Complete β-oxidation of valproate: cleavage of 3-oxovalproyl-CoA by a mitochondrial 3-oxoacyl-CoA thiolase

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The β-oxidation of valproic acid (VPA; 2-n-propylpentanoic acid) was investigated *in vitro* in intact rat liver mitochondria incubated with ^{3}H-labelled VPA. The metabolism of [4,5-$^3H_2$]VPA and [2-^{3}H]VPA was studied by analysing the different acyl-CoA intermediates formed by reverse-phase HPLC with radiochemical detection. Valproyl-CoA, $\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-oxovalproyl-CoA (labelled and non-labelled) were determined using continuous on-line radiochemical and UV detection. The formation of these intermediates was investigated using the two tritiated precursors in respiratory states 3 and 4. Valproyl-CoA was present at highest concentrations under both conditions. Two distinct labelled peaks were found, which were identified as ^3H_2O and [4,5-$^3H_2$]3-oxo-VPA. The formation of ^3H_2O strongly suggested that VPA underwent complete β-oxidation and that [4,5-$^3H_2$]3-oxo-VPA was formed by hydrolysis of the corresponding thioester. The hypothesis that 3-oxovalproyl-CoA undergoes thiolytic cleavage was investigated further. For this purpose a mitochondrial lysate was incubated with synthetic 3-oxovalproyl-CoA, carnitine and carnitine acetyltransferase for subsequent monitoring of the formation of propionylcarnitine and pentanoylcarnitine using electrospray ionization tandem MS. The detection of these compounds demonstrated unequivocally that the intermediate 3-oxovalproyl-CoA is a substrate of a mitochondrial thiolase, producing propionyl-CoA and pentanoyl-CoA, thus demonstrating the complete β-oxidation of VPA in the mitochondrion. Our data should lead to a re-evaluation of the generally accepted concept that the biotransformation of VPA by mitochondrial β-oxidation is incomplete.

Key words: fatty acid β-oxidation, mitochondrial thiolases, valproate metabolism, valproic acid.

INTRODUCTION

Fatty acid β-oxidation is inhibited by valproic acid (VPA; 2-n-propylpentanoic acid) in both rats and humans [1–4], either due to sequestration of free CoA [5] or via direct inhibition of one or more of the enzymes or transporters involved. Previous studies on the mitochondrial metabolism of VPA provided evidence that it is first activated into valproyl-CoA [1,4] and then dehydrogenated to form $\Delta^{2(E)}$-valproyl-CoA by 2-methyl-branched-chain acyl-CoA dehydrogenase [6], followed by hydration to 3-hydroxyvalproyl-CoA and dehydrogenation to 3-oxovalproyl-CoA (3-ketovalproyl-CoA) [7]. The available evidence indicates that VPA does not undergo complete β-oxidation, since cleavage of 3-oxovalproyl-CoA does not appear to occur [7,8]. This would imply accumulation of 3-oxovalproyl-CoA in the mitochondria, slowly producing the free acid form by hydrolysis. In line with this concept, 3-oxo-VPA is the mitochondrial metabolite of VPA that is found to be excreted *in vivo* in large amounts [9,10].

We have recently reported quantitative data on valproate acyl-CoA intermediates in intact respiring mitochondria [11]. In the present paper we have re-investigated the mitochondrial metabolism of VPA in order to elucidate the fate of these β-oxidation intermediates and the enzymic basis of the reactions involved. The results presented here show for the first time that the intermediate 3-oxovalproyl-CoA undergoes thiolytic cleavage by a mitochondrial 3-oxoacyl-CoA thiolase, thereby demonstrating the complete β-oxidation of valproate.

EXPERIMENTAL

Materials

VPA, BSA (fatty acid free), bicinchoninic acid, propionyl-CoA, L-carnitine and other standard biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $\Delta^{2(E)}$-VPA (2-n-propyl-2-pentenoic acid) was kindly donated by Professor D. Lindhout (Erasmus University of Rotterdam, The Netherlands). Carnitine acetyltransferase (CAT; 80 units/mg), CoA (grade II; trilithium salt), ADP and ATP were supplied by Boehringer (Mannheim, Germany). Lichrosolv®-grade solvents for HPLC were from Merck (Darmstadt, Germany).

^3H_2O was obtained from Amersham Pharmacia Biotech. [4,5-$^3H_2$]VPA (specific radioactivity 50.0 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, U.S.A.), and [2-^{3}H]VPA (sodium salt; specific radioactivity 15.0 Ci/mmol) was acquired from ARC-Sanver Tech (St. Louis, MO, U.S.A.). Ultima flow AP scintillation cocktail was purchased from Packard (Groningen, The Netherlands).

Abbreviations used: VPA, valproic acid (2-n-propylpentanoic acid); $\Delta^{2(E)}$-VPA, 2-n-propyl-2-pentenoic acid; CAT, carnitine acetyltransferase; radio-HPLC, HPLC with radiochemical detection; ESI-MS/MS, electrospray ionization tandem MS.

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Preparation of mitochondria from rat liver

Adult male Wistar rats (approx. 250 g) were starved for 18 h and then used for the preparation of mitochondria according to an established procedure [11]. Mitochondrial protein concentration was determined using the bicinchoninic acid (BCA) assay [12] with BSA as a reference substance.

Synthesis of valproyl-CoA, $\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-oxovalproyl-CoA

The CoA thioesters of VPA, $\Delta^{2(E)}$-VPA and 3-hydroxy-VPA were prepared by the anhydride method using ethylchloroformiate, adapted from the method of Rasmussen et al. [13]. $\Delta^{2(E)}$-Valproyl-CoA was used as a precursor for the synthesis of 3-oxovalproyl-CoA, using solubilized rat liver mitochondria as a source of enzyme catalysing the production of 3-oxovalproyl-CoA from $\Delta^{2(E)}$-valproyl-CoA. The detailed procedures for the synthesis, identification, purification and quantification of valproyl-CoA, $\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-oxovalproyl-CoA have been published elsewhere [11].

Mitochondrial incubations with [^{3}H]VPA

Incubations were performed at 37 °C in a final volume of 250 μl containing freshly isolated mitochondria (5 mg/ml) suspended in the basal isotonic isolation buffer at pH 7.4 (250 mM sucrose, 5 mM NaMops and 2 mM EGTA) held in a shaking water bath. Incubations for the analysis of intermediates were started by the addition of 20 pmol of [4,5-$^3H_2$]VPA or 60 pmol of [2-^{3}H]VPA after previous evaporation of the ethanol present in the stock solution and redissolution in the incubation buffer.

Studies were performed with and without the simultaneous inclusion of non-labelled VPA (0.1–2.0 mM). Reactions were stopped at appropriate times and samples were prepared, as described previously [11], for analysis by HPLC with radiochemical detection (radio-HPLC). When the acyl-CoA esters were to be analysed in the two fractions (pellet and supernatant), reactions were terminated at appropriate times by immediate centrifugation (17000 ***g***, 3 min, 4 °C). After separation of the supernatant and redissolution of the pellet in 100 μl of incubation buffer, both fractions were acidified with 25 μl of 2 M HCl. After centrifugation (17000 ***g***, 5 min, 4 °C), the supernatants of both fractions were neutralized. The pH was checked, and after a further centrifugation (17000 ***g***, 5 min, 4 °C), the supernatants were collected and kept at −20 °C until analysis by radio-HPLC. An aliquot (75 μl) of each sample was applied to the column of the HPLC system. Prior to radio-HPLC analysis, the radioactivity of an aliquot of each series of samples was measured in a liquid scintillation counter (Beckman).

Rat liver mitochondria were also incubated in state 3, using both ^{3}H-labelled VPA substrates, with and without non-labelled VPA, in the presence of 4 mM $MgCl_2$, 0.5 mg/ml BSA, 0.5 mM malate, 1 mM ADP and 4 mM ATP. Under these conditions, the effect of the respiratory substrate succinate (10 mM; plus 5 μg/ml rotenone) on the acyl-CoA profiles of VPA β-oxidation intermediates was also analysed.

Alkaline hydrolysis of the mitochondrial incubations was performed by adding the medium (250 μl) to 100 μl of 2 M NaOH followed by heating at 50 °C for 30 min. After this period, 100 μl of 2 M HCl was added to the samples and the pH was brought to 6 by the addition of 1 M acetate buffer. Protein was removed by centrifugation (17000 ***g***, 5 min, 4 °C) and the supernatant was analysed by radio-HPLC.

Radio-HPLC analysis of VPA acyl-CoA esters

A Waters model 510 HPLC pump was used as the solvent delivery system, with a Waters gradient controller model 680. The samples were introduced by an automated Gilson 231 injector. The column was a Supelcosil LC-18-S (5 μm; 250 mm × 4.6 mm). Resolution of the different valproyl-CoA metabolites was achieved by linearly increasing the acetonitrile content of the 16.9 mM sodium pyrophosphate (pH 6.9) elution buffer from 10 % to 45 % (v/v) in 20 min at a flow rate of 1 ml/min. The column was washed for 5 min with 70 % acetonitrile and then re-equilibrated for 15 min under the starting conditions in a total run time of 42 min.

Acyl-CoA esters were detected with an LDC/Milton Roy SM 4000 UV detector (λ 258 nm), and the radioactivity of the eluted compounds was measured on-line with a radiomatic 525 TR flow scintillation analyser (Packard) containing a mixing cell of 500 μl volume. The UV detector and the radioactivity monitor were connected in series. The time lag between the detectors was determined with the valproate acyl-CoA ester intermediates (labelled and non-labelled).

Identification of ^3H_2O

At appropriate times, samples were acidified and neutralized after centrifugation. Samples were loaded on top of an ion-exchange column (Dowex 1 × 8-200), which was then washed with 2 × 1 ml of water. The total eluate was divided into three aliquots. The radioactivity of the first aliquot was measured directly in a liquid scintillation counter. The second aliquot was injected on to the radio-HPLC system, and the retention time of the observed peak was compared with that of a pure standard of ^3H_2O. The third fraction was evaporated to dryness under a nitrogen stream after adjusting the pH to 7–8; the radioactivity remaining in the dry residue was then determined.

Identification of 3-oxo-VPA by GC-MS

Incubations identical to those described above were performed for a 1 h period using non-labelled VPA as substrate. Samples were subsequently analysed using HPLC with UV detection. In order to obtain enough material for GC-MS analysis, repeated injections were performed and fractions containing the unknown intermediate were combined. Collection was based on a previously established and precise retention time window, since the compound did not absorb at 258 nm. The isolated 3-oxo-VPA was extracted further and derivatized for GC-MS analysis. First the acetonitrile content of the fraction was removed by evaporation under nitrogen at 37 °C, and the remaining aqueous solution was acidified to pH 1–2 with 4 % (w/v) HCl. Then 50 μl of an internal standard (3-phenylbutyric acid) and 200 μl of *o*-ethylhydroxylamine hydrochloride were added. After 2 h at room temperature, the NaCl-saturated solution was subjected to repeated extraction (× 3) with ethyl acetate. The organic phases were collected, dried with anhydrous sodium sulphate and decanted. The solvent was evaporated to dryness under nitrogen at 37 °C. Then 50 μl of derivatization reagent consisting of bis(trimethylsilyl)trifluoroacetamide/pyridine/trimethylchlorosilane (5:1:0.01, by vol.) was added, and the solution was left at room temperature for 1 h. An aliquot of 1 μl of the final solution was analysed by capillary GC (CPSil-19CB) coupled to a quadrupole mass detector in electron-impact mode (QP 2000; Shimadzu, Kyoto, Japan). A blank was run in parallel which corresponded to mitochondria incubated in the absence of VPA.

Metabolism of 3-oxovalproyl-CoA

Isolated mitochondria kept at −70 °C were thawed in iced water, diluted with PBS and subjected to mild sonication (2 × 10 s, 80 W, 4 °C) to ensure complete lysis of the organelles. Incubations were carried out in a shaking water bath at 37 °C in a final volume of 100 μl. Reactions were started by the addition of 3-oxovalproyl-CoA (75 μM) to a mixture, preincubated for 5 min at 37 °C, containing mitochondrial lysate (5 mg/ml), 100 mM Tris/HCl, pH 8.3, 25 mM $MgCl_2$, 0.2 mg/ml BSA, 100 μM CoA, 5 μl of CAT and 2.5 mM carnitine, with or without 50 mM KCl. Incubations were allowed to proceed and then terminated at appropriate times (0–4 h) as described below. The progress of the reactions was monitored by the measurement of both acyl-CoA intermediates using HPLC with UV detection and acylcarnitines using electrospray ionization tandem MS (ESI-MS/MS). For the measurement of acyl-CoA esters, the mixture was acidified to pH 1.0 with 2 M HCl to stop the reaction, centrifuged (17000 ***g***, 5 min, 4 °C), neutralized to pH 5–6 with 2 M KOH/0.6 M Mes and centrifuged again for 5 min. The supernatant was used for injection into HPLC. For the measurement of acylcarnitines, reactions were stopped by the addition of 100 μl of acetonitrile. Precipitated protein was removed by centrifugation (17000 ***g***, 5 min, 4 °C) and 100 μl of the supernatant was processed further for ESI-MS/MS.

In parallel, similar incubations with 3-oxovalproyl-CoA were performed in the absence of carnitine and CAT. Incubations were allowed to proceed for the appropriate times, stopped with HCl and neutralized as described above. A part of this sample was used directly for acyl-CoA analysis by HPLC, and another aliquot was added in a 10:1 (v/v) ratio to a solution containing 50 mM carnitine and 5 μl of CAT. Thereafter, incubations were allowed to proceed for 60 min at 37 °C, stopped by the addition of acetonitrile and then centrifuged, and acylcarnitines were measured in the supernatant by ESI-MS/MS as described below. In some experiments propionyl-CoA was used instead of 3-oxovalproyl-CoA.

Analysis of acylcarnitines by ESI-MS/MS

After addition of 50 μl of a standard mixture (5 μmol/l [$^2H_3$]propionylcarnitine, 2 μmol/l [$^2H_3$]octanoylcarnitine and 2 μmol/l [$^2H_3$]palmitoylcarnitine in acetonitrile) to the samples, they were deproteinized with acetonitrile. Following centrifugation, the respective supernatants were dried under a stream of nitrogen at 45 °C and subsequently derivatized with 100 μl of 3 M butanol/HCl for 15 min at 60 °C. The samples were dried under nitrogen at 45 °C and redissolved in 300 μl of acetonitrile. Prior to injection, 70 μl of the acetonitrile solution was mixed with 30 μl of water.

Acylcarnitines were measured as precursor ions of mass 85. Scanning was performed from 200 to 550 Da for 2 min on a Micromass Quattro II triple-quadrupole mass spectrometer, using a Gilson 231 XL autosampler and a Hewlett-Packard HP-1100 HPLC pump, according to published procedures [14].

RESULTS

Radio-HPLC analysis and identification of VPA-derived metabolites

In the experiment depicted in Figure 1, isolated rat liver mitochondria were incubated with [4,5-$^3H_2$]VPA for 10 min and the reactions were terminated by the addition of HCl. After neutralization to pH 6.0, the acyl-CoAs in the supernatant were analysed by HPLC. Figure 1(A) shows the profile of radioactivity

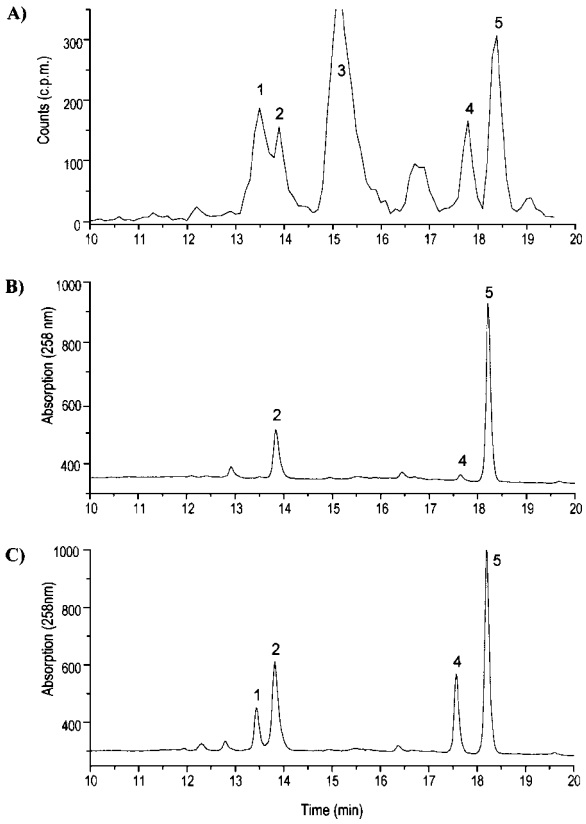


Figure 1 HPLC profiles obtained for radiochemical and UV detection of VPA-acyl-CoA intermediates

See the Experimental section for details. Identification of peaks: 1, 3-hydroxyvalproyl-CoA; 2,3-oxovalproyl-CoA; 3, VPA (remaining substrate); 4, $\Delta^{2(E)}$-valproyl-CoA; 5, valproyl-CoA. (**A**) Radiochromatogram of VPA acyl-CoA metabolites derived from rat liver mitochondria (respiration state 4) incubated with [4,5-$^3H_2$]VPA for 10 min (numbers correspond to the labelled analogues of the above-identified peaks). (**B**) HPLC profile obtained by UV detection of the above incubation mixture after addition of 0.1 mM non-labelled VPA. (**C**) Re-chromatography after spiking the above sample with a standard mixture of VPA-acyl-CoA esters (2.5 μM).

eluted from the column, and Figure 1(B) depicts UV absorbance at 258 nm, showing one major peak (peak 5) and one minor peak (peak 2). Figure 1(C) shows the A_{258} profile of a sample spiked with a mixture of the four mitochondrial VPA-derived acyl-CoA esters, i.e. valproyl-CoA, $\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-oxovalproyl-CoA. The results show that valproyl-CoA is the main metabolite formed, in both respiration state 3 and state 4.

A time course study was undertaken to analyse the metabolism of [4,5-$^3H_2$]VPA. Figure 2(a) shows the decrease in the concentration of [4,5-$^3H_2$]VPA with time, indicating its uptake and further biotransformation within mitochondria. Radio-HPLC was used to monitor the formation of the different acyl-CoA esters over time, and the four β-oxidation intermediates of VPA were compared. As shown in Figure 2(b), valproyl-CoA was the major metabolite, reaching its highest concentration within 6–10 min, followed by its progressive disappearance. This time course curve was obtained by UV detection after incubating rat liver mitochondria with ^{3}H-labelled substrate and 0.1 mM non-labelled VPA. For incubations performed with only the labelled substrate, similar progress curves were obtained for the four intermediates (results not shown), although the maximum values at 10 min were much lower.

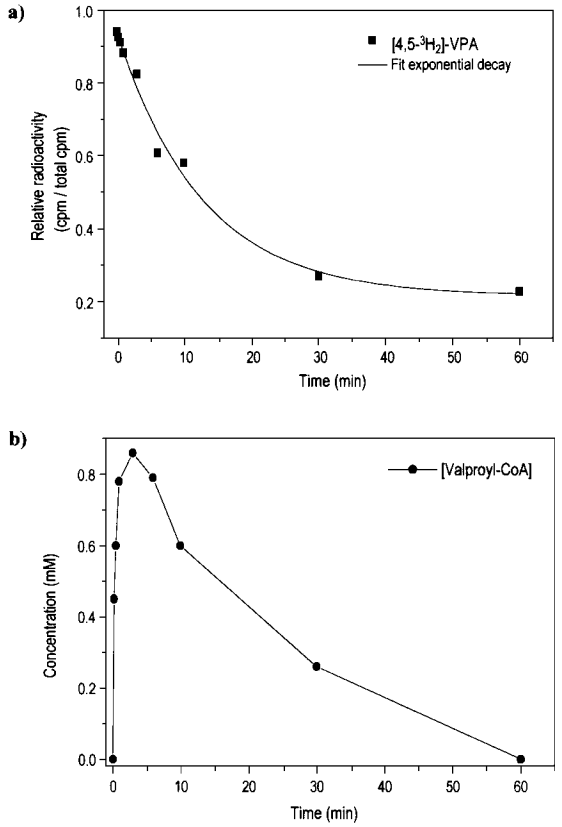


Figure 2 Consumption of [4,5-$^3H_2$]VPA (a) and formation of valproyl-CoA (b) with time

Rat liver mitochondria were incubated in the standard reaction medium supplemented with [4,5-$^3H_2$]VPA (state 4). The disappearance of [4,5-$^3H_2$]VPA and the formation of valproyl-CoA were determined as described in the Experimental section.

Of the four acyl-CoA derivatives, 3-hydroxyvalproyl-CoA ester was the least retained compound, eluting at approx. 13 min (Figure 1). However, two unknown compounds eluting earlier than 3-hydroxyvalproyl-CoA were observed by radiodetection. Inspection of the UV profile indicated that these compounds did not absorb at 258 nm, implying that they are probably not acyl-CoAs. As depicted in Figure 3, where they are identified as peaks A and B, the formation of these metabolites increased with time up to 2 h of incubation.

The unknown metabolite A (Figure 3) was identified as ^3H_2O by the following observations. First, the compound identified as peak A had exactly the same retention time as authentic ^3H_2O. Furthermore, the material was not retained on either a cation- or an anion-exchange column, indicating its non-ionic nature. A different precursor labelled at the α-position in the molecule, [2-^{3}H]VPA, represented a more straightforward substrate for the characterization of peak A. Figure 4 shows the VPA acyl-CoA intermediates formed during incubation of rat liver mitochondria with this substrate. As expected, two main products were formed from this acid, i.e. ^3H_2O and [2-^{3}H]valproyl-CoA.

The unknown metabolite B (Figure 3) was identified as [4,5-$^3H_2$]3-oxo-VPA by GC-MS analysis of the corresponding HPLC peak fraction. The mass spectrum of the isolated compound proved to be identical with that of 3-oxo-VPA, as described in literature for the trimethylsilyl derivative [15].

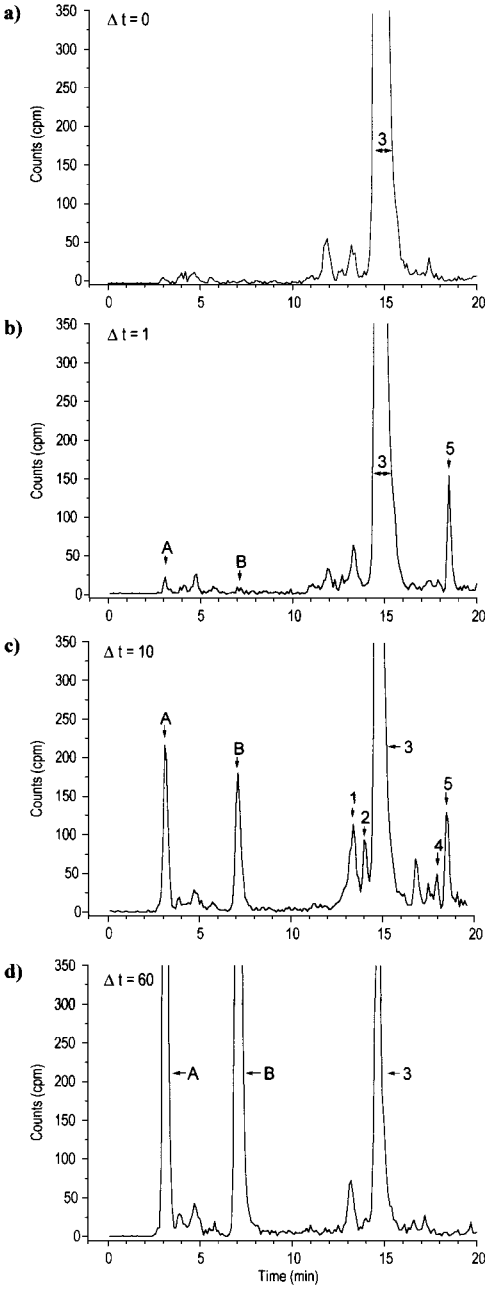


Figure 3 Time course of the formation of VPA metabolites (peaks A and B) derived from incubation of rat liver mitochondria with [4,5-$^3H_2$]VPA (state 4), detected by radio-HPLC

Incubations were carried out for (**a**) 0 min, (**b**) 1 min, (**c**) 10 min and (**d**) 60 min. Identification of peaks: A, ^3H_2O; B, [4,5-$^3H_2$]3-oxo-VPA; 1, [4,5-$^3H_2$]3-hydroxyvalproyl-CoA; 2, [4,5-$^3H_2$]3-oxovalproyl-CoA; 3, [4,5-$^3H_2$]VPA; 4, [4,5-$^3H_2$]Δ^2-valproyl-CoA; 5, [4,5-$^3H_2$]valproyl-CoA.

Study of the thiolytic cleavage of 3-oxovalproyl-CoA

The formation of ^3H_2O from [4,5-$^3H_2$]VPA suggests that 3-oxovalproyl-CoA is cleaved by a mitochondrial thiolase, giving rise to propionyl-CoA and pentanoyl-CoA. Based on the findings that these two short-chain CoA esters were not detected using our methods of HPLC and radio-HPLC, we developed a new system (Scheme 1) to test this hypothesis. The method is based

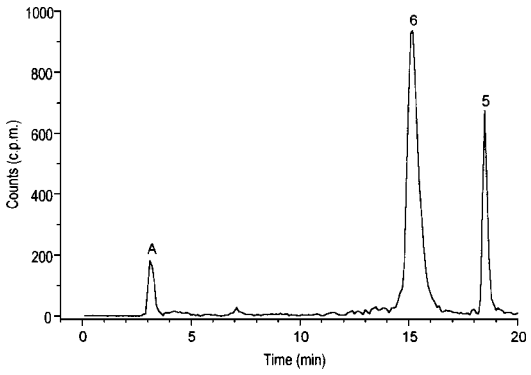


Figure 4 Radio-HPLC analysis of VPA acyl-CoA intermediates formed on incubation of rat liver mitochondria with [2-^{3}H]VPA for 10 min (state 4)

Identification of peaks: A, ^3H_2O; 6, [2-^{3}H]VPA (remaining substrate); 5, [2-^{3}H]valproyl-CoA.

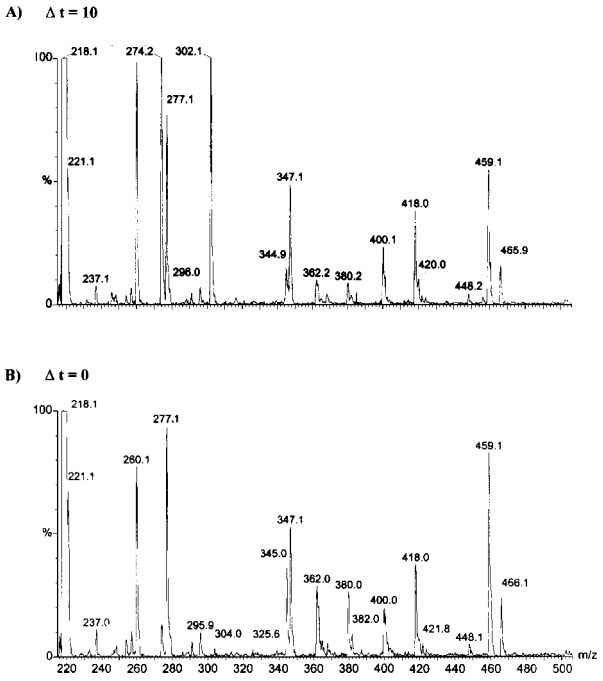


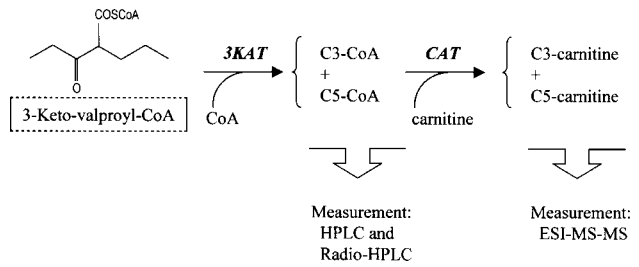
Figure 5 Profile of acylcarnitines produced from 3-oxovalproyl-CoA incubated for 10 min with rat liver mitochondrial lysate, CAT and carnitine, analysed by ESI-MS/MS using *m/z* 85 precursor ion scans

See the Experimental section for details. (**A**) Incubation time of 10 min; (**B**) incubation time of 0 min (basal conditions). Identification: carnitine, *m/z* 218.1; acetylcarnitine, *m/z* 260.1; propionylcarnitine, *m/z* 274.2; [$^2H_3$]propionylcarnitine (internal standard), *m/z* 277.1; pentanoylcarnitine, *m/z* 302.1; [$^2H_3$]octanoylcarnitine (internal standard), *m/z* 347.1; [$^2H_3$]palmitoylcarnitine (internal standard), *m/z* 459.1.

on the addition of CAT and carnitine to the reaction medium to ensure conversion of the short-chain acyl-CoAs into propionylcarnitine and pentanoylcarnitine. The acylcarnitine esters produced in this system were then analysed by ESI-MS/MS. The validity of the method employed was tested in parallel using different acyl-CoA esters, including propionyl-CoA, acetoacetyl-CoA, 2-methylacetoacetyl-CoA and 3-oxopalmitoyl-CoA, instead of 3-oxovalproyl-CoA. Measurement of the respective acylcarnitines established the usefulness and efficiency of the method (results not shown). CAT was able to convert all of the acyl-CoAs, even the C_{16} substrate, into their carnitine esters.

Figure 5 depicts the acylcarnitine profiles measured in a mitochondrial lysate incubated with 3-oxovalproyl-CoA, CAT and carnitine. The clear increases in the mass peaks at 274.2 and 302.1 indicate the progressive production of propionylcarnitine and pentanoylcarnitine respectively, which shows unequivocally that 3-oxovalproyl-CoA undergoes thiolytic cleavage in mitochondria. The decrease in the amount of substrate was confirmed in parallel incubations monitored by HPLC analysis. The rate of consumption of 3-oxovalproyl-CoA and the rate of production of pentanoylcarnitine, during the first 5 min of incubation, were calculated. Approximate values of 0.04 nmol/min per mg of protein for consumption and 0.016 nmol/min per mg of protein for production were established. These data suggest that only 40% of the substrate undergoes thiolytic cleavage and that the remaining 60% disappears, most probably due to hydrolysis.

Mitochondria contain several distinct 3-oxoacyl-CoA thiolases, including a K^+-dependent acetoacetyl-CoA thiolase [16,17]. In order to investigate whether 3-oxovalproyl-CoA is metabolized by this thiolase, we studied the cleavage of 3-oxovalproyl-CoA with omission of potassium from the incubation medium (results not shown). The amounts of both propionylcarnitine and pentanoylcarnitine produced from 75 μmol/l substrate, with and without the addition of potassium, reached a maximum of 10–15 μmol/l after 60 min, and these values were virtually unaffected by exogenous potassium.



Scheme 1 Experimental design for the study of the thiolytic cleavage of 3-oxovalproyl-CoA

Abbreviations: 3KAT, 3-oxoacyl-CoA thiolase; C3, propionyl; C5, pentanoyl. 3-Keto-valproyl-CoA = 3-oxovalproyl-CoA.

DISCUSSION

In the present study the metabolism of VPA in rat liver mitochondria was addressed, with specific emphasis on the question of whether VPA undergoes full β-oxidation to propionyl-CoA and pentanoyl-CoA. A combined approach was used, based on HPLC with radiochemical and UV detection, to resolve the various VPA-derived acyl-CoA esters and radiolabelled VPA to trace the VPA-derived compounds. Two distinctly labelled substrates, [4,5-$^3H_2$]VPA and [2-^{3}H]VPA, were incubated with intact rat liver mitochondria in respiration state 3 or state 4. The first of the two substrates allows the study of all four sequential steps of β-oxidation, since ^{3}H will be retained in all VPA-derived CoA esters. On the other hand, use of

[2-^{3}H]VPA gives information on the first step of VPA β-oxidation, which is catalysed by the enzyme short-branched-chain acyl-CoA dehydrogenase [6].

The observation that ^3H_2O was formed from [4,5-$^3H_2$]VPA was a first clue that valproate undergoes full mitochondrial β-oxidation, thus implying the thiolytic cleavage of 3-oxovalproyl-CoA. However, the direct products of such a cleavage, propionyl-CoA and pentanoyl-CoA, were not detected, most probably due to their rapid subsequent metabolism in intact mitochondria. In addition to ^3H_2O, we also detected 3-oxovalproate, which is probably produced from 3-oxovalproyl-CoA via some acyl-CoA thioesterase present in mitochondria [18]. The resulting decrease in the amount of substrate available for further cleavage may also contribute to the inability to detect the respective reaction products.

In order to verify the initial hypothesis of the full β-oxidation of VPA, a new approach was then devised using mitochondrial lysates and authentic 3-oxovalproyl-CoA [11] along with carnitine plus CAT to allow rapid trapping of propionyl-CoA and pentanoyl-CoA in the form of their corresponding carnitine esters. In these experiments, the detection of propionylcarnitine and pentanoylcarnitine provided direct evidence that 3-oxovalproyl-CoA indeed undergoes thiolytic cleavage to propionyl-CoA and pentanoyl-CoA. An advantage of this method is the elimination of potential problems introduced by the presence of mitochondrial acyl-CoA thioesterases, which may convert propionyl-CoA and pentanoyl-CoA into the free acids. Indeed, when we added propionyl-CoA and/or pentanoyl-CoA to sonicated mitochondria, both CoA esters underwent rapid hydrolysis to free CoA-SH and the two corresponding acids. These findings provide an explanation for the earlier results of Li et al. [7], who did not detect the formation of propionyl-CoA or pentanoyl-CoA during their β-oxidation studies, which was one of the main reasons leading to their conclusion that 3-oxovalproyl-CoA does not undergo thiolytic cleavage.

Furthermore, VPA branched-chain acyl-CoA esters, as compared with straight-chain acyl-CoAs, seem to have a greater resistance to hydrolysis. This observation has also been reported by others [7], and it is probably due to the steric hindrance caused by the n-propyl branch within the respective structures. The lower activities of the acyl-CoA thioesterases towards valproyl-CoA and branched-chain metabolites ($\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 3-oxovalproyl-CoA) are, however, probably still higher than that of the thiolase acting on 3-oxovalproyl-CoA, which would explain the accumulation of 3-oxo-VPA and the less extensive thiolytic cleavage of 3-oxovalproyl-CoA.

The results obtained also suggest that the potassium concentration in the mitochondrial matrix does not significantly affect the activity of the 3-oxoacyl-CoA thiolase involved in β-oxidation of VPA. This may exclude the possibility of the participation of acetoacetyl-CoA thiolase and long-chain 3-oxoacyl-CoA thiolase in the metabolism of VPA.

Based on the results described in the present paper, we propose that valproate may undergo two distinct routes of mitochondrial degradation, which follow a common pathway until 3-oxovalproyl-CoA but then branch. One pathway involves the thiolytic cleavage of 3-oxovalproyl-CoA by one of the mitochondrial thiolases to form propionyl-CoA and pentanoyl-CoA, accomplishing one complete cycle of β-oxidation. The other route involves the hydrolysis of 3-oxovalproyl-CoA by some unknown acyl-CoA thioesterase to produce 3-oxo-VPA plus CoA-SH. This finding is relevant to the understanding of the mitochondrial metabolism of valproate, because it provides grounds to question one of the main assumptions in current thinking, namely that thiolytic cleavage does not occur in valproate β-oxidation. This will therefore open up new research perspectives on the complex metabolic pathways of valproate.

This work was partially funded by Fundação para a Ciência e a Tecnologia, Programa Praxis XXI (project PSAU/27/96), and by a grant (BD/3809/94) awarded to M. F. B. S.

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Received 20 July 2001/16 November 2001; accepted 19 December 2001