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ROLE OF ISOVALERYL-CoA DEHYDROGENASE AND SHORT BRANCHED-CHAIN ACYL-CoA DEHYDROGENASE IN THE METABOLISM OF VALPROIC ACID: IMPLICATIONS FOR THE BRANCHED-CHAIN AMINO ACID OXIDATION PATHWAY

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Running title: Valproate and the metabolism of branched-chain amino acids.

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Abbreviations: VPA: 2-*n*-propylpentanoic acid or valproic acid; $\Delta^{2(E)}$-VPA: 2-*n*-propyl-2-pentenoic acid; CoA: coenzyme A; DephCoA: dephosphocoenzyme A; BCAAs: branched-chain amino acids; VP-CoA: valproyl-CoA; VP-DephCoA: valproyl-dephosphoCoA.

Abbreviations of enzymes: IVD: Isovaleryl-CoA dehydrogenase (EC 1.3.99.10); IBD: Isobutyryl-CoA dehydrogenase (EC 1.3.99.3) and SBCAD: Short branched-chain acyl-CoA dehydrogenase (EC 1.3.99.12).

ABSTRACT

Many biological systems including the oxidative catabolic pathway for branched-chain amino acids (BCAAs) are affected *in vivo* by valproate therapy. In this study we investigated the potential effect of valproic acid (VPA) and some of its metabolites on the metabolism of BCAAs. *In vitro* studies were performed using isovaleryl-CoA dehydrogenase (IVD), isobutyryl-CoA dehydrogenase (IBD) and short branched-chain acyl-CoA dehydrogenase (SBCAD), enzymes involved in the degradation pathway of leucine, valine and isoleucine. The enzymatic activities of the three purified human enzymes were measured using optimized HPLC procedures and the respective kinetic parameters were determined in the absence and presence of VPA and the corresponding CoA and dephosphoCoA conjugates. Valproyl-CoA and valproyl-dephosphoCoA inhibited IVD activity significantly by a purely competitive mechanism with K_i values of 74±4 µM and 170±12 µM, respectively. IBD activity was not affected by any of the tested VPA esters. However, valproyl-CoA did inhibit SBCAD activity by a purely competitive mechanism with a K_i of 249±29 µM. In addition, valproyl-dephosphoCoA inhibited SBCAD activity via a distinct mechanism (K_i=511±96 µM) which appeared to be of the mixed type. Furthermore, we also show that both SBCAD and IVD are active using valproyl-CoA as a substrate. The catalytic efficiency of SBCAD turned out to be much higher than of IVD, demonstrating that SBCAD is the most probable candidate for the first dehydrogenation step of VPA β-oxidation. Our data explain some of the effects of valproate on the branched-chain amino acid metabolism and shed new light on the biotransformation pathway of valproate.

INTRODUCTION

Valproic acid (VPA; 2-*n*-propylpentanoic acid) is a simple branched-chain fatty acid that is known worldwide for its anticonvulsant properties. It is mostly used for the treatment of several types of seizures, bipolar disorders, various psychiatric syndromes and migraine (Peterson and Naunton, 2005; Perucca, 2002; Bialer and Yagen, 2007). In addition, VPA has recently emerged as a drug that shows potential in cancer treatment (Duenas-Gonzalez, 2008). Although VPA has a broad range of clinical use, it is associated with several adverse effects. Hepatotoxicity is a well recognized complication of VPA therapy (Gerber et al., 1979; Bissell et al., 2001; Sztajnkrycer, 2002). Knowledge of the pathophysiological mechanisms underlying this hepatotoxicity is incomplete. Inhibition of mitochondrial catabolic pathways, for instance fatty acid β-oxidation, has strongly been implicated in the hepatotoxicity of VPA (Silva et al., 2008; Bjornsson, 2008; Silva et al., 2001a). In patients receiving valproate, an increase in serum levels of the branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) and their intermediates as well as an increase in urinary excretion of these amino acids have been reported (Silva et al., 2001a; Anderson et al., 1994). These findings suggest that valproate adversely affects BCAAs metabolism.

VPA and the intermediates in the degradation of the two BCAAs, isoleucine and valine undergo β-oxidation (Silva et al., 2002) generating structurally similar metabolites which suggest that VPA might use key enzymes of the BCAAs catabolic pathways for its own oxidation (figure 1). Isovaleryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA, the intermediates in BCAAs catabolism, are converted to 3-methylcrotonyl-CoA, tiglyl-CoA, and methacrylyl-CoA by isovaleryl-CoA dehydrogenase (IVD, EC 1.3.99.10), short branched-chain acyl-CoA dehydrogenase (SBCAD, EC 1.3.99.12), and isobutyryl-CoA dehydrogenase (IBD, EC 1.3.99.3), respectively. These enzymes are members of the acyl-CoA dehydrogenase family (ACD), which consists of homologous mitochondrial

flavoproteins that catalyze the α,β-dehydrogenation of acyl-CoA thioester substrates to the corresponding *trans*-2-enoyl-CoAs (Battaile et al., 2004). These enzymes share many similar molecular and catalytic properties but differ with respect to their substrate specificities regarding length and configuration of the carbon backbone of their substrates.

Previous work from Li *et al.* (Li et al., 1991), Ito *et al* (Ito et al., 1990) and our group (Silva et al., 2002 ; Silva et al., 2001b; Silva et al., 2004), has led to the partial resolution of the mitochondrial β-oxidation pathway of VPA. It was concluded that the enzymes involved in the oxidation of straight-chain fatty acids including very long-, long-, medium- and short-chain acyl-CoA dehydrogenases are not involved in the first dehydrogenation reaction of VPA. Ito *et al*. (Ito et al., 1990) originally showed that the rat 2-methyl-branched-chain acyl-CoA dehydrogenase was active with valproyl-CoA (VP-CoA) as substrate, but Willard *et al* (Willard et al., 1996) subsequently demonstrated that the human homologue was much less active with VP-CoA than its rat counterpart. In the current study we have investigated the involvement of the three BCAA acyl-CoA dehydrogenases with respect to the first step of the β-oxidation of VPA. We report the kinetic characterization of the SBCAD reaction with VP-CoA as substrate using the human purified enzyme. We also demonstrate that IVD is able to produce $\Delta^{2(E)}$-valproyl-CoA ($\Delta^{2(E)}$-VP-CoA), although with a much lower catalytic efficiency. Furthermore, we have investigated the potential inhibitory effect of VPA and some of its mitochondrial metabolites especially in the form of the corresponding acyl-CoA intermediates, VP-CoA and valproyl-dephosphoCoA (VP-DephCoA) (Silva et al., 2001b; Silva et al., 2004), on the activity of IVD, IBD and SBCAD. The implications of these findings with respect to the treatment of patients by VPA are discussed.

MATERIAL AND METHODS

Materials

Valproic acid, human and bovine serum albumin, bicinchoninic acid, ferrocenium hexafluorophosphate, FAD, isovaleryl-CoA and isobutyryl-CoA were obtained from Sigma Chemical Co. (St. Louis, MO). Tris was obtained from Merck (Darmstadt, Germany). Heterologously expressed IVD, IBD and SBCAD were obtained as described before (Mohsen and Vockley, 1995; Nguyen et al., 2002; Gibson et al., 2000). VP-CoA (Silva et al., 2001b), VP-DephCoA (Silva et al., 2004), and 2-methylbutyryl-CoA (Rasmussen et al., 1990) were synthesized as described previously.

Enzymatic activity measurement of the branched-chain acyl-CoA dehydrogenases

Experimental conditions were optimized for each enzyme in terms of protein, time and pH. The reaction mixture contained 200 mM Tris·HCl pH 8.0, 50 µM FAD, 400 µM ferrocenium hexafluorophosphate, 0.1 mg/mL bovine serum albumin and substrate (isovaleryl-CoA, isobutyryl-CoA, 2-methylbutyryl-CoA) or VP-CoA. This mixture was added to an enzyme solution containing 0.1 mg/mL BSA. Incubations were carried out at 37ºC for variable time periods depending on the enzyme and the substrate used. The reactions were terminated by adding 10 µL 2 M HCl and afterwards the samples were placed on ice. After neutralization with (2 M KOH)/(1 M MES pH 6.0), 10 µL 10 mM L-cysteine and 30 µL methanol were added to the mixture. L-cysteine is added in order to reduce oxidized ferrocinium hexafluorophosphate which may interfere with the chromatographic separation. The samples were centrifuged at 20,000 x *g* for 5 min and the metabolites in the supernatants were analysed by HPLC.

Sample analysis by HPLC

Acyl-CoA esters were quantified by HPLC. Separation was performed at room temperature with a Perkin Elmer pump (PE series 200) and a Gilson 234 auto-sampling injector. A frit C-402X (Upchurch scientific), a 4.6 mm x 250 mm Supelcosil LC-18-DB (5 µM) column (Supelco) and a guard column (4.6 mm x 20 mm) filled with the same packing material, were used. For gradient elution of branched-chain acyl-CoAs a binary system of methanol and 50 mM potassium phosphate pH 5.3 was used, whereas for the analysis of VPA intermediary metabolites a system of acetonitrile and 17 mM sodium phosphate pH 6.9 was used. Acyl-CoA esters were detected with a UV detector (SPD-10A VP UV-VIS, Shimadzu) at 260 nm.

Identification of Δ^2-VP-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 x 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 tertiary system using solvent A (50 mM ammoniumacetate, pH 7.0), solvent B (100% acetonitrile) and solvent C (20 mM ammoniumbicarbonate).

Separation was performed at 40ºC 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 350ºC, with optimal collision energy of 30 eV. Acyl-CoA's were measured using multiple reaction monitoring (MRM) in the negative ionization mode, using

the transitions: m/z 445.5 → 79.0 for C_8-CoA (VP-CoA) and m/z 444.5 → 79.0 for $C_{8:1}$-CoA (Δ^2-VP-CoA). The system was controlled by Xcalibur Software 2.0.

Data analysis

The characterization of IVD, SBCAD activities in the absence and presence of different inhibitors was performed by plotting the measured reaction rates as function of the substrate concentration (at a fixed concentration of the remaining components of the reaction). The steady state kinetic data of IVD, IBD and SBCAD activities were determined by nonlinear regression analysis using the SigmaPlot® 10.0 Technical Graphing Software (Systat, Inc.) and the Enzyme Kinetics Module (v1.3). The Michaelis-Menten equation was used to calculate kinetic parameters (K_m and V_{max}) of the enzyme reaction using the respective substrates. The inhibition constant K_i was calculated by nonlinear regression of the respective inhibition curves, using the mentioned software.

RESULTS

Effects of VP-CoA and VP-DephCoA on the activity of IVD, IBD and SBCAD

Kinetic studies were performed with the three heterologously expressed and purified acyl-CoA dehydrogenases IVD, IBD and SBCAD. The corresponding activity was determined with their natural substrates, isovaleryl-CoA, isobutyryl-CoA and 2-methylbutyryl-CoA, respectively. The kinetic constants K_m and V_{max} were calculated and are summarized in table 1.

The effect of VP-CoA and VP-DephCoA was tested on the three enzymes. The activities of IVD and SBCAD as measured at 0.2 mM isovaleryl-CoA and 0.2 mM 2-methylbutyryl-CoA, were decreased by about 75% and 45% respectively, at 1 mM VP-CoA. After incubation with 1 mM VP-DephCoA, both enzyme activities were decreased about 45%. The activity of IBD was not affected in the presence of VP-CoA and VP-DephCoA. In order to further characterize the inhibition mechanism, the activity of IVD and SBCAD was determined as a function of the inhibitor concentration.

The obtained results were analysed by three linearization methods of the Michaelis-Menten equation namely, Lineweaver-Burk, Eadie Hofstee and Hanes-Woolf plots. Analysis of the data pointed to a purely competitive mechanism of inhibition of both VP-CoA and VP-DephCoA with respect to IVD, as depicted in figure 2. However, with respect to SBCAD, these CoA esters appeared to have different inhibitory mechanisms. Indeed, whereas VP-CoA was found to inhibit SBCAD by a purely competitive mechanism, VP-DephCoA appeared to be a mixed-type inhibitor, as shown in figure 3. The Lineweaver-Burk linearization plots and corresponding Dixon plots (Dixon, 1953) are exhibited in parallel, either in figure 2 or figure 3.

Assuming a purely competitive or mixed mechanism of inhibition, the inhibition constant (K_i) was calculated. With respect to IVD activity, a K_i value of 74 ± 4 µM was estimated for VP-CoA and of 170 ± 12 µM for VP-DephCoA. The inhibition constant (K_i) of VP-CoA and VP-DephCoA for the activity of SBCAD was of 249 ± 29 µM and 511 ± 96 µM, respectively.

Involvement of branched-chain acyl-CoA dehydrogenases in the dehydrogenation of VP-CoA

In order to identify the metabolizing enzyme of the first dehydrogenation step of the oxidative metabolism of VPA, the purified enzymes (IVD, IBD and SBCAD) were incubated with 1 mM of VP-CoA. As shown in figure 4, $\Delta^{2(E)}$-VP-CoA was not observed using IBD as enzyme (Fig. 4-B), suggesting that IBD is not involved in the metabolism of VPA. However, both IVD (Fig. 4-A) and SBCAD (Fig. 4-C) showed activity with VP-CoA as a substrate, amounting to 0.13 nmol/mg protein·min and 1.20 nmol/mg protein·min, respectively. Even though SBCAD dehydrogenates VP-CoA at a much lower rate than its natural substrate, *i.e.* 2-methylbutyryl-CoA, the kinetic parameters were determined (K_m = 304 ± 51 µM and V_{max} = 0.27 ± 0.02 nmol/mg protein·min).

An extra incubation was performed with SBCAD to identify the product of VP-CoA. Figure 5-B shows the HPLC chromatograms related with the synthesis of $\Delta^{2(E)}$-VP-CoA, where SBCAD was incubated for 1 hour with VP-CoA. In figure 5-A the same incubation was performed for 0 hour and consequently there is no product formation. The same samples were analysed by HPLC-ESI-MS/MS using multiple reaction monitoring in the negative ionization mode. Figure 5-C and 5-D shows the mass spectra of singly charged ions which are formed more abundantly. The mass of a $C_{8:1}$-CoA (889.8) was detected in samples where SBCAD was incubated for 1 hour with VP-CoA (Fig. 5-D). This mass corresponds to $\Delta^{2(E)}$-VP-CoA, the dehydrogenation product of VP-CoA.

DISCUSSION

This study shows that valproate interferes with enzymes involved in the oxidative metabolism of leucine and isoleucine. Specifically, two branched-chain acyl-CoA dehydrogenases (BCADs),

IVD and SBCAD, were found to participate in the oxidation of VPA. We have shown that human IVD is able to convert VP-CoA into $\Delta^{2(E)}$-VP-CoA, although at a lower rate than with its natural substrate, isovaleryl-CoA. VP-CoA has previously been shown to be oxidized at a significant rate by SBCAD from rat liver mitochondria (Ito et al., 1990) and by bacterially expressed rat and human SBCAD (Willard et al., 1996). We have used human SBCAD expressed in *E. coli* (Willard et al., 1996) to confirm and characterize the active role of SBCAD in the β-oxidation of VPA. These results are in agreement with the inhibition studies performed with VP-CoA and VP-DephCoA and the BCADs. Both VPA metabolites inhibit IVD by a purely competitive mechanism. IBD activity was not affected by these VPA metabolites. However, VP-CoA did inhibit SBCAD activity by a purely competitive mechanism whereas VP-DephCoA inhibited SBCAD activity through a distinct mechanism.

The observed inhibitory effects of the VPA metabolites as tested in this study, on the activity of the BCADs are most probably due to structural similarities between the substrates of these enzymes and the valproate metabolites VP-CoA and VP-DephCoA. IVD oxidizes β-branched acyl-CoAs such as isovaleryl-CoA, but both SBCAD and IBD oxidize short-chain acyl-CoAs with a branched α-position (figure 6). Due to its α-branched configuration, VP-CoA would have appeared to be a good substrate analogue for both SBCAD and IBD. However, VP-CoA is a substrate analogue for SBCAD but not for IBD. The natural substrate of IBD is isobutyryl-CoA, which has a smaller acyl moiety than the VP-CoA acyl moiety, hence the apparent lack of interaction between VP-CoA and IBD is probably because of the limited size of the isobutyryl moiety binding pocket (Battaile et al., 2004). SBCAD is able to oxidize α-branched acyl-CoAs such as 2-methyl-butyryl-CoA and straight chain substrates as butyryl-CoA and hexanoyl-CoA (He et al., 2003), and therefore its active site seems to be the best accommodating for the dehydrogenation of VP-CoA.

Unexpectedly, VP-CoA was found to be oxidized by IVD. Although the reaction occurs at a very low rate, it is still rather surprising since this enzyme handles β- and not α-branched acyl-CoAs. IVD has a larger binding pocket than IBD (Battaile et al., 2004), which apparently allows the enzyme to act upon VP-CoA with its five carbon backbone. This result is in agreement with the competitive mechanism of inhibition seen for IVD when VP-CoA was used as an inhibitor.

Since SBCAD dehydrogenates VP-CoA to $\Delta^{2(E)}$-VP-CoA and is significantly inhibited by both VP-CoA and VP-DephCoA, it would be expected that patients under VPA therapy would accumulate the endogenous substrate of SBCAD, 2-methylbutyryl-CoA. However, no studies have reported the increase of metabolites derived from 2-methylbutyryl-CoA perhaps because IBD also has activity towards this substrate (Nguyen et al., 2002) substituting, at least in part, SBCAD activity in case of its malfunction or inhibition.

It has been shown that a dysregulated BCAA metabolism makes an independent contribution to development of insulin resistance and glucose intolerance in obese humans (Newgard et al., 2009). Insulin resistance associated to weight gain has also been reported during VPA treatment (Masuccio et al., 2010; Verrotti et al., 2010). However, it is unknown to what extent the interference of valproate on the BCAA oxidation shown in this paper is related with the significant weight gain potentially associated with VPA. It remains to be established if the inhibitory effects, as observed in this study, are enough to account for the well-recognized potential liver toxicity of VPA. In this respect, it is important to mention that although there are a significant number of patients suffering from side effects, VPA-induced hepatotoxicity seems to be idiosyncratic and only affects a small group of patients.

In summary, we have shown that VPA can interfere with the activity of some enzymes from the ACD family, namely, IVD and SBCAD. Taking into account that ACDs have overlapping activity with different substrates (Battaile et al., 2004; Tiffany et al., 1997), the drug might exacerbate the impairment of genetically affected routes and elicit a toxic condition (Silva et al.,

2008). In fact, it has been suggested already that the intake of VPA should be avoided in patients with inborn errors affecting mitochondrial metabolism (Silva et al., 2008). For that reason, we conclude that VPA administration should be avoided in cases of inborn deficiencies affecting certain ACDs or affecting the leucine and isoleucine oxidative pathways (Vockley and Ensenauer, 2006; Korman, 2006).

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Luís PB, Ruiter J, IJlst L, Duran M, Wanders RJ and Silva MF

Conducted experiments: Luís PB

Contributed new reagents or analytical tools: Mohsen A-W and Vockley J

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Wrote or contributed to the writing of the manuscript: Luís PB, IJlst L, Almeida IT, Duran M, Vockley J, Wanders RJ and Silva MF

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LEGENDS FOR FIGURES:

Figure 1 – Schematic representation of the catabolic pathways of the branched-chain amino acids (leucine, isoleucine and valine) and valproic acid, highlighting the reactions of the first cycle of β-oxidation and corresponding enzymes of the initial dehydrogenation (1- IVD: Isovaleryl-CoA dehydrogenase; 2- SBCAD: Short branched chain acyl-CoA dehydrogenase; 3- IBD: Isobutyryl-CoA dehydrogenase)

Figure 2 – Inhibitory effect of VP-CoA and VP-DephCoA on the activity of heterologously expressed human isovaleryl-CoA dehydrogenase (IVD). Lineweaver Burk linearization plots of IVD activity with isovaleryl-CoA as a substrate in the presence of VP-CoA (**A**) and VP-DephCoA (**B**). Dixon plots of IVD activity in the presence of VP-CoA (**C**) and VP-DephCoA (**D**). Incubations were carried at 37ºC, pH 8, for 10 min.

Figure 3 – Inhibitory effect of VP-CoA and VP-DephCoA on the activity of heterologously expressed human short branched-chain acyl-CoA dehydrogenase (SBCAD). Lineweaver Burk linearization plots of SBCAD activity with 2-methylbutyryl-CoA as a substrate in the presence of VP-CoA (**A**) and VP-DephCoA (**B**). Dixon plots of SBCAD activity in the presence of VP-CoA (**C**) and VP-DephCoA (**D**). Incubations were carried at 37ºC, pH 8, for 30 min.

Figure 4 – Activity of the branched-chain acyl-CoA dehydrogenases (IVD, IBD and SBCAD) using VP-CoA as a substrate. HPLC analysis of the incubation mixtures with IVD (**A**), IBD (**B**) and SBCAD (**C**) using 1 mM VP-CoA as substrate. Incubations were carried out at 37ºC for 30 min (——) and 0 min (- - -). Peaks: 1: VP-CoA and 2: $\Delta^{2(E)}$-VP-CoA.

Figure 5 – Identification of the oxidation product of VP-CoA using SBCAD. (A) and (B): HPLC chromatograms of incubations of SBCAD with VP-CoA, carried out at 37º for 0h or 1h, respectively. (C) and (D): MS spectra (singly charged ions) of incubations of SBCAD with VP-CoA, carried out at 37º for 0h or 1h, respectively. Metabolites: 1: VP-CoA, 2: $\Delta^{2(E)}$-VP-CoA.

Figure 6 – Chemical structures of isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA, intermediates from the leucine, isoleucine and valine pathways, respectively, and VP-CoA, the CoA thioester of valproic acid.

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Table 1 – Kinetic parameters (K_m and V_{max}) of IVD, IBD and SBCAD activities using the substrates, isovaleryl-CoA, iso butyryl-CoA a nd 2-methylbutyryl-CoA, r espectively. Incubations were carried at 37ºC, pH 8 for 10 min for IVD assay and for 30 min for IBD and S BCAD assays. (T he r esults ar e t he mean +/- SD from t wo t o t hree i ndependent experiments).

Enzyme	Substrate	K_m (µM)	V_{max} (µmol/(mg protein·min))
IVD	Isovaleryl-CoA	125 ± 2.3	77 ± 6.7
IBD	Isobutyryl-CoA 24	± 0.6	32 ± 1.5
SBCAD	2-Methylbutyryl-CoA	12 ± 2.0	12 ± 0.2

Figure 1

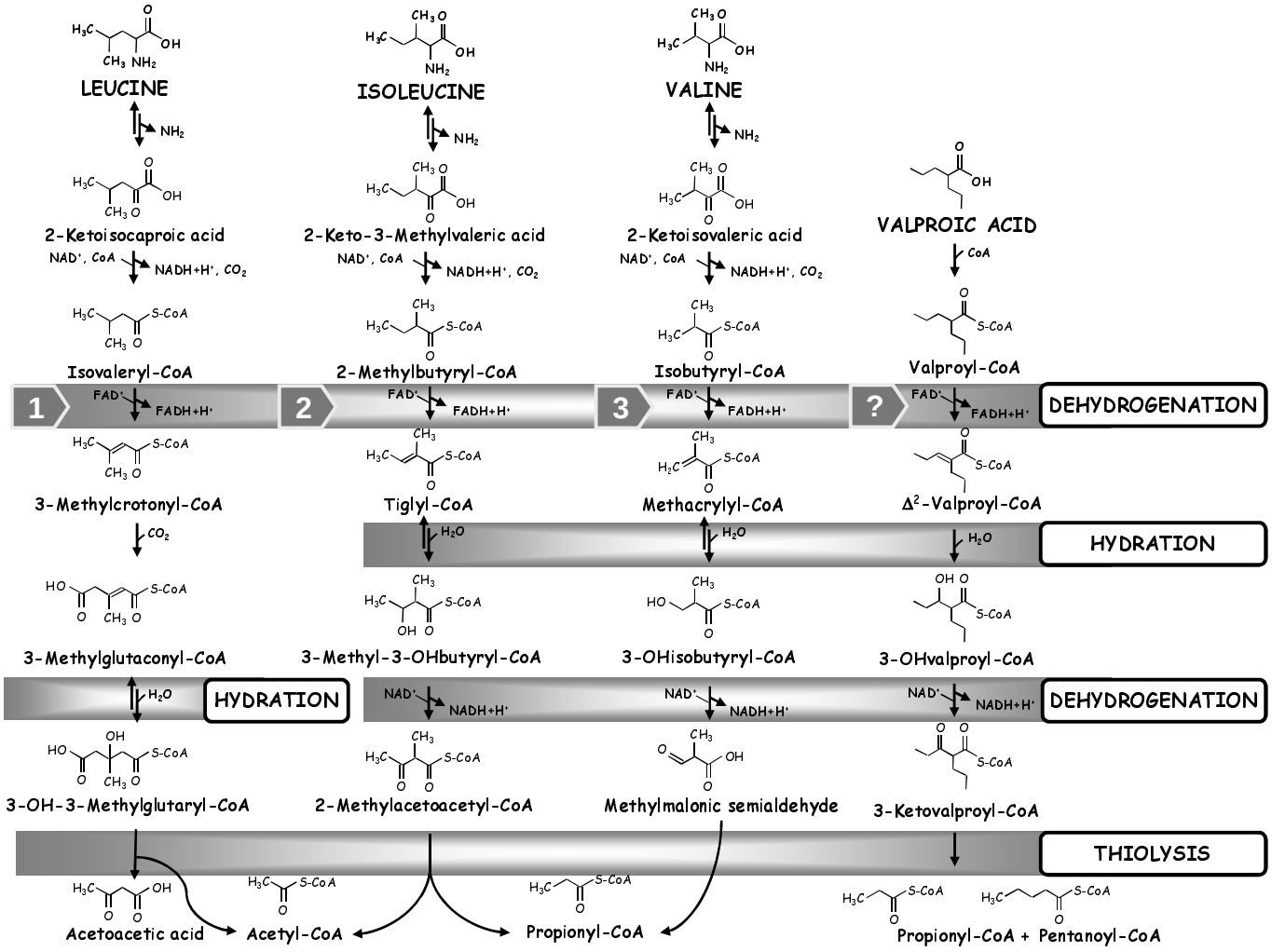
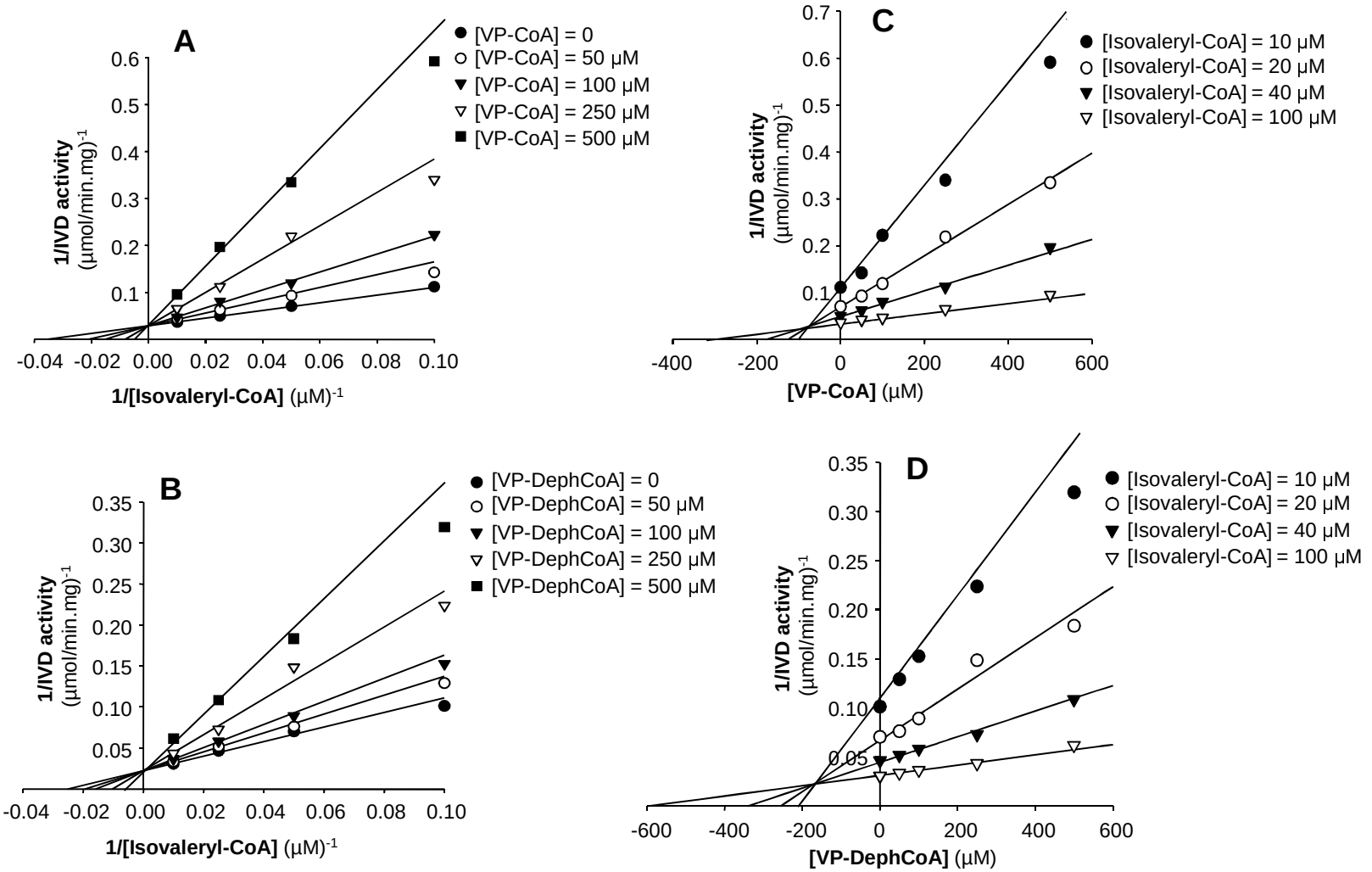


Figure 2



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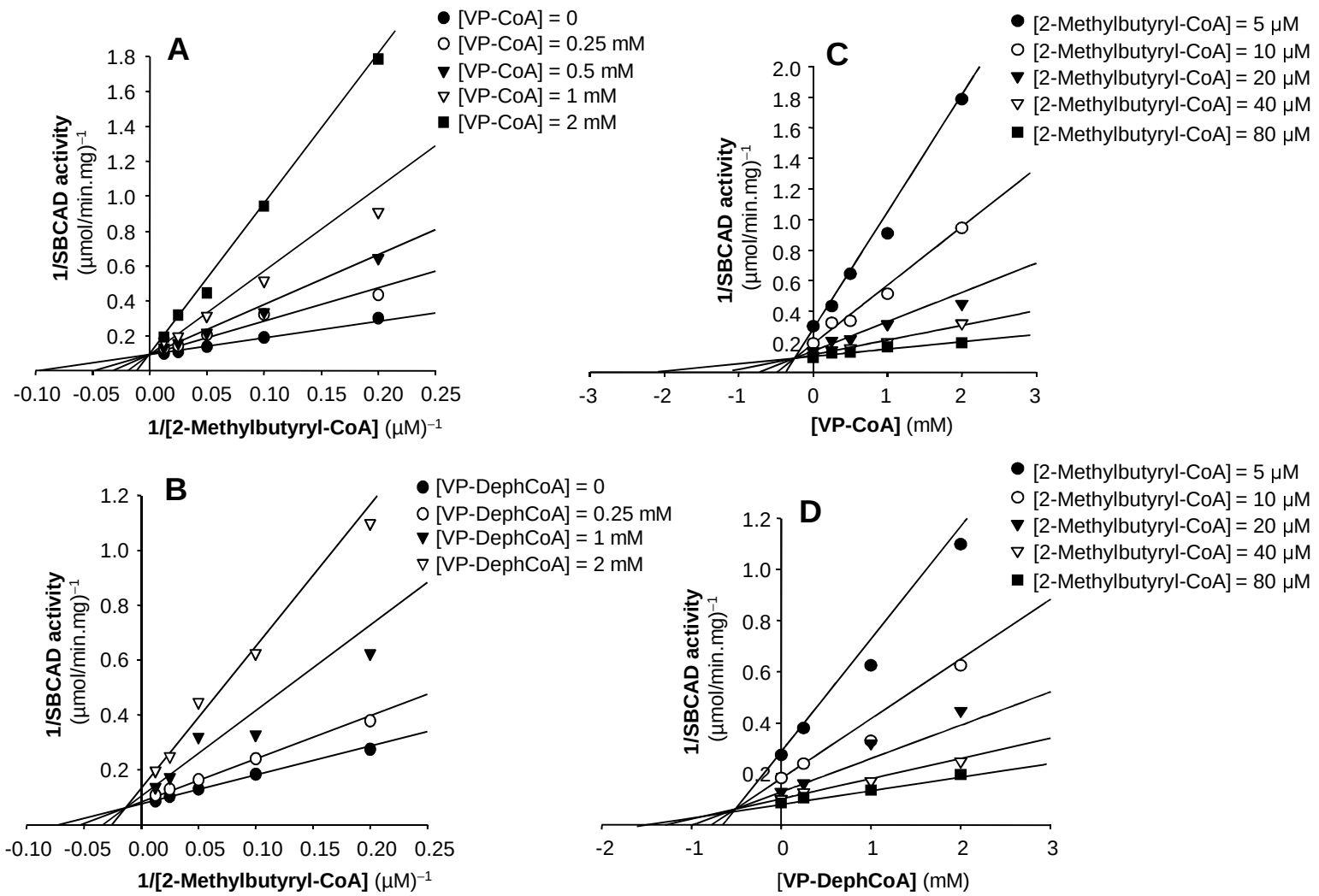


Figure 4

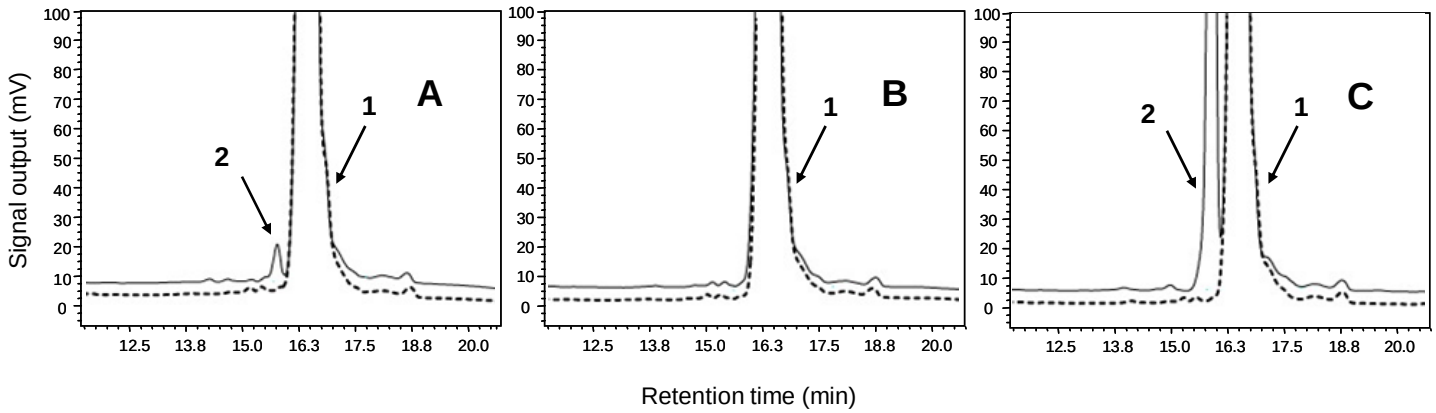


Figure 5

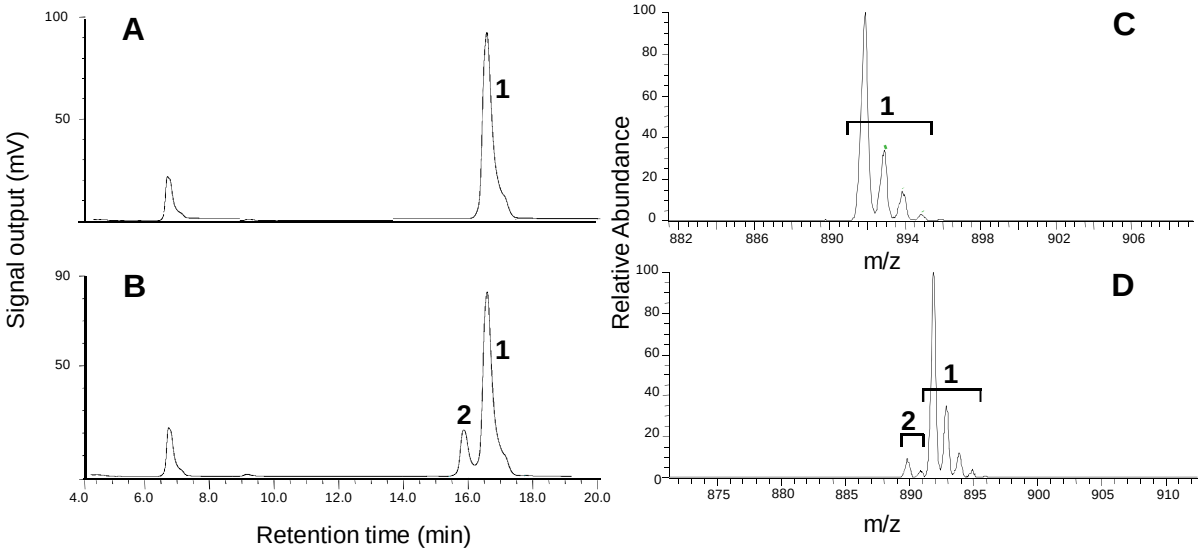
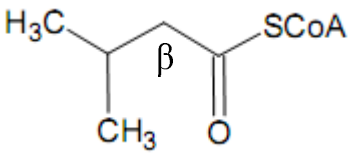
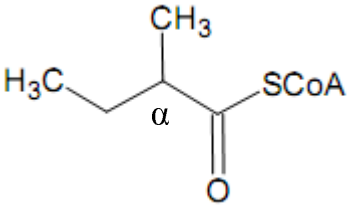


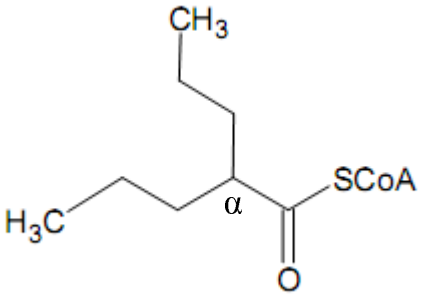
Figure 6



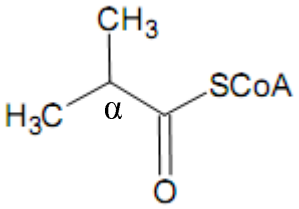
Isovaleryl-CoA



2-Methylbutyryl-CoA



Valproyl-CoA



Isobutyryl-CoA