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VALPROATE-INDUCED HYPERAMMONEMIA OF RENAL ORIGIN

EFFECTS OF VALPROATE ON GLUTAMINE TRANSPORT IN RAT KIDNEY MITOCHONDRIA

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Abstract—The antiepileptic sodium valproate (VPA) systematically induces an asymptomatic hyperammonemia of renal origin in fasting normal human volunteers and in fasting rats, accompanied by an increased renal glutamine uptake. Fasting rats were injected with VPA and their mitochondria isolated, or isolated mitochondria of fasting rats were incubated with VPA. Transmembranal mitochondrial glutamine uptake and activities for five mitochondrial and three cytosolic enzymes involved in ammoniagenesis were measured. In VPA-incubated mitochondria, glutamine transport increased for VPA concentrations between 10^{-3} and 10^{-5} M; enzyme activities did not change. In mitochondria of VPA-treated rats, K_m and V_{max} were unaffected. These findings reflect membrane effects of VPA observed in other experimental settings.

Sodium valproate (VPA)§, a widely used antiepileptic [1], always induces a well-tolerated hyperammonemia of renal origin in the fasting human and in fasting rats, unaccompanied by abnormalities of the acid-base status or of hepatic and pancreatic functional tests [2–5]. The liver can be involved in the hyperammonemia, but in other circumstances [3, 6, 7]. The mechanisms of renal valproate-induced hyperammonemia are still unclear but there is evidence that glutamine uptake increased with VAP [2, 5, 8]. The aim of this study was to determine the possible biochemical mechanism of the hyperammonemia of renal origin. As glutamine plays a major role in renal ammoniagenesis, and as VPA can interfere with mitochondrial function [9–11], we have examined the effects of VPA on kidney mitochondria, especially its effects on glutamine transport and on certain enzymes involved in glutamine metabolism.

MATERIALS AND METHODS

Mitochondrial isolation and VPA treatment. Male Wistar rats weighing 250–300 g were fasted for 18 hr but were free to drink. One group was injected intraperitoneally (i.p.) with VPA (LCG 211 17, Labaz Laboratories, France) 200 mg/kg, in 0.9% NaCl, pH 7.4. At this dose, VPA protects against convulsions; its concentration has been measured as 2.52 ± 0.38 (mean ± SD) μg/mg protein in kidney, and 0.29 μg/mg protein in kidney mitochondria [11]. Another group of rats was injected with 0.9% NaCl alone; their isolated mitochondria were the "controls". All rats were killed by decapitation 15 min after the injection; arterial ammonium concentration of VPA-injected rats is then twice the normal [5]. To obtain VPA-incubated mitochondria, rats of a third group were killed and their isolated renal mitochondria were incubated with VPA at 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8} or 1×10^{-10} M for 20 min at 4°.

Kidney mitochondria were isolated by the method of Adam and Simpson [12] in a medium containing sucrose 70 mM, mannitol 220 mM, HEPES 2 mM, bovine serum albumin 0.5 g/l, pH 7.4. The final pellet was resuspended in a small volume of sucrose 0.3 M, HEPES 5 mM. The respiration was measured to ascertain mitochondrial integrity. All steps in the isolation procedure were carried out at 4°. Proteins were assayed by the method of Lowry *et al.* [13].

Glutamine transport. Glutamine transport was assayed using the procedure of Simpson and Adam [14]. Mitochondria, 2–5 mg of protein, were incubated in a mixture of KCl 115 mM; sucrose 20 mM; HEPES 18 mM (pH 7.4); $MgSo_4$ 0.5 mM; sodium arsenite 1 mM; rotenone 1 μg/ml; glutamine at different concentrations, 1, 2.5, 5, 7.5 or 10×10^{-4} M, for determination of K_m and V_{max}; radioactive L-[U-^{14}C]glutamine 0.5 μCi/1 ml incubation mixture (10.7 GBq/mmol, Amersham, France, SA) and tritiated [6,6'(n)-3H] sucrose 1 μCi/1 ml incubation mixture (555 GBq/mmol, Amersham). Incubation was carried out for 2 min at 37°.

A volume of 150 μl of $HClO_4$ 1.25 N were placed in microcentrifuge tubes and bromododecane 200 μl was layered on top of the perchloric acid. Mitochondrial incubation mixture (200 μl) was layered on top of the bromododecane. The tube was immediately centrifuged in a Eppendorf type microcentrifuge (Janetzki TH 12) at 12,000 *g* for 2 min. A

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§ Abbreviations: VPA, sodium valproate; i.p., intraperitoneally.

Table 1. $1/V_{max}$ and $1/K_m$ values for glutamine transport in rat kidney mitochondria

	$1/V_{max}$ (/pmol)	$1/K_m$ (/mM)
Controls	0.62 ± 0.14	1.22 ± 0.28
Mitochondria of VPA-injected rats	0.55 ± 0.10 NS	1.24 ± 0.15 NS
VPA-incubated mitochondria VPA concentration (M)		
10^{-3}	0.48 ± 0.97 NS	1.85 ± 0.23 $P < 0.01$
10^{-4}	0.69 ± 0.24 NS	1.79 ± 0.32 $P < 0.01$
10^{-5}	0.56 ± 0.14 NS	1.67 ± 0.30 $P < 0.05$
10^{-6}	0.65 ± 0.14 NS	1.61 ± 0.30 NS
10^{-7}	0.64 ± 0.20 NS	1.32 ± 0.27 NS
10^{-8}	0.63 ± 0.15 NS	1.27 ± 0.21 NS
10^{-10}	0.63 ± 0.08 NS	1.28 ± 0.12 NS

Values expressed are mean ± SD. For statistical analysis: see Materials and Methods. NS, not significant.

blank sample was simultaneously prepared by adding 200 μl of the incubation mixture without glutamine on top of the bromododecane. The two upper layers were then removed; the perchloric-acid extracts of mitochondria were immediately neutralized with a solution of equal parts of potassium phosphate 0.1 N, pH 6.2, and KOH 1 N. Radioactivity was then measured with a scintillation counter (Beckman LS 9000). Radioactivity of the blank was subtracted from the total of the perchloric acid extracts.

Enzyme activities. The following enzyme activities were determined as described previously [15]: in the mitochondria, glutamate dehydrogenase (EC 1.4.1.3), aspartate aminotransferase (EC 2.6.1.1.), phosphate-dependent glutaminase (EC 3.5.1.2) and γ-glutamyl transpeptidase (EC 2.3.2.2); in the cytosol, γ-glutamyl transpeptidase, adenosine deaminase (EC 3.5.4.4) and glutamine synthetase (EC 6.3.1.2). These enzyme activities were determined *in vitro*: mitochondria and cytosol were incubated for 20 min at 4° with the same VPA concentrations as for the glutamine transport experiments.

Ammonia concentration was measured in mitochondria by an automated microassay utilizing the colorimetric method of Berthelot [16]. Statistical analysis was done using Student's *t*-test for comparing enzymes activities and the one-way ANOVA followed by a multiple range test for comparing K_m and V_{max}, and amounts of glutamine transported.

RESULTS

Glutamine transport

For kidney mitochondria of VPA-treated rats, V_{max} and K_m of control and experimental mitochondria did not differ statistically (Table 1, Fig. 1). The amount of glutamine transported was often higher in these experimental mitochondria than in the controls, but not to a statistically significant degree.

For VPA-incubated mitochondria, glutamine transport increased, apparently competitively. The maximum transport rate, V_{max}, had practically the same value as controls for all VPA concentrations studied. The affinity constant, K_m, decreased. This

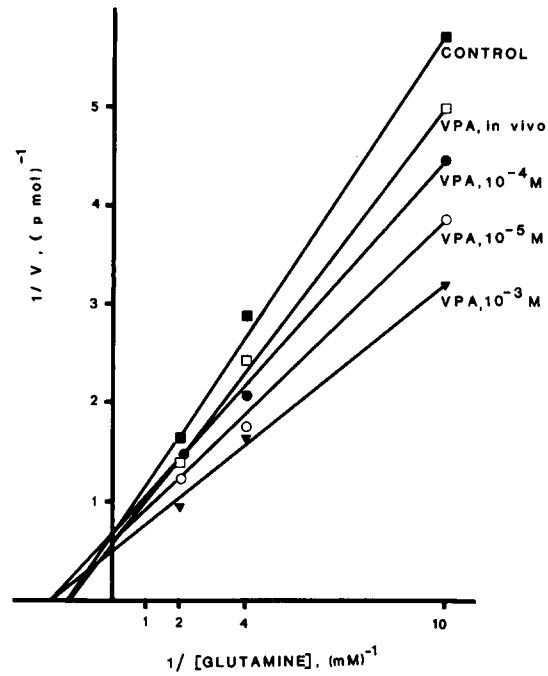

Fig. 1. Lineweaver–Burk plot of rat mitochondrial glutamine transport. Control; VPA *in vivo*, values for mitochondria of VPA-injected rats; others, values for mitochondria incubated with VPA at concentrations giving results statistically different from controls (see Table 1). Only three concentrations of glutamine are graphed for clarity. Lines calculated by least squares method.

effect was most evident at VPA concentrations between 10^{-3} and 10^{-5} M: the multiple range analysis showed a statistically significant difference between controls and experimental mitochondria at concentrations of 10^{-3} M ($P < 0.01$), 10^{-4} M ($P < 0.01$) and 10^{-5} M ($P < 0.05$); but at [VPA] 10^{-6} M, $P < 0.1$. At higher concentrations, results were extremely variable. At low concentrations, $>10^{-6}$ M, control and VPA-incubated mitochondria did not differ. Ammonia production was greater in VPA-incubated mitochondria compared to controls for

Table 2. Specific activities of enzymes involved in glutamine metabolism in rat kidney cell mitochondria (Mt) and cytosol (Cyt)

Enzyme	Glutaminase	Glutamate dehydrogenase	Glutamine synthetase	Glutamine synthetase	Aspartate aminotransferase	Glutamyl transpeptidase	Glutamyl transpeptidase	Adenosine deaminase
Cell fraction	Mt	Mt	Mt	Cyt	Mt	Mt	Cyt	Cyt
Controls								
Sp. act.	1.28	19.1×10^{-2}	7.51×10^{-3}	14.9×10^{-2}	79.9×10^{-2}	52.0×10^{-2}	19.4×10^{-3}	61.2
SD	0.37	6.6×10^{-2}	0.66×10^{-3}	2.1×10^{-2}	4.3×10^{-2}	9.3×10^{-2}	4.0×10^{-3}	3.6
N	12	10	12	12	12	12	14	11
VPA-injected rats								
Sp. act.	1.28	19.1×10^{-2}	7.57×10^{-3}	17.2×10^{-2}	73.5×10^{-2}	59.9×10^{-2}	24.2×10^{-2}	65.4
SD	0.28	6.9×10^{-2}	0.99×10^{-3}	1.3×10^{-2}	8.9×10^{-2}	4.6×10^{-2}	5.12×10^{-3}	7.8
N	7	5	7	7	5	5	9	6
VPA-incubated Mt or VPA-incubated Cyt, VPA concentration, M								
1×10^{-3} M								
Sp. act.	1.32	20.4×10^{-2}	7.56×10^{-3}	15.2×10^{-2}	82.5×10^{-2}	60.0×10^{-2}	22.4×10^{-3}	58.8
SD	0.47	7.2×10^{-2}	0.72×10^{-3}	3.2×10^{-2}	10.6×10^{-2}	10.3×10^{-2}	6.7×10^{-3}	9.0
1×10^{-4}								
Sp. act.	1.30	21.6×10^{-2}	7.98×10^{-3}	16.3×10^{-2}	76.3×10^{-2}	57.6×10^{-2}	20.8×10^{-3}	67.2
SD	0.38	6.8×10^{-2}	1.03×10^{-3}	2.3×10^{-2}	9.6×10^{-2}	12.8×10^{-2}	5.2×10^{-3}	18.0
1×10^{-5} M								
Sp. act.	1.26	18.5×10^{-2}	7.95×10^{-3}	17.5×10^{-2}	84.8×10^{-2}	55.6×10^{-2}	20.2×10^{-3}	65.4
SD	0.58	8.1×10^{-2}	1.21×10^{-3}	1.9×10^{-2}	12.5×10^{-2}	9.8×10^{-2}	6.3×10^{-3}	7.2
1×10^{-6} M								
Sp. act.	1.18	20.3×10^{-2}	6.82×10^{-2}	14.8×10^{-2}	72.8×10^{-2}	56.4×10^{-2}	23.8×10^{-3}	64.8
SD	0.48	5.6×10^{-2}	1.01×10^{-3}	2.5×10^{-2}	6.2×10^{-2}	10.2×10^{-2}	9.2×10^{-3}	4.2
1×10^{-7} M								
Sp. act.	1.30	20.9×10^{-2}	6.50×10^{-3}	14.2×10^{-2}	74.9×10^{-2}	60.8×10^{-2}	21.8×10^{-2}	57.0
SD	0.35	6.2×10^{-2}	0.95×10^{-3}	1.9×10^{-2}	7.2×10^{-2}	13.5×10^{-2}	4.8×10^{-3}	7.8
1×10^{-8} M								
Sp. act.	1.26	19.2×10^{-2}	6.72×10^{-3}	16.5×10^{-2}	78.9×10^{-2}	60.7×10^{-2}	19.0×10^{-2}	62.4
SD	0.39	7.0×10^{-2}	0.87×10^{-3}	2.1×10^{-2}	8.4×10^{-2}	12.8×10^{-2}	5.8×10^{-3}	10.8
1×10^{-10} M								
Sp. act.	1.22	19.1×10^{-2}	7.29×10^{-3}	16.2×10^{-2}	80.1×10^{-2}	59.2×10^{-2}	19.7×10^{-2}	60.6
SD	0.26	6.9×10^{-2}	0.78×10^{-3}	2.3×10^{-2}	8.7×10^{-2}	8.9×10^{-2}	6.9×10^{-3}	5.4
N	5	5	5	5	5	5	5	5

Sp. act., specific activities for glutaminase, μmol NAD^+ formed per 15 min per mg protein; glutamate dehydrogenase, μmol NAD^+ formed per min per mg protein; glutamine synthetase, μmol hydroxamate formed per min per mg protein; aspartate aminotransferase, μmol NADH oxidized per min per mg protein; glutamyl transpeptidase, μmol *p*-nitroanilide formed per min per mg protein and adenosine deaminase, *n* mol substrate transformed per min per mg protein. Values expressed as mean ± SD. Mt, mitochondria; Cyt, cytosol; N, number of experiments. There was no statistically significant difference between controls and VPA injected rats or VPA-mitochondria and VPA-incubated cytosol.

VPA concentrations of 10^{-3}, 10^{-4} and 10^{-5} ($P < 0.05$).

Enzyme activities

Enzyme activities did not vary significantly between mitochondria of VPA-injected rats and their controls or the relevant experimental and control cytosols, nor between the VPA-incubated mitochondria and their controls or the relevant experimental and control cytosols (Table 2).

DISCUSSION

This study shows that VPA is responsible for an increased glutamine transport across the mitochondria membranes of the kidney in the fasting rat, unaccompanied by changes in the activities of the enzymes involved in ammoniagenesis. Three conclusions may be drawn.

The effect of VPA on glutamine transport may be related with a membrane-disordering effect of VPA. VPA is known to have an effect on membranes, first suggested by electrophysiological studies [17, 18] and, more recently, by physicochemical evidence. VPA induces a disordering of the brain cell membranes [19, 20], modifies the protein–lipid structure of liver and kidney mitochondrial membranes, as shown by electron paramagnetic resonance studies [11], and inhibits the intramitochondrial transport of pyruvate [9] and succinate [21].

The VPA-induced hyperammonemia of renal origin, during which renal glutamine uptake increases [5, 8], may be related to an increased glutamine transport in kidney mitochondria. It is well known that mitochondria play an important role during renal ammoniagenesis [22, 23]. Our findings in valproate-induced hyperammonemia of renal origin are not in favour of an enzymatic hypothesis involved in ammoniagenesis [22, 23], insofar as we saw no direct changes of the enzymatic activities involved in ammoniagenesis, we measured in either mitochondria of VPA-injected rats or in rat mitochondria incubated with VPA. On the other hand, the lowered K_m suggests that VPA increases the affinity of the substrate for the transport system, the number of sites involved in the transport remaining constant.

However, this higher glutamine transport cannot alone account for the hyperammonemia. First, glutamine transport is not modified in VPA-treated rats, although this negative finding is perhaps due to the time interval between mitochondrial preparation and transport measurement. Second, the experiments were performed on isolated mitochondria, whereas it has been shown that enzyme activity of mitochondria does depend on the presence of cytosol: mitochondrial functions can be altered when the organelles are not in their natural environment [23].

The fundamental physiopathological mechanism for the increase of intramitochondrial glutamine transport remains to be clarified. Which hypothesis, as put by Preuss *et al.* [24], best describes the phenomenon, a "push", where enhanced glutamine transport into the mitochondria is followed by deamination to glutamine which is then deaminated, or a "pull", where glutamine deamination at first precedes deamination? Our observations favor the first: since glutaminase activity did not change (as observed by others [8]), glutamate, a powerful inhibitor of glutaminase [22], probably did not accumulate in the mitochondrial matrix. The VPA-induced inhibition of succinate transport in kidney mitochondria [21] could also be a factor modifying renal ammoniagenesis, to the extent that glutamine incorporation is linked with the dicarboxylate transport system [25].

The VPA-induced hyperammonemia of renal origin in fasting normal human volunteers and in fasting rats is well-tolerated, probably because the liver successfully detoxifies the ammonia. VPA can, however, modify the liver's ammonia metabolism in other circumstances. Different biochemical mechanisms have been proposed for the increase of ammonia production. Following i.p. injection of an amino-acid mixture, VPA-treated rats can develop a hyperammonemia persisting in the binephrectomized animal and accompanied by a decrease of hepatic mitochondrial carbamyl phosphate synthetase I activity [3]. The liver's importance has also been emphasized when VPA administration is associated with other antiepileptics [26–28], a result others do not confirm [29], or with a particular food intake pattern [30, 31]. The hyperammonemia may be related to ureagenesis inhibition, as when VPA induces secondary clinical effects [6, 32, 33]. The effect of ammoniagenesis is probably not specific to VPA: other short-chain fatty acids can induce a hyperammonemia of hepatic origin [34], and we have observed that short, branched-chain fatty acids, but not straight-chain fatty acids induce hyperammonemia.

In conclusion, VPA modifies ammonia metabolism in the kidney and in the liver by different but not mutually exclusive biochemical pathways. In the kidney, VPA induces a modification of the mitochondrial membrane permeability and increases glutamine transport across the internal mitochondrial membrane. These effects may be one explanation for VPA-induced, asymptomatic hyperammonemia of renal origin in the fasting rats.

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