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COMMENTARY

VALPROATE AND MITOCHONDRIA

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Historical introduction

Valproic acid (*n*-dipropylacetate) (VPA†) was used as an organic solvent until the chance discovery by Meunier and coworkers [1] that the compound had a potent antiepileptic activity, which has led to the widespread use of the sodium salt in the treatment of seizures. The precise mechanism of action of the compound within the CNS remains unknown and is the subject of intense research activity [2]. However, in order not to deceive the general reader of this type of article, we would like to make it clear at this early point that the mode of action of valproate is not our immediate concern. Rather, it is one of the drawbacks of valproate therapy that originally engendered our interest in the effects of the compound on intermediary metabolism.

Valproate toxicity

Although valproate therapy is generally well tolerated and effective, there does exist a relatively small group of patients who suffer from a hepatotoxic reaction to such treatment, which has proved to be fatal in some cases [3, 4]. The clinical and biochemical features in these patients include hepatic microvesicular steatosis and encephalopathy and are similar to those observed in cases of Reye's syndrome, a condition in which short-chain fatty acids have been implicated [5]. It was the observation of this toxic side-effect of valproate that stimulated a new field of research, distinct from the investigation of the mode of action, which now includes the effects of valproate on metabolism, teratogenicity, cell development and the metabolism of valproate itself.

Apart from the histopathological features of the intoxication, several clinical observations led to the investigation of the effects of valproate on intermediary metabolism. One of the first was the observation by Mortensen *et al.* [6] of a dicarboxylic aciduria in 75% of patients during valproate therapy, suggesting an interference with the β-oxidation of fatty acids within the mitochondria. This interference with fatty acid metabolism was confirmed in rats, which inevitably presented with a typical dicarboxylic aciduria [6]. A second feature was the persistent hyperammonaemia observed during valproate intoxication, indicating an effect on mitochondrial function [7–9].

Further investigations of these effects were performed using the ubiquitous rat liver in the form of homogenates, isolated hepatocytes or mitochondrial fractions. Perhaps the most revealing of these studies were those of Becker and Harris [10] and Turnbull *et al.* [11], who demonstrated for the first time an inhibition of the β-oxidation of fatty acids in isolated hepatocytes. This effect was confirmed later in liver homogenates [12] and also in mitochondrial fractions [13, 14]. In their report, Becker and Harris also showed inhibitions of ketogenesis, fatty acid synthesis in isolated rat hepatocytes, and gluconeogenesis in both hepatocytes and renal tubules *in vitro*. This latter effect was attributed to a marked fall in the concentration of acetyl-CoA, the allosteric effector of pyruvate carboxylase. Other groups have confirmed the effect of the drug on both ketogenesis [15–17] and gluconeogenesis [18] in rat liver. To complete their report, Becker and Harris also described the appearance in the livers of valproate-treated rats of a novel CoA ester, which they identified as valproyl-CoA, that was associated with a drastic decrease in the levels of free CoA. From this observation they attributed the inhibitory effects of valproate to a sequestration of free CoA in the form of valproyl-CoA, thereby blocking CoA-dependent metabolic pathways, as well as possible direct enzyme inhibition by the CoA ester itself.

Several reports from other groups have extended these observations on valproate effects, including the inhibition of ureagenesis in hepatocytes [11, 19], which was also suggested to be due to the decreased cellular concentration of acetyl-CoA [11].

Mitochondrial metabolism of valproate

In vivo administration of valproate leads to a major excretion of valproate metabolites in the urine, the principal elimination pathway being glucuronidation (70% of the administered dose) in the endoplasmic reticulum [20]. Being a fatty acid itself, valproate is prone to be metabolized by the mitochondria. Indeed, intermediates of the β-oxidation of the drug including the CoA esters of valproate, 2-propyl-pent-2-enoate (2-ene-VPA), 3-hydroxy-2-propylpentanoate (3-OH-VPA) and propyl-2-oxo-3-pentanoate (3-oxo-VPA) have been

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† Abbreviations: VPA, valproic acid; 3-OH-VPA, 3-hydroxy-2-propylpentanoate; 3-oxo-VPA, propyl-2-oxo-3-pentanoate; 2-ene-VPA, 2-propyl-pent-2-enoate; 4-ene-VPA, 2-propyl-pent-4-enoate; AoA, aminooxyacetic acid.

identified in isolated mitochondria incubated with valproate [21, 22]. β-Oxidation is the second most important route of the biotransformation of the drug and is essentially mitochondrial, although evidence for peroxisomal β-oxidation has been presented [23]. Other extramitochondrial pathways, including ω, ω-1, ω-2 hydroxylations, and δ- and γ-dehydrogenations, are quantitatively minor but there is growing evidence that these routes of catabolism produce some metabolites of the drug that may contribute to its hepatotoxic properties [15, 24].

It was Gerber *et al.* [25] who originally drew attention to the similarity of the structures of certain valproate metabolites, such as 2-propyl-pent-4-enoate (4-ene-VPA), to known hepatotoxic compounds, namely methylene cyclopropylacetic acid, the active principle of hypoglycin which causes Jamaican vomiting sickness [26], and pent-4-enoate [27]. All these compounds cause similar biochemical and histological disturbances in cases of intoxication, with hepatic steatogenesis being the major effect. This prompted studies on the effect of valproate metabolites on mitochondrial metabolism [28], as will be discussed later.

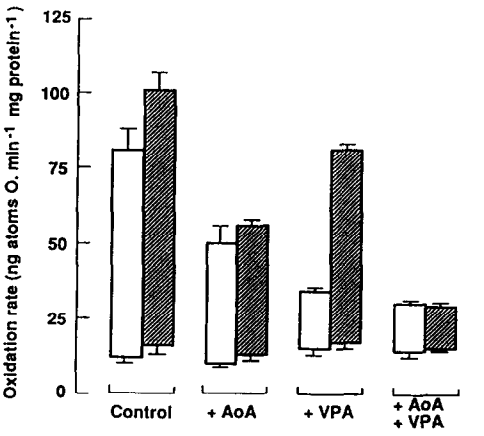

Fig. 1. Effects of 1 mM aminooxyacetic acid (AoA) and 1 mM VPA on the oxidation of 10 mM glutamate (open bars) and 10 mM glutamate + 1 mM malate (hatched bars) by rat liver mitochondria. The upper part of each bar represents the state 3 oxidation rate (+ADP), the lower part the state 4 oxidation rate (after consumption of the ADP). Values are means ± SEM for three different preparations. Reproduced from Ref. 14.

In vitro *effects on mitochondrial oxidations*

Effect of valproate on the oxidation of glutamate, succinate, α-ketoglutarate and pyruvate. Direct effects of valproate on mitochondria *in vitro* have been demonstrated by several groups, the first being Haas *et al.* [29]. They showed that valproate had inhibitory effects on mitochondrial respiration rates at concentrations as low as 24 μM with glutamate as substrate, and 50% inhibition of state 3 rates at about 240 μM. A 10-fold higher concentration was necessary to produce a 20% decrease in the rate with succinate, this concentration also producing a significant uncoupling evidenced by increased state 4 rates with all substrates. Rates in mitochondria uncoupled by the addition of dinitrophenol were inhibited in a similar fashion to the coupled rates. Becker and Harris [10] reported similar effects in rat liver mitochondria with pyruvate/malate supported respiration, and no effect on the succinate oxidation rate. However, they did not find any inhibition of glutamate-supported respiration. There were no significant effects on state 4 or the ADP:O ratios at the lower concentrations and oxygen consumption with ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was not affected at any of the concentrations tested [29].

We investigated the effects of valproate in isolated mitochondria with a variety of substrates in order to confirm these observations [14]. In our hands preincubation with valproate inhibited mitochondrial respiration with all substrates tested, but to markedly different extents. Oxygen consumption rates with glutamate, pyruvate/malate, α-ketoglutarate/malate and fatty acids were all very sensitive to the inhibition. There was 50% inhibition of glutamate oxidation and β-oxidation at about 125 μM, while pyruvate/malate oxidation was more sensitive, being decreased by 50% at 24 μM. Succinate-supported respiration was only decreased by 20% at 1 mM valproate, the maximum concentration used. However, when malate was added together with glutamate, the inhibition was markedly less, to the same order as that against succinate. The oxidation rate with malate alone was very sensitive to valproate, and malate was routinely added with pyruvate and α-ketoglutarate, which were not "protected" against valproate inhibition. This effect of malate was explained using aminooxyacetic acid (AoA), a specific inhibitor of aminotransferases, which could thus differentiate between the two mitochondrial metabolic pathways for glutamate oxidation, namely dehydrogenation by glutamate dehydrogenase or the aminooxyacetic acid (AoA) sensitive transamination with oxaloacetate (derived from malate). As shown in Fig. 1, AoA had similar inhibitory effects on glutamate oxidation with and without malate, while valproate was much more effective in the absence of malate. However, the combination of AoA and valproate effectively inhibited that part of glutamate oxidation which was insensitive to valproate with malate. These results suggest that valproate inhibits glutamate oxidation through the glutamate dehydrogenase pathway, an effect which we will discuss later. The important point to note—and which was already evident from the differences in effects on glutamate in the absence and presence of malate—is that valproate appears to specifically inhibit oxidative processes within the mitochondrial matrix, and not the glutamate transport through the inner membrane nor the subsequent oxidation of the NADH generated from these matrix reactions. Thus, at the risk of appearing too pedantic, we wish to emphasise that *valproate* in vitro *does not inhibit oxidative phosphorylation*, which is the process by which ATP is generated through the oxidation of NADH and $FADH_2$ by the electron transport chain in the inner membrane. The inhibitions described above are the consequence of decreased generation

of these nucleotides due to interference with substrate oxidations within the matrix.

The exact nature of these inhibitions remains to be elucidated, but several possibilities have been indicated. Valproate derivatives, among which are valproyl-CoA, 2-ene-VPA and its CoA ester, cause inactivation and inhibition of purified beef brain α-ketoglutarate dehydrogenase [30]. This is significant as this effect could explain the observed inhibitions of respiration with not only α-ketoglutarate, but also the specific inhibition of glutamate oxidation through the glutamate dehydrogenase pathway, the product of which is α-ketoglutarate. It has not yet been determined whether the effect on α-ketoglutarate dehydrogenase is responsible for the smaller inhibition of succinate oxidation through decreased activity of the Kreb's cycle, or indeed whether this influences pyruvate oxidation. Valproyl-CoA has also been reported to inhibit purified pig heart pyruvate dehydrogenase [11], and human short- and medium-chain acyl-CoA dehydrogenases [31].

Effect of valproate on the β-oxidation of fatty acids. Valproate was also shown to inhibit β-oxidation in isolated mitochondria [13, 14, 28], as we have already mentioned, with a K_i similar to that of glutamate (125 μM). The typical valproate-induced dicarboxylic aciduria suggests that this may be the most important inhibition realized *in vivo*. Our studies on the *in vitro* effects of valproate have demonstrated the necessity of coupling for the observation of the full inhibitory effects [14, 28]. β-Oxidation of palmitoylcarnitine in uncoupled mitochondria was inhibited to the same degree as coupled rates provided that the preincubation with valproate was performed in coupled conditions (65% inhibition). Uncoupling before the addition of valproate decreased the inhibition to 28%. This requirement for coupled mitochondria to generate the reactive species, and the formation of valproyl-CoA with the associated decrease in free CoA levels within the matrix support the original concept of an inhibition due to CoA sequestration. Indeed, all the oxidations that can be described as being valproate-sensitive, i.e. pyruvate, glutamate, α-ketoglutarate and β-oxidation, involve CoA-dependent reactions. The possibility of a common mechanism of action therefore suggests itself. However, the time courses of CoA sequestration and valproate inhibitions are very different, sequestration being essentially complete within 30 sec, whereas full inhibition of β-oxidation by valproate requires 2.5 min [28]. Thus, the sequestration of free CoA itself may not be sufficient to explain all the inhibitory effects of valproate. Furthermore, uncoupling mitochondria before the preincubation with valproate, thereby decreasing the available ATP for the acyl-CoA synthetase, only decreases the inhibition, but does not totally eliminate this effect of valproate. It is still necessary to explain this remaining inhibition.

Effect of valproate on metabolite transport. Effects of valproate on substrate transport through the mitochondrial inner membrane have been demonstrated. Benavides *et al.* [32] found an inhibition of coupled and uncoupled respiration in isolated rat brain mitochondria by valproate when pyruvate was the substrate. Respiration with other substrates was not affected, and the inhibition was lost when the mitochondria were osmotically disrupted. They demonstrated that valproate inhibits the pyruvate transporter in a competitive manner with a K_i of 0.17 mM. The *in vitro* and *in vivo* administration of valproate has been shown to affect the transport of succinate into renal mitochondria [33]. However, as we have already indicated, the drug appears to have only a minor effect on the oxidation rates with succinate in hepatic mitochondria.

Effect of valproate metabolites on mitochondrial oxidations. Valproate metabolites known to be generated in the mitochondria were tested by us for their ability to sequester free CoA and to inhibit oxygen consumption with various substrates [28]. Surprisingly, none of those tested was found to either decrease free CoA or to inhibit the oxidation rates with glutamate, pyruvate, α-ketoglutarate or palmitoyl-CoA. However, both 2-ene-VPA and 3-oxo-VPA inhibited the β-oxidation rate with decanoate as substrate, whereas only 2-ene-VPA affected decanoyl-CoA oxidation [28]. It appears, therefore, that mitochondrial metabolites of valproate affect medium-chain acyl-CoA synthetase, while at least one (2-ene-VPA) also inhibits β-oxidation of medium-chain fatty acids by a mechanism not involving CoA-sequestration or acyl-CoA synthetase.

In the same study we included non-mitochondrial valproate metabolites, i.e. those that have been shown to be produced by ω- or γ-dehydrogenation. Of the tested compounds the products of γ-dehydrogenation (4-ene-VPA and 2,4-diene-VPA) and to a lesser extent the final product of ω-oxidation (2-propylglutarate) were the most interesting, having inhibitory profiles similar to that of the parent molecule against glutamate, pyruvate/malate, α-ketoglutarate/malate and palmitoylcarnitine oxidations. Furthermore, all three compounds were very effective in decreasing free CoA levels in isolated mitochondria, indicating that they have relatively easy access into the mitochondrial matrix and are substrates for the acyl-CoA synthetase. It is clear, therefore, that in the intact cell it would be feasible for valproate to be metabolized in the cytosol, and the products of such metabolism could enter the mitochondrial matrix to sequester the mitochondrial pool of free CoA. Interestingly, the effects of uncoupling on these inhibitors were identical to those on valproate, being equally effective against uncoupled respiration provided they were preincubated with coupled mitochondria, but the inhibitions being markedly less when the mitochondria were uncoupled before the addition of the inhibitor [28]. Thus, these extra-mitochondrial metabolites also rely, at least in part, on an activation to an inhibitory species, by an ATP-dependent mechanism.

It appears that the inhibitions of mitochondrial oxidations by valproate *in vitro* are due to multiple causes. The sequestration of free CoA by valproate and valproate metabolites has been clearly demonstrated but can explain only part of the inhibitory effects of the drug. Other mechanisms are involved in the inhibitions observed among which direct inactivation of mitochondrial enzymes including α-

ketoglutarate dehydrogenase and pyruvate dehydrogenase have been demonstrated, and also inhibition of metabolite transporters such as those for pyruvate in rat brain mitochondria [32] and succinate in kidney mitochondria [33].

Chronic administration of valproate

The effects described above were obtained in isolated mitochondrial fractions exposed to ranges of valproate concentrations similar to the circulating levels found after administration to whole animals. However, valproate has also been described as having mitochondrial effects following *in vivo* administration. Rumbach *et al.* [34] reported decreased respiratory capacity in coupled and uncoupled mitochondria from rat brain and liver following 5 days of valproate administration. These effects were more pronounced in liver than brain, probably due to differences in tissue concentrations. It is notable that the inhibitions were observed with both succinate and glutamate/malate as substrates, these being the oxidations which are least sensitive *in vitro*. The same group demonstrated that valproate interacts with hepatic and renal mitochondrial membranes by modifying the conformation of membrane proteins, a change that occurred when the drug was administered to the whole animal or isolated mitochondria [35]. This interaction was subsequently shown to inhibit the rate of succinate transport through the inner membrane [33], and may also explain the effects on pyruvate transport in brain mitochondria previously reported by Benavides *et al.* [32].

Hayasaka *et al.* [36] found a 17% decrease in the succinate-supported respiration rate of hepatic mitochondria from rats following 5 days of subcutaneous administration of valproate. This decrease was attributed to a loss of cytochrome *c* oxidase activity associated with a significant loss of cytochrome aa_3 from these mitochondria. They also noted that the incorporation of radioactive leucine into proteins was slower in valproate-treated mitochondria, suggesting an effect on protein synthesis.

We have confirmed the effects of chronic valproate treatment [1% (w/w) in the diet] and the changes that occur to the hepatic mitochondria, over a longer period of administration. Our initial results showed that a relatively long-term administration (10 weeks) of valproate led to a significant decrease (30%) in coupled respiratory activity with succinate and glutamate/malate as substrates, which was due to a marked loss (82%) of cytochrome *c* oxidase activity and cytochrome aa_3 content [37]. There was no difference between control and valproate-treated animals when the respiration was measured as the rate of antimycin-sensitive ferricyanide reduction [37], a method which does not include cytochrome *c* oxidase in the reaction, confirming the latter to be the site of inhibition of the oxygen consumption rate. Finally, a major surprise was the observation that the rates of oxygen consumption in uncoupled mitochondria were not different from controls. This indicates that the decreased capacity of cytochrome *c* oxidase was still sufficient for normal rates of electron flow through to oxygen, but that this

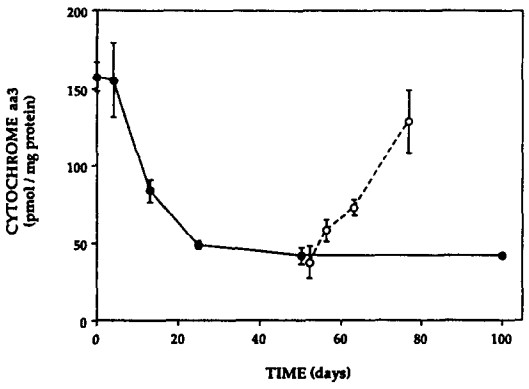

Fig. 2. Decrease of cytochrome aa_3 content assayed spectrophotometrically in mitochondrial fractions from livers of rats chronically treated with valproate [1% (w/w) in the diet] (●). The recovery of these cytochromes after interruption of the chronic treatment is also shown (○). Results are expressed in pmol/mg protein (means ± SEM for N = 5–13 preparations). Data in this figure were obtained from previously unpublished observations.

rate was controlled by the coupling to proton-translocation within Complex IV; our data also suggested that chronic administration of the drug inhibited this proton-pumping activity in Complex IV. Subsequent studies revealed the time-course of these changes, with a relatively rapid loss of cytochrome aa_3, to 50% within 2 weeks (Fig. 2). However, although continuing the treatment further decreased the cytochrome aa_3 content, to <20% of controls after 50 days, the respiration rates with succinate and glutamate/malate remained at a "plateau" value of 70% of controls. Removal of valproate from the diet led to a progressive return of oxidation rates and cytochrome aa_3 content to control values over 10–24 days (Fig. 2). It is clear that valproate may be of interest as a research tool to those workers currently studying the mode of action of Complex IV. It remains to be seen whether the diminished cytochrome aa_3 content is due to effects on the synthesis or assembly of Complex IV.

In contrast to the effects on mitochondrial respiration, chronic administration of valproate increases the mitochondrial β-oxidation capacity in the liver, with an increase in palmitoyl-CoA dehydrogenase activity [38]. Indeed, treated rats display a hepatomegaly which includes an increase in the mitochondrial fractional volume of the hepatocyte [23, 38] associated with an increase in mitochondrial protein in the liver [36–38]. Thus, overall mitochondrial synthesis is increased and the decrease in cytochrome aa_3 content must be a specific effect, and not a general inhibition of protein synthesis as suggested by Hayasaka *et al.* [36].

Species specificity

The effects of valproate which we have described have all been defined in one experimental animal, the rat. However, valproate has also been tested on other species with different results. The proliferation of peroxisomes in livers of mice was shown to be

much less than at the equivalent doses in rat, and guinea-pig livers appeared insensitive to this effect [39]. We have investigated the effects of valproate on hepatic mitochondria from mice and guinea-pigs and compared them with our results on rats [39]. The effects of valproate *in vitro* were similar in rats and guinea-pigs, while β-oxidation was affected to a lesser extent in mice. However, the small decrease (20%) in hepatic mitochondrial oxidation rates observed in rats *in vivo* was not observed in guinea-pigs* following chronic administration for 2 weeks, while there was a small effect in mice. These results are similar to those observed with other peroxisome-proliferating compounds [40], and suggest that the differences observed between the *in vitro* and *in vivo* effects of valproate are due to differences in the *in vivo* metabolism of the drug.

Conclusion

The influence of valproate on mitochondrial metabolism is extremely complex, and there are obvious differences between effects *in vitro* and *in vivo*, notably the lack of effect on the respiratory chain following *in vitro* administration, in contrast to the loss of cytochrome *c* oxidase following valproate feeding. Furthermore, the species differences in sensitivity to valproate, as exemplified by the results in the rat and guinea-pig, must be explained. The results in guinea-pig also raise the question of the differences in effects on hepatic mitochondria following the *in vitro* administration, which produces a particularly marked inhibition, and the *in vivo* administration of valproate, which has no apparent influence on either mitochondrial or peroxisomal function.

Several mechanisms must be evoked to explain the inhibitions observed *in vitro* in isolated mitochondrial fractions. It is clear that both the sequestration of free CoA by the drug and some of its metabolites, the inactivation of mitochondrial enzymes by valproate derivatives, and the interference with substrate carriers are the prevalent mechanisms, which most probably interact to produce the final inhibition. The most important consequence of chronic *in vivo* administration of the drug in rat liver is probably the inhibition of oxidative phosphorylation due to an interference with the proton-pumping activity of Complex IV, although the appearance of a dicarboxylic aciduria is proof of a significant inhibition of β-oxidation. The importance of these effects in relation to the pathogenicity of the drug remains to be established. However, it is clear that if the effects observed in rodents also occur in humans, the use of valproate in patients with compromised mitochondrial function must be minimized. It also remains to be seen whether the valproate-fed rat can serve as a useful model in the study of the mechanism of action of Complex IV. Thus, despite the intense research activity of the past 10 years the nature of valproate toxicity is still undefined, each answer posing a new question.

* We have also noted that there was no effect on hepatic cytochrome aa_3 content or cytochrome oxidase activity in the guinea-pig after 25 days of valproate feeding.

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