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Valproic acid utilizes the isoleucine breakdown pathway for its complete β-oxidation

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ABSTRACT

Valproic acid (VPA) is a simple branched medium-chain fatty acid with expanding therapeutic applications beyond its prime anticonvulsant properties.

Aims: (1) To resolve the underlying basis for the interference of valproate with the isoleucine degradative pathway and (2) to shed new light on the enzymology of the β-oxidation pathway of valproate.

Methods: Urine organic acids were analyzed by gas chromatography/mass spectrometry. In vitro studies were performed with heterologously expressed human 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) and fibroblasts from controls and a patient with MHBD deficiency using 2-methyl-3-hydroxybutyryl-CoA and 3-hydroxyvalproyl-CoA as substrates. The respective enzymatic activities were measured using optimized HPLC procedures. Short-chain enoyl-CoA hydratase (ECHS1) immunoprecipitation in a human liver homogenate was performed and hydratase activity was measured in the supernatants by HPLC, using crotonyl-CoA and $\Delta^{2(E)}$-valproyl-CoA as substrates.

Results: Patients on valproate therapy had a moderately increased urinary excretion of the isoleucine metabolite 2-methyl-3-hydroxybutyric acid. MHBD was found to convert 3-hydroxyvalproyl-CoA into 3-ketovalproyl-CoA. MHBD activity in control fibroblasts was comparable using both 2-methyl-3-hydroxybutyryl-CoA and 3-hydroxyvalproyl-CoA as substrates. In fibroblasts of a patient with MHBD deficiency, there was no detectable MHBD activity when 3-hydroxyvalproyl-CoA was used as substrate. Samples with immunoprecipitated crotonase had no detectable hydratase activity using both crotonyl-CoA and $\Delta^{2(E)}$-valproyl-CoA as substrates.

Discussion: This work demonstrates for the first time, that MHBD is the unique enzyme responsible for the dehydrogenation of 3-hydroxyvalproyl-CoA. Furthermore, we show that crotonase is the major, if not the single hydratase involved in VPA β-oxidation, next to its role in isoleucine catabolism.

1. Introduction

Valproic acid (VPA; 2-*n*-propylpentanoic acid) is an effective anticonvulsant which is widely used for the treatment of several types of seizures. Other clinical indications for VPA treatment include bipolar disorders, various psychiatric syndromes and migraine [1–3]. In addition, VPA has also been tested in the management of certain types of cancer [4] and in patients infected with the human immunodeficiency virus [5]. Despite its broad range of clinical use, VPA is associated with liver failure [6–8]. The physiological mechanisms underlying this hepatotoxicity have not been elucidated yet. It has been reported, however, that VPA interferes with several metabolic processes in mitochondria such as oxidative phosphorylation [9,10], gluconeogenesis [11], ureagenesis

Abbreviations: VPA, 2-*n*-propylpentanoic acid or valproic acid; $\Delta^{2(E)}$-VPA, 2-*n*-propyl-2-pentenoic acid; CoA, coenzyme A; MHBA, 2-methyl-3-hydroxybutyric acid; 3-OHVP-CoA, 3-hydroxyvalproyl-CoA; 2-Me-3-OHBut-CoA, 2-methyl-3-hydroxybutyryl-CoA; MHBD, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.178); ECHS1, short-chain enoyl-CoA hydratase or crotonase (EC 4.2.1.17); SBCAD, short branched-chain acyl-CoA dehydrogenase (EC 1.3.99.12).

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[12,13], branched-chain amino acid (BCAA) catabolism [14,15] and fatty acid oxidation (FAO) [16,17].

Considering its structure of a simple branched-chain fatty acid, VPA is a substrate for FAO, which takes place predominantly in mitochondria [18–22]. The mechanism used by VPA to cross the mitochondrial membrane is not clearly defined yet. However a carnitine independent process seems to be the most likely mechanism. Its biotransformation by means of β-oxidation involves the activation of valproic acid into valproyl-CoA inside the mitochondria, most probably by a medium-chain acyl-CoA synthetase [17]. In vitro studies have also reported the activation of VPA in the extramitochondrial compartment [23]. In the mitochondria, valproyl-CoA is converted into $\Delta^{2(E)}$-valproyl-CoA predominantly by short branched-chain acyl-CoA dehydrogenase (SBCAD) [15,24] and to a lesser extent by isovaleryl-CoA dehydrogenase (IVD), as recently demonstrated by our group [15]. $\Delta^{2(E)}$-valproyl-CoA is hydrated into 3-hydroxyvalproyl-CoA which is subsequently oxidized into 3-ketovalproyl-CoA. Both enzymatic reactions have been demonstrated [19,20]. However, the enzymes catalysing the hydration of $\Delta^{2(E)}$-valproyl-CoA and the oxidation of 3-hydroxyvalproyl-CoA have not been identified yet. The final reaction of the β-oxidation of VPA consists of the thiolytic cleavage of 3-ketovalproyl-CoA by a mitochondrial thiolase [21]. Although the complete β-oxidation of VPA has been reported [17,21], there is still a gap in knowledge regarding the enzymes involved in the intermediate steps of mitochondrial oxidation of VPA.

The aim of this study was: (1) to resolve the underlying mechanism for the interference of valproate with the isoleucine degradative pathway and (2) to shed new light on the enzymology of the valproate β-oxidation pathway. This work demonstrates that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) participates in the oxidative metabolism of VPA and is the unique enzyme oxidizing 3-hydroxyvalproyl-CoA into 3-ketovalproyl-CoA. Furthermore, we have investigated the potential inhibitory effect of 3-hydroxyvalproyl-CoA, on the activity of MHBD, tested with its endogenous physiological substrate. In addition, we show that short-chain enoyl-CoA hydratase (ECHS1) also known as crotonase is the only enzyme hydrating $\Delta^{2(E)}$-valproyl-CoA into 3-hydroxyvalproyl-CoA. The importance of these findings with respect to the interference with the BCAA oxidation pathway will be discussed as well as the implications for the treatment of patients with VPA.

2. Materials and methods

2.1. Materials

Valproic acid, human and bovine serum albumin, bicinchoninic acid, *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA), ferrocenium hexafluorophosphate, flavin adenine dinucleotide disodium salt hydrate (FAD), trimethylchlorosilane (TMCS), protein A–sepharose, 2-methylacetoacetyl-CoA, crotonase and crotonyl-CoA were obtained from Sigma Chemical Co. (St. Louis, MO). Pyridine was obtained from Pierce Chemicals (Rockford, IL). 2-Phenylbutyric acid and ethoxyamine hydrochloride were obtained from Fluka Chemie (AG, Buchs SG, Switzerland). Sodium chloride, ethyl acetate, sodium sulphate anhydrous, Tris and potassium hydroxide were obtained from Merck (Darmstadt, Germany). Lichrosolv-grade solvents for high performance liquid chromatography (HPLC) were obtained from Biosolve (Valkenswaard, The Netherlands). All other reagents were of analytical grade. All media and supplements for fibroblasts culture were obtained from Gibco (Grand Island, NY). Polyclonal antibodies were raised in rabbits against pig crotonase. Heterologously expressed MHBD [25] and SBCAD [26] were generated as described before.

2.2. Human samples

Urine samples were collected from 14 patients under long-term treatment with VPA and from 37 control individuals. In general, 12 h had elapsed since the last valproate intake in the group under treatment. These samples were obtained through a standard clinical protocol established between the Hospital Pediátrico de Coimbra and the Research Institute for Medicines and Pharmaceutical Sciences.

Human skin fibroblasts were cultured in Ham's F10 medium with glutamine, 10% fetal bovine serum, 1% mixture of penicillin, streptomycin, fungizone and incubated in a humidified CO_2 incubator (5% CO_2, 95% air) at 37 °C. Primary human fibroblast cell lines were obtained from normal control subjects and a patient with MHBD deficiency, a clinical case that has been reported before by Ofman et al. [25]. The diagnosis in this patient was established using clinical, biochemical and molecular methods. Cells were harvested with trypsin and washed twice with PBS and once with 0.9% NaCl. Cell pellets were stored at −80 °C until further use.

Human liver biopsy material as used in this study was from a patient undergoing abdominal surgery with informed consent of the patient to use this material for scientific research as described elsewhere [27].

2.3. Synthesis of valproyl-CoA, $\Delta^{2(E)}$-valproyl-CoA, 3-hydroxyvalproyl-CoA and 2-methyl-3-hydroxy-butyryl-CoA

Valproyl-CoA and 3-hydroxyvalproyl-CoA were prepared by the mixed anhydride method using the following precursors: VPA, 3-hydroxyVPA and CoA, as published earlier [20]. 3-Ketovalproyl-CoA was synthesized enzymatically as described before [21]. SBCAD was used for the synthesis of $\Delta^{2(E)}$-valproyl-CoA using valproyl-CoA as a substrate [15]. The reaction mixture contained 0.2 mM valproyl-CoA, 0.4 mM ferrocenium hexafluorophosphate, 0.1 mM FAD and 200 mM Tris–HCl buffer pH 8.0. The reaction was started by adding 0.24 mg of SBCAD and the incubation was allowed to proceed at 37 °C for about 9 h. The reaction was terminated with 2 M HCl and afterwards neutralized using a solution containing 2 M KOH plus 1 M MES pH 6.0.

In order to synthesize 2-methyl-3-hydroxybutyryl-CoA a reaction mixture containing 350 μM 2-methylacetoacetyl-CoA, 10 mM KPi plus 5 mM MES pH 6.35 and 1 mM NADH was incubated with 3 mg of MHBD, purified as described by Ofman et al. [25] for 3 h at 37 °C. The reaction was stopped with 2 M PCA and neutralized with a solution containing 2 M KOH plus 1 M MES, pH 6.0. Both enzymatic synthesis products were further purified by high-performance liquid chromatography (HPLC).

2.4. Metabolite analysis

Organic acids were analyzed in urine samples from VPA-treated patients and controls. To this end, an internal standard, i.e. 2-phenylbutyric acid (0.5 μmol), was added to about 1 mL of urine followed by acidification with 2 M HCl. Ketoacid stabilization was carried out with ethoxyamine hydrochloride (0.4 g/L) and the samples were incubated at 60 °C for 30 min. The solution was saturated with sodium chloride and the organic acids were extracted twice with ethyl acetate. The organic phases were dried with anhydrous sodium sulphate and subsequently evaporated at room temperature under constant nitrogen gas flow. The dried residue was derivatized with a mixture of *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA), pyridine and trimethylchlorosilane (TMCS) (5:1:0.05) for 1 h at 60 °C. The organic acids were analyzed by gas chromatography (GC) coupled to mass spectrometry, GC–MS (Shimadzu, Mod QP5050).

2.5. MHBD activity in human fibroblasts

The protein concentration of human skin fibroblasts suspensions was determined using the bicinchoninic acid (BCA) assay [28] and human serum albumin was used as reference. Fibroblasts were sonicated with 2 cycles of 40 J under constant cooling in ice-water. The standard enzyme assay mixture contained 25 mM Tris–HCl pH 8.3, 1 mM NAD, 5 mM pyruvate, 0.1 mg/mL BSA, 10.2 U/mL lactate dehydrogenase (LDH), plus 2-methyl-3-hydroxybutyryl-CoA or 3-hydroxyvalproyl-CoA. Reactions were started by adding the previous mixture to the fibroblast suspension. After 30 or 15 min at 37 °C, the reactions were terminated by adding 10 μL of 2 M HCl and afterwards the samples were placed on ice. After neutralization with a solution containing 2 M KOH plus 1 M MES, pH 6.0, the samples were centrifuged at 14,000 rpm for 5 min and the metabolites in the supernatants were analyzed by HPLC.

2.6. Activity measurement of purified MHBD

Similar conditions as described above were used to determine the activity of the purified human MHBD. The reaction mixture contained 2-methyl-3-hydroxybutyryl-CoA (0.5–10 μM) or 3-hydroxyvalproyl-CoA (15–120 μM). This mixture was added to 23.7 μg of purified MHBD. Incubations were carried out at 37 °C for 15 min. The same experimental procedure used for the measurement of MHBD activity in fibroblasts samples was followed using the human enzyme.

2.7. Quantitative analysis of acyl-CoA esters using HPLC

Acyl-CoA esters were analyzed by an automated high-performance liquid chromatography (HPLC) method with reverse-phase separation and UV-detection (Gilson 234 auto-sampling injector; Perkin Elmer 200 solvent delivery system and a Shimadzu UV-detector SPD-10A VP UV-VIS). Separation was performed at room temperature on a 4.6 mm × 250 mm Supelcosil LC-18-DB (5 μM) and a 4.6 mm × 20 mm guard column filled with the same packing material was used. The acyl-CoA esters were detected at 260 nm. For the separation of branched-chain acyl-CoAs a gradient elution based on a binary system of methanol and 50 mM potassium phosphate (V/V, solvent A: 10/90, solvent B: 50/50), pH 5.3, was used. The acyl-CoAs were eluted at a flow rate of 2 mL/min using a linear gradient of 25–35% of solvent B in 30 min. For the analysis of VPA intermediary metabolites the solvent used was acetonitrile and 16.9 mM sodium phosphate (V/V, solvent A: 10/90, solvent B: 70/30), pH 6.9. The CoA esters of VPA were eluted at a flow rate of 3 mL/min using a linear gradient of 0–34% of solvent B in 16 min. Peaks of interest were integrated using Chromeleon software and the respective areas were used for quantitation, based on purified compounds previously synthesized [20].

2.8. Identification of 3-ketovalproyl-CoA by HPLC–ESI-MS/MS

HPLC–ESI-MS/MS analysis was performed using a triple-quadrupole TSQ Quantum HPLC tandem mass spectrometer (MS/MS) from Thermo Finnigan in the negative electrospray ionization (ESI) mode. The samples were injected onto an YMC-Pack Pro C_4 column (2.1 mm × 100 mm, YMC Europe GMBH) using a HPLC system consisting of a Surveyor MS-pump with degasser, a Surveyor autosampler and a column oven. The flow rate was set at 250 μL/min. Elution of acyl-CoAs was achieved with a tertiary system using solvent A (50 mM ammonium acetate, pH 7.0), solvent B (100% acetonitrile) and solvent C (20 mM ammonium bicarbonate).

Separation was performed at 40 °C and nitrogen was used as nebulizing gas while argon was used as collision gas at a pressure of 1.5 mTorr. The capillary voltage was 2.5 kV and the respective temperature was 350 °C, with optimal collision energy of 30 eV. Acyl-CoAs were measured using multiple reaction monitoring (MRM) in the negative ionization mode, using the transitions: m/z 453.5 → 79.0 for 3-OHC_8-CoA and m/z 452.5 → 79.0 for 3-KetoC_8-CoA. The system was controlled by Xcalibur Software (v. 2.0).

2.9. Immunoprecipitation of human crotonase

Immunoprecipitation was carried out using human liver homogenate. Protein–A sepharose beads were incubated overnight end-over-end at 4 °C with variable amounts (0–60 μL) of antibody, i.e. anti-crotonase, to form the complex protein A–anti-crotonase. Incubations were also performed with pre-immune rabbit serum as a control. Afterwards, 100 μL of 0.7 mg/mL of human liver homogenate was incubated with the different quantities of the complex protein A–anti-crotonase, end-over-end, for 2.5 h at 4 °C. After centrifugation of the samples, the supernatants were analyzed for crotonyl-CoA, $\Delta^{2(E)}$-valproyl-CoA hydratase activities.

2.10. Hydratase activity using crotonyl-CoA and $\Delta^{2(E)}$-valproyl-CoA as substrates

The respective supernatants were added to a reaction mixture of 100 mM Tris pH 8.0 and $\Delta^{2(E)}$-valproyl-CoA. Incubations were carried out at 37 °C for 10 min and the reactions were stopped with the addition of 2 M HCl, neutralized with a solution containing 2 M KOH plus 1 M MES, pH 6.0. 20 μL of acetonitrile was added to the mixtures and after centrifugation at 14,000 rpm for 5 min, the product, 3-hydroxyvalproyl-CoA, was analyzed by HPLC.

Crotonase activity was measured spectrophotometrically by following the decrease in time, of the unsaturated substrate, i.e. crotonyl-CoA at 263 nm using a molar extinction coefficient of 6.200 M^{-1} cm^{-1} [29]. The reaction mixture contained 100 mM Tris–HCl (pH 8.0) and 100 μM crotonyl-CoA. Incubations were carried out at 37 °C.

2.11. Data analysis

The characterization of MHBD activity in the absence and presence of 3-hydroxyvalproyl-CoA was performed by plotting the measured reaction rates as a function of the 2-methyl-hydroxybutyryl-CoA concentration (at a fixed concentration of the remaining components of the reaction). The kinetic data were evaluated by nonlinear regression analysis using the SigmaPlot® 10.0 Technical Graphing Software (Systat, Inc.) and the Enzyme Kinetics Module (v 1.3). The Michaelis–Menten equation was used to calculate kinetic parameters (K_m and V_{max}).

The significance of data was determined using two-tailed unpaired t test with the confidence intervals set at 95%. Data with p values less than 0.05 are marked in the figures with an asterisk.

3. Results

3.1. Urinary excretion of 2-methyl-3-hydroxybutyric acid in patients on valproate therapy

The possible interaction of VPA with the in vivo metabolism of isoleucine oxidative metabolism was investigated by studying the profile of organic acids in urine collected from patients under valproate therapy using GC–MS. As shown in Fig. 1, a significant increase of 2-methyl-3-hydroxybutyric acid (2-MHBA) was observed in patients under drug therapy (63 ± 30 mmol/mol creatinine for VPA-treated patients (n = 14) versus 42 ± 29 mmol/mol creatinine for controls (n = 37) $p < 0.05$). These results indicate that VPA probably interferes with the oxidative pathway of

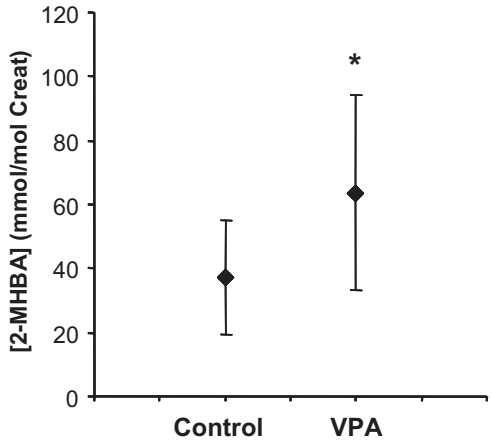


Fig. 1. Excretion of 2-methyl-3-hydroxybutyric acid (2-MHBA) analyzed by GC–MS in patients under treatment with VPA ($n = 14$) as compared to controls ($n = 37$). *Significant difference between represented values ($p < 0.05$), which are the mean and respective standard deviation.

isoleucine, most likely at the level of the enzyme MHBD which catalyses the conversion of 2-methyl-3-hydroxybutyryl-CoA into 2-methyl-3-hydroxyacetoacetyl-CoA. Given the structural similarities between 2-methyl-3-hydroxybutyryl-CoA and 3-hydroxyvalproyl-CoA, we hypothesized that both substrates may be handled by MHBD so that competitive inhibition takes place. This was investigated using purified MHBD.

3.2. Involvement of MHBD in the oxidation of 3-hydroxyvalproyl-CoA

Purified MHBD was incubated with 3-hydroxyvalproyl-CoA at different concentrations. Fig. 2 shows the formation of 3-ketovalproyl-CoA after a 15 min incubation period. The product of these incubations was identified by HPLC–ESI-MS/MS using multiple reaction monitoring in the negative ionization mode. The unequivocal presence of single charged ions identifies the two VPA metabolites. In fact, the apparent mass of a single charged ion for 3-ketoC_8-CoA (m/z 905.7) was detected in samples where MHBD was incubated with 3-hydroxyvalproyl-CoA. This mass corresponds to 3-ketovalproyl-CoA, the oxidation product of 3-hydroxyvalproyl-CoA (m/z 907.7). The presence of salts in the samples interferes with the MS/MS measurement of double charged ions which are much less abundant. However, the presence of ions with approximate masses of 453.2 and 452.2 was detected on the spectra of 3-hydroxyvalproyl-CoA and 3-ketovalproyl-CoA, respectively. The kinetic parameters of MHBD were subsequently determined using 3-hydroxyvalproyl-CoA as a substrate ($K_m = 63 \pm 6$ μM and $V_{max} = 187 \pm 6$ nmol/mg protein/min).

In vitro studies using fibroblasts of controls and patients with MHBD deficiency were performed in order to find out if MHBD is the unique enzyme that is able to oxidize 3-hydroxyvalproyl-CoA into 3-ketovalproyl-CoA. As shown in Fig. 3, 3-hydroxyacyl-CoA dehydrogenase activity measurements in homogenates prepared from control fibroblasts revealed comparable specific activities using either the natural substrate, i.e. 2-methyl-3-hydroxybutyryl-CoA or 3-hydroxyvalproyl-CoA (0.57 ± 0.11 and 0.56 ± 0.17 nmol/mg protein/min, respectively). The same assay was performed in fibroblasts of a patient in whom MHBD genetic deficiency was established earlier using enzymatic and molecular methods. The specific activity of MHBD as measured in fibroblasts was 0.041 ± 0.02 nmol/mg protein/min, when 2-methyl-3-hydroxybutyryl-CoA was used as a substrate (<10% of normal values). There was no detectable MHBD activity when 3-hydroxyvalproyl-CoA was used as a substrate, which indicates that MHBD is the predominant, if not the only enzyme involved in the dehydrogenation of 3-hydroxyvalproyl-CoA.

3.3. Effect of 3-hydroxyvalproyl-CoA on the activity of MHBD

The finding that 3-hydroxyvalproyl-CoA and 2-methyl-3-hydroxybutyryl-CoA are both handled by the same enzyme would suggest that the accumulation of 2-methyl-3-hydroxybutyric acid

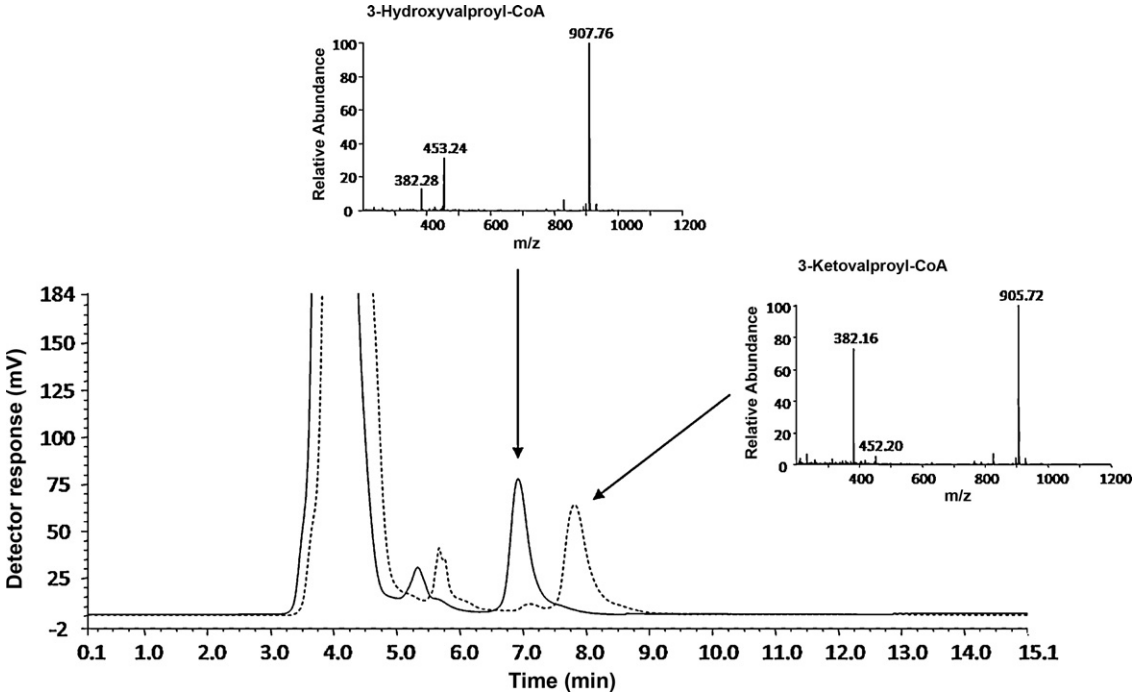


Fig. 2. HPLC chromatogram showing the activity of MHBD using 1 mM 3-hydroxyvalproyl-CoA as substrate. Incubations were carried out at 37 °C for 15 min (dashed line) and corresponding blank at 0 min (solid line). The peaks are identified by the respective MS/MS spectrum corresponding to 3-hydroxyvalproyl-CoA (m/z 907.76) and 3-ketovalproyl-CoA (m/z 905.72).

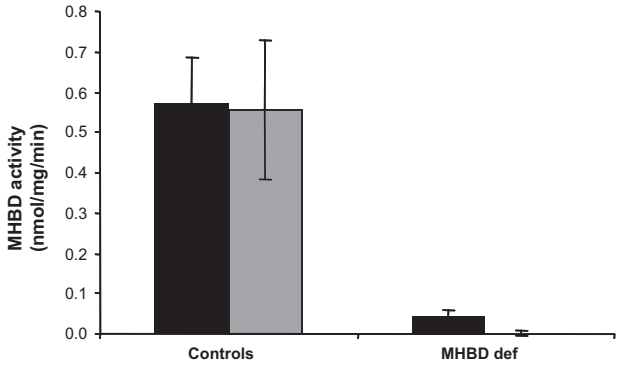


Fig. 3. MHBD activity in control and MHBD deficient fibroblasts using 0.05 mM 2-methyl-3-hydroxybutyryl-CoA (■) or 0.2 mM 3-hydroxyvalproyl-CoA (▩). Incubations were carried out at 37 °C, pH 8.3 for 30 min.

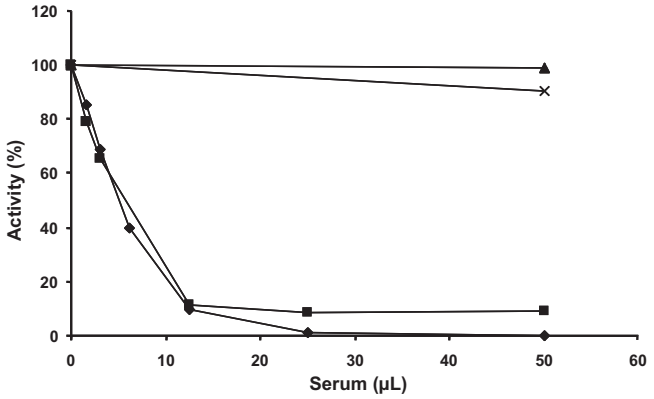


Fig. 5. Hydratase activity (%) in human liver homogenate incubated with increasing amounts of the complex protein A–anti-crotonase (0–50 µL) (◆ and ■) and protein A-preimmune serum (0 and 50 µL) (▲ and ×). Hydratase activity was determined in supernatants after immunoprecipitation using $\Delta^{2(E)}$-valproyl-CoA (◆ and ▲) and crotonyl-CoA (■ and ×) as substrates. The absolute values for 100% activity correspond to 0.05 nmol/min/mg and 2.45 nmol/min/mg for $\Delta^{2(E)}$-valproyl-CoA and crotonyl-CoA, respectively.

in patients on valproate could be explained based on the competitive inhibition of flux through MHBD by 3-hydroxyvalproyl-CoA. To investigate this further, we first determined the activity of the purified enzyme with the natural substrate, 2-methyl-3-hydroxybutyryl-CoA, and the respective kinetic parameters were determined ($K_m = 0.69 \pm 0.17$ µM and $V_{max} = 73 \pm 16$ nmol/mg protein/min). Subsequently, the effect of 3-hydroxyvalproyl-CoA was tested on the activity of MHBD. The respective enzyme activity decreased about 35% in the presence of 250 µM 3-hydroxyvalproyl-CoA. In order to further characterize the inhibition mechanism, the activity of MHBD was determined as a function of the inhibitor concentration.

The results obtained were analyzed by different linearization methods of the Michaelis–Menten equation, where only the Lineweaver–Burk and the Dixon plots are shown in Fig. 4. The pattern of results does not provide an unambiguous indication of the type of inhibition, but overall graphic analysis of the data pointed to a competitive mechanism of inhibition of MHBD by 3-hydroxyvalproyl-CoA.

3.4. Human crotonase converts $\Delta^{2(E)}$-valproyl-CoA into 3-hydroxyvalproyl-CoA

After having established that MHBD catalyses the third step in the β-oxidation of valproyl-CoA, we subsequently investigated the nature of the hydratase. Since crotonase was a likely candidate, we first focussed on this enzyme and performed immunoprecipitation experiments. To this end, human liver homogenates were incubated with anti-crotonase and pre-immune serum was used as a control. Fig. 5 shows the enoyl-CoA hydratase activity using both $\Delta^{2(E)}$-valproyl-CoA and crotonyl-CoA as substrates. Hydratase activity, as determined with the two substrates, decreased in similar fashion upon addition of anti-crotonase reaching zero activity with increasing amounts of antibody. Incubations performed with and without the highest amount of pre-immune serum showed similar hydratase activities using the two CoA esters as substrates.

4. Discussion

It has been known for many years that treatment with valproate interferes with the metabolism of the amino acid leucine as demonstrated by the increased urinary excretion of 3-hydroxyisovaleric acid [30]. Careful quantitative analysis of urinary organic acids in patients on valproate therapy has now revealed that the isoleucine pathway is also compromised. In contrast to the unresolved issue how VPA interferes with leucine metabolism, we have now successfully clarified the interaction between valproate and the isoleucine breakdown pathway. The present study describes the elucidation of two of the enzymatic steps involved

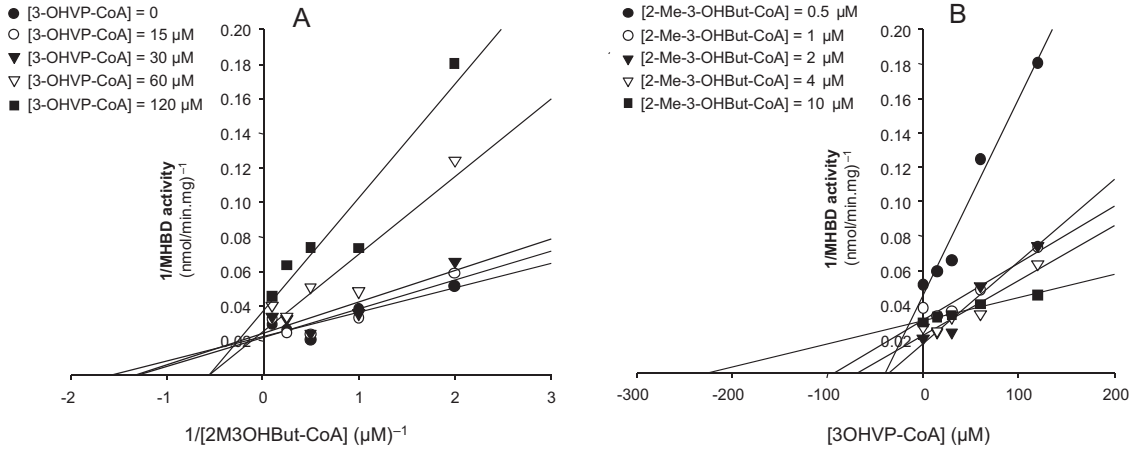
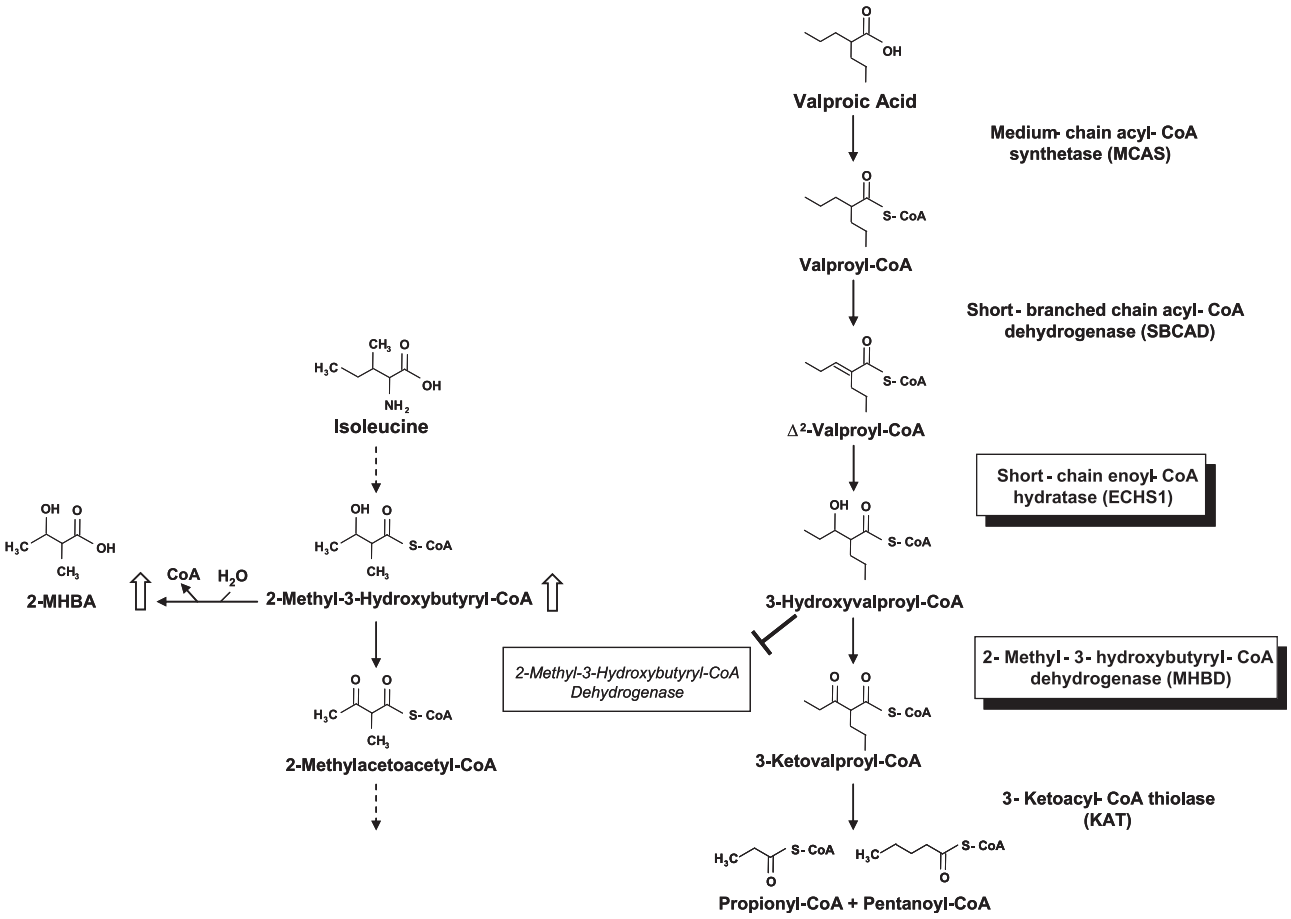


Fig. 4. Inhibitory effect of 3-hydroxyvalproyl-CoA on the activity of heterologously expressed human MHBD. Lineweaver–Burk (A) and Dixon (B) plots of MHBD activity with 2-methyl-hydroxybutyryl-CoA as a substrate in the presence of 3-hydroxyvalproyl-CoA. Incubations were carried at 37 °C, pH 8.3, for 15 min.



Scheme 1. Schematic representation of the mitochondrial β-oxidation of valproic acid incorporating the two novel metabolizing enzymes: short-chain enoyl-CoA hydratase (ECHS1) and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD). The potential interference with isoleucine oxidative pathway is also represented. MHBD participates in metabolism of VPA, handling 3-hydroxyvalproyl-CoA as a substrate. The competitive inhibition of MHBD explains the increased in vivo excretion of 2-methyl-3-hydroxybutyric acid (2-MHBA) shown in urine of patients under valproate therapy.

in the mitochondrial β-oxidation of VPA, i.e. the hydration of $\Delta^{2(E)}$-valproyl-CoA into 3-hydroxyvalproyl-CoA and the further dehydrogenation into 3-ketovalproyl-CoA (Scheme 1).

The in vitro formation of 3-hydroxyvalproyl-CoA has been reported previously in rat liver mitochondria [19,20] but the enzyme catalysing the second step of the β-oxidation of VPA had not been identified until now. Several mitochondrial enzymes with enoyl-CoA hydratase activity have been characterized: (1) crotonase, localized in the mitochondrial matrix [31,32]; (2) mitochondrial long-chain enoyl-CoA hydratase as part of the membrane-bound mitochondrial trifunctional protein (MTP) [31] and (3) 3-methylglutaconyl-CoA hydratase [33]. Crotonase plays a key role in mitochondrial fatty acid metabolism by catalysing the second step of the β-oxidation cycle. The enzyme is most active towards short-chain substrates and acts with decreasing efficiency on 2-enoyl-CoAs from crotonyl-CoA to hexadecenoyl-CoA, which is only hydrated at a rate 1–2% of the rate of crotonyl-CoA [31]. The results described in this study show that crotonase is the only enzyme responsible for the hydration of $\Delta^{2(E)}$-valproyl-CoA into 3-hydroxyvalproyl-CoA (Fig. 5).

Subsequently, we have investigated the dehydrogenation of 3-hydroxyvalproyl-CoA. The identity of the enzyme responsible for this step has not been established before. In vitro studies demonstrated no dehydrogenation of 3-hydroxyvalproyl-CoA using purified pig heart short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) or peroxisomal bifunctional protein [19]. The enzyme responsible for this oxidation step had been suggested to be an unknown NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase (3HAD) [17]. We have used human MHBD expressed heterologously in *Escherichia coli* [25] to elucidate the second dehydrogenation step of the β-oxidation of VPA. MHBD is a mitochondrial matrix enzyme which is active against a broad range of substrates with a preference for short-chain methyl-branched acyl-CoAs. Hence it has been postulated as the enzyme responsible for the dehydrogenation of 2-methyl-3-hydroxybutyryl-CoA in the isoleucine oxidative pathway [34]. Our results show that MHBD is also responsible for the oxidation of 3-hydroxyvalproyl-CoA into 3-ketovalproyl-CoA. The K_m of MHBD using 3-hydroxyvalproyl-CoA is about 10 fold the K_m using 2-methyl-3-hydroxybutyryl-CoA suggesting that the drug metabolite has considerable affinity towards MHBD as compared to its natural substrate. We have also proven that there is no other human enzyme capable of using 3-hydroxyvalproyl-CoA as a substrate, at least in human skin fibroblasts. Furthermore, our data shows that 3-hydroxyvalproyl-CoA inhibits MHBD most likely due to structural similarities between the substrates, 3-hydroxyvalproyl-CoA and 2-methyl-3-hydroxybutyryl-CoA. This result is in agreement with the significantly increased excretion of 2-methyl-3-hydroxybutyric acid in urine samples of patients under valproate therapy since the metabolic origin of this organic acid derives from the accumulation of the endogenous isoleucine intermediary, 2-methyl-3-hydroxybutyryl-CoA [34].

Our results also provide new insights to the previous work performed by our group reporting the interference of the drug with BCAAs catabolism [15]. In these studies human SBCAD expressed in *E. coli* was used to characterize the active role of this enzyme in the β-oxidation of VPA. Besides the participation of SBCAD, the present

report on the involvement of ECHS1 and MHBD in the biotransformation of VPA may provide an in-depth contribution to the mitochondrial dysfunction and potential hepatotoxicity often reported in VPA-treated patients.

The question arises whether it is advisable to treat patients with inborn errors of the isoleucine pathway with valproate. Our results have clearly indicated that valproate may seriously perturb the isoleucine pathway; therefore any valproate administered to a patient with e.g. MHBD-deficiency will result in a further accumulation of 2-methyl-3-hydroxybutyrate, which has been demonstrated in vitro to compromise energy metabolism in cerebral cortex of developing rats [35]. Due to the possible neurotoxicity of this substance, it is perhaps safer to choose another first-line anti-epileptic drug in these patients. On the other hand, it is always warranted to screen for organic acidurias in patients who are candidates for valproate therapy. Although VPA-induced hepatotoxicity seems to be idiosyncratic and only affecting a small group of patients, VPA therapy should be carefully prescribed in cases of inborn deficiencies affecting not only FAO but also the isoleucine oxidative pathway.

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