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Short Communication

Broad specificity of carnitine palmitoyltransferase II towards long-chain acyl-CoA β -oxidation intermediates and its practical approach to the synthesis of various long-chain acylcarnitines

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Long-chain fatty acid β -oxidation defects involving 3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) or mitochondrial trifunctional protein (MTP) (Pollitt 1995) are characterized by abnormal urinary organic acids and specific plasma acylcarnitine profile during crises (Dorland et al 1995). The same atypical acylcarnitine esters have also been found in *in vitro* studies comprising the incubation of patient's cells (Nada et al 1995; Schaefer et al 1995) with long-chain fatty acids. An explanation for the finding of these abnormalities might be that the different acyl-CoA esters that accumulate within the mitochondrial matrix are exported to the cytosol in the acylcarnitine form. The mechanism associated with this process is unclear but may primarily involve carnitine palmitoyltransferase (CPT) II catalysing the conversion of the intramitochondrial long-chain acyl-CoA esters into the corresponding acylcarnitines followed by export from the mitochondria via the acylcarnitine/carnitine carrier. While studying the specificity of CPT towards palmitoyl-CoA and its β -oxidation intermediates, we found that CPT II accepts as substrates not only acyl-CoA esters but also 2,3-unsaturated, 3-hydroxy and 3-keto acyl-CoA esters.

In the present paper we have made use of the reactivity of CPT II towards 3-hydroxypalmitoyl-CoA to synthesize 3-hydroxypalmitoylcarnitine enzymatically. The synthesis of this compound and other 3-hydroxyacylcarnitines is important for the qualitative and quantitative analysis of the acylcarnitine profile in LCHAD and MTP deficiencies. These

substances may also be useful for experiments directed towards solving the problems of the peculiar clinical findings in LCHAD deficiency.

METHODS

Measurement of the CPT II activity towards 3-hydroxypalmitoyl-CoA: Measurement of CPT II activity was based on the work of Scholte and coworkers (1979). To a standard mixture composed of 50mmol/L Hepes–NaOH pH 7.4 (Sigma), 150mmol/L KCl, 1mmol/L EDTA (Merck), 1mmol/L dithiothreitol (DTT) (Boehringer), 0.5mmol/L L-carnitine (Sigma) and 200000dpm of L-[N-methyl-¹⁴C]carnitine-HCl (Nec, Dupont) several concentrations of 3-hydroxypalmitoyl-CoA (enzymatically synthesized by methods developed in our laboratory) were added from a stock solution in 20mmol/L MES buffer (Sigma) pH 6.0. The incubations at 37°C were started by the addition of 1.3U/μl of purified CPT II (generously offered by L.L. Bieber, Michigan State University, Michigan, USA). After 5min the reactions were stopped with 0.6mol/L HCl and the [¹⁴C]acylcarnitines produced were extracted with n-butanol. The organic phase was collected into a scintillation counting vial and radioactivity was determined. These assays were performed in the presence or absence of 20μmol/L bovine serum albumin (BSA).

Synthesis of 3-hydroxypalmitoylcarnitine: To the standard mixture described above (with the exception of DTT) 20μmol/L BSA, 500μmol/L L-carnitine and 100μmol/L of 3-hydroxypalmitoyl-CoA (in 20mmol/L MES pH 6.0) were added. The synthesis (37°C) was started by the addition of 1.3U/μl of purified CPT II. The production of 3-hydroxypalmitoylcarnitine was followed by the release of CoA, measured in several aliquots taken from the reaction medium after a 10min incubation period, by the colorimetric reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Fluka) (412nm). After reaching a plateau, the reaction was stopped with 0.6mol/L HCl and the mixture neutralized to pH 6–7. The product was then applied to a C8 (6ml) column (Baker) equilibrated with 3ml methanol and 3ml 50mmol/L Tris-HCl pH 8.0. The column was washed with 5×1 ml 50mmol/L Tris-HCl pH 8.0 and 5×1 ml methanol–50mmol/L Tris-HCl pH 8.0 (70/30) and the acylcarnitine (free of CoA) was eluted with 5×1 ml methanol–50mmol/L Tris-HCl pH 8.0 (90/10). To achieve further separation between 3-hydroxypalmitoyl-CoA and 3-hydroxypalmitoylcarnitine we used the acylcarnitine extraction procedure developed by Costa et al (in press) based on the use of a PRS-propylsulfonic acid (strong cation exchange) column. After elution, samples were taken in acetonitrile (for FAB-MS analysis) or 20mmol/L MES buffer pH 6.0.

After the purification steps described above, the content of acylcarnitine was measured by the measurement of carnitine after hydrolysis described by Barth et al (1983). To identify and verify the purity of the 3-hydroxypalmitoylcarnitine synthesized, the compound was derivatized to a butyl ester and further analysed by fast-atom bombardment mass spectrometry (FAB-MS) (Dorland et al 1995).

RESULTS AND DISCUSSION

We have shown that carnitine palmitoyltransferase II accepts as substrates not only acyl-CoA esters but also other intermediates of fatty acid β-oxidation, in particular

Figure 1 Activity of purified carnitine palmitoyltransferase II towards 3-hydroxypalmitoyl-CoA and the effect of addition of 20 $\mu\text{mol/L}$ of bovine serum albumin to the reaction medium

3-hydroxyacyl-CoA esters (Figure 1). These findings led us to develop an enzymatic method for the synthesis of 3-hydroxyacylcarnitines, which are important compounds for the analysis of the acylcarnitine profile in long-chain fatty acid β -oxidation disorders. Using purified CPT II and in the presence of L-carnitine and BSA (to reduce the substrate inhibition observed in its absence (Figure 1)), it was possible to convert 3-hydroxypalmitoyl-CoA into 3-hydroxypalmitoylcarnitine with an overall recovery rate of about 10%. The FAB-MS spectrum of the 3-hydroxypalmitoylcarnitine synthesized in this way showed that it is of high purity, thus suggesting that the method developed is effective. Since CPT II may also be reactive with other 3-hydroxyacyl-CoAs, we suggest that the method described here can also be used for the synthesis of other 3-hydroxyacylcarnitines. A disadvantage of the method presented is the need for purified CPT II as a source of enzyme. Other enzymatic sources such as partially purified mitochondrial membranes, which are a good source of CPT II activity, were also tried (results not shown). However, the purity of the final product was much less than observed with purified CPT II. The availability of CPT II cDNA will enable easy production of CPT II, thus providing enough enzyme for the synthesis of larger amounts of 3-hydroxyacylcarnitines and other long-chain acylcarnitine β -oxidation intermediates.

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