI deficiency (hepatic form).

MATERIALS AND METHODS

Fibroblasts from controls and CPT-deficient patients were cultured as previously described (18). Cell lines A and B were from patients with hepatic presentation (15, 16) and cell lines C and D from patients with muscular presentation. Muscular CPT deficiency of these patients was previously described. Human livers were from kidney transplantation donors. Livers were removed within 30 min after circulation arrest, immediately frozen in solid CO_2, and stored at −80°C before use.

CPT activity. CPT activity was assayed by the measurement of palmitoyl L-(methyl^{14}C) carnitine formation from palmitoyl CoA and L-(methyl^{14}C) carnitine. Assays I and II were performed as previously described (16) on fibroblast homogenates. Assay I, performed in nondetergent conditions, was carried out with 110 μM palmitoyl CoA complexed with 1% albumin, 550 μM L-carnitine and, if noted, 50 μM malonyl CoA. CPT activity, assayable in detergent conditions, was measured by assay II conducted without albumin, with 1 mM palmitoyl-D-carnitine, 40 μM palmitoyl CoA, and 1 mM L-carnitine. CPT activity assayable in detergent conditions was also measured in the 100 000 g supernatant of fibroblasts, muscle, and liver solubilized with 1% Tween 20 and 500 mM KCl. Incubation mixture was similar to that of assay I except that albumin was omitted.

Immunoinhibition experiments. Aliquots of 100 000 g supernatants of muscle, liver, and fibroblast homogenates solubilized with 1% Tween 20 in 500 mM KCl were preincubated for 60 min with IgG fraction (19) of preimmune or anti-CPT immune serum in 50 mM KH_2PO_4, pH 7.4, 50 mM NaCl, and 20 μL of protein A Sepharose diluted with an equal vol of 10 mM KH_2PO_4, pH 7.4, at 25°C. Then the mixture was centrifuged for 4 min and CPT activity was measured by assay III in the supernatant.

Table 1. *CPT activities of fibroblasts from patients with hepatic (A and B) and muscular (C and D) presentation**

| | | Controls | Patients | | | |
			A	B	C	D
Assay I		2.42 ± 0.70 (1.20–3.35) (n = 15)	0.39 ± 0.04†	0.56 ± 0.18†	1.35 ± 0.20	3.1 ± 0.4
	+ Malonyl CoA 50 μM	0.22 ± 0.06 (0.12–0.31) (n = 15)	0.21 ± 0.05†	0.26 ± 0.03†	0.15 ± 0.02	0.17 ± 0.02
Assay II		2.20 ± 0.35 (n = 7)	2.50 ± 0.45†	2.02 ± 0.28†	0.35 ± 0.08	0.31 ± 0.06
Assay III		1.5 ± 0.3 (n = 6)	1.5 ± 0.15	1.6 ± 0.20	0.28 ± 0.03	0.19 ± 0.02

* CPT activities were assayed by the measurement of palmitoyl-L-(methyl ^{14}C)carnitine formation from palmitoyl CoA and L-(methyl ^{14}C)carnitine in three distinct assays as indicated in Materials and Methods. Assay I conducted in nondetergent conditions was performed on homogenate. Assays II and III, respectively, performed on homogenate preincubated with 1 mM palmitoyl-D-carnitine and on 100 000 g supernatant of fibroblasts solubilized with 1% Tween 20 in 0.5 M KCl were carried out in detergent conditions. Results (mean ± SD) are expressed as nmol of palmitoyl-L-(methyl ^{14}C)carnitine produced · min^{-1}/mg protein. n = number of different control cell lines. Three independent experiments were performed in deficient strains. Range of control values is given in parentheses.
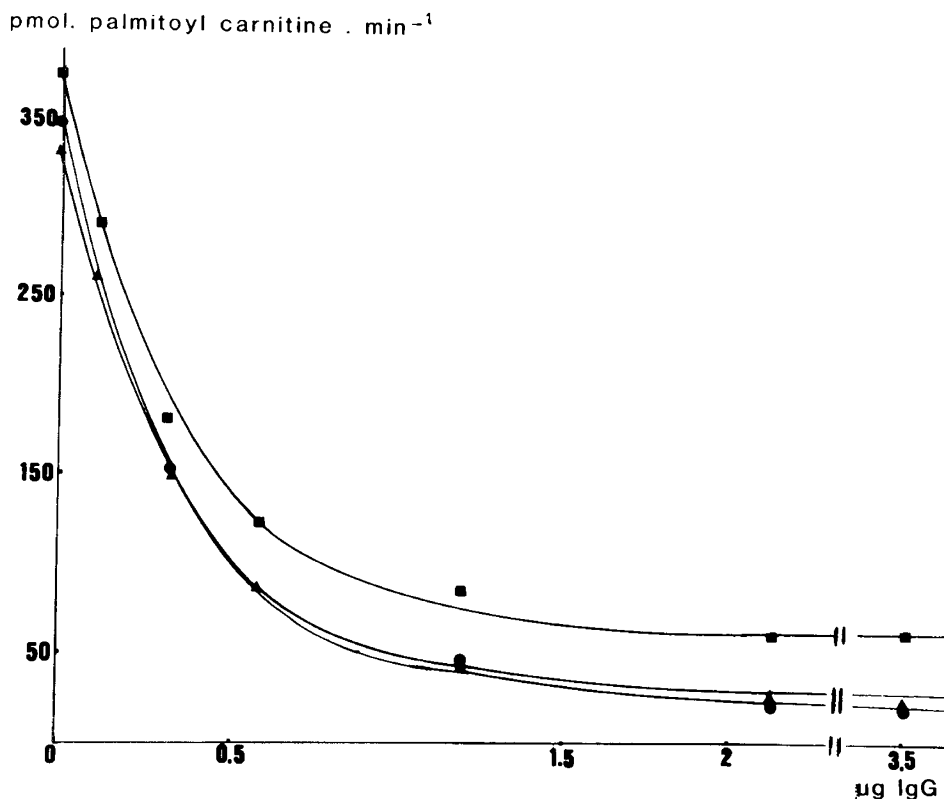
† Taken from Table 1 of Ref. 16.

Fig. 1. Immunoinhibition of CPT activity from liver, fibroblasts, and skeletal muscle. Aliquots of 100 000 g supernatants of liver (■), fibroblasts (●), and skeletal muscle (▲) solubilized with 1% Tween 20 in 0.5 KCl were incubated for 60 min at 25°C with various amounts of IgG from anti-CPT immune serum. Then the incubation mixtures were centrifuged at 12 000 × g for five min. CPT activity was measured in the supernatant. Incubation with nonimmune IgG did not affect CPT activity. Result of a typical experiment.

Immunoprecipitation experiments. Immunoprecipitation experiments were performed as described by Fenton *et al.* (20). 0.15 M NaCl, 10 mM EDTA, 0.2% SDS, and 10 mM KH₂PO₄, pH 7.4, were added to 100 000 g supernatants of homogenates of muscle, liver, or fibroblasts, solubilized with 1% Tween 20 in 500 mM NaCl. Aliquots (corresponding respectively to 300–500 μg, 600–1200 μg, and 600–1200 μg protein for liver, fibroblasts, and muscle), were then incubated with 0.5–2.0 μL of preimmune or anti-CPT immune serum and 20 μL of protein A Sepharose

for 15 h at 4°C. Pellets of protein A Sepharose were washed four times with 0.5 M NaCl, 10 mM EDTA, 1% Tween 20, 0.1% SDS, and 10 mM KH₂PO₄, pH 7.4. proteins bound to protein A Sepharose were released by incubation of the pellets for 10 min at 100°C with 50 μL of 200 mM Tris, pH 6.8, containing 30% glycerol, 3% SDS, and 15% β-mercaptoethanol; they were analyzed by SDS-PAGE carried out as described (21). Then proteins were electrotransferred to nitrocellulose sheets (22). Treatment and revelation were performed as described (23) with
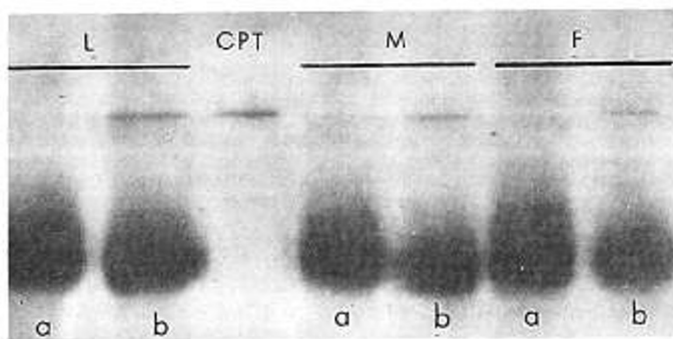
Fig. 2. Analysis of CPT of liver, fibroblasts, and skeletal muscle. Solubilized samples of liver (L), skeletal muscle (M), and fibroblasts (F) were incubated for 15 h at 4°C with nonimmune (a) or anti-CPT immune (b) serum and protein A Sepharose as described in Materials and Methods. Proteins bound to protein A Sepharose were then released and analyzed by SDS-PAGE and immunoblotting. Their electrophoretic mobility was compared to that of purified liver CPT. CPT activities (assay III) of samples incubated with preimmune or immune serum were, respectively, 4.0, 1.25, and 1.0 nmol·min^{-1} in liver, skeletal muscle, and fibroblasts.
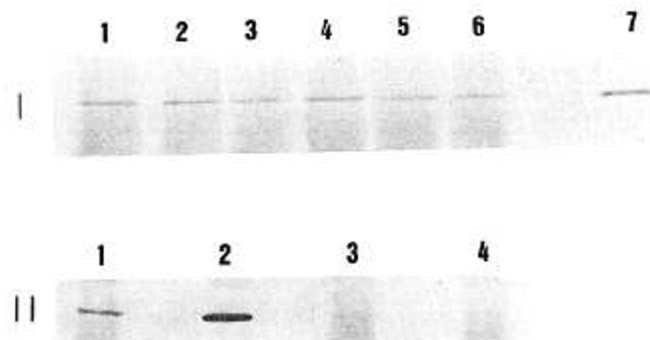

Fig. 3. Immunoquantitative analysis of CPT of fibroblasts from patients with CPT defects. Samples (1 mg protein) of solubilized fibroblasts from control cell lines and from patients with hepatic (patients A and B) and muscular (patients C and D) forms of CPT defect were incubated with anti-CPT immune serum and protein A Sepharose as described in Materials and Methods. Proteins released from protein A Sepharose were analyzed by SDS-PAGE and immunoblotting. I: Experiments performed in duplicate. Lines 1 and 2, patient A; lines 3 and 4, patient B; lines 5 and 6, control; line 7, purified liver CPT (0.5 μg). II: line 1, control; line 2, purified liver CPT (1 μg); line 3, patient C; line 4, patient D. Similar results were obtained in two other separate experiments.

some modifications: nitrocellulose sheets were blocked 60 min at 37°C and 15 h at 4°C. Then they were incubated for 60 min at 37°C and overnight at 4°C with a 1/200 dilution of antiserum in the same buffer as for blocking. Protein was determined as described (24).

Palmitoyl CoA, L-carnitine, malonyl CoA and BSA fatty acid-free were purchased from Sigma Chemical Co. (St. Louis, MO), protein A Sepharose from Pharmacia, (Fine Chemicals, Piscataway, NJ), L-(methyl^{14}C) carnitine and ^{35}S-methionine from Amersham Corp. (Arlington Heights, IL), and palmitoyl-D-carnitine was a gift from Sigma Tau (Paris, France).

RESULTS AND DISCUSSION

CPT deficiency realizes two different clinical forms. Most patients with CPT defect exhibit muscular symptoms (acute episodes of rhabdomyolysis after prolonged exercise or occasionally after fasting), which are ascribed to muscular deficiency (14). Another clinical form of CPT deficiency (hepatic form) was diagnosed by Bougneres et al. (15). It consists of hypoglycemia with inappropriate ketonemia during fasting, resulting from he-

patic CPT defect. Until now, only two patients have been diagnosed with this clinical form of CPT deficiency (15, 16). We studied (16) CPT activities of fibroblasts from two patients with the muscular form and the only two patients diagnosed with the hepatic form. We showed that CPTI was decreased in patients with the hepatic form, whereas it was normal and CPTII decreased in patients with the muscular form.

As shown in Table 1, study of CPT activities of fibroblast homogenates from two other patients with the muscular form of CPT defect gave the same figure. As compared with controls, these cell lines exhibited normal CPTI activity measured by assay I performed in nondetergent conditions; the suppressibility of CPT activity by malonyl CoA, a specific inhibitor of CPTI (25), was similar in cell lines of controls and patients. CPT activity assayed in detergent conditions is considered to measure CPTII due to the suppressibility of CPTI by detergents (6, 7). Patients with C and D cell lines exhibited a decreased CPTII activity measured with assay II conducted without albumin and with 1 mM palmitoyl-D-carnitine as detergent (26). Similar results were obtained if the assay was performed with other detergents such as Tween 20 or Triton X100 (results not shown). A reduced CPT activity assayable in detergent conditions was also evidenced in the 100 000 g supernatant of patients' fibroblasts solubilized with Tween 20 (assay III). These results strengthen the previous observation (16) that the muscular form of CPT deficiency may result from CPTII defect.

It must be stressed that mutations exhibited by the two sets of patients only affect CPTI activity in the hepatic form and CPTII activity in the muscular form. This result could suggest that CPTI and CPTII activities are borne by distinct proteins. Indeed, whether CPTI and CPTII are supported by a single or two different proteins is still discussed (8–13).

We previously purified, according to the procedures described by Fiol and Bieber (17), a protein supporting acylcarnitine transferase activity (Demaugre F, Cepanec C, Leroux JP, unpublished data) from human liver mitochondrial membranes solubilized with Triton X100. Antibodies against this protein were raised in rabbits. Its mol wt, estimated by SDS-PAGE, was 66 000 D. It exhibits enzymatic activity (negligible with acetyl or butyryl CoA and maximal with lauryl CoA as substrates) in presence of detergents. This protein likely supports CPTII activity (discussed below). As shown in Figure 1, IgG fraction of immune serum acts similarly on CPT activity measured in human liver, fibroblasts, and skeletal muscle solubilized with Tween 20; curves of suppression of CPT activity were similar in all tissues. In actual conditions of assay, more than 80% of CPT activity was suppressed at saturating concentration of Ig. Immunoprecipitation experiments followed by SDS-PAGE and Western blot were performed to analyze the material recognized by immune serum. As shown by Figure 2, immune serum recognized in liver, fibroblasts, and muscle a single protein of electrophoretic mobility similar to that of purified CPT. These data suggest that the protein supporting CPT activity assayable in extracts solubilized with detergents—presumably CPTII—is similar if not identical in these tissues. Similar findings concerning CPTII of rat liver and muscle were reported by Woeltje et al. (9).

Immunoprecipitation experiments (Fig. 3) were performed with fibroblasts from the two patients with the hepatic form and two patients with the muscular form of CPT deficiency. As compared with controls, immune serum recognized a single protein with normal electrophoretic mobility but in decreased amount in fibroblasts from patients with the muscular form of CPT deficiency. CRM estimated by densitometry was decreased by nearly 90% in cell line C and by more than 95% in cell line D. The amount of CRM correlated with the residual CPTII activity. CPTI was unaffected in these cells. This result suggests that immune serum only recognizes the protein supporting CPTII activity. One can postulate that the CPTII defect of these cells results from a decreased production of protein with normal affinity for the antibody, although a decreased affinity for the

antibody cannot be excluded. Studies in progress capable of probing the intimate structure of CPT genes and mRNA are required to differentiate these possibilities.

In fibroblasts from patients with the hepatic form of the defect, immunoprecipitation experiments did not reveal any difference between controls and patients (Fig. 3). Mutations causing the CPT defect exhibited by these cells (16; Table 1) do not affect the expression of the protein recognized by the immune serum, likely CPTII. The CRM of these cells does not correlate with the residual CPTI activity, but correlates with CPTII activity, which is unaffected.

The hypothesis of distinct proteins supporting CPTI and CPTII activities could explain results of enzymatic studies and immunoprecipitation experiments performed with both sets of patients. The mutations exhibited by "hepatic" and "muscular" cell lines would affect the expression of different proteins supporting CPTI and CPTII activities, respectively. Our results strengthen recent data advocating distinct proteins supporting CPT activities. Contrary to CPTII, CPTI activity is suppressed if assayed with detergents (6, 7, 9). Antibodies raised against a purified protein supporting CPT activity assayable in detergent conditions do not recognize the protein supporting CPTI activity (9), identified by its capacity to bind tetradecylglycidyl CoA, a specific inhibitor of CPTI activity. The mol wt of the protein supporting CPTI activity estimated either by SDS-PAGE (7) or by radiation inactivation experiments (8), was nearly 90 000 D, whereas the mol wt of the protein bearing CPTII activity estimated by the same methods was nearly 70 000 D (7, 8). As a consequence, CPT purified by classical methods using detergents (11–13, and Demaugre F, Cepanec C, Leroux JP, unpublished data) only supports CPTII activity. Moreover, it can be deduced from the data of Murthy and Pande (27) that proteins supporting CPTI and CPTII activities are not localized in the same subcellular structure: contrary to CPTII, CPTI activity might not be associated with the inner but with the external mitochondrial membrane.

As previously reported (28), the absence of membrane components may decrease CPTI activity. As a consequence, one cannot exclude that CPTI deficiency might be the result of mutations concerning such components. Further investigations must be performed to clarify this point.

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