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Research Article

In vivo effect of sodium valproate on mouse liver

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Abstract. The in vivo effect of sodium valproate (SV) on the activity of uridine diphosphate glucuronosyltransferase (UDP-GT) and hepatotoxicity in the mouse liver was studied. Mice were injected intraperitoneally (IP) with SV at doses varying from 50 to 800 mg/kg per day, for six consecutive days (dose-response group) or at a standard dose of 300 mg/g per day for 2–10 days (time-response group), whereas the controls were injected with normal saline. Valproic acid levels had a positive correlation to the dose ($P < 0.001$) and duration of drug administration ($P = 0.006$). A gradual increase in UDP-GT activity was observed in doses of up to approximately 400 mg/kg per day, whereas in higher doses the enzyme activity gradually decreased. The time course of UDP-GT activity at the standard dose of 300 mg/kg per day increased progressively, with a maximum up to the sixth day and then had a gradual reduction. Hepatic necrosis (which was unrelated to the dose or the duration of drug administration) was found in 13% of the SV-treated animals and in none of the controls. We conclude that at an optimal dose (300–400 mg/kg per day) and at a time course of 6 days, SV causes liver UDP-GT induction, whereas in higher doses and longer duration of administration, UDP-GT activity is gradually reduced. SV also causes hepatotoxicity unrelated to dose and time course.

Key words. Activity; liver; mouse; sodium valproate; uridine diphosphate glucuronosyltransferase.

Sodium valproate (SV) is a commonly used anticonvulsant with various side effects [1] of which the more serious and occasionally irreversible are those of hepatic involvement [2–4]. Deaths due to hepatotoxicity have been reported, mainly in children [2, 4, 5]. Although hepatic failure is uncommon, hepatic dysfunction indicated by an asymptomatic rise in serum liver enzymes occurs in patients during SV treatment [3, 6, 7]. The relatively common transient and asymptomatic rise in serum levels of transaminases, (usually unaccompanied by an increase of serum bilirubin levels) [3, 8] is an interesting observation and could be explained by an increase of uridine diphosphate glucuronosyltransferase (UDP-GT) activity effected by SV. This fact, coupled with the uncertainty involved in the mechanism by which SV induces liver damage, was our rationale in determining its in vivo effect on the liver of mice. In the present study, we investigated the changes in UDP-GT activity in liver homogenates, the serum valproic acid and alanine aminotransferase levels, as well as the liver histology of mice treated with different doses of SV during different time periods.

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Material and methods

Animals and treatment procedures. Male mice (1–2 months old, weighing 15–20 g) received SV intraperitoneally (IP) dissolved in normal saline in a final volume of 1 ml in varying doses of 50, 100, 200, 300, 400, 500, 600 and 800 mg/kg per day, for six consecutive

days; these constituted the dose-response groups. The time-response groups received a standard dose (300 mg/kg per day) for time periods varying from 2, 4, 6, 8 and 10 days, whereas the controls were injected, IP, with 1 ml of normal saline. The mice were permitted free access to food and water. At the end of the experiments, a blood sample was taken 30 min after the last SV injection; the animals were then decapitated, and their livers were immediately excised for histology, homogenization and enzyme assay.

Materials. SV (in purified powder form) was kindly donated by the Sanofi Company (France). Serum valproic acid (VPA) levels were estimated using an enzyme multiple immunotechnic (EMIT) kit (Syva Company, Atlanta, GA, USA). Serum alanine aminotransferase (ALT) was measured by a kinetic assay with reagents from Guilford Pharmaceuticals (USA). Ethyl anthranilate, methyl *n*-propyl ketone propan-1-ol and *n*-butyl acetate were purchased from British Drug Houses (Poole, UK). All other chemicals used were conventional commercial products (Sigma Chemicals, St. Louis, MO).

Methods. Serum VPA levels were estimated using the EMIT kit. Serum ALT levels were determined using a modified version of the Wrobleski-LaDue-Henry kinetic method [9]. Hepatic UDP-GT activity was assayed on liver homogenates utilizing the method of Black et al. [10]. All assays were performed in duplicate. Liver specimens (0.25 g wet weight) were homogenized with a Teflon head in 0.25 M sucrose, 1 mM ethylenediaminetetraacetate (EDTA, pH 7.4) in a final volume of 1 ml; an equal volume of 2% digitonin suspension was added. The incubation mixture contained 200 µl of bilirubin-albumin substrate, 40 µl of 125 mM $MgCl_2$, 40 µl of triethanolamine buffer (pH 7.4) and 20 µl of uridine diphosphate-glucuronic acid (UDPGA) (containing 1.67 nmol). The tubes were incubated in a shaking incubator at 37 °C for 30 min. Finally, the reaction was initiated by adding the enzyme preparation and terminated by soaking the tubes in chipped ice and immediately adding 1 ml of glycine-HCl buffer (pH 2.8). The diazo-reagent was added, and extraction of azo-pigments was carried out as described by Black et al. [10]. UDP-GT activity was expressed as micrograms of bilirubin conjugated per milligram of protein per hour. The protein concentration was measured using the method described by Lowry and colleagues [11]. All histology was assessed by the same histopathologist. The liver tissue samples were fixed in 10% formaldehyde, embedded in paraplast, and the histological sections were stained with hematoxylin-eosin and Masson's trichrome.

Statistical analysis. Linear and curvilinear regression was used to estimate the relation of serum VPA and UDP-GT levels to dose and duration of administration. Predicted values and their 95% confidence intervals were calculated. The Student's *t* test was used to test for differences between the groups.

Results

Preliminary studies. It has been shown that oestrogens cause an induction in liver enzymes [12]. To determine whether or not such an induction exists in hepatic UDP-GT activity in female mice, we conducted preliminary experiments using mice of both sexes. One millitre of normal saline was injected daily IP for 10 days, in four male and four female mice. The mean value $\pm$ SD of hepatic UDP-GT activity in male mice was 4.58 ± 1.66 µg of bilirubin conjugated per milligram of protein per hour, and in female mice, 11.77 ± 1.36 µg ($0.001 < P < 0.01$). Thus, all of the following experiments were done on male animals.

Dose response. In this series of experiments, SV in different doses was injected daily, IP, for six consecutive days in eight subgroups of mice. The subgroup injected with 800 mg/kg per day SV initially included

Table 1. Dose-response group.

SV dose (mg/kg per day)	*n*	Serum VPA levels (µg/ml)			UDP-GT activity (µg bilirubin conjugated per			Predicted UDP-GT activity mg protein per hour)	
		mean	SD	SE	mean	SD	SE	predicted mean	95% CI for mean
50	7	15.50	8.53	3.48	7.35	1.94	0.73	7.08	2.88–11.27
100	8	44.57	36.34	13.74	7.53	1.04	0.37	7.75	3.63–11.87
200	6	74.80	53.81	24.07	8.60	2.94	1.20	8.73	4.62–12.85
300	6	316.80	152.23	68.08	8.88	2.37	0.97	9.25	5.11–13.40
400	7	432.00	233.51	88.26	10.03	2.09	0.79	9.30	5.16–13.43
500	4	465.50	41.68	20.84	8.33	2.16	1.08	8.88	4.75–13.00
600	7	779.00	165.60	67.71	7.97	1.76	0.66	7.98	3.72–12.25
Total	45				8.35	2.11	0.31		
Controls	32				5.60	1.65	0.29		

SD, standard deviation; SE, standard error; CI, confidence interval.

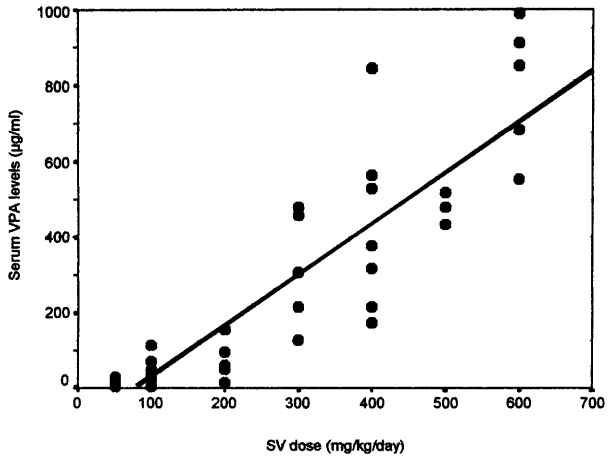

Figure 1. Serum VPA levels and SV dose.

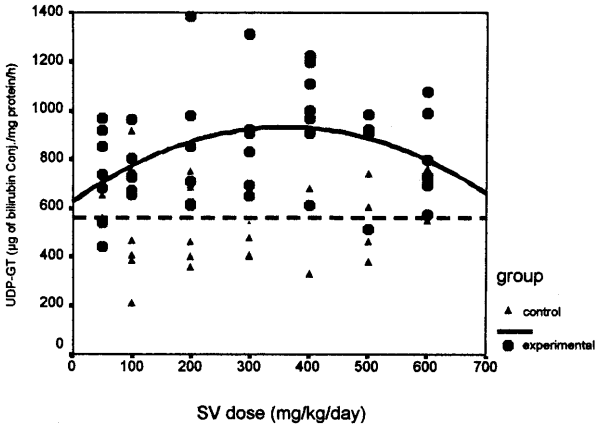

Figure 2. UDP-GT activity and SV dose.

seven mice, but only one survived up to the 6th day; the rest probably died from drug overdose. Serum VPA levels (table 1) correlated significantly with the dose administered ($r = 0.888$, $P < 0.001$). The regression line of VPA levels and dose is shown in figure 1. There was a curvilinear relation between UDP-GT levels (table 1) and the dose administered ($P = 0.219$ for linear regression and $P = 0.037$ for fitting a quadratic polynomial). The predicted mean UDP-GT activity according to the dose injected and the corresponding 95% confidence interval (CI) is shown in table 1. The regression curve is shown in figure 2, along with the line depicting the mean UDP-GT level in the controls. A gradual increase of the UDP-GT enzyme was observed in doses up to 400 mg/kg per day, while in higher doses, enzyme activity gradually decreased. ALT levels (table 2) were not correlated to SV dose ($r = -0.134$, $P = 0.451$). The mean SD value of ALT was 113.74 ± 118.51 U/l in the 34 SV-treated mice that were measured; the corresponding values were 99.78 ± 104.89 U/l in the 32 controls (the difference was not statistically significant, $t_{64} = 0.51$, $P = 0.615$).

Time response. In this series of experiments, 300 mg/kg per day of SV was injected IP for 2, 4, 6, 8 or 10 days and normal saline in the corresponding controls. Serum VPA levels (table 3) correlated significantly with the duration of administration ($r = 0.658$, $P = 0.006$). The regression line of serum VPA levels and time course is shown in figure 3. There was a curvilinear relation (table 3) between UDP-GT levels and the duration of drug administration ($P = 0.380$ for linear regression, and $P = 0.076$ for fitting a quadratic polynomial). The predicted mean UDP-GT activity according to the dose injected and the corresponding 95% CI is shown in table 3. The regression curve is shown in figure 4, along with the line depicting the mean UDP-GT level in the controls. A gradual increase of the UDP-GT enzyme was observed for an administration time of up to 6 days, whereas for > 6 days, enzyme activity gradually decreased.

Histological findings. Histology was performed on the livers of 44 mice treated with an IP SV injection and 24 controls. In 6 animals (13%) of the SV-treated group, the livers showed focal areas of degeneration and recent liver-cell necrosis, with an accompanying inflammatory reaction of the adjacent parenchyma. Prominent microvesicular steatosis was present but without specific localization, and there was no cholestasis or cirrhosis (fig. 5). These findings were unrelated to the dose or duration of the drug administration. Since Kupffer's cell hyperplasia, mild microvesicular steatosis and increased numbers of inflammatory cells in the portal triads and adjacent liver parenchyma were also present in the controls, these were interpreted as nonspecific findings. Four out of 6 animals with hepatic necrosis were treated with SV (50 mg/kg per day); their UDP-

Table 2. Serum ALT levels according to SV dose.

SV dose (mg/kg per 24 h)	Animals *n*	ALT Mean	U/l SD	SE
50	5	105.60	88.73	39.68
100	8	154.22	151.17	50.39
200	4	97.75	60.37	30.18
300	7	122.00	153.55	58.04
400	4	21.50	17.21	8.61
500	5	124.00	105.76	47.30
600	0	–	–	–
Total	34	113.74	118.51	20.32
Controls	32	99.78	104.89	18.54

Table 3. Time-response group.

Time (days)	*n*	Serum VPA levels (μg/ml)			UDP-GT activity (μg bilirubin conjugated per mg protein per hour)			Predicted UDP-GT activity	
		mean	SD	SE	mean	SD	SE	predicted mean	95% CI for mean
2	4	265.50	60.07	30.04	5.79	0.03	0.02	5.85	2.80–8.90
4	3	280.00	108.22	62.48	7.53	1.60	0.92	7.50	4.78–10.23
6	3	292.00	27.06	15.62	8.38	0.82	0.47	8.27	5.51–11.03
8	3	416.00	55.75	32.19	7.96	1.17	0.67	8.14	5.42–10.86
10	3	420.00	87.43	50.48	7.20	1.66	0.96	7.12	4.21–10.04
Total	14				7.49	1.35	0.36		
Controls	13				5.44	1.36	0.38		

GT level was $6.0193 \pm$ SD 1.347 compared with 9.132 ± 0.568 in those without necrosis that received the same dose ($P = 0.014$).

Discussion

In the mouse, orally administered SV is very rapidly absorbed, with maximum serum levels occurring between 5 and 30 min after treatment [13]. Plasma VPA levels of mice that received SV subcutaneously have been reported to be dose-related (30 min following the last injection) [14]. In the rat, maximal brain levels have been observed 30 min after IP drug administration [15]. In the present study, the serum levels of VPA were dose- and time-course related. Dickinson et al. [16] studied the disposition of VPA in the rat after intravenous administration and found a dose-dependent decline in blood concentration, whereas the magnitude and duration of bile stimulation closely followed serum VPA concentration.

Mild and reversible hepatic involvement, common (up to 44%) [3, 6, 7] during SV treatment, is dose-related and generally consists only of abnormalities in liver enzymes without clinical symptoms. Occasionally, the hepatotoxicity is irreversible [2–5], non-dose-dependent [17] and it appears to be idiosyncratic [2, 3]. Major risk factors include a young age, high drug dosage, concurrent use of multiple medications and underlying metabolic disorders [2–5].

Kingsley et al. [18] documented the hepatotoxicity of SV in rat hepatocyte cultures and found that it was dose-related; this finding does not concur with our study. In the present study, hepatotoxicity (in the form of hepatic necrosis) was found only in the treatment group, and this was unrelated to the dose or duration of drug administration; our findings are in agreement with other studies in humans. In a study of 23 fatal cases of valproate-induced hepatic injury, Zimmerman and Ishaak [2] speculated that the fatty liver might be produced by the idiosyncratic exaggeration of a particular metabolic pathway.

The mechanism by which VPA causes hepatic damage is uncertain; hepatotoxicity is suspected to result from the formation of toxic VPA metabolites [3–5, 19–22], or altered antioxidant enzyme activities [23]. Glucuronidation is the main metabolic route for valproate [24]. The conjugated drug is rapidly excreted in the urine, and about 70% of the administered dose is eliminated by this route within 24 h. In mice, as early as 15 min after the administration of 14C-valproate, radioactivity is found in the bile and urine [25]. The higher the dose of VPA, the greater the percentage metabolized by glucuronidation [26]; this concurs with the increase of UDP-GT activity according to the SV dose in our study. VPA can undergo mitochondrial or peroxisomal β-oxidation, whereas ω and ω_1 hydroxylation are associated with microsomes (i.e. with cytochrome P-450) and are of less importance than glucoronidation and β-oxidation. The impairment of VPA β-oxidation and the increase in metabolites of alternative metabolic pathways (ω and ω_1 hydroxylation) have been the most frequent findings in the serum

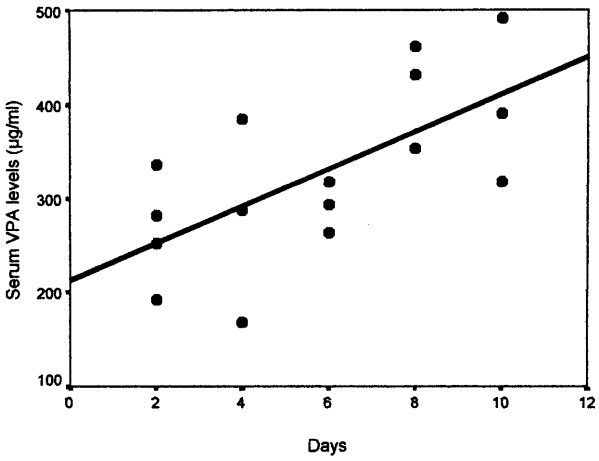

Figure 3. Serum VPA levels and time course.

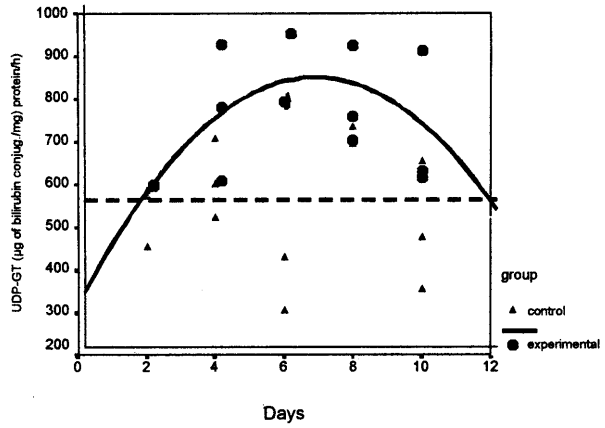

Figure 4. UDP-GT activity and time course.

of patients with VPA-associated hepatotoxicity [21, 22, 26, 27]. Microvesicular steatosis (which characterizes VPA-associated liver injury) possibly occurs via interference with the process of fatty acid β-oxidation (through reactive intermediates which inhibit key enzymes in the β-oxidation cycle) [28] or by the possible idiosyncratic production of toxic VPA metabolites [29]. Studies in experimental animals have revealed high doses of VPA-induced hepatic steatogenesis without necrosis in the rat [30] and fatty liver with necrosis in the mouse [31], as was found in our study. The present study showed a non-dose-related hepatotoxicity but cannot resolve the mechanism by which it was caused. Conjugation with glucuronic acid is dependent on UDP-GT enzyme activity as well as on the accessibility of UDPGA [32]. In humans, glucuronide conjugates and the oxidation products represent the most abundant metabolites of VPA in the urine [33]. VPA has been shown to interact with all major antiepileptic drugs through displacement from albumin binding sites and the inhibition of drug metabolism [33]. More recently, evidence has shown that VPA inhibits the elimination of drugs metabolized by glucuronide conjugation [34]. In mice injected with VPA in a dose- and time-dependent fashion, hepatic UDPGA was reduced by 90% [35]. Furthermore, Granneman et al. [36] have reported a dose-dependent increase in urinary excretion of VPA glucuronide and a decrease in urinary excretion of 3-oxo-VPA, a β-oxidative metabolite of VPA. These findings are compatible with those of the present study, which showed an enhancement of UDP-GT activity according to the dose and duration of SV treatment. Following IP galactosamine treatment in rats, Gregus et al. [37] found a decrease in hepatic UDPGA levels, delayed plasma clearance of VPA, reduction in plasma concentrations as well as a reduction in the biliary excretion of its glucuronide conjugates by 97%. In our study, after IP injection of SV for six consecutive days, a progressive increase of UDP-GT activity (with maximum activity at a dose of 400 mg/kg per day) was observed; at higher doses, the activity of the enzyme was slightly decreased. This finding could either be secondary to liver damage due to large doses of SV, or the conjugate could act by a negative feedback mechanism on the UDP-GT activity. The finding that UDP-GT levels were lower in the group with hepatic necrosis (compared to those with normal histology) could support the first hypothesis.

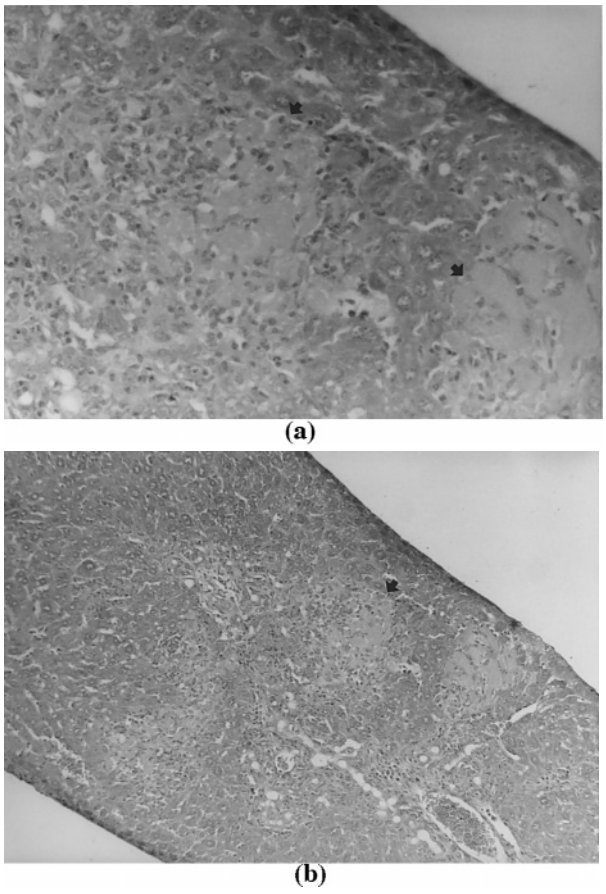

Figure 5. (*a*) Foci of degeneration and necrosis of liver cells (H.E. × 25). (*b*) Degeneration of liver cells accompanies by a mild inflammatory reaction (H.E. × 100).

Clinical studies have demonstrated consistent results in that serum bilirubin levels were significantly reduced in patients receiving phenobarbital, carbamazepine and phenytoin [38]. The mechanism for the reduction of bilirubin level by these drugs has been suggested to be an induction of hepatic microsomal enzyme, glucuronyl transferase [39] or the increased excretion of bilirubin from hepatic cells into bile [40]. The marginally significant reduction in bilirubin levels in patients treated with VPA which was found by Gough et al. [38] has been speculated to be the result of increased excretion of bilirubin from the hepatic cell into the bile. According

to our study, induced bilirubin conjugation by an inducing effect of VPA on the hepatic enzyme UDP-GT would be a reasonable explanation for the occurrence of reduced bilirubin. An induction effect of VPA on hepatic UDP-GT could be another explanation for the steady-state plasma total bilirubin concentration, which was lowered by 40 and 55% after an intravenous (IV) bolus dose of sodium valproate of 50 and 200 mg/kg, respectively, in guinea pigs with steady-state hyperbilirubinaemia, as reported by Yu et al. who speculated that it was caused by decreased plasma binding [41]. The increase of UDP-GT activity, found in the present study, which could be the result of enzyme induction by SV, may also explain the usual anicteric state of some patients on sodium valproate treatment, although they have serum transaminase elevation. To our knowledge, this in vivo effect of VPA on UDP-GT on mouse liver has not been previously studied.

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