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Valproic acid metabolites inhibit dihydrolipoyl dehydrogenase activity leading to impaired 2-oxoglutarate-driven oxidative phosphorylation

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Abstract

The effect of the antiepileptic drug valproic acid (VPA) on mitochondrial oxidative phosphorylation (OXPHOS) was investigated in vitro. Two experimental approaches were used, in the presence of selected respiratory-chain substrates: (1) formation of ATP in digitonin permeabilized rat hepatocytes and (2) measurement of the rate of oxygen consumption by polarography in rat liver mitochondria. VPA (0.1–1.0 mM) was found to inhibit oxygen consumption and ATP synthesis under state 3 conditions with glutamate and 2-oxoglutarate as respiratory substrates. No inhibitory effect on OXPHOS was observed when succinate (plus rotenone) was used as substrate. We tested the hypothesis that dihydrolipoyl dehydrogenase (DLDH) might be a direct target of VPA, especially its acyl-CoA intermediates. Valproyl-CoA (0.5–1.0 mM) and valproyl-dephosphoCoA (0.5–1.0 mM) both inhibited the DLDH activity, acting apparently by different mechanisms. The decreased activity of DLDH induced by VPA metabolites may, at least in part, account for the impaired rate of oxygen consumption and ATP synthesis in mitochondria if 2-oxoglutarate or glutamate were used as respiratory substrates, thus limiting the flux of these substrates through the citric acid cycle.

Keywords: Valproic acid; Oxidative phosphorylation; Dihydrolipoyl dehydrogenase; Valproyl-CoA; Mitochondrial dysfunction; Drug-induced hepatotoxicity

The association between valproic acid, an effective anticonvulsant drug, with hepatotoxicity and steatosis is well documented [1–3]. After acetaminophen and trogliatzone, valproate drug has been referred as the third most common xenobiotic suspected of causing death due to liver injury [4].

Besides the inhibition of mitochondrial fatty acid β-oxidation, the impairment of oxidative phosphorylation (OXPHOS) is an important mechanism leading to mitochondrial dysfunction, which plays a central role in valproate-induced steatosis and steatohepatitis [5,6]. OXPHOS is regulated by a complex variety of factors and any xenobiotic that may inhibit this pathway will compromise normal mitochondrial function. However, the mechanisms involved in the drug-induced effects on OXPHOS are still far from being resolved.

Valproic acid (VPA) and its metabolites have been reported to inhibit mitochondrial OXPHOS as first described by Haas et al. who demonstrated a direct effect of VPA on mitochondrial substrate oxidation in vitro [7–9]. This effect was also studied following the in vivo administration of VPA [10–12]. The reported data are, however, quite variable, depending on the approach, on the type of substrate used and on the degree of

Abbreviations: VPA, 2-*n*-propylpentanoic acid or valproic acid; $\Delta^{2(E)}$-VPA, 2-*n*-propyl-2-pentenoic acid; Δ^{4}-VPA, 2-*n*-propyl-4-pentenoic acid; CoA, coenzyme A; dephCoA, dephosphoCoA; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid; SEM buffer, Sucrose/EGTA/MOPS buffer; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; BCA, bicinchoninic acid; PDHC, pyruvate dehydrogenase complex; 2-OGDHC, 2-oxoglutarate dehydrogenase complex; BCODHC, branched chain 2-oxoacid dehydrogenase complex; DLDH, dihydrolipoyl dehydrogenase (dihydrolipoamide dehydrogenase or subunit E_3: EC 1.8.1.4)

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inhibition induced by VPA. In the present work, the in vitro effects of VPA on mitochondrial OXPHOS were re-evaluated in an attempt to further explain one of our previous findings [13] of a striking effect of the drug on mitochondrial pyruvate oxidation. The effects of VPA and of two unsaturated metabolites, $\Delta^{2(E)}$-VPA and Δ^{4}-VPA, were studied on the energy metabolism driven by two substrates, glutamate and 2-oxoglutarate. Furthermore, we studied the hypothesis whether the obtained effects were due to a specific inhibition at the level of dihydrolipoamide dehydrogenase (DLDH), the common component (E_3) of the multi-enzyme complexes 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase and the branched-chain 2-oxoacid dehydrogenase [14]. The measurement of the activity of this individual enzyme was performed in the presence of two potential inhibitors: valproyl-CoA and valproyl-dephosphoCoA (dephCoA). The former is the acyl-CoA ester of VPA that is formed in higher concentration in the mitochondrial matrix [15] and the strongest inhibitor of the glycine cleavage system (GCS) as compared with VPA [16,17]. The second VPA metabolite was identified by our group [18] as being a potential dephosphorylation product of the adenosine moiety of valproyl-CoA.

1. Materials and methods

Reagents and chemicals: VPA was obtained from Sigma Chemical Co. (St. Louis, MO, USA) as well as D,L-lipoamide, bovine serum albumin (fatty acid free) (BSA), bicinchoninic acid (BCA) and other biochemical standards. $\Delta^{2(E)}$-VPA and Δ^{4}-VPA were a kind gift of Prof. Dr. D. Lindhout (University Medical Center, Utrecht). Valproyl-CoA and valproyl-dephosphoCoA were synthesised as described previously [15,18]. 2-Oxoglutarate dehydrogenase (2-OGDH) (from porcine heart) was obtained from Sigma Chemical Co (St. Louis, MO, USA). ADP, ATP, NAD and NADH were obtained from Boehringer Mannheim GmbH (Germany).

1.1. Preparation of hepatocytes and rat liver mitochondria

Male Wistar rats (200–250 g) were given a standard laboratory diet. Hepatocytes were isolated according to established procedures essentially using the method of Berry and Friend [19] and subsequently suspended in Krebs Henselheit buffer (pH=7.4). Cell viability was routinely tested by trypan blue (0.4% w/v) exclusion and phase contrast microscopy. Preparations with more than 10% damaged cells were discarded. Protein was determined by the bicinchoninic acid (BCA) assay [20] using bovine serum albumin as a reference substance. Cell viability was tested in parallel incubations. The inclusion of VPA, $\Delta^{2(E)}$-VPA or Δ^{4}-VPA did not induce a decrease in cell viability compared with the control conditions, within the used incubation time at 37 °C.

Adult male Wistar rats (about 250 g) were starved for 18 h and used for preparation of mitochondria. After decapitation, the livers were rapidly removed and rinsed into ice-cold homogenization medium (250 mM manitol, 5 mM Tris–HCl, 0.5 mM EGTA; pH=7.4). Rat liver mitochondria were prepared according to a published procedure [15]. The mitochondria were finally resuspended in the homogenization medium (25–50 mg/mL). Mitochondrial protein content was measured using the BCA assay and BSA as a reference substance. All incubations with VPA and its metabolites were performed in freshly isolated mitochondria.

1.2. Synthesis of ATP using rat hepatocytes

Measurement of ATP synthesis in digitonin permeabilized hepatocytes was essentially based on the method described by Wanders et al. [21]. The cells were suspended in Krebs Henselheit buffer (pH=7.4). Hepatocytes (final conc. 0.4 mg/mL) were pre-incubated for 10 min at 25° C, in the presence and absence (controls) of VPA or its unsaturated metabolites, $\Delta^{2(E)}$-VPA and Δ^{4}-VPA. As the solution of $\Delta^{2(E)}$-VPA was prepared in DMSO, DMSO was added to the control incubations at the same final concentration (1%). Reactions were started by adding ADP (1 mmol/L) and allowed to proceed for 30 min at 25 °C in a cytosol-mimicking medium containing 15 mg/mL digitonin (optimal concentration previously established for hepatocytes; results not shown), 150 mM KCl, 25 mM Tris–HCl (pH=7.4), 2 mM EDTA, 10 mM potassium phosphate buffer (pH=7.4), 0.1% bovine serum albumine and the respiratory substrates: 10 mM succinate (plus 20 μg/mL rotenone), 10 mM L-glutamate (plus or minus 5 mM L-malate) and 10 mM 2-oxoglutarate. VPA was added to the reaction medium from a stock solution at pH=7.4. For each respiratory substrate, additional negative controls were tested in the presence of 50 mM 2,4-dinitrophenol (DNP). Reactions were stopped with 2 M perchloric acid with vortexing. After 15 min on iced water, samples were centrifuged for 5 min at 10,000*g* (4 °C) and the supernatants neutralised to pH=6–7 with 2 M KOH/ 0.6 M MOPS. The ATP produced was measured fluorimetrically in the protein-free supernatants according to a standard procedure [22].

1.3. Respiration oxidation rates using rat liver mitochondria

The effect of VPA and Δ^{4}-VPA on mitochondrial respiratory function was assessed by means of a polarographic technique using intact rat liver mitochondria in the presence of the following respiratory substrates: succinate (plus rotenone), L-glutamate (plus or minus L-malate) and 2-oxoglutarate.

Oxygen consumption was measured at 25 °C in a Clark-type membrane coated oxygen electrode (Hansatech, Instr.) in a 0.5 mL incubation reaction vessel. The medium had the following composition: 250 mM mannitol, 5 mM Tris–HCl, 0.5 mM EGTA, 10 mM potassium phosphate buffer (pH=7.4) and 0.5% BSA. Mitochondrial protein was used at a concentration of 0.4 mg/mL, with 10 mM succinate (plus 3 μM rotenone) or 10 mM glutamate (plus or minus 5 mM malate), and at 1.0 mg/mL when 10 mM 2-oxoglutarate was used as substrate. VPA was added to the reaction medium from a stock solution at pH=7.4. After an equilibration period in the presence of the respective substrate and eventually the inhibitor (plus or minus VPA or Δ^{4}-VPA), ADP (0.2 mM) was added to initiate state 3 oxidation. Evaluation of states 3 and 4 and the subsequent calculation of the respiratory control indexes and P/O-ratios were essentially done according to Estabrook [23], assuming an oxygen concentration of 0.253 μmol/mL (O_2) under the conditions described above.

1.4. Measurement of dihydrolipoamide dehydrogenase (E_3 subunit) activity using purified 2-OGDH

Dihydrolipoamide dehydrogenase activity was measured according to a method adapted from Reed *et al.* [24]. Considering that the kinetics of the reaction catalysed by DLDH in the forward direction (a) is technically very difficult to measure [14], in the present work the enzyme was measured in the reverse direction (b):

$$\underset{}{NAD^+} + \underset{\text{(dihydrolipoamide)}}{Lip(SH)_2} \overset{(a)}{\underset{(b)}{\rightleftarrows}} \underset{\text{(Lipoamide)}}{Lip(S)_2} + NADH^+ + H^+$$

The assay is based on the spectrophotometric determination of the rate of NADH oxidation (at 340 nm) in the presence of the dehydrogenase and lipoamide. The standard assay mixture contained 200 μM NADH in 150 mM potassium phosphate buffer (pH=6.7), water and enzyme solution, making a total volume of 500 μL. Reactions were initiated by adding 25 μL of 3 mM lipoamide dissolved in 4.8% ethanol (solution made immediately before use) to the mixture described above. The temperature was maintained at 37 °C. Stock solution of the enzyme complex contained 0.28 U/mg for 2-OGDH. The respective enzyme solution (2-OGDH) was prepared in phosphate buffered saline (PBS) immediately before the assay to avoid loss of catalytic activity.

Valproyl-CoA and valproyl-dephosphoCoA were added to the medium and the decrease in absorbance was monitored for 5 min before starting the reaction. The addition of the substrate lipoamide was therefore done after a pre-incubation of 5 min at 37 °C. A complete inactivation of the enzyme was achieved using arsenite (10 mM) which gave an enzyme activity less than 3% of the control, measured under the same conditions but in the absence of the inhibitor.

1.5. Data analysis

The parameters (K_m and V_{max}) of enzyme kinetics were obtained by four methods of calculation: three linearizations of the Michaelis–Menten equation $V = [S] \times V_{max}/([S] + K_m)$(Lineweaver – Burk $1/V = f(1/[S])$; Eadie – Hofstee $V = f(V/[S])$; and Hanes-Woolf $[S]/V = f([S])$) and the direct linear plot of Cornish Bowden $V_{max} = f(K_m)$ [25].

The best-fit curves of the Michaelis–Menten equation to the experimental data were obtained through non-linear regression analysis by the computer software Berkeley Madonna (v. 8.0®, Macey and Oster).

2. Results

2.1. Effect of VPA, $\Delta^{2(E)}$-VPA and Δ^4-VPA on the rate of ATP synthesis

In order to study the effect of VPA (0.1–2.0 mM) and its unsaturated metabolites, $\Delta^{2(E)}$-VPA (1 mM) and Δ^4-VPA (1 mM), on ATP-synthesis in mitochondria, rat hepatocytes were permeabilized with digitonin and incubated with different substrates, including succinate (plus rotenone), L-glutamate (plus L-malate), L-glutamate and 2-oxoglutarate under state 3 conditions. The incubations made without the parent drug or its metabolites were considered as controls and the obtained oxidation rates were taken as 100%. The absolute values for reference ranges of the rates of ATP synthesis, expressed in nmol ATP/min/mg protein, were: 41.05–41.23, 48.02–49.82, 34.74–35.50 and 18.59–18.90 respectively for succinate (plus rotenone), L-glutamate (plus L-malate), L-glutamate and 2-oxoglutarate.

VPA was found to inhibit the rate of ATP synthesis in the presence of glutamate and 2-oxoglutarate as respiratory substrates, respectively (Fig. 1A–I). No inhibitory effect was observed on succinate driven-oxidative phosphorylation and only a very mild one (20–25%) with glutamate/malate as substrate couple.

Fig. 1A-II shows that ATP synthesis was also selectively inhibited by Δ^4-VPA depending on the respiratory substrate used. As observed with VPA, ATP synthesis remained practically unaffected by Δ^4-VPA with succinate/rotenone as substrate, whereas ATP synthesis was clearly inhibited when 2-oxoglutarate or L-glutamate was used as a respiratory substrate. These results suggest that VPA and Δ^4-VPA may have a similar mechanism of inhibition most probably different from that of $\Delta^{2(E)}$-VPA, which induced a much milder inhibitory effect on ATP synthesis driven by the same substrates (70–90% of control).

2.2. Effect of VPA and Δ^4-VPA on the oxygen consumption rate

The effects of VPA and Δ^4-VPA on the oxidation rate of succinate (plus rotenone), glutamate (plus or minus malate),

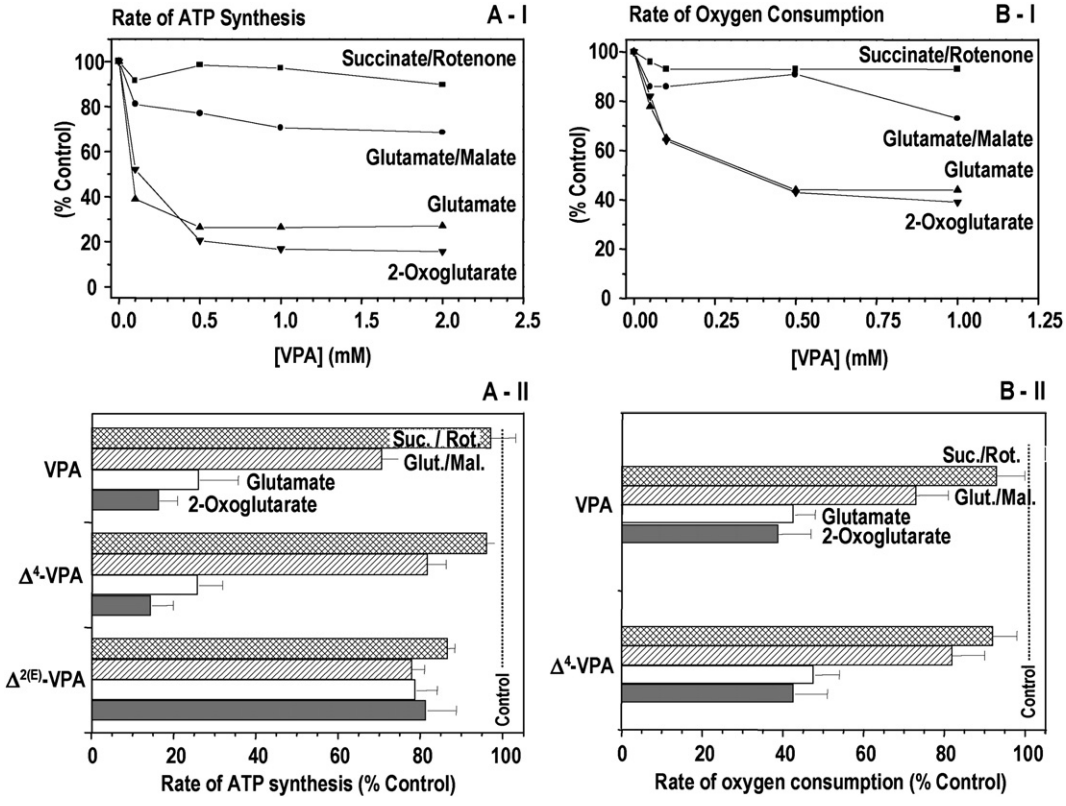

Fig. 1. Effect of VPA (variable concentration) (graphs A-I and B-I) and its unsaturated metabolites, Δ^4-VPA and $\Delta^{2(E)}$-VPA (graphs A-II and B-II, all at conc. = 1 mM), on the: (A) ATP production rate, in rat hepatocytes, (B) oxygen consumption in rat liver mitochondria, both driven by different respiratory substrates: succinate (plus rotenone) –■–, glutamate (plus malate) –●–, glutamate –▲–, and 2-oxoglutarate. –▼– (see Materials and methods for experimental details; the data shown are from a representative experiment; similar results were obtained in three other experiments; the data shown in graphs A-II and B-II represent the mean value and the respective error bar. The estimated value for the standard deviation (%) for all points (graphs I and II) was below 12.7%.).

and 2-oxoglutarate, were subsequently determined in coupled rat liver mitochondria. In all experiments, the mitochondria were pre-incubated with the drug for 5 min. This time period is sufficient to give the maximum inhibitory effect for all substrates [12], before initiation of state 3 oxidation. The addition of ADP was delayed for the same period of time in the controls. The absolute reference values for the oxidation rates, expressed in nmol O_2/min/mg protein, were: 52.1, 40.9, 42.8, and 20.4 respectively for succinate (plus rotenone), L-glutamate (plus L-malate), L-glutamate and 2-oxoglutarate.

In agreement with the results obtained when ATP synthesis was studied, VPA inhibited glutamate and 2-oxoglutarate oxidation (state 3) to a similar extent (Fig. 1B-I). The oxygen consumption with succinate/rotenone was not significantly affected by VPA and only a very mild effect (20–25%; 1 mM VPA) was obtained with glutamate/malate as substrate couple. State 4 oxygen consumption remained practically unchanged with all substrates as compared to control values, except for glutamate where a slight increase was found. In accordance with the ATP results, Fig. 1B-II shows that Δ^4-VPA (at 1 mM) inhibited the oxidation of the same substrates to a similar extent as VPA (1 mM).

2.3. Effect of valproyl-CoA and valproyl-dephosphoCoA on the enzyme activity of E_3 subunit

The dihydrolipoamide dehydrogenase activity (E_3) was linear up 0.1 U/mL. Thus further experiments on substrate dependence and the effect of inhibitors on the E_3 activity were performed using a protein concentration of 0.05 U/mL. The effect of increasing concentration of NADH (0–400 μM) on E_3 activity was also studied, providing an estimated K_m of 37.3 μM and a V_{max} of 2.5 μmol/min/mg respectively. Further experiments were performed using a fixed concentration of 150 μM of NADH, thus ensuring that NADH would not be limiting flux through the enzyme. The activity of E_3 as a function of increasing concentration of lipoamide (0.25–3.0 mM) was determined. The kinetic constants K_m and V_{max} were estimated by the linearizations of the Michaelis–Menten equation and by the fit of this equation to the experimental data points using the non-linear regression software. A K_m of 0.49 mM and a V_{max} of 1.0 μmol/min/mg were respectively obtained.

The activity of DLDH was determined in the presence of the potential inhibitors valproyl-CoA (0.5 and 1.0 mM) and valproyl-dephCoA (0.5 and 1.0 mM) at various lipoamide concentrations. Fig. 2 displays the kinetic data of E_3 activity

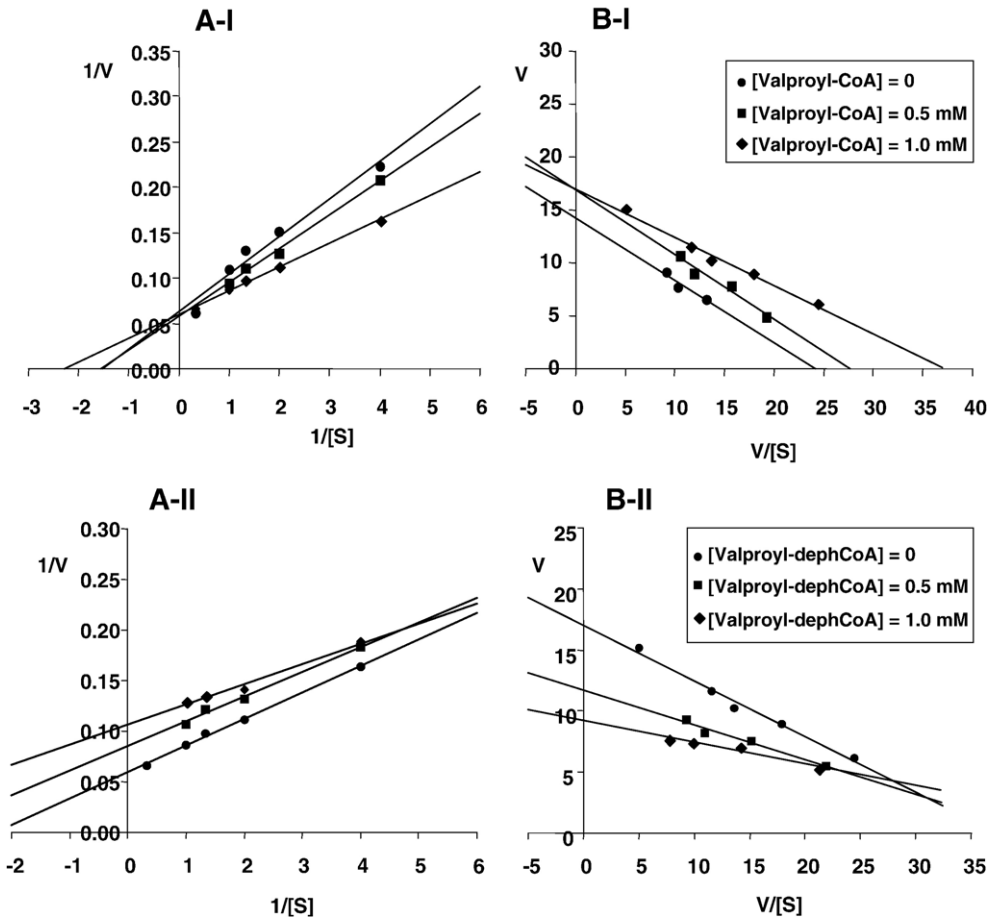

Fig. 2. Linearization plots of dihydrolipoamide dehydrogenase (DLDH) activity in the presence of valproyl-CoA (graphs A-I and B-I) and in the presence of valproyl-dephosphoCoA (graphs A-II and B-II): graph A (I and II)—Lineweaver–Burk plot ($1/V = f(1/[S])$) graph B (I and II)—Eadie–Hofstee plot ($V = f(V/[S])$).

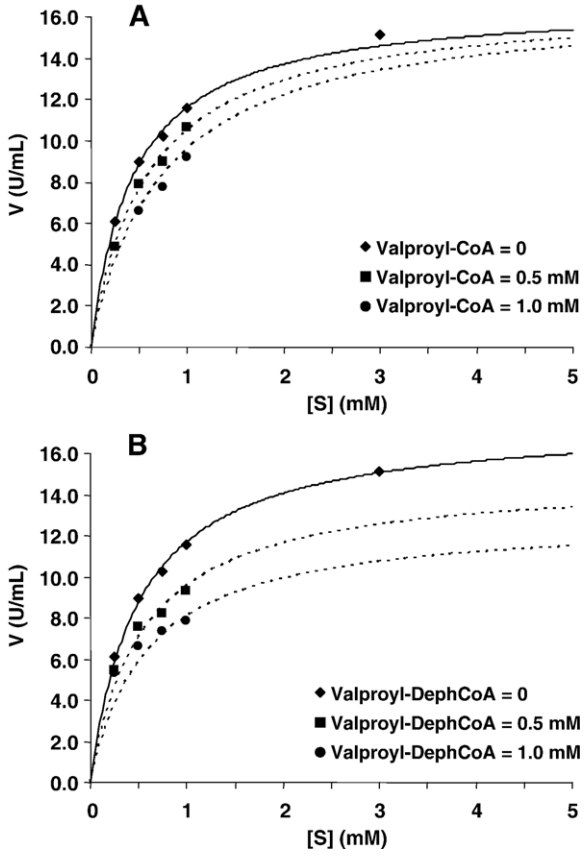

Fig. 3. Measurement of dihydrolipoamide dehydrogenase activity as a function of substrate (lipoamide) concentration (0.25 to 3.0 mM) in the absence and in the presence of valproyl-CoA (A) and valproyl-dephCoA (B). The dashed lines represent the curve simulations based on the estimated kinetic parameters obtained with the inhibitors, superimposed to our experimental data, in parallel to the best-fits obtained in the absence of inhibitor (upper curves) (see Materials and methods and Results for experimental conditions and data analysis).

obtained in the presence of both VPA metabolites according to the linearization procedures of Lineweaver–Burk (Fig. 2 AI and AII) and Eadie–Hofstee (Fig. 2 BI and BII). The Hanes–Woolf linearization and the direct linear plot of Cornish Bowden were also used to analyse the experimental data (plots not shown).

The results clearly show an inhibition of DLDH activity induced by valproyl-CoA (Fig. 2 AI and B-I) and valproyl-dephCoA (Fig. 2 A-II and B-II) at physiological concentrations. On the basis of the overall graphical analysis, our data suggest a clear distinction between the mechanisms of inhibition assigned to the two tested compounds: valproyl-CoA seems to interact as a pure competitive inhibitor and valproyl-dephCoA appears to be an uncompetitive inhibitor.

The kinetic parameters (V'_{max} and K'_m, expressed in μmol/min/mg and mM respectively) for each inhibitor were calculated according to the different linearization procedures. The following average values were obtained: valproyl-CoA, 0.5 mM (V'_{max} = 1.01 and K'_m=0.63) and 1.0 mM (V'_{max} =0.85 and K'_m=0.61), valproyl-dephCoA, 0.5 mM (V'_{max} =0.69 and K'_m=0.29) and 1.0 mM (V'_{max} =0.54 and K'_m=0.17). To further confirm the inhibition mechanism, the average values were used to calculate the ratios: $\frac{V'_{max}}{V_{max}}\cdot\frac{K_m}{K'_m}$ and $(V'_{max}/V_{max})/(K'_m/K_m)$ which are bilinear functions of the inhibitor concentration [I] [25].

The graphic representation of our data according to these functions was in accordance with the assumption that valproyl-CoA and valproyl-dephCoA inhibit E_3 activity by the two presumed models of inhibition (data not shown). Therefore, the respective values of K_I were calculated for valproyl-CoA and valproyl-dephCoA following the respective inhibition models.

A K_I of 1.60 mM for valproyl-dephCoA was calculated through the graphic representation of the ratio $\frac{K'_m}{K_m}$ versus $[I]$ $\left(as\ \frac{K'_m}{K_m}=1+\frac{[I]}{K_i}\right)$, where K_I is the obtained reciprocal of the slope. For valproyl-CoA, the K_I obtained by the bilinear function was further confirmed with another graphic representation (method of Dixon) [26] given by $1/V=f([I])$ and a value of 1.43±0.29 mM was obtained.

Using the kinetic parameters calculated as described above (valproyl-CoA: K_I=1.43 mM and valproyl-dephCoA: K_I=1.60 mM) the hyperbolic curves of DLDH activity versus [S] in the presence of both inhibitors were simulated for the various values of K'_m and V'_{max}. Fig. 3 represents the curve simulations obtained with the inhibitors superimposed to our experimental data, in parallel to the best-fits obtained in the absence of inhibitor. As shown in Fig. 3A, in the presence of 0.5 mM and 1.0 mM of valproyl-CoA, the apparent K_m tends to increase and V_{max} is not significantly affected, as expected for a competitive inhibitor. However, as shown in Fig. 3B, valproyl-dephCoA at 0.5 and 1.0 mM inhibits E_3 activity by an uncompetitive mechanism, affecting both the values of K_m that increases and V_{max} that decreases.

3. Discussion

Valproate and its unsaturated metabolite Δ^4-VPA were found to be clear inhibitors of 2-oxoglutarate- and glutamate-driven oxidative phosphorylation and of the respiration rate sustained by these substrates. Interestingly, no inhibitory effect was found on respiration and ATP synthesis when succinate, in the presence of rotenone, and glutamate, in the presence of malate, were used as respiratory substrates.

The present data on the effect of VPA on OXPHOS show that VPA is not affecting the electron transport chain *per se* (after complex II) or strictly the phosphorylation system, as we have previously reported [13]. In fact, since the ATP production rate obtained with succinate/rotenone as substrate remained unaffected by valproate, the drug does not affect the synthesis of ATP from ADP and phosphate. However, the effects induced by VPA observed in the presence of 2-oxoglutarate and glutamate as single respiratory substrates are clearly indicative of a common underlying mechanism of inhibition. These substrates share some common aspects in their metabolism. In hepatocytes, glutamate is transported from the cytosol into the mitochondria where it undergoes oxidative deamination catalysed by L-glutamate dehydrogenase present in the matrix, yielding 2-oxoglutarate and ammonia. 2-Oxoglutarate can be also imported into mitochondria directly by a specific carrier in the mitochondrial membrane and converted *via* the citric acid cycle until full oxidation to CO_2 and H_2O.

Several hypotheses were formulated to explain the obtained results and to establish the linkage between these substrates.

The observed inhibitory effects of VPA and Δ^4-VPA on mitochondrial ATP synthesis in the presence of pyruvate, 2-oxoglutarate and glutamate is likely due to an inhibitory effect on their respective mitochondrial oxidation and/or on their specific transport systems embedded in the mitochondrial inner membrane. Since the hypothesis of the defective import into mitochondria would implicate an effect on at least three different transport systems (pyruvate carrier, 2-oxoglutarate carrier and glutamate transport systems), which seemed unlikely, we reasoned that specific inhibition of dihydrolipoamide dehydrogenase (E_3 subunit) would be the most likely mechanism to investigate. Fig. 4 depicts the integrated scheme of the metabolic relationships referred above. As illustrated, an interaction of the intramitochondrial metabolites of VPA with the E_3 subunit activity would impair the oxidation rates of both 2-oxoglutarate and glutamate as reported in this work and that of pyruvate as reported earlier by our group [13]. In order to find evidence for this, the activity of E_3 subunit was studied and characterized in the presence of valproyl-CoA, the most abundant CoA ester of VPA in the mitochondrial matrix [15] and in the presence of its dephosphorylated product [18]. The present work reports a VPA associated decrease on ATP synthesis rate driven by specific respiratory substrates and it has been previously reported by our group that any intramitochondrial depletion on ATP would increment the conversion rate of valproyl-CoA into valproyl-dephosphoCoA with its subsequent concentration increase in the matrix [18]. Our data suggest that DLDH is apparently one potential target of toxicity of this metabolite. Thus the results obtained with the second ester of VPA are of great importance, since the understanding of the potential toxic significance of valproyl-dephosphoCoA formation in mitochondria, remains to be fully elucidated.

Due to the very limited amount of Δ^4-VPA, not commercially available, it was not possible to study its direct effect on E_3 activity or to synthesize its CoA ester. Therefore further work is required to evaluate the potential inhibitory role of Δ^4-VPA or Δ^4-VPA-CoA on this enzyme.

Our present results show that valproyl-CoA and valproyl-dephCoA formed intramitochondrially after activation of VPA, both inhibit the activity of DLDH. Being the common component (E_3) of the multienzyme complexes pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (as well as branched chain 2-oxoacid dehydrogenase) we may predict that these mitochondrial enzymes will also be affected by VPA and/or valproyl-CoA (or -dephCoA). The subsequent reduced rate of NADH and possibly $FADH_2$ production in the mitochondrial matrix may explain the reported decrease of oxygen consumption induced by VPA, in the presence of the substrates mentioned above.

Kinetic analysis suggests that valproyl-CoA inhibits DLDH by a purely competitive mechanism whereas valproyl-dephCoA interacts with the enzyme by a purely uncompetitive mechanism. A deeper insight into the binding mode of these acyl thioesters related with enzyme structural data needs further investigation in order to shed some light to this differential mode of inhibition.

In agreement with our findings, a competitive inhibition of beef brain 2-OGDHC by valproyl-CoA and $\Delta^{2,3}$-valproyl-CoA (1 mM) was reported earlier by Luder et al. [27]. Moreover, patients receiving valproate have been reported to have higher

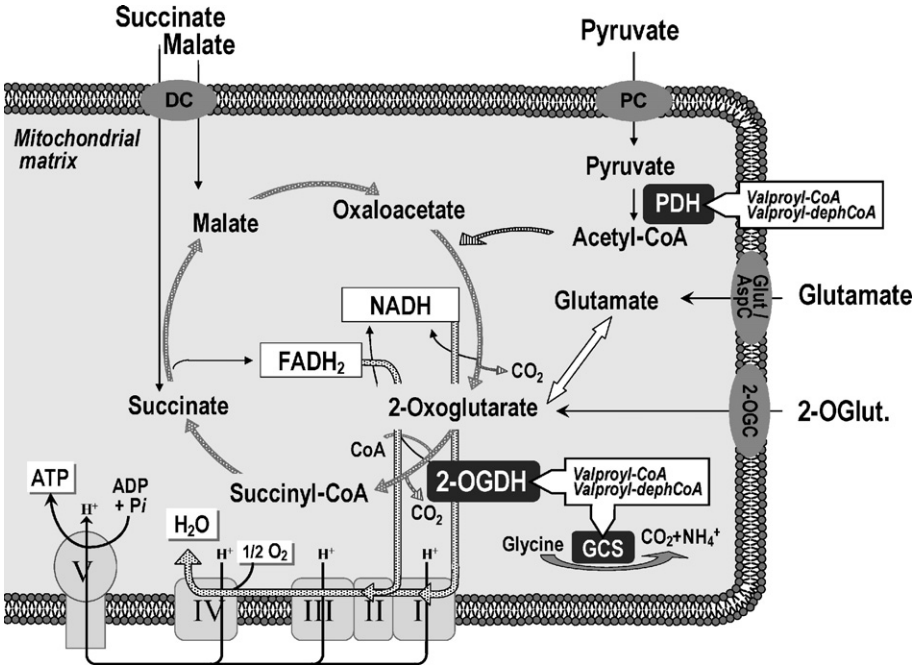

Fig. 4. A simplified schematic representation of the inhibitory effect induced by valproyl-CoA and valproyl-dephCoA on mitochondrial dihydrolipoyl dehydrogenase, affecting the oxidative metabolism of 2-oxoglutarate, glutamate and pyruvate as well as the catabolism of glycine. (Abbreviations of enzymes and carriers: PDH: pyruvate dehydrogenase complex; 2-OGDH: 2-oxoglutarate dehydrogenase complex; GCS: glycine cleavage system; DC: dicarboxylate carrier; PC: pyruvate carrier; Glut/AspC: glutamate/aspartate carrier; 2-OGC: 2-oxoglutarate carrier).

serum branched-chain amino acids and a significant increase in the excretion of the deaminated acid metabolites of valine, isoleucine and leucine [28]. These in vivo effects clearly indicate an interaction between valproate and branched-chain amino acid metabolism, where the possible inhibition on BCODHC has to be considered. Concerning the effect of valproate on PDHC activity, although it has been evoked as a possible interaction site [13,29], to our knowledge however, no direct evidence showing the interference of VPA with this enzyme has been reported.

Although rarely mentioned, DLDH also participates in the glycine cleavage system (GCS) by reoxidising the dihydrolipoyl moiety of the hydrogen carrier protein of the system, which is required for the metabolism of glycine to N^5, N^{10}-methylene-tetrahydrofolate, CO_2 and NH_4^+ [14]. Interactions of VPA with the GCS have also been demonstrated in the literature [16,17], but none of those reports have assigned the inhibitory effects of VPA on GCS with a potential VPA-induced decrease in DLDH activity. Moreover, our results may partly explain the reported effects of VPA on the mitochondrial glycine cleavage system and the hyperglycinemia associated with VPA treatment. This phenomenon has remained unexplained so far.

It is interesting to verify the clinical and biochemical spectrum of abnormalities that may eventually exist between a genetic and an acquired (drug-induced) deficiency in the E_3 subunit. Inherited defects in lipoamide dehydrogenase are rather rare disorders in humans and the impairment of the enzyme is much more striking than the mild in vitro effect that we report in this work. The clinical presentation and course is variable, ranging from acidemia in early childhood, infantile neurodegenerative disease and death in many cases. But interestingly, hepatic impairment has occurred in a case of a genetic defect of E_3 [30] and a clinical picture with Reye-like liver involvement without mental retardation has also been described in the Ashkenazi-Jewish population [31,32]. A mild DLDH deficiency has been reported in an adult case only with hepatocellular injury and without central nervous system involvement [33]. In fact, a benign or severe form of liver dysfunction has been documented in many cases of valproate associated hepatotoxicity [34].

Furthermore, studies with three cell lines (fibroblasts) of DLDH deficient patients showed that ATP production rates were decreased in all of them, suggesting in addition that there is an ATP synthesis threshold below which serious neurological disease becomes evident [35]. According to the authors, a slight increment of ATP synthesis rate was likely sufficient for moderate brain protection. Transposing these considerations to our results, we would like to find the answer to one immediate question: what would the critical ATP synthesis threshold be for liver disease and hepatotoxicity? Considering that ATP is at the crossroad of the major metabolic pathways, our present findings may have interesting implications in cell toxicity associated with VPA therapy.

Although other mechanism(s) may account for the notorious effect on 2-oxoacids-driven OXPHOS and respiration rates observed in vitro in the presence of VPA, these reported effects may well be explained, at least in part, by the inhibition of DLDH by the CoA (or dephCoA) esters of the parent drug.

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