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A case of carnitine palmitoyltransferase II deficiency in human skeletal muscle

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A 20-year-old man was shown to have a deficiency of carnitine palmitoyltransferase (CPT) II in skeletal muscle. The evidence was: (i) there was no significant oxidation of [9,10-^{3}H]palmitate or of [1-^{14}C]palmitate in mitochondrial fractions from fresh skeletal muscle from the patient; (ii) all the CPT activity in a homogenate of fresh muscle from the patient was overt (CPT I) with no increase in activity after the inner membrane was disrupted; (iii) all the CPT activity in the patient's muscle was inhibited by malonyl-CoA; and (iv) an immunoreactive peptide of 67 kDa corresponding to CPT II, present in mitochondria from controls, was absent in those from the patient.

Carnitine palmitoyltransferase deficiency; Carnitine palmitoyltransferase II; Mitochondria; (Skeletal muscle)

1. INTRODUCTION

The mitochondrial oxidation of long-chain fatty acids involves the formation of their CoA esters in the outer mitochondrial membrane and the endoplasmic reticulum. In the presence of carnitine their acyl groups are then transferred to CoA in the matrix by the concerted action of CPT I, the carnitine/acylcarnitine exchange carrier and CPT II. CPT II is located on the inner face of the inner membrane [1] and it was believed that CPT I is located on the outer face [1], and that CPT I and CPT II are the same protein with different kinetic properties due to their different locations in the mitochondrion [2]. However, recent work indicates that CPT I in skeletal muscle and liver mitochondria is located in the outer mitochondrial membrane and is a different protein from CPT II [3–8]. CPT I, but not CPT II, is inhibited by malonyl-CoA [4].

CPT deficiency is an uncommon cause of muscle disease in man. Patients with impaired CPT activity in skeletal muscle normally present with pain after prolonged exercise often associated with myoglobinuria [9]. Because of the variety of assay procedures used it is often uncertain, however, whether other workers have measured CPT I, CPT II or both (see [10,11]). Here, we report a case of unequivocal CPT II deficiency in human muscle.

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Abbreviations: CPT, CPT I, CPT II, carnitine palmitoyltransferase, its overt and latent forms

2. MATERIALS AND METHODS

2.1. *Case history*

A 20-year-old male medical student was referred to Newcastle for investigation. He presented with a history of exertional muscle pain and myoglobulinuria. Ingestion of carbohydrate relieved the severity of his symptoms, suggesting a defect of fatty acid oxidation in skeletal muscle. Histologically his skeletal

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muscle was normal except for a minor excess of neutral lipid. A defect of the respiratory chain was excluded [11].

2.2. *Materials*

Most biochemicals were obtained from Sigma (Poole, Dorset) and BCL (Lewes, Sussex). Malonyl-CoA was obtained from PL Biochemicals (Milwaukee, WI). L-Carnitine was a gift from Sigma-tau (Rome). 1-L-[*methyl*-^{3}H]Carnitine, 1-DL-[*methyl*-^{14}C]carnitine, [1-^{14}C]palmitate and [9,10-^{3}H]palmitate were obtained from Amersham International (Amersham, Bucks). Labelled carnitine was dissolved in 1.0 M HCl and extracted 5 times with isobutanol to remove background radioactivity. Palmitoyl-CoA and palmitoyl-DL-[^{14}C]carnitine were prepared and characterised [12]. Antibody to bovine liver CPT II (latent CPT) was raised in rabbits and precipitated from immune serum with $(NH_4)_2SO_4$ [3].

2.3. *Preparation of homogenates and mitochondrial fractions from skeletal muscle*

Samples of muscle (1–5 g) were obtained from the left vastus lateralis from the patient and from subjects with no evidence of muscle disease, and used immediately or stored at −85°C.

2.3.1. Mitochondrial fractions

Mitochondrial fractions were prepared from 3–5 g fresh muscle in a KCl-based medium [13]. The respiratory control ratios measured with 10 mM glutamate plus 1 mM malate as substrate [11] were in the range 3–5. Protein was determined by a modified Lowry method [11].

2.3.2. Homogenates

Fresh or frozen muscle (0.1–0.8 g) was disrupted using a Polytron homogeniser for 5 s with a rheostat setting of 9 in 12 vols of 50 mM potassium phosphate, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP (pH 7.6) at 0°C. Samples of homogenates of fresh muscle were freeze-thawed 3 times to expose CPT II. Citrate synthase activity [11] was increased by over 90% indicating that most mitochondria were intact before freeze-thawing. Citrate synthase activity was not increased further by 0.1% Triton X-100 (v/v).

2.4. *Assay of β-oxidation*

Incubations were carried out at 30°C and pH 7.0 in 1.0 ml of 110 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 10 mM potassium phosphate, 5 mM ATP, 1 mM L-carnitine, 100 μM CoA, 1 mM EGTA, 0.2 mg cytochrome *c* and mitochondrial fractions (0.3–0.6 mg protein) and the reaction was initiated with 0.12 mM palmitate containing 22 kBq [9,10-^{3}H]palmitate and 3 kBq [1-^{14}C]palmitate complexed to defatted bovine serum albumin in a molar ratio of 5:1. Incubations were quenched by the injection of 200 μl of 5 M $HClO_4$, followed by 100 μl of 0.5 M $KHCO_3$. The $^{14}CO_2$ and [^{14}C]acetylcarnitine formed were measured [13]. A sample of the acid-quenched medium was centrifuged at 10000 × *g* for 10 min, and the supernatant adjusted to pH 12 with 2 M KOH and left at 20°C for 45 min to hydrolyse labelled acetylcarnitine formed. The solution was then neutralised with 1.2 M HCl and passed down a 0.5 ml Dowex I column (acid form, 200 mesh). The eluant, which contained only the ^3H_2O formed, was counted. The release of ^3H_2O from [9,10-^{3}H]palmitate is a good indicator of the flux through β-oxidation [14].

2.5. *Assay of carnitine palmitoyltransferase activities*

2.5.1. Forward assay

CPT activity was measured in the direction of palmitoylcarnitine formation essentially as described by Zierz and Engel [15]. Muscle homogenate (50 μl) was added to 100 mM potassium phosphate, 2 mM KCN, 1 mM dithiothreitol, 80 μM palmitoyl-CoA and 2.5 mM 1-L-[*methyl*-^{3}H]carnitine (18.5 kBq) and 0.1% (w/v) of defatted bovine serum albumin in a final volume of 1.0 ml at 30°C and pH 7.6. After an appropriate time the reaction was stopped by adding 1.0 ml of 1.2 M HCl at 0°C. Palmitoyl[^{14}C]carnitine (2 kBq) was added as an internal standard. Palmitoyl[^{3}H]carnitine formed and the palmitoyl[^{14}C]carnitine standard were extracted into 2.0 ml of water-saturated isobutanol by shaking for 10 min and then centrifugation at 2000 rpm for 5 min. The isobutanol layer was washed twice with 5 ml isobutanol-saturated water to remove free [^{3}H]carnitine, and 1.0 ml was added to 8 ml scintillant (NE260 general purpose, Nuclear Enterprises, Sighthill, Edinburgh) and counted for ^{3}H and ^{14}C using a dual-channel programme. The internal standard confirmed that the extraction of labelled palmitoylcarnitine was complete (99 ± 1.7%, mean ± SD, n = 18). The reaction was linear for 10 min with all preparations.

For determination of malonyl-CoA inhibition of CPT activity, the assay was scaled down to a final volume of 0.1 ml containing 2.5 mM [^{3}H]carnitine (18.5 kBq).

2.5.2. Isotope exchange assay

This assay depends on the rate of incorporation of [^{3}H]carnitine into a pool of palmitoylcarnitine in the presence of CoA [15]. The assay medium (final volume 1.0 ml at 30°C) was the same as that used for the forward assay except that the substrates were 2.5 mM L-[^{3}H]carnitine (18.5 kBq), 100 μM or 0.5 mM palmitoylcarnitine and 0.2 mM CoA. The reaction was started by addition of homogenate and stopped by 1.2 M HCl. The assay was linear for 20 min.

2.6. *Immunoblot analysis*

The proteins in mitochondrial fractions were separated on 10% SDS-polyacrylamide gels [16] and then transferred to nitrocellulose paper [17] with 0.1% SDS (w/v) in the transfer buffer. Immunoreactive peptides were visualised by the immunoperoxidase method [18] using antiserum to CPT II diluted 1:1000 with 50 mM Tris, 0.15 M NaCl (pH 7.4).

3. RESULTS

3.1. *β-Oxidation*

There was virtually no oxidation of [9,10-^{3}H]palmitate by skeletal muscle mitochondrial fractions from the patient compared with those from the controls (fig.1); the very low rate detected may have been due to contaminating microperoxisomes [19]. No significant formation of [^{14}C]acetylcarnitine (patient, 0.20 nmol · min^{-1} · mg protein^{-1}; controls, 16.8 ± 2.2, mean ± SD, n = 3) or of $^{14}CO_2$ was observed (patient,

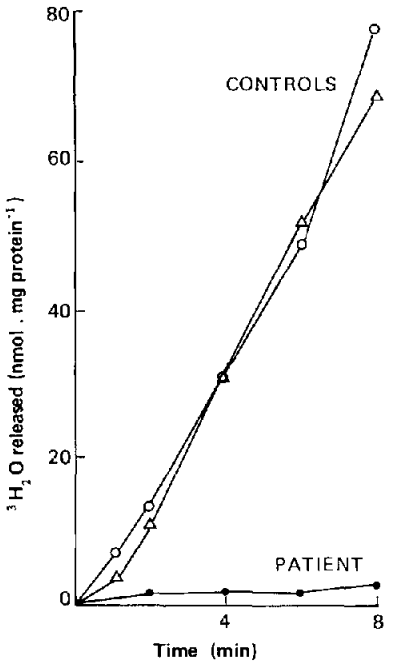

Fig.1. Oxidation of [9,10-^{3}H]palmitate by skeletal muscle mitochondrial fractions.

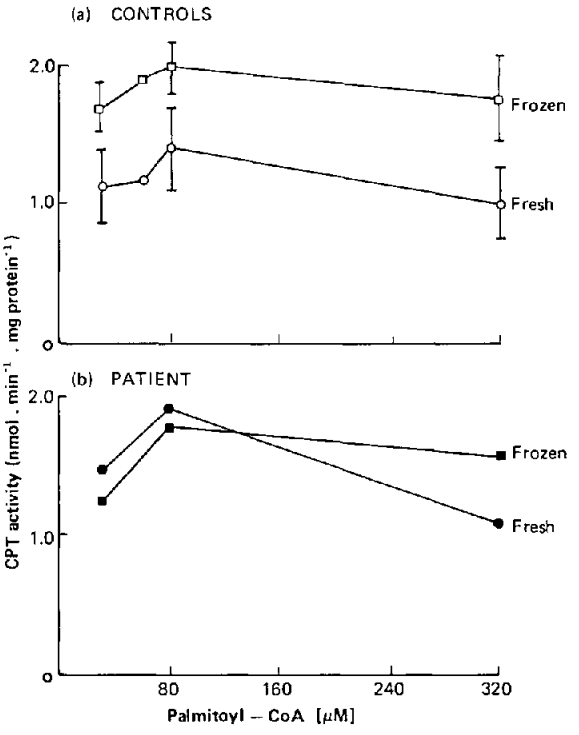

Fig.2. Carnitine palmitoyltransferase activities in fresh and freeze-thawed skeletal muscle homogenates measured in the direction of palmitoylcarnitine formation.

0.05 nmol·min^{-1}·mg protein^{-1}; controls, 1.40 ± 0.75, mean ± SD, n = 3).

3.2. *Carnitine palmitoyltransferase activities*

3.2.1. Forward assay

CPT activity was similar in homogenates of fresh muscle from the patient and controls in which the mitochondria were mainly intact (fig.2). By contrast, the activity in control homogenates increased by a third after freeze-thawing while it did not increase in the homogenates of muscle from the patient (fig.2). CPT activity in intact mitochondria is due to CPT I while that in disrupted mitochondria is due to CPT I plus CPT II [1].

CPT I is strongly inhibited by malonyl-CoA while CPT II is insensitive [4]. Malonyl-CoA virtually abolished CPT activity in freeze-thawed homogenates of muscle from the patient, but only halved that in homogenates of control muscle (fig.3).

3.2.2. Exchange assay

CPT activities (nmol·min^{-1}·mg protein^{-1})

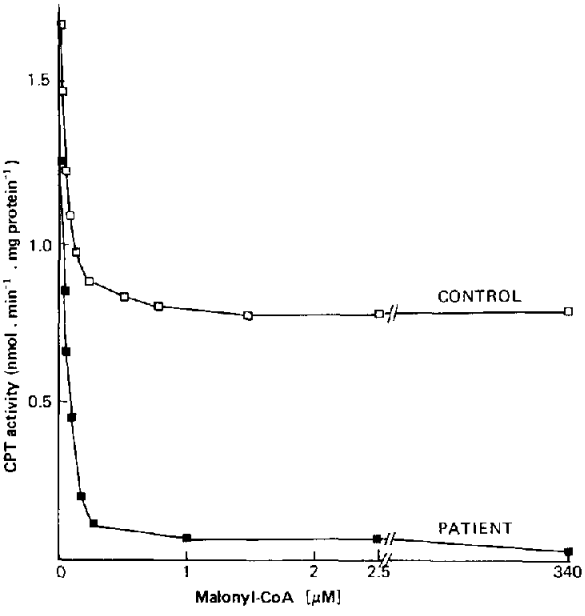

Fig.3. Effect of malonyl-CoA on carnitine palmitoyltransferase activities measured in the direction of palmitoylcarnitine formation in freeze-thawed homogenates of skeletal muscle.

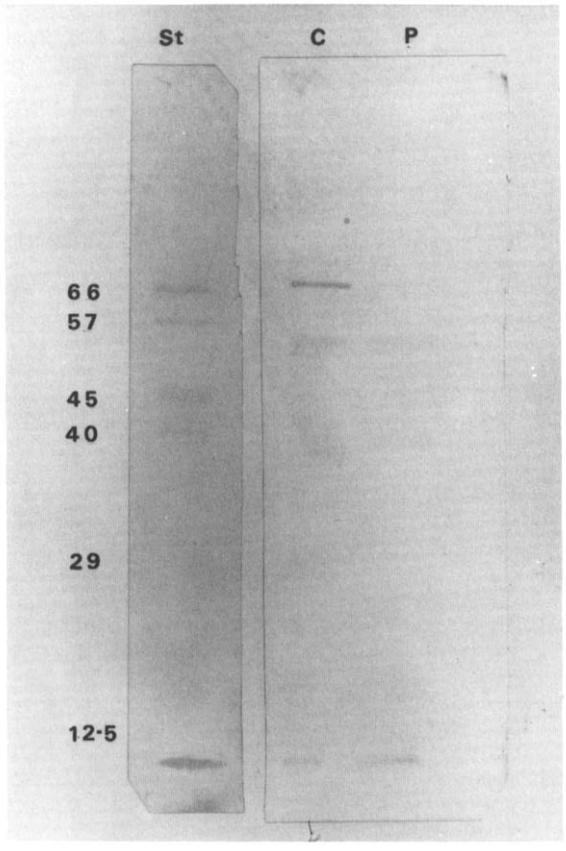

Fig.4. Immunoblot analysis of CPT II. Human skeletal muscle mitochondrial muscle proteins (200 μg) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted. Lane: ST, molecular mass standards (kDa); C, control; P, patient. A band corresponding to CPT II present in mitochondria from the control was absent in those from the patient. A band corresponding to CPT II was also present in skeletal muscle mitochondria from another individual (not shown).

measured in freeze-thawed homogenates were: (using 0.1 mM palmitoylcarnitine) patient, 0.46; controls, 0.59 ± 0.09 (means ± SD, n = 8); (using 0.5 mM palmitoylcarnitine) patient, 0.13; controls, 0.68 ± 0.10).

3.3. *Immunoblot analysis of CPT II*

An immunoreactive peptide of molecular mass 67 kDa corresponding to CPT II [8] was detected in skeletal muscle mitochondrial fractions from a control but was not in the fraction from the patient (fig.4). In contrast, an unidentified immunoreactive peptide of 56 kDa was present in similar amounts in mitochondria from both control and patient.

4. DISCUSSION

Our results unambiguously demonstrate the absence of CPT II activity and immunologically active CPT II in skeletal muscle from our patient and provide further evidence that CPT I and CPT II are different proteins in skeletal muscle. The inability of mitochondrial preparations from the patient's muscle to oxidise palmitate can be explained by the defect of CPT II activity, although CPT I activity was apparently normal.

There are several reports of CPT deficiency in muscle (see [11,12]); of these, one case was thought to be a defect of CPT I and a number to be defects of CPT II [10,20–28]. The interpretation of these reports is often uncertain because of the different assays used. High concentrations of palmitoylcarnitine, usually included in the exchange assay (>0.5 mM) and routinely in hydroxamate assays (>4 mM), inhibit CPT I [11,12]. Low activity due to deficiency of CPT II may therefore be interpreted as a combined deficiency. Most workers have used frozen samples of muscle and CPT activities in preparations from fresh and frozen muscle do not appear to have been compared in previous studies (fig.2).

Some workers have measured CPT by both forward and exchange assays. Scholte et al. measure CPT I and CPT II selectively in disrupted mitochondria. CPT I is assayed in the forward direction with 10 μM palmitoyl-CoA and 0.25 mM L-carnitine as substrates, when only CPT I is measured because of its low K_m for carnitine [11]. The exchange assay measures both CPT I and CPT II at low concentrations (<50 μM) of palmitoylcarnitine, however, at >0.7 mM, and in the presence of 2.5 mM carnitine, most of the activity is due to CPT II [11]. Zierz and Engel assayed CPT in muscle homogenates from 7 cases of CPT deficiency after freeze-thawing and sonication using both the forward and exchange assays employing a range of palmitoyl-CoA and palmitoylcarnitine concentrations. They assumed that CPT I and CPT II are the same protein and concluded that there is a defective form of CPT with altered kinetics which is abnormally sensitive to inhibition by detergents, palmitoyl-CoA,

palmitoylcarnitine and malonyl-CoA, rather than the presence of normal CPT I and absence of CPT II [17].

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