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INHIBITION OF HUMAN RED BLOOD CELL GLUTATHIONE REDUCTASE BY VALPROIC ACID

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Abstract—Glutathione reductase (GR) one of the enzymes of the glutathione redox cycle, plays a salient role in maintaining appropriate cellular levels of reduced glutathione. The enzyme in human red blood cells is inhibited *in vitro* by the anticonvulsant drug valproic acid (VPA). The inhibition is dose-dependent, reversible, uncompetitive and does not depend on the redox state of the enzyme. VPA also inhibits red blood cell GR activity in children being treated with the drug. The level of serum VPA correlates significantly with the suppression of GR activity.

Treatment with valproic acid (VPA‖) is frequently prescribed for the management of various seizure disorders. However, the use of the drug has occasionally been associated with significant adverse effects including a number of fatalities [1]. Signs of VPA toxicity include both morphologic and biochemical changes in the liver [2]. Depletion of hepatic glutathione is one of the mechanisms by which drugs may evoke toxicologic responses [3]. Glutathione in its reduced (GSH) and oxidized (GSSG) forms is the major thiol redox system of the cell, providing protection against peroxidative damage. GR, one of the enzymes of the glutathione redox cycle, has a critical role in maintaining a high cellular GSH:GSSG ratio. It contains FAD and a redox active disulfide at its active site and catalyses the reduction of GSSG at the expense of NADPH. GR is thought to be an important drug target.

In a previous work [4] we provide evidence that rat liver GR activity is depressed in a dose-dependent manner following administration of VPA. It is not clear whether GR inhibition is restricted to liver tissue. Therefore, the aim of this study was to explore the effect of VPA on human red cell GR.

MATERIALS AND METHODS

In vitro *studies in man.* Blood from apparently healthy unrelated subjects was drawn into EDTA vacutainer tubes. After centrifugation, the red cells were suspended in ice-cold isotonic sodium chloride and washed twice in 10 vol. of the same solution. The packed cells were mixed with 4 vol. of a stabilizing solution consisting of 2.7 mM EDTA (pH 7.0) and 0.7 mM 2-mercaptoethanol. The haemolysate was rapidly frozen to −70°, and then thawed in a water bath, diluted 1:20 with stabilizing solution and used immediately after preparation. VPA was added to haemolysate aliquots to yield 0, 0.27, 0.54, 1.08 and 1.8 mmol/L, and the mixture was incubated at 37°. Samples were withdrawn for assay from incubation mixtures at zero time and also at 5, 11, 16, 22, 28, 33, 38 and 70 min. For substrate kinetics, the concentrations of GSSG ranged from 0.27 to 2.3 mM at constant cofactor concentration of 0.35 mM. For NADPH kinetics, the concentrations ranged from 0.019 to 0.33 mM, at constant substrate concentration of 2.3 mM. Each data point represents the average of at least six independent determinations, each run in duplicate. Double-reciprocal plots were used to assess the nature of the interaction between the enzyme and VPA.

The effect of NADPH and FAD on the course of GR inhibition by VPA was examined as follows: NADPH or FAD were added to haemolysate aliquots to yield 0.35 mmol/L or 1 μmol/L, respectively, and the mixture was incubated at 37° for 30 min (pre-incubation mixture). Then VPA (1.8 mmol/L) or the same volume of saline were added and the mixtures were incubated for 40 min more. Samples were withdrawn for GR assay immediately after VPA or saline addition and at 5, 20 and 40 min (Fig. 2). The reversibility of GR inhibition was tested by dialysis. Haemolysate aliquots were incubated for 30 min at 37° in the absence and presence of VPA (1.8 mmol/L). Samples were then dialysed overnight against the stabilizing solution (4°). Two samples in which the dialysis step was omitted were run in parallel. At the end of the interval GR activity and VPA concentration were measured in all the samples (Table 1).

In vivo *studies in man.* A group of 21 children and a group of nine adults on VPA monotherapy were included in the study. The control group consisted of 19 age-matched healthy children, and 10 healthy adults, respectively (Table 2). Blood was sampled for VPA concentration at the end of a dosage interval, just prior to the next dose. Red blood cell GR activity was measured in the same blood samples.

Assay procedures. GR activity was measured at

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‖ Abbreviations: VPA, valproic acid; GSH, reduced glutathione; GSSG; oxidized glutathione; GR, glutathione reductase.

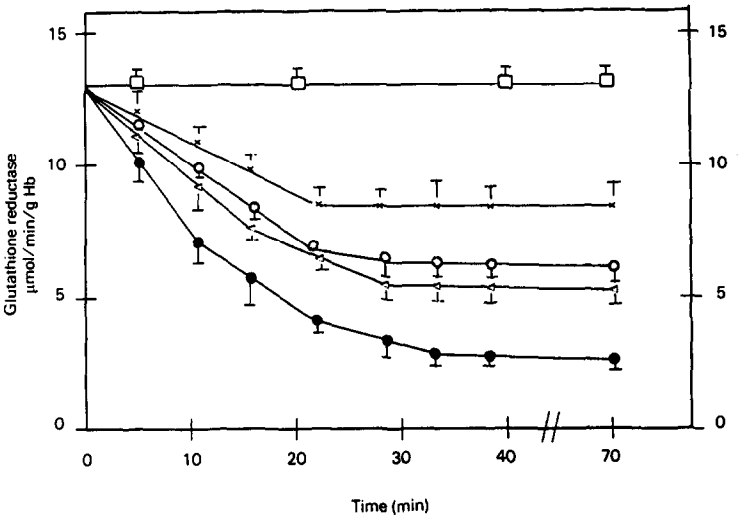

Fig. 1. Inhibition of human red blood cell GR by varying concentrations of VPA as a function of time. Results are means ± SD of six independent experiments. For assay conditions see details in text. VPA (mmol/mL): (□) 0; (×) 0.27; (○) 0.54; (△) 1.08; (●) 1.8.

37° by following NADPH oxidation at 340 nm [5]. The reaction mixture consisted of 0.05 mM Tris–HCl/0.25 mM EDTA, pH 8.0, 2.3 mM GSSG; 0.35 mM NADPH; 20 µL of 1:20 haemolysate. The values are expressed as µmol NADPH oxidized per min/g hemoglobin at 37°. VPA in treated patients was quantified by a commercial kit (Emit, Syva, Palo Alto). In this assay the total (protein-bound plus unbound) VPA concentration in plasma is measured. The therapeutic levels range between 0.3 and 0.6 mmol/L; toxic levels > 1.2 mmol/L. Metabolites of VPA (2-propyl-2-pentenoic acid, 2-propyl-4-pentenoic acid, 2-propyl glutaric acid) do not interfere with the test.

Statistics. Results are given as the mean ± SD. Data were analysed by one-way analysis of variance followed by Bonferroni's test. P values of less than 0.05 were considered as significant. Analysis of enzyme kinetic data was performed according to Cleland [6]. The relationship between serum VPA level and GR activity was assessed by linear regression analysis.

RESULTS

Inhibition of red blood cell GR by VPA in vitro

GR is inhibited by VPA in a concentration-dependent manner. The inhibition ranges from 35% (0.27 mmol/L VPA) to 80% (1.8 mmol/L VPA). Concentration–effect curves are given in Fig. 1. An initial decrease in the reaction velocity is seen within 20–25 min, after which the progress curve is linear. Preincubation with NADPH has no effect on the inhibitory action of VPA thus suggesting that the redox state of the enzyme does not influence the reaction (Fig. 2).

Preincubation with FAD does not affect the inhibitory effect of VPA (Fig. 2). The lost GR activity is restored after dialysis (Table 1). The effect

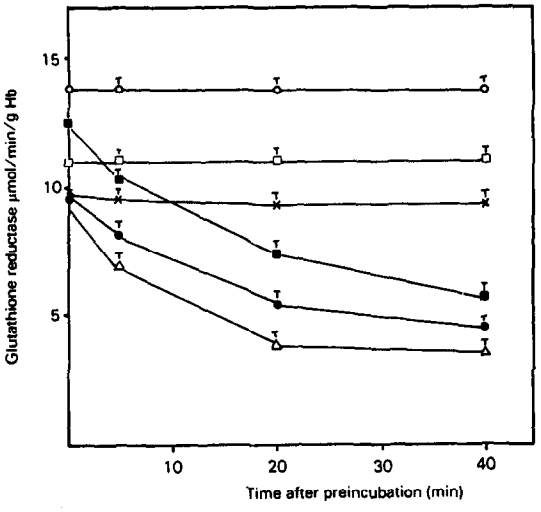

Fig. 2. Inhibition of human red blood cell GR by VPA after preincubation with NADPH or FAD. All values are means ± SD from six independent experiments. For assay conditions see details in text. (□) Saline; (×) NADPH + saline; (○) FAD + saline; (●) VPA; (△) NADPH + VPA; (■) FAD + VPA.

of VPA on GR activity at constant NADPH and increasing GSSG concentrations or at constant GSSG and increasing NADPH concentrations are given in Figs 3 and 4, respectively. Double-reciprocal plots of enzyme velocity versus substrate or NADPH concentrations in the presence of different constant amounts of VPA are straight lines parallel to those yielded in the absence of inhibitor.

Inhibition of red blood cell GR by VPA in vivo

Red blood cell GR activity in children and adults

Table 1. Effect of dialysis on the restoration of GR activity

	VPA concentration (mmol/L)		GR activity (μmol/min/gHb)	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Haemolysate + VPA	0.01	1.8	6.1 ± 0.36	1.48 ± 0.60
Haemolysate + saline	—	—	6.0 ± 0.40	6.05 ± 0.34

Results are mean ± SD of three independent determinations.
For assay conditions see details in text.

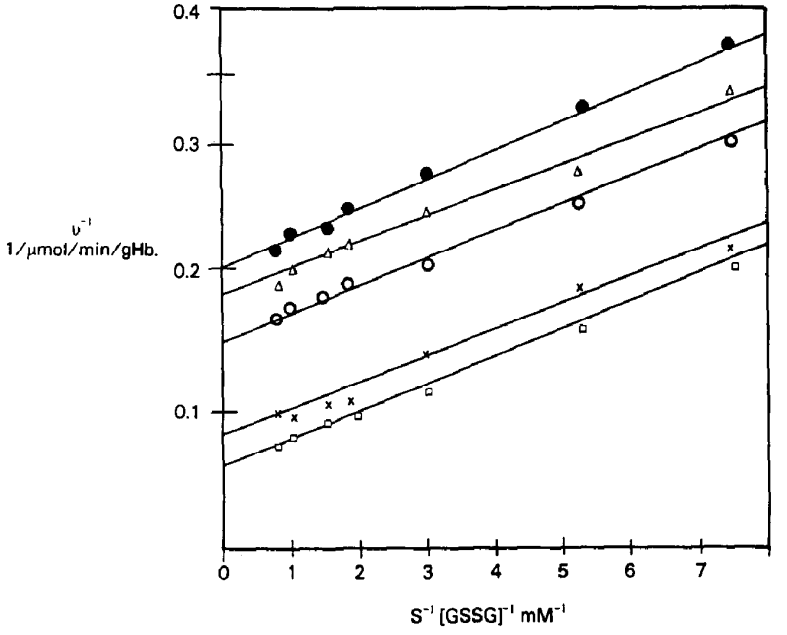

Fig. 3. Effect of different constant concentrations of VPA on human red blood cell GR activity (v) at constant NADPH and increasing GSSG concentrations. For assay conditions see details in text. VPA (mmol/L): (□) 0; (×) 0.27; (○) 0.54; (△) 1.08; (●) 1.8.

receiving VPA monotherapy is summarized in Table 2.

Red blood cell GR activity in children receiving VPA monotherapy is significantly reduced as compared to normal controls ($P < 0.01$). No change in GR activity could be detected in the group of adults on VPA.

The regression line for the correlation of GR activity versus the amount of total serum VPA in treated children is plotted in Fig. 5. A statistically significant correlation ($r = -0.82$) between the concentration of serum VPA and the suppression of GR activity can be seen in this group.

DISCUSSION

The results reported here provide evidence that VPA is able to inhibit GR activity of human red blood cells *in vitro*. The VPA concentrations needed to suppress GR activity are in the therapeutic range or higher than the drug concentrations in plasma of patients being treated with the drug. This inhibition is not limited to red blood cell GR, the drug also inhibiting the activity of the enzyme in rat liver [4] or the purified rabbit muscle enzyme (unpublished data). The inhibition is dependent on VPA concentration, is restored by dialysis and does not depend on the redox state of the enzyme. The double-reciprocal plots of enzyme velocity versus GSSG or NADPH concentrations, as generated by least squares fit on the data, are consistent with an uncompetitive inhibition. VPA also inhibits red blood cell GR activity in young patients being treated with the drug. The extent of inhibition is correlated with the serum concentrations of VPA. No inhibition whatsoever is seen in VPA-treated adults. Our data regarding the *in vivo* inhibition of GR activity in young patients are in contrast to the results of Pippenger *et al.* [7] who found significant alterations of erythrocyte free radical scavenging enzyme activities in children on VPA therapy, though not of GR.

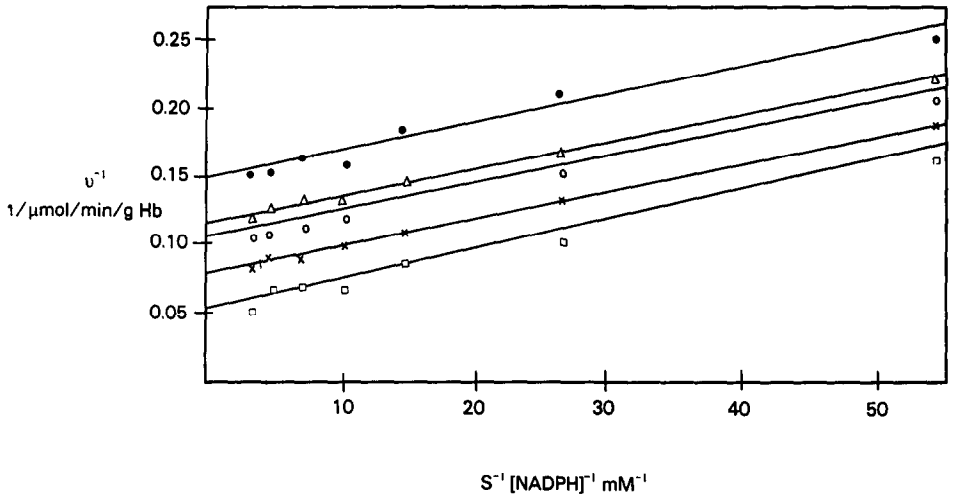

Fig. 4. Effect of different constant concentrations of VPA on human red blood cell GR activity (v) at constant GSSG and increasing NADPH concentrations. For assay conditions see details in test. VPA (mmol/L): (□) 0; (×) 0.27; (○) 0.54; (△) 1.08; (●) 1.8.

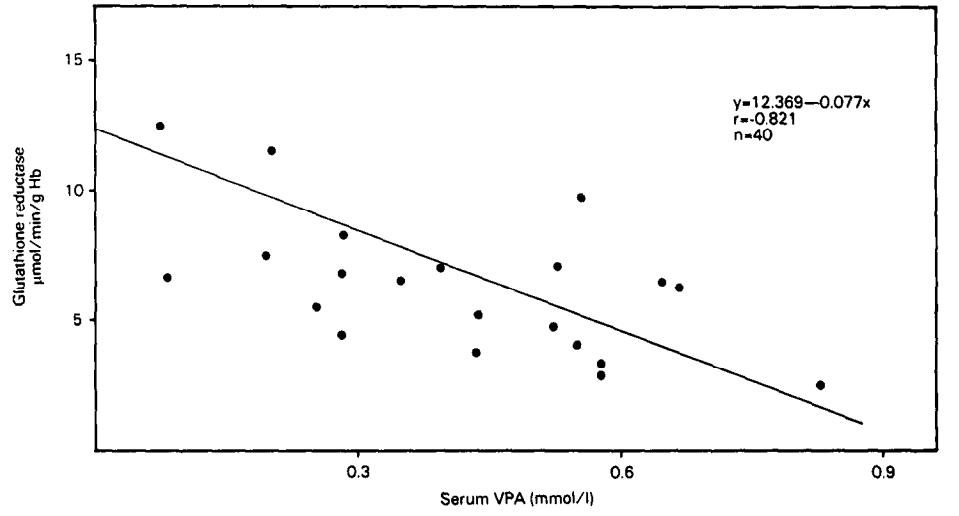

Fig. 5. Relationships between red blood cell GR activity and the concentration of serum VPA in children on the drug.

Table 2. Red blood cell GR activity in children and adults treated with VPA

Group	Age (years)	Dose (mg/day)	Plasma VPA (mmol/L)	GR activity (μmol/min/gHb)
Children on VPA (N = 21)	4–16	200–800	0.42 ± 0.2	6.5 ± 2.6*
Children (control) (N = 19)	5–15	—	—	12.9 ± 2.0
Adults on VPA (N = 9)	25–78	400–1500	0.44 ± 0.21	14.1 ± 3.8
Adults (control) (N = 10)	23–65	—	—	13.4 ± 3.6

Results are mean ± SD.
* $P < 0.01$.

Our findings raise questions about possible physiological implications in young patients being treated with VPA. GR maintains appropriate cellular levels of GSH and the VPA-mediated suppression of the enzyme may limit its availability for reaction with free radicals and peroxides. The metabolism of VPA generates an increased body burden of free radicals [8, 9]. Failure to remove this highly reactive species before they can inflict damage on various cellular constituents may be expressed by an increased susceptibility to the drug. Indeed, young children have been shown to be at a higher risk to develop undesired effects upon treatment with VPA.

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