

Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: A review

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Summary Valproic acid (VPA; 2-*n*-propylpentanoic acid) is widely used as a major drug in the treatment of epilepsy and in the control of several types of seizures. Being a simple fatty acid, VPA is a substrate for the fatty acid β -oxidation (FAO) pathway, which takes place primarily in mitochondria. The toxicity of valproate has long been considered to be due primarily to its interference with mitochondrial β -oxidation. The metabolism of the drug, its effects on enzymes of FAO and their cofactors such as CoA and/or carnitine will be reviewed. The cumulative consequences of VPA therapy in inborn errors of metabolism (IEMs) and the importance of recognizing an underlying IEM in cases of VPA-induced steatosis and acute liver toxicity are two different concepts that will be emphasized.

Abbreviations

CoA	coenzyme A
DILI	drug-induced liver injury
FAO	fatty acid β -oxidation
FFA	free fatty acids
IEM	inborn error of metabolism
RLM	rat liver mitochondria
VPA	valproic acid

Introduction

The control of a wide spectrum of seizure disorders is of particular interest for many inborn errors of metabolism (IEMs). Since its introduction into clinical use, valproic acid (VPA) has become one of the most widely prescribed antiepileptic drugs worldwide. Despite the undisputed pharmacological importance and effectiveness of VPA, its potential hepatotoxicity is still a major concern. In fact, the more general problem of drug-induced liver injury (DILI) is a recognized, but unresolved problem with significant clinical and economic impact (Watkins and Seeff 2006). The exact mechanisms of DILI remain largely unknown (Holt and Ju 2006). Their elucidation will be enhanced by a translational research effort, namely involving the identification of genetic determinants such as an underlying IEM. Certainly the understanding of those mechanisms is crucial to predicting and preventing DILI. Steatosis due to drugs and/or other primary aetiologies is one possible presentation of this liver injury. The microvesicular fatty liver triggered by a variety of drugs, including VPA, has been unequivocally associated with mitochondrial dysfunction (Begrich et al 2006).

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Because mitochondrial dysfunction is central in the pathophysiology of steatosis, the study of hepatic metabolism *per se*, either in the dynamics of free fatty acid turnover or in its active role in VPA detoxification, is a focus of our laboratories' interest.

Although the elucidation of the interactions mentioned above is difficult, this paper will focus solely on the endogenous mitochondrial fatty acid β -oxidation (FAO) in humans, highlighting the mechanisms that intersect with the oxidative metabolism of VPA.

Chemistry and pharmacology of VPA

Valproic acid was first synthesized in 1882 (Burton 1882) but its anticonvulsant activity was discovered serendipitously by Pierre Eymard and his group. Unexpectedly, they found an anticonvulsant effect of vehicle alone (VPA) tested as a control. This finding was published in 1963 (Meunier et al 1963) and it is considered a milestone in the history of antiepileptic treatment (Löscher 1999). VPA is a simple eight-carbon branched-chain carboxylic acid with properties of a weak acid (pK_a 4.95). Today, valproic acid is commonly prescribed worldwide, as a broad-spectrum antiepileptic drug with specific indications for many forms of epilepsy and many types of seizures, affecting both children and adults.

The mechanism of the antiepileptic action of VPA involves the regional changes in the concentration of the neurotransmitter γ -aminobutyric acid (GABA), (Perucca 2002; Shorvon 1990). Several studies have shown that VPA administration is associated with increased levels of GABA in the brain, thereby potentiating the GABAergic transmission in specific brain regions (Owens and Nemeroff 2003). Whether this effect is due to activation of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, or to inhibition of the catabolic enzymes succinic semialdehyde dehydrogenase (SSAD) and GABA transaminase (GABA-T) remains unclear (Davis et al 1994; Sztajnkrycyer 2002). In addition, VPA may exert a direct effect on the potassium channels of the neuronal membrane (Porter and Meldrum 2001). VPA reduces the release of the epileptogenic γ -hydroxybutyric acid and attenuates the neuronal excitation mediated by activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. However, the neurochemical and neurophysiological effects of valproate as potential mechanisms for anticonvulsive action are quite complex in nature and are not well-defined (Löscher 2002). It should be noted, however, that the same can be said for virtually all anticonvulsants, despite progress in

mechanistic understanding (Owens and Nemeroff 2003).

Pharmacokinetic aspects

Several comprehensive reviews that discuss the major aspects of the pharmacokinetic properties of valproic acid have been presented (Cotariu and Zaidman 1988; Davis et al 1994; DeVane 2003; Gugler and van Unruh 1980; Shenn 1999; Vajda et al 1981; Zaccara et al 1988). These properties are predominantly determined by the mode of VPA administration, and the condition of the patient (particularly age) (Battino et al 1995; Dutta et al 2004). Detailed information concerning VPA absorption, distribution and elimination can be found in these reviews and other sources (see Abbott Laboratories 2007), and will not be discussed here.

Absorption: VPA is rapidly absorbed after oral administration (bioavailability $\geq 80\%$). While the absorption rate of valproate from the gastrointestinal tract and the fluctuation in plasma valproate concentrations vary with the dosing regimen, formulation and conditions of use, the efficacy of valproate as an anticonvulsant in chronic use is unlikely to be affected.

Distribution: VPA is extensively ($\geq 90\%$) bound to plasma proteins, mainly albumin, similarly to endogenous free fatty acids (FFA). The extent of binding decreases with increasing drug concentration, resulting in an increase of the free fraction of the drug. Chemical equilibrium is established between the different forms of circulating VPA: the protein-bound fraction, the unbound or free fraction, the ionized valproate fraction, and the non-ionized fraction. In principle, as with any xenobiotic, only the free fraction of VPA will cross membranes and enter the cell. The therapeutic concentration of VPA in plasma for most patients during chronic oral treatment ranges from 40 to 100 $\mu\text{g/ml}$ (280–700 $\mu\text{mol/L}$). The simultaneous concentration of valproate in brain will be 6–27 $\mu\text{g/g}$ (42–190 $\mu\text{mol/g}$).

Elimination: Less than 3% of VPA is excreted unchanged into the urine. Hence, VPA undergoes extensive biotransformation, mainly in the liver, via five major metabolic pathways: mitochondrial β -oxidation, microsomal ω - and (ω -1)-hydroxylation, glucuronidation and other minor conjugation reactions. Hepatic metabolism is therefore the major route of valproate elimination. The excretion of VPA and its metabolites occurs primarily in the urine, with trace amounts in the bile, faeces and expired air.

Toxicological aspects

VPA is well tolerated by the vast majority of patients. However, a wide range of adverse effects has been reported in the literature since its introduction into clinical use. Undoubtedly, its potential teratogenicity and hepatotoxicity are the major undesirable effects (Radatz and Nau 1999; Schmidt 1999). The most common adverse effects of valproate include gastrointestinal disturbances, tremor and weight gain. Other rare serious complications may occur in some patients receiving VPA chronically, including fatal haemorrhagic pancreatitis, bone marrow suppression and hyperammonaemic encephalopathy.

Hepatic failure resulting in fatalities has occurred in several patients receiving valproic acid (Bryant and Dreifuss 1996; Dreifuss et al 1989; Scheffner et al 1988). This rare form of hepatotoxicity, often irreversible, is acute and idiosyncratic, characterized by microvesicular steatosis sometimes accompanied by necrosis, and histological characteristics similar to Reye syndrome. Children under the age of 2 years, on a multi-drug antiepileptic regimen are primarily at risk, with a reported incidence of 1/500–1/800 in contrast to an incidence of 1/20 000 in the general population (Perucca 2002).

Mental retardation, neurological diseases, viral infections and inherited metabolic diseases have also been associated with fatality in this group. Therefore, young children may be at higher risk because of immature hepatic function and, most probably, because of a recognized (or undiagnosed) metabolic abnormality that could trigger the hepatic failure. However, adults with VPA-associated fatal hepatotoxicity (17–62 years) have also been reported (König et al 1994, 1999).

A second and different type of hepatotoxicity has also been related to VPA therapy, especially in a chronic regimen. It is a much milder and reversible form of toxicity that seems to be dose-dependent and associated with the start of treatment, where up to 44% of patients have elevated levels of liver enzymes without clinical symptoms (Radatz and Nau 1999). Thus, caution should be exerted in the clinical and biochemical monitoring of patients treated with VPA and the drug should be discontinued with significant hepatic dysfunction.

The pathogenesis of these forms of VPA hepatotoxicity is still not clear, but the observation of microvesicular steatosis is consistent with the VPA-induced impairment of mitochondrial function and fatty acid metabolism (Begrache et al 2006; Pessayre et al 1999).

Biotransformation of VPA

Valproate is almost entirely metabolized by the liver, the organ that is also the dominant target organ of valproate toxicity. The minor contribution of VPA biotransformation in extrahepatic tissues (e.g. skin, gastrointestinal tract, lung and kidney) is not known with certainty.

In principle the elimination of a xenobiotic depends on its conversion into more water-soluble products by biotransformation, resulting in a decrease of its pharmacological effect and a 'detoxification' for the organism. However, in the case of VPA, there are metabolite(s) which also exert a pharmacological effect whereas other metabolite(s) of the drug potentially induce a toxic effect.

The multiple metabolic pathways involved in VPA biotransformation give rise to more than 50 known metabolites of the parent drug (Abbott and Anari 1999). The availability of increasingly sensitive analytical methods has enabled the identification and quantification of numerous VPA metabolites. The simple molecular structure of VPA argues against such a complex biotransformation, which is commonly divided into two main phases: phase I, oxidative reactions, mainly oxidations, reductions and hydrolysis; and phase II, conjugation reactions, including glucuronidation and conjugation with glutathione, carnitine, coenzyme A and/or amino acids such as glycine or glutamic acid. The conjugates formed are highly hydrophilic, which promotes their excretion.

The relative contribution of each individual metabolic pathway to the overall metabolism of VPA has been roughly estimated in adult patients on monotherapy: (1) 30–50% of dose appears in the urine as a glucuronide conjugate; (2) mitochondrial β -oxidation typically accounts for over 40% of the dose; (3) less than 15–20% of the dose is eliminated by other oxidative mechanisms.

Special emphasis will be given here to the mitochondrial metabolism of the drug, which involves activation to a coenzyme A ester and subsequent β -oxidation (see below).

Most if not all of the extramitochondrial phase I oxidative biotransformation of VPA is ascribed to the cytochrome P450 enzyme system, responsible for a small part of VPA metabolism. The desaturation of VPA to the terminal olefin Δ^4 -VPA is an important P450-catalysed reaction of this drug metabolism (Rettie et al 1987). The isoforms CYP2C9 and CYP2A6 (Sadeque et al 1997) as well as CYP2B6 (Abbott and Anari 1999) mediate the formation of Δ^4 -VPA. Even though Δ^4 -VPA was found to be a

minor metabolite in the plasma and urine of experimental animals and humans, it has been the object of a number of studies over the years (Baillie 1988; Levy et al 1990; Rettenmeier et al 1985, 1986). The structural similarity between Δ^4 -VPA and the known hepatotoxin 4-pentenol acid led to development of the hypothesis that this VPA metabolite might also be involved in liver injury (Gerber et al 1979; Zimmerman and Ishak 1982). Subsequently Δ^4 -VPA was found to be a potent inducer of hepatic microvesicular steatosis and an inhibitor of fatty acid β -oxidation (Granneman et al 1984; Kesterson et al 1984).

Glucuronic acid conjugation is the principal pathway of hepatic extramitochondrial metabolism of VPA, both for the parent drug and also for many other primary metabolites of VPA formed through various oxidative phase I reactions (Abbott and Anari 1999). The reaction is mediated by uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT1A6) (Burchell 1999). The glucuronide conjugate of VPA is its major metabolite excreted *in vivo* mainly in the urine and bile (Booth et al 1996). VPA-glucuronide is, like other acyl-glucuronide conjugates, capable of undergoing a number of reactions including hydrolysis, rearrangement and covalent binding to proteins (Spahn-Langguth and Benet 1992), giving rise to adducts which are weakly immunogenic in humans (Bailey and Dickinson 1996; Williams et al 1992).

The formation of several glutathione (GSH) and *N*-acetylcysteine (NAC) conjugates of VPA metabolites has been demonstrated in rats treated with VPA, Δ^4 -VPA and $\Delta^{2(E),4}$ -VPA (Gopaul et al 2000; Kassahun et al 1991, 1994) using LC-MS/MS and gas chromatography–mass spectrometry (GC-MS). In these studies, Kassahun *et al.* highlighted the role of mitochondrial CoASH-dependent reactions and proposed that metabolic activation of Δ^4 -VPA and $\Delta^{2(E),4}$ -VPA to their respective CoA esters is obligatory for GSH conjugation.

Conjugation with amino acids such as glycine, which takes place in mitochondria via glycine *N*-acylase, has been demonstrated to be the least significant secondary metabolic pathway of VPA in the rat (Granneman et al 1984). In humans, valproylglycine has been identified as a urinary metabolite of VPA (Abbott and Anari 1999) along with VPA-glutamate conjugate.

Although it seems not to be essential for VPA mitochondrial β -oxidation, the respective valproyl-L-carnitine conjugate has been identified in the urine of three paediatric patients (Millington et al 1985), where it accounted for less than 1% of the VPA dose. Since carnitine acetyltransferase seems to have no activity in catalysing this reaction *in vitro* (Becker and Harris 1983), carnitine palmitoyltransferase I might be the

enzyme responsible for the synthesis of valproylcarnitine. Our own unpublished results do not support this hypothesis. It has not been elucidated whether it occurs inside mitochondria, via CPT II. The carnitine conjugate of the dicarboxylic acid metabolite 2-*n*-propylglutaric acid (propylglutaryl-carnitine) has been reported by our group in the plasma of patients under VPA monotherapy (Silva et al 2001c). The specific enzyme mediating the reaction, possibly a dicarboxyl-CoA synthetase, and its subcellular localization have not been defined.

Mitochondrial β -oxidation

β -Oxidation is the most significant pathway of oxidative metabolism of therapeutic VPA levels, accounting for nearly 70% of phase I reactions (Sztajnkrzyer 2002). Early studies on the metabolic fate of VPA (Kuhara et al 1978; Matsumoto et al 1976) suggested the drug to be a substrate for the mitochondrial β -oxidation enzyme system, deduced from the identification of 3-hydroxy-VPA and 3-keto-VPA in the urine of VPA-treated rats, and $\Delta^{2(E)}$ -VPA in humans. The three products represented three VPA β -oxidation intermediates. Further studies on VPA metabolites formed *in vivo* (Jakobs and Löscher 1978; Löscher et al 1981; Nau and Löscher 1982; Nau et al 1981, 1984; Rettenmeier et al 1987) reinforced the idea that β -oxidation is a major oxidative pathway of VPA metabolism.

With the aid of purified mitochondrial enzymes, valproyl-CoA was shown to be dehydrogenated by the enzyme 2-methyl branched-chain acyl-CoA dehydrogenase producing 2-propyl-2,3-pentenoyl-CoA ($\Delta^{2(E)}$ -valproyl-CoA) in rat (Ito et al 1990).

Becker and Harris were the first to demonstrate the formation of valproyl-CoA, identifying this conjugate by HPLC analysis in extracts of the liver of rats treated with VPA (Becker and Harris 1983).

Direct proof of VPA β -oxidation, including the subsequent reactions and metabolites, was obtained through *in vitro* studies performed with isolated rat liver mitochondria (Bjorge and Baillie 1991; Li et al 1991). A simplified scheme of the pathway, including the chemical structure of the intermediates, is shown in Fig. 1. Using intact rat hepatocytes and ^{13}C -labeled analogues of VPA as chiral probes, some stereochemical aspects of the reactions of β -oxidation were elucidated (Shirley et al 1993). More recently, the results of our group (Silva et al 2001a, 2002, 2004) provided additional insight with respect to the mitochondrial metabolism of this drug.

As depicted in Fig. 1, and by comparison with the metabolism of dietary/endogenous saturated fatty

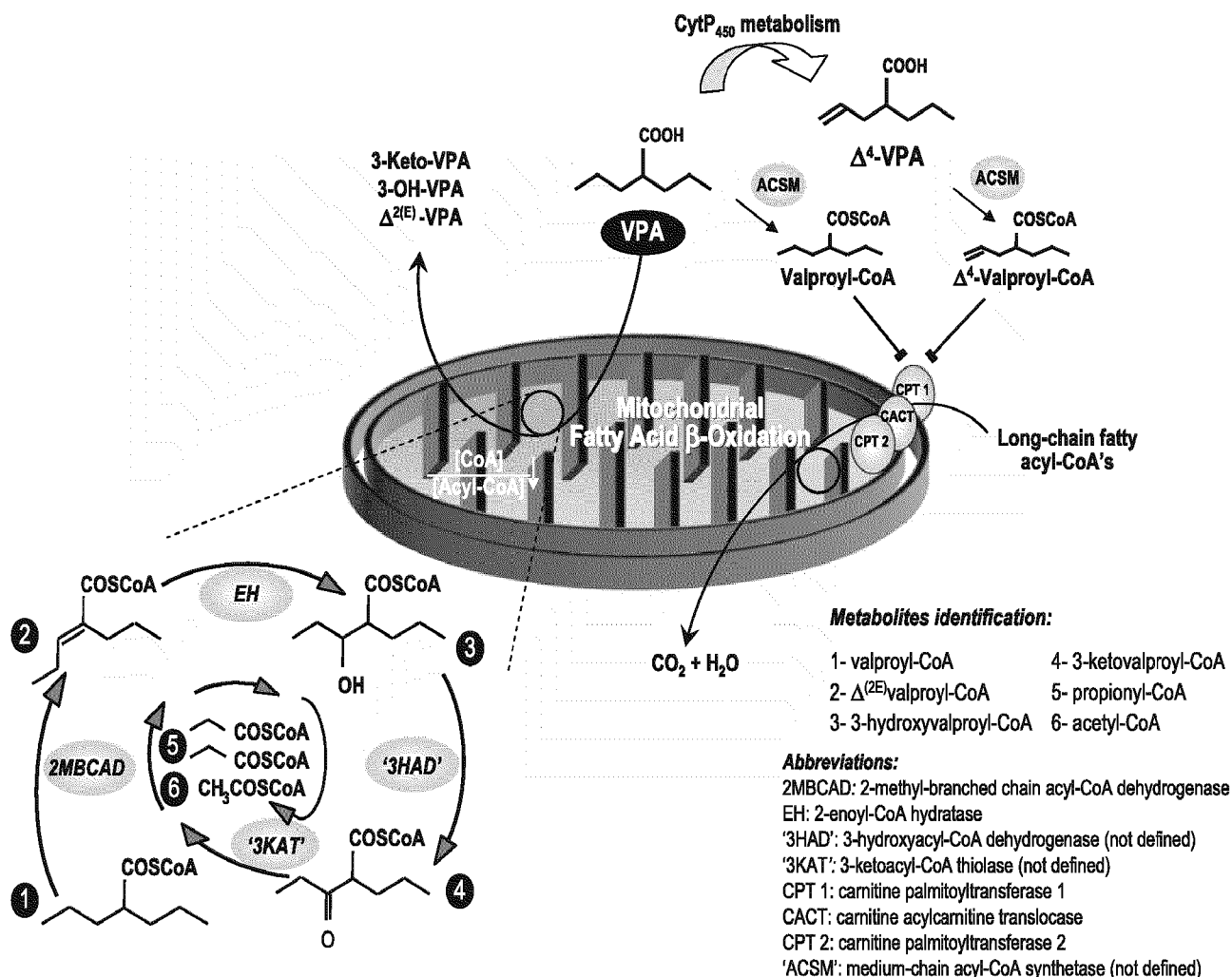


Fig. 1 Schematic representation of mitochondrial β -oxidation of valproic acid (VPA) and potential interaction with the carnitine shuttle proteins

acids, each cycle of valproate β -oxidation involves four consecutive reactions catalysed by separate enzymes after the entry into mitochondria and activation to the valproyl-CoA ester.

Activation of VPA to valproyl-CoA and sites of activation

As VPA is a simple branched medium-chain fatty acid, the crossing of the highly regulated, outer and inner, mitochondrial membranes is not clearly defined. However, it has been assumed that a predominantly carnitine-independent process is a more likely mechanism. Once inside the mitochondria, VPA must be converted to an active intermediate, valproyl-CoA, in the presence of ATP and CoA, in order to gain access to the β -oxidation system. The activation of VPA is the only step in VPA degradation requiring ATP. The enzyme(s) responsible for the activation of VPA have not yet been defined. However, medium-chain acyl-CoA synthetase (EC 6.2.1.2) is the major candidate.

The existence of cytosolic acyl-CoA synthetases prompted us to investigate whether VPA can also be activated in the extramitochondrial compartment. Indeed, valproyl-CoA was detected by HPLC in both liver mitochondria and cytosol isolated from VPA-treated rats, and also after *in vitro* incubation of both cellular compartments with VPA and cofactors (ATP and CoA) (Aires et al 2007). The *in vitro* activation of Δ^4 -VPA to Δ^4 -valproyl-CoA was also demonstrated. The recognition of the extramitochondrial activation of VPA in conjunction with the intramitochondrial reaction has consequences for further biotransformation of the drug, since the metabolic fate of the extramitochondrial valproyl-CoA is still unknown. As depicted in Fig. 1, the valproyl-CoA and Δ^4 -valproyl-CoA formed in the cytosol may enter the mitochondria via the carnitine shuttle (carnitine palmitoyltransferase I (CPT I), carnitine-acylcarnitine translocase (CACT) and carnitine palmitoyltransferase II (CPT II)). The presence of these VPA acyl-CoA esters in two different subcellular compartments may act as potential inhibitors at distinct targets (Aires et al 2007).

VPA β -oxidation reactions and drug metabolizing enzymes

In the mitochondrial matrix the first oxidative step in the sequence is the conversion of valproyl-CoA to $\Delta^{2(E)}$ -valproyl-CoA, a reaction that has been shown to be mediated by 2-methyl-branched-chain acyl-CoA dehydrogenase (2MBCAD) (Ito et al 1990) using the enzyme purified from rat liver mitochondria. $\Delta^{2(E)}$ -Valproic acid, the hydrolysed form of $\Delta^{2(E)}$ -valproyl-CoA, is the second major mitochondrial metabolite found in plasma.

The second step of the β -oxidation involves hydration of $\Delta^{2(E)}$ -valproyl-CoA to 3-hydroxyvalproyl-CoA. This reaction was studied *in vitro* and it was shown that purified bovine liver crotonase caused a decrease in the absorbance at 263 nm of a solution of the substrate (Li et al 1991). Our own experimental results clearly demonstrated the intramitochondrial formation of 3-hydroxyvalproyl-CoA (Silva et al 2001a). However, the enzyme catalysing the hydration of $\Delta^{2(E)}$ -valproyl-CoA, is unknown. It cannot be excluded that other mitochondrial enzymes than crotonase, with enoyl-CoA hydratase activity and higher affinity for medium or long-chain substrates, can also use $\Delta^{2(E)}$ -valproyl-CoA as substrate.

The identity of the third enzyme catalysing the conversion of 3-hydroxyvalproyl-CoA to 3-ketovalproyl-CoA has not been established. No dehydrogenation of 3-hydroxyvalproyl-CoA was observed (Li et al 1991) with purified pig heart SCHAD (short-chain 3-hydroxyacyl-CoA dehydrogenase) or peroxisomal bifunctional protein. However, when solubilized rat liver mitochondria (RLM) or a mitochondrial membrane fraction was used as source of enzyme, in the presence of NAD, there was a clear production of 3-ketovalproyl-CoA. These results suggested an unknown NAD⁺-dependent 3-hydroxyacyl-CoA dehydrogenase (3HAD) that is active towards the branched-chain substrate. Our own unpublished results support this concept, since the activity of 3HAD was not significantly affected in the presence of 3-hydroxyvalproyl-CoA and purified SCHAD could not catalyse the reverse reaction (formation of 3-hydroxyvalproyl-CoA from 3-ketovalproyl-CoA). This reaction in the β -oxidation of VPA requires further investigation, including the elucidation of the catalytic role of either LCHAD (long-chain

3-hydroxyacyl-CoA dehydrogenase) or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD), the latter involved in the mitochondrial oxidation of 2-methyl branched-chain fatty acids and isoleucine (Zschocke et al 2000).

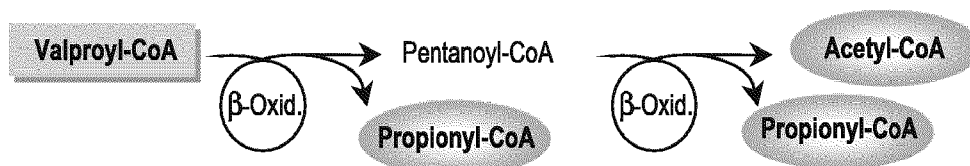
The final reaction of the β -oxidation cycle of straight-chain fatty acids consists of the thiolitic cleavage of 3-ketoacyl-CoA derivatives, a reaction catalysed by the 3-ketoacyl-CoA thiolases producing a chain-shortened acyl-CoA and acetyl-CoA. 3-ketoVPA is the major mitochondrial metabolite excreted *in vivo* (Battino et al 1995; Katayama et al 1998), and the measurement of the combined excretion of Δ^2 -VPA and 3-ketoVPA correlates with the β -oxidation of VPA *in vivo* (Rettenmeier et al 1987). Because of the efficient excretion of 3-keto-VPA, produced by hydrolysis of 3-ketovalproyl-CoA, the cleavage of 3-ketovalproyl-CoA was considered nonexistent. However, with radiolabelling studies, we provided clear evidence that 3-ketovalproyl-CoA undergoes thiolitic cleavage and is a substrate of a mitochondrial thiolase (Silva et al 2002). Using a ³H-labelled substrate, [4,5-³H₂]VPA, it was possible to follow the flow of metabolites along the β -oxidation pathway beyond the 3-ketovalproyl-CoA stage and the formation of ³H₂O supported full oxidation. In addition, we demonstrated the thiolitic cleavage of this substrate and the subsequent formation of *n*-pentanoyl-CoA and *n*-propionyl-CoA by the detection of pentanoylcarnitine and propionylcarnitine using electrospray ionization tandem mass spectrometry.

Through another cycle of β -oxidation, pentanoyl-CoA will be further cleaved into acetyl-CoA and propionyl-CoA as shown in Fig. 2. Both metabolites will ultimately enter the tricarboxylic acid cycle to complete oxidation to CO₂ and H₂O. The identification of the mitochondrial thiolase that acts upon 3-ketovalproyl-CoA remains undefined.

VPA and its effect on mitochondrial β -oxidation

The interference of VPA (or its metabolites) with fatty acid metabolism, mainly mitochondrial β -oxidation, is supported by several experimental findings. Many conditions leading to microvesicular steatosis are characterized by decreased mitochondrial fatty acid β -oxidation

Fig. 2 Schematic representation of the complete β -oxidation of valproyl-CoA



(Fromenty and Pessayre 1997). Inhibition of FAO associated with VPA has been well documented *in vitro* (Ponchault et al 1992; Silva et al 2001b). *In vivo*, several arguments account for this mitochondrial dysfunction: (1) the Reye-like syndrome presentation of VPA-associated liver toxicity; (2) the dicarboxylic aciduria observed in VPA-treated patients (Mortensen et al 1980); and (3) the decrease in plasma ketone bodies in animals and humans after administration of the drug (Becker and Harris 1983; Granneman et al 1984; Thurston et al 1983; Turnbull et al 1983).

Several mechanisms have been proposed to explain the VPA-induced hepatotoxicity including (1) formation of reactive metabolites of VPA; (2) drug-induced coenzyme A deficiency; (3) carnitine deficiency; (4) an underlying inborn error of metabolism; (5) hyperammonaemia; and (6) oxidative stress as a result of compromised free-radical scavenging activity or enhanced production of reactive oxygen species (Chang and Abbot 2006; Tong et al 2005), the latter beyond the scope of this paper.

CoA sequestration

Patients with Reye syndrome and compromised fatty acid oxidation were reported to have hepatic accumulation of short- and medium-chain acyl-CoA esters with severe depletion of free CoASH (Corkey et al 1988).

In the case of VPA therapy, the formation of valproyl-CoA and its intramitochondrial metabolites would sequester the limited pool of mitochondrial free CoA, thereby inhibiting CoA-dependent metabolic processes (Becker and Harris 1983; Silva et al. 2001a). The observed increase in the acyl-CoA/CoA ratio associated with a lower acetyl-CoA concentration is thought to be the consequences of either valproyl-CoA β -oxidation in the mitochondrial matrix or the inhibition of FAO. Furthermore, the branched-chain acyl-CoA esters formed from VPA metabolism appear to be more resistant to hydrolysis than straight-chain acyl-CoAs, probably owing to steric hindrance (Li et al 1991; Moore et al 1988; Silva et al 2002), a fact that would exacerbate CoA depletion.

Experiments conducted in rats treated with VPA or its unsaturated metabolites (Δ^4 -VPA, $\Delta^{2,4}$ -VPA, Δ^3 -VPA, $\Delta^{2(E)}$ -VPA) confirmed the above hypothesis (Kesterson et al 1984). In liver, free CoA levels diminished in all treatment groups while total CoA remained essentially unchanged. Levels of hepatic acetyl-CoA were reduced in rats receiving VPA and Δ^4 -VPA, whereas medium-chain acyl-CoAs increased in all groups. Subsequently, it was reported that VPA

and three of its metabolites (Δ^4 -VPA, $\Delta^{2,4}$ -VPA and 2-PGA) decreased the level of free CoA after incubation with coupled liver mitochondria (Ponchault et al 1992). Thus, the VPA-induced depletion of intramitochondrial CoA would explain why the β -oxidation of different chain-length fatty acids (long-, medium- or short-chain) is affected with subsequent impairment of ATP production. Furthermore, the distribution of total cellular CoA between the intra- and extramitochondrial compartments is not identical in different tissues. In liver, the two pools are almost equal, whereas in heart only 5% is present in the cytosol (Yao et al 1994). The formation of valproyl-CoA beyond the mitochondria (Aires et al 2007) suggests that the extramitochondrial pool of CoA may also be depleted, resulting in a cumulative inhibitory effect on long-chain fatty acid β -oxidation.

Carnitine metabolism

Depletion of free (and total) carnitine would also result in an inhibition of mitochondrial FAO. VPA-induced plasma carnitine deficiency has been documented in some studies and case reports, but hypocarnitinaemia has not been confirmed in all studies (Lheureux et al 2005).

VPA can deplete carnitine stores, especially during long-term or high-dose therapy, via different mechanisms, including: (1) presumed formation of valproyl-carnitine; (2) decreasing tubular reabsorption of both free and acylcarnitine; (3) reduction of the carnitine biosynthesis by inhibition of selected biosynthetic enzyme(s); (4) inhibition of the organic cation transporter 2 (OCTN2), resulting in a decreased transport of extracellular carnitine into the cell; and (5) impairment of carnitine recycling from long-chain acylcarnitines by CPT II, due to the VPA-induced decrease of mitochondrial free coenzyme A level (Lheureux et al 2005).

The formation of valproylcarnitine seems to be of no quantitative importance *in vivo* (Silva et al 2001c) since its excretion accounts for less than 1% of the total acylcarnitine elimination in urine. In contrast, other acylcarnitines may be formed in higher amounts, including 3-hydroxyisovalerylcarnitine (C5OH) and 2-propylglutarylcarnitine as demonstrated in VPA-treated adults (Silva et al 2001c). This was confirmed by identification of an increase of C₅OH-carnitine after VPA treatment in paediatric patients, in conjunction with a decrease of free carnitine (C₀) (Werner et al 2007).

Inhibition of carnitine biosynthesis has been reported after a single intraperitoneal dose of VPA in rats (Farkas

et al 1996). Total carnitine in liver was decreased, whereas the carnitine precursor butyrobetaine was increased. However, under *in vitro* conditions, VPA did not inhibit the enzyme butyrobetaine hydroxylase.

VPA impaired carnitine uptake in cultured control human skin fibroblasts, which was completely reversed with removal of drug from the culture medium (Tein and Xie 1994).

Reports on the effect of VPA on the activity of the OCTN2 transporter involved renal tubular reabsorption of carnitine but reveal conflicting results. In one study, VPA did not inhibit carnitine transport (Wagner et al 2000), whereas in another study, VPA was transported by OCTN2, and exhibited a significant inhibitory effect (Ohashi et al 1999; Wu et al 2004). Further investigations concerning the effect of this drug on carnitine transport are needed.

Carnitine depletion induced by VPA has several adverse consequences. Since this depletion can impair the transport of long-chain fatty acids into the mitochondria, it decreases their β -oxidation and the production of acetyl-CoA and ATP. As a consequence of its own capacity to inhibit β -oxidation, VPA metabolism can be shifted into microsomal oxidation, resulting in the formation of Δ^4 -VPA, a metabolite implicated in the VPA-induced hepatotoxicity previously described. Additionally, carnitine depletion can also impair the urea cycle, resulting in hyperammonaemia, a common manifestation of VPA toxicity. As acetyl-CoA stores are depleted due to impairment of mitochondrial FAO, the synthesis of *N*-acetylglutamic acid (NAG), an obligatory cofactor of carbamoyl phosphate synthase (CPS), is also decreased (Lheureux et al 2005; Sztajnkrzyer 2002).

However, there are also reports describing no carnitine abnormalities in patients receiving VPA in monotherapy as compared with a control group (De Vivo et al 1998; Raskind and El-Chaar 2000). Therefore, there is still no consensus concerning carnitine bioavailability during VPA therapy and especially concerning the effectiveness of carnitine supplementation (Russell 2007). In fact, many studies have focused on the benefits of carnitine supplementation in VPA-treated patients leading to the recommendation by the Pediatric Neurology Advisory Committee in 1996 (De Vivo et al 1998) for the use of carnitine supplementation in chronic VPA treatment. In their review, Evans and Fornasini stress that, despite the considerable data available on the use of L-carnitine in primary and secondary carnitine deficiency, very few studies have been reported assessing the pharmacokinetics of exogenous L-carnitine in these patients. The alterations in the absorption/distribution/excretion that

induced deficiency would also be expected to alter the pharmacokinetics of exogenous L-carnitine administration. Consequently, the use of pharmacokinetic analysis would help to clarify the variability within and between patients in relation to the plasma-to-tissue levels of L-carnitine and the ratios of acyl-L-carnitine to L-carnitine (Evans and Fornasini 2003).

In conclusion, the co-administration of carnitine may play a role in preventing VPA hepatotoxicity. It seems reasonable to use carnitine for documented VPA toxicity, particularly in cases of an overdose and when the patient presents with coma and rising ammonia. However there is still doubt whether current literature provides enough evidence-based data to support this supplementation (Bohan et al 2001; Raskind and El-Chaar 2000; Russell 2007; Verrotti et al 2002).

Effect of VPA on β -oxidation enzymes

The formation of different VPA acyl-CoA esters, with potential toxicity, underlies the hypothesis that they modulate the activity of selected enzymes and transport systems. VPA and its metabolites might impair mitochondrial β -oxidation of fatty acids by a direct inhibition of FAO enzymes. The work of Ito and colleagues revealed *in vitro* that 0.3 mmol/L valproyl-CoA, inhibited human short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD) (Ito et al 1990), while VPA itself did not significantly affect the activities of the various acyl-CoA dehydrogenases.

In vitro experiments using also the free forms of VPA or its metabolites, but not the CoA esters, revealed a reversible binding of the metabolite $\Delta^{2,4}$ -VPA to the α -subunit of the trifunctional protein with ensuing FAO inhibition. However, binding of VPA itself or the metabolites Δ^2 -VPA, Δ^4 -VPA and 3-hydroxy- Δ^4 -VPA was negligible (Baldwin et al 1996).

Using a different experimental approach, the mRNA levels of the various (short-, medium- and long-) acyl-CoA dehydrogenases (ACD), which catalyse the first step of β -oxidation, were shown to be increased in liver, in skeletal muscle and especially in the heart of VPA-treated rats fed *ad libitum*, with an apparent decrease noted after 3 days of starvation. The enhanced expression of fatty ACD mRNA and proteins in VPA-treated rats accounted for a feedback mechanism related to the inhibition of β -oxidation in rats fed *ad libitum* (Kibayashi et al 1999).

Gene expression profiles were also studied in liver of VPA-treated mice using micro-array analysis. Data clustering revealed that gene expression changes

depended on the time rather than the dose of VPA treatment, and genes associated with lipid and fatty acid metabolism were strikingly altered, up- and down-regulated (Lee et al 2007).

In line with the structural analogy between Δ^4 -VPA and 4-pentenoic acid, which was shown to inhibit 3-ketoacyl-CoA thiolase (Fong and Schulz 1978), it has been postulated by several authors that Δ^4 -VPA (or its CoA ester) would be a potent inhibitor of enzymes of the FAO pathway (Sadeque et al 1997). However, no systematic studies have been performed to test this hypothesis.

Interference with mitochondrial carnitine-shuttle proteins induced by the extramitochondrial valproyl-CoA and Δ^4 -valproyl-CoA, a hypothesis from our group (Aires et al 2007), is currently under study. We have obtained evidence (unpublished data) that valproyl-CoA is a competitive inhibitor of carnitine palmitoyl-transferase I (CPT I) activity *in vitro*. This activity was evaluated using ESI-MS/MS, determined as the rate of synthesis of palmitoylcarnitine from the substrate [^{13}C]palmitoyl-CoA, in control human fibroblasts and recombinant rCPT I. Valproyl-CoA interferes with the regulatory role exerted by malonyl-CoA on CPT I activity, inducing an increase of the K_i value with increasing valproyl-CoA concentration. Malonyl-CoA is the first intermediate of the fatty acid synthetic pathway, which regulates fatty acid oxidation under fed conditions. This targeted effect of valproyl-CoA may account for the decreased rate of long-chain fatty acid oxidation previously reported (Silva et al 2001b). In conclusion, our findings may be crucial in understanding the VPA-induced hepatotoxicity (characterized by microvesicular steatosis) and the weight gain associated with VPA therapy.

VPA and Inborn errors of metabolism

As previously stated, VPA affects many biochemical systems in the mitochondrion, some of them important in energy metabolism. If a patient has an underlying inborn error, manifested with epileptic episodes or other seizures types, the intake of this drug as an anticonvulsant may stress vital pathways, exacerbating the impairment of the genetically affected route and triggering the toxic syndrome.

A number of case reports describe the association of VPA therapy in patients with inborn errors, especially those affecting mitochondrial metabolism, such as fatty acid oxidation defects, multiple acyl-CoA dehydrogenase (MAD) (Papadimitriou and Servidei 1991) or CPT II deficiencies (Kottlors et al 2001), mitochondrial

diseases (Chabrol et al 1994; Delarue et al 2000; Lam et al 1997; König et al 1999; Krähenbühl et al 2000) and urea cycle disorders (Hjelm et al 1986; Leão 1995; Oeschner et al 1998; Sewell et al 1995). In all cases, there is a consensus for avoiding the administration of VPA. Therapeutic strategies including valproic acid should also be avoided in patients with 2-MBCAD or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiencies, since the potential active role of both enzymes on VPA β -oxidation might result in an adverse reaction.

Interestingly, two different situations can be gleaned from reported cases. First, VPA triggered the clinical onset of the metabolic disease, which was later diagnosed. Second, there were cases attributed to valproate toxicity, such as a fatal idiosyncratic hepatic failure, that have been studied later and identified as a genetic defect. In the latter situation, it was the drug that precipitated and exacerbated the complications of the inherited metabolic disease.

The improvement of diagnostic tools and analytical techniques in the last decade has enabled the 'explosion' in the characterization (at both biochemical and molecular levels) of genetic diseases, and elucidated the possible aetiologies of undefined disorders, such as Reye and Reye-like syndromes. In addition, considering the multiplicity of enzymes and biochemical systems within human cells, a high number of new genetic metabolic diseases remain to be identified. Thus, as suggested by other authors, it could be hypothesized that a genetic underlying cause for VPA-associated hepatic toxicity might exist.

Final remarks

VPA directly or indirectly affects fatty acid β -oxidation. The most probable hypothesis is that the hepatotoxicity associated with this drug may develop not from a single causative insult but due to the interaction of multiple genetic and metabolic factors, and to the pleiotropic effects of the drug in mitochondrial metabolism. It is difficult to establish the relative contribution of the parent drug or its metabolites, as well as the discrepancies between data obtained *in vitro* and *in vivo*.

Given that the widespread use of VPA will certainly continue, its valuable clinical spectrum and expanding pharmacological use, including its recent consideration as a first-generation HDAC (histone deacetylase) inhibitor (Kuendgen and Gattermann 2007), further studies of VPA metabolism are essential to clarify its interactions and minimize its potential for inhibition of mitochondrial metabolism and ensuing adverse effects.

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