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# No evidence for inhibition of human glutathione reductase by valproic acid

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**Abstract**—The human red blood cell enzyme glutathione reductase (GR) was reported to be inhibited by the anticonvulsant drug valproic acid (VPA) [Cotariu *et al.*, *Biochem Pharmacol* **43**: 425–429, 1992]. When attempting to reproduce and extend these experiments, we could not detect any significant effect of VPA on glutathione reductase in haemolysates from 20 healthy children and 10 children under VPA therapy, no matter which concentration of the drug (0.9 or 1.8 mM in a haemolysate diluted by a factor of 50 or 1.8 mM directly in the assay), which incubation time (0–60 min) and which assay system were chosen. An influence of VPA on FAD-free apoglutathione reductase was also excluded. GR-activities of 10 children under VPA therapy (1.08 ± 0.14 U/mL blood or 7.57 ± 0.94 U/g Hb) were almost identical with the activities of age- and sex-matched controls (1.04 ± 0.17 U/mL or 7.79 ± 1.32 U/g Hb). No correlation between erythrocyte GR activity and serum levels of VPA was observed. Finally, incubation of crystalline human GR with VPA did not lead to enzyme inhibition; rather, in most experiments the enzyme was stabilized by incubation with VPA. Possible explanations for the discrepancies between the results of Cotariu *et al.* and our data are discussed.

*Key words:* epilepsy; glutathione reductase; valproic acid

The primary function of the human flavoenzyme GR* (EC 1.6.4.2; NADPH + GSSG + $H^+ \rightarrow NADP^+ + 2GSH$) is the maintenance of a reducing intracellular milieu by keeping the antioxidant glutathione to more than 99% in its reduced form [1]. We are interested in specific GR inhibitors that are effective at low concentrations, e.g. by interfering with the folding process of the homodimeric enzyme [2], or by converting it into an oxidase [3]. Such compounds lead to increased oxidative stress which is fatal for intracellular parasites [3, 4]. Recently, Cotariu *et al.* [5] described an inhibitory effect of the anticonvulsant drug VPA on red blood cell GR. By incubating diluted haemolysates of children with VPA the authors observed an inhibition of GR which they reported to be uncompetitive, dose-dependent, and maximal (≤80%) after an incubation period of 40 min; this inhibition could be reversed by dialysis [5].

Since the branched fatty acid VPA (2-propylpentanoic acid) is a drug widely employed in clinical paediatrics, we tried to verify the described findings by different approaches: we studied the haemolysate of healthy and VPA-treated children using the procedures of Cotariu *et al.* as well as other methods. In addition, the effect of the drug on isolated GR from erythrocytes was tested.

## *Patients and Methods*

*Patients.* Effects of VPA on GR *in vitro* were determined in haemolysates from 20 healthy children (aged 3 months to 14 years, median: 8 years) and from 10 children (aged 7 to 14 years, median 12 years) under therapy with sodium valproate (600–1200 mg/day) [6].

*Materials.* All reagents, obtained from Baker (Deventer, The Netherlands), Biomol (Hamburg, F.R.G.) and Fluka (Buchs, Switzerland) were of the highest available purity. Digitonin, GSSG, FAD and BSA were purchased from Serva (Heidelberg, F.R.G.). VPA was obtained from Pharma Wernigerode (F.R.G.) and Merck/Schuchardt (Hohenbrunn, F.R.G.).

*Solutions.* pH 6.9-Assay buffer (50 mM potassium phosphate + 200 mM potassium chloride + 1 mM EDTA, pH 6.9). In experiments on whole blood, 40 mg/L digitonin was added as haemolysing agent to this buffer [7–9]. pH 8.0-Assay buffer as used in Ref. 5 (50 mM Tris–HCl + 0.25 mM EDTA, pH 8.0). Stabilizing solution was prepared as described in Ref. 5 (2.7 mM EDTA + 0.7 mM β-mercaptoethanol, pH 7.0). NADPH (4 mM), GSSG (20 mM), and FAD (1 mM) were dissolved in the corresponding assay buffer. VPA (90 mM) was freshly made up and diluted in the buffer used in the subsequent assay. Serum levels of VPA were determined as described previously [10].

*Methods for measuring GR in haemolysates.* We followed two experimental protocols: Procedure 1: according to the preparation described by Cotariu *et al.* [5], erythrocytes were washed twice in 0.9% NaCl and resuspended in 4 vol. of stabilizing solution. After cell lysis by three times freezing and thawing the haemolysate was diluted 20-fold with the stabilizing solution. Before and 40 min after incubation with VPA, 20 μL haemolysate were transferred into a 1 mL cuvette, and GR activity was measured at 37° in the pH 8.0-assay buffer with 0.35 mM NADPH and 2.3 mM GSSG as substrate concentrations [5].

Procedure 2: following our established method for determining GR-activity [7–9], 100 μL blood were mixed with 4.9 mL pH 6.9-assay buffer containing digitonin for haemolysis. We directly incubated this haemolysate (980 μL) with VPA (0, 10 or 20 μL of a 90 mM VPA stock solution plus 20, 10 or 0 μL buffer) in order to maintain the dilution chosen in Ref. 5. The incubations were carried out in closed plastic vials at 37° in a water bath. Samples of 100 μL were drawn at 0, 20, 40 and 60 min. GR activity was subsequently assayed at 25° in a total volume of 1 mL containing pH 6.9-assay buffer, 100 μM NADPH and 1 mM GSSG with which the reaction was started [7–9]. The activities were calculated from the change in absorbance due to the oxidation of NADPH, the range being $0.01 \leq \Delta A_{340nm}/\text{min} \leq 0.07$.

*Methods for preparing and measuring crystalline GR.* GR from erythrocytes was prepared and assayed as described by Krohne-Ehrich *et al.* [8]. The purified enzyme had a specific activity of 225 U/mg and was stored as a pellet in 90% ammonium sulfate. An aliquot of the GR pellet was dissolved in pH 6.9-assay buffer and dialysed exhaustively against this buffer. The retentate was diluted

* Abbreviations: GR, glutathione reductase; GSSG, glutathione disulfide; GSH, reduced glutathione; Hb, haemoglobin; VPA, valproic acid.

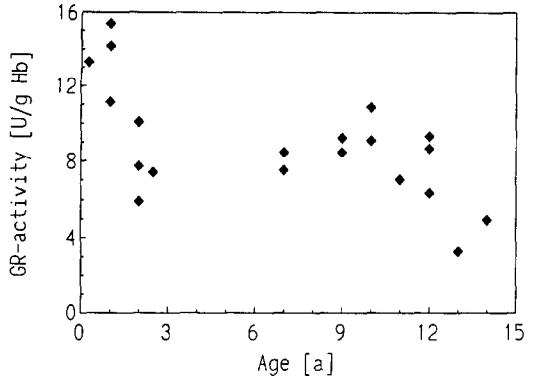


Fig. 1. Correlation of GR activity (U/g Hb) with age in 20 healthy children aged 3 months to 14 years.

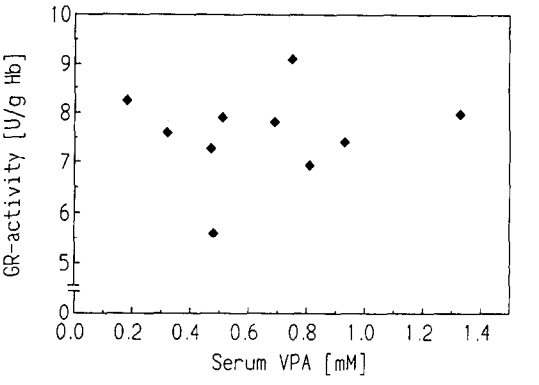


Fig. 2. VPA serum concentrations (mM) of 10 children under VPA therapy in correlation with the activity of glutathione reductase (U/g Hb). No correlation is detectable.

with assay buffer to give a GR stock solution of 9 $\mu$M (approx. 100 U/mL).

*Incubation of the purified enzyme with VPA.* Crystalline GR (2 nM) was incubated with 2.3 mM VPA in the presence of 3 $\mu$M BSA as a stabilizer of the diluted enzyme. Alternatively, we used 200 nM GR, which is stable for more than 24 hr, and varied the VPA concentration between 2.3 $\mu$M and 230 mM. These conditions also exclude possible interactions between VPA, being a branched fatty acid, and BSA. In control mixtures, VPA was replaced by an equal volume of buffer. Incubations were carried out at 37° for 40 min, which is the time reported to result in maximal inhibition [5]. At $t = 0$ min and $t = 40$ min, samples of a suitable volume were drawn and their activities were measured in the pH 6.9-assay system. Each experiment was reproduced twice.

*Results and Discussion*

When attempting to reproduce the glutathione reductase assay conditions of Ref. 5, we realized that the total absorption at 340 nm was above 2.5 and that the expected absorption changes ranged between 0.001 and 0.005 $min^{-1}$. Moreover, the GSSG concentration of 2.3 mM in this assay causes substrate inhibition of GR [1, 7]. These conditions, implying a low signal to noise ratio, are unfavourable for measuring enzyme activity and enzyme inhibition. Consequently, we started our search for an inhibitory effect of VPA on glutathione reductase using the established assay of Worthington and Rosemeyer (see Ref. 7 and Procedure 2 in Patients and Methods).

*Influence of VPA on GR from haemolysates.* GR activities in the blood of 10 children under treatment with sodium valproate were compared with 10 age- and sex-matched controls. Almost identical values were observed for the activities in full blood (VPA group: 1.08 ± 0.14 U/mL blood corresponding to 7.57 ± 0.94 U/g Hb; controls: 1.04 ± 0.17 U/mL blood and 7.79 ± 1.32 U/g Hb, respectively). As a fringe benefit of these measurements, the average GR activity at 25° in the blood from children between 3 months and 14 years of age was determined: taking all data together (N = 20) we found a value of 1.12 ± 0.30 U/mL blood and 8.18 ± 2.47 U/g Hb, respectively. This agrees well with the results described for the controls by Cotariu *et al.* [5] if one takes into account that their assays were carried out at 37°. As shown in Fig. 1, infants (≤1 year) appear to have higher GR activities (U/g Hb) than older children, which can at least partially be explained by higher Hb values of the latter.

No correlation between GR activity (in U/g Hb or in U/mL blood) and serum levels of VPA, which covered the therapeutic range of 0.15–1.00 mM (20–140 $\mu$g/mL serum) [6], was observed (Fig. 2). These results contrast with the data of Cotariu *et al.* [5] who detected significantly reduced GR activity in children receiving VPA therapy (6.5 ± 2.6 U/g Hb vs 12.9 ± 2.0 U/g Hb in controls) and a significant correlation of GR activity (U/g Hb) with serum VPA ($r = -0.82$).

Incubation of the haemolysates from 20 controls and 10 patients under valproate therapy with 0.9 or 1.8 mM VPA for up to 60 min did not result in any significant inhibition of the enzyme (Table 1). Rather, a slight activation was observed for long incubations and high VPA concentrations. An inhibition of GR activity reaching a maximum of 80% after 40 min incubation with 1.8 mM VPA, as described in Ref. 5, was not observed.

In another set of experiments using blood from 10 healthy children, VPA (final concentration 1.8 mM) was added to 1 mL assay mixture which contained 100 $\mu$L haemolysate representing 2 $\mu$L blood. The samples were incubated for 40 min and assayed. Compared to the initial control activity which differed not significantly from the VPA-containing sample, the latter showed 103.4 ± 8.8% after the incubation period, the corresponding value of the VPA-free controls being 98.8 ± 4.2%. It should be emphasized that the VPA concentration was as high as 1.8 mM also in the course of the enzyme assay. This exceeds the maximal therapeutic plasma concentration by a factor of 2 [6].

In red blood cells, glutathione reductase is not fully saturated with FAD [9]. An effect of VPA on the FAD-free apoenzyme was tested by incubating haemolysates from five healthy children with VPA for 60 min. The proportion of apoglutathione reductase was measured according to Ref. 9 and is given as percentage of total glutathione reductase (total GR = holo GR + apo GR). At $t = 0$ min, the VPA-treated haemolysate contained on average 22.7% and the control haemolysates 17.3% apoenzyme. At $t = 60$ min the corresponding values were 8.2% and 2.8%. This suggests that VPA has a protecting effect on the labile apoenzyme *in vitro*.

Finally, the following parameters were systematically exchanged between procedure 1 [5] and procedure 2: mode of haemolysis, incubation conditions with prolonged incubation times (up to 26 hr), assay buffer and assay temperature. Under no condition an inhibition of glutathione reductase by VPA was observed (data not shown).

Table 1. Influence of incubation with VPA on holoGR activities in blood of 20 healthy children (a) and of 10 children under VPA therapy (b)

| Incubation | Time (min) | | | |
|---|---|---|---|---|
| | 0 | 20 | 40 | 60 |
| (a) | | | | |
| No VPA | 100 | 101.3 ± 6.1 | 100.1 ± 4.8 | 102.0 ± 7.4 |
| VPA 0.9 mM | 98.5 ± 2.0 | 99.6 ± 5.8 | 98.8 ± 6.6 | 101.4 ± 4.2 |
| VPA 1.8 mM | 99.7 ± 6.5 | 102.4 ± 4.9 | 99.3 ± 6.4 | 104.7 ± 8.9 |
| (b) | | | | |
| No VPA | 100 | 98.8 ± 4.2 | 102.1 ± 5.4 | 99.1 ± 3.9 |
| VPA 1.8 mM | 101.6 ± 5.3 | 97.4 ± 5.0 | 97.2 ± 3.7 | 99.0 ± 6.2 |

All values are given as percentage of the corresponding control at zero time. Diluted (50-fold) haemolysate (D = 50) was incubated with 0.9 or 1.8 mM VPA for up to 60 min at 37°; subsequently 100 μL aliquots were assayed in 1 mL reaction mixture.

Table 2. Influence of VPA on purified human erythrocyte holoGR after an incubation period of 40 min

| GR (nM) | Concentrations in the incubation mixture VPA | | | | | |
|---|---|---|---|---|---|---|
| | 2.3 μM | 23 μM | 230 μM | 2.3 mM | 23 mM | 230 mM |
| 2 | | | | 100* | | |
| | | | | 101† | | |
| 200 | 118 | 107 | 105 | 101 | 101 | 108 |
| | 107‡ | 104‡ | 105‡ | 110‡ | 100‡ | |

Values are given in per cent of controls. All values were reproducible within a range of 5% error.

* Incubation in the presence of 3 μM GR-stabilizing BSA.

† Preincubation for 30 min with 100 μM NADPH in the presence of BSA.

‡ Coincubation with 100 μM NADPH.

It cannot be excluded that decomposition products of VPA might act as the actual inhibitors. This hypothesis was studied by using VPA after preincubation in water adjusted with acetic acid to pH 4.0 or in 1 mM EDTA (pH 7.0) at 37° for 2 to 20 hr. However, measurements with different concentrations of the preincubated VPA showed no inhibition either.

Finally, there was no difference between VPA for clinical use (from Pharma Wernigerode) and VPA for experimental purposes (from Merck/Schuchardt).

*Influence of VPA on crystalline GR.* The results obtained by the incubation of highly purified GR (2 nM with 3 μM BSA or 200 nM without BSA) with VPA yielded no significant inhibition. Rather, in 90% of the experiments VPA led to a stabilization of the enzyme (Table 2). When 2 nM GR was incubated with 2.3 mM VPA in the absence of BSA, this mixture lost 10% less activity in 40 min than the VPA-free control. A concentration of 2 nM was chosen in order to simulate the incubation experiments with haemolysates [5], the concentration of GR subunits in erythrocytes being approx. 200 nM [3].

According to Ref. 5, the inhibition by VPA was not influenced by preincubation with NADPH. As NADPH alters the redox state of GR and could modulate inhibitor action [3] we pre- and coincubated the enzyme with 100 μM NADPH—without any effect of VPA subsequently occurring (Table 2). We also excluded the possible influence of plastic vials on the postulated inhibitory effect of VPA by testing parallel incubation mixtures (with isolated enzyme or blood) in glass tubes.

*Conclusions.* Our results indicate that VPA and its degradation products are not inhibitors of human glutathione reductase. It is possible that the effects observed by Cotariu *et al.* [5] are due to the particular assay conditions chosen. Another explanation would imply the presence of a compound contaminating their VPA and acting as an inhibitor of GR. The occurrence of a VPA-sensitive glutathione reductase in their patients and controls is less likely since gene polymorphism of this enzyme is rare [3].

The paper on VPA [5] is reminiscent of a report on 2,4-dihydroxybenzylamine as a specific inhibitor of glutathione reductase [11]. Also in the latter case the inhibitory effects were not reproducible in our laboratory (Schönleben and Kirsch, personal communication). Apart from our interest in glutathione reductase, we feel obliged to repeat studies on promising inhibitors since biochemists and pharmacologists should produce results on which clinicians and cell biologists can rely.

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