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SHORT COMMUNICATIONS

Increased microsomal irreversible binding of phenytoin by valproic acid

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Valproic acid (VPA) and valpromide are antiepileptic drugs now known as a new class of epoxide hydrolase inhibitors [1]. The clinical significance of the inhibition of epoxide hydrolase is best demonstrated in the interaction of valpromide and valproic acid with carbamazepine and its active metabolite, carbamazepine-10,11-epoxide [2–5].

Valproic acid is commonly used together with other antiepileptic drugs, especially phenytoin. Polytherapy with valproic acid has been reported as a major contributing factor in fatal hepatic toxicity [6]. Induction of valproic acid metabolism by other antiepileptic drugs which increases the reactive metabolite, 4-ene-valproate, has been reported [7].

Phenytoin is known to be metabolized to form *p*-hydroxyphenyl-phenyl-hydantoin in humans and in animals. An arene oxide intermediate was proposed [8]. Phenytoin was found to bind to macromolecules irreversibly in microsomal preparations, possibly mediated by the reactive arene oxide intermediate [9, 10]. The arene oxide intermediate was considered toxic and to be responsible for phenytoin teratogenicity [11, 12], cytotoxicity [13], hepatotoxicity [14] and other toxic effects [15]. It is possible that valproic acid may increase phenytoin toxicity by inhibiting epoxide hydrolase and increasing the arene oxide intermediate. To test this hypothesis, we measured the effect of valproic acid on the irreversible binding of phenytoin to microsomal proteins.

Materials and Methods

Chemicals and animals. [^{14}C]Diphenylhydantoin (sp. act. 55.8 mCi/mmol) and tissue solubilizing reagent (Protosol) were purchased from New England Nuclear (Boston, MA). Phenytoin, valproic acid, and 1,2-epoxy-3,3-trichloropropene (TCPO) were from the Sigma Chemical Co. (St. Louis, MO). NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and scintillation fluid were from E. Merck (Darmstadt, Germany). Glutathione (reduced form, GSH) was from Boehringer Mannheim GmbH (Mannheim, Germany). Untreated male Sprague–Dawley rats bred in the Animal Center of National Cheng Kung University were used in the study. They were maintained on a standard diet of Purina laboratory chow and water *ad lib.*

Preparation of microsomes. Rats (200–300 g) were killed by decapitation and the livers were excised, pooled and washed with 1.15% KCl. The livers were minced and homogenized at 0° in 4 vol. of cold 1.15% KCl solution using a Potter–Elvehjem homogenizer with a Teflon pestle. Microsomes were prepared by standard methods. The protein concentration was determined by the method of Lowry *et al.* [16] with bovine serum albumin as the standard. Cytochrome P450 content was measured as described by Omura and Sato [17].

Irreversible binding of phenytoin to microsomal proteins. A method of measuring the irreversible binding of phenytoin developed by Pantarotto *et al.* [9] was adapted. In brief, the incubation mixture in a final volume of 2 mL contained an NADPH-regenerating system, 0.05 M phosphate buffer (pH 7.4), 5 mM magnesium chloride, 0.15 M potassium chloride and 10 mg of microsomal protein. Valproic acid (0.7 or 7 mM), TCPO (0.6 mM), or GSH (1 mM) was added to make a final concentration as listed. After a 5-min preincubation at 37°, the reaction was started by the addition of 10 μL of an ethanol solution of labeled phenytoin to obtain a final substrate concentration of 0.1 mM. The labeled phenytoin was diluted with the cold drug to obtain a specific activity of 2 mCi/mmol. Incubation was at 37° for 2 hr. The reaction was stopped by the addition of 5 mL of ethanol. The samples were centrifuged, the supernatant was discarded, and the precipitate was extracted three times with 5 mL of the following solvent: 70% ethanol, three times with ethanol, boiling ethanol, benzene/ethanol (1:4, v/v), twice with acetone/chloroform (4:1), acetone/ethanol (5:1), diethyl ether/ethanol (5:1), twice with ethyl acetate/ethanol (5:1), ethanol, and ethanol/water (2:3). All the extraction supernatants were tested for radioactivity. No radioactivity could be removed after this exhaustive extraction procedure. The precipitate was dissolved in 0.5 mL of Protosol and transferred into a counting vial containing 10 mL of scintillation fluid. Radioactivity was measured in a liquid scintillation counter (model LS 5000TA; Beckman Instrument Co., Fullerton, CA). The values obtained were corrected for quenching by the external standardization method.

Results and Discussion

Valproic acid increased the irreversible binding of phenytoin to microsomal proteins. The phenomenon was similar to the effect of TCPO (Table 1). Phenytoin forms

Table 1. Effects of inhibitors of epoxide hydrolase on the irreversible protein binding of [^{14}C]phenytoin in rat liver microsomal preparation*

	Phenytoin binding (nmol/10 mg protein)		
	−NADPH	+NADPH	+NADPH + GSH
Control	0.13 ± 0.011	0.76 ± 0.014	0.41 ± 0.002
0.7 mM Valproate	0.12 ± 0.004	0.88 ± 0.017†	0.36 ± 0.021
7 mM Valproate	0.10 ± 0.002	1.03 ± 0.022†	0.35 ± 0.011
0.6 mM TCPO	0.11 ± 0.003	1.10 ± 0.062†	0.46 ± 0.044

* Values are means ± SEM, N = 3.
† $P < 0.05$ in comparison to control.

the major metabolite *p*-hydroxyphenyl-phenyl-hydantoin via an arene oxide intermediate. The arene oxide is highly electrophilic and may react with microsomal proteins to form irreversible adducts [9, 10]. The fact that valproic acid increased the irreversible binding and that the effect could be reversed by adding 1 mM glutathione suggests an increase of arene oxide by valproic acid. The result is consistent with our hypothesis that valproic acid may increase phenytoin toxicity by inhibiting the epoxide hydrolase.

At a therapeutic range of valproic acid (0.4 to 0.7 mM), irreversible binding of phenytoin to microsomal proteins increased significantly, but not to a great extent. At a high concentration (7 mM), the effect of valproic acid was comparable to that of TCPO (0.6 mM), a well known epoxide hydrolase inhibitor. In the clinical situation, the combined use of valproic acid and phenytoin does not necessarily increase the irreversible binding of phenytoin to hepatic proteins because of the presence of glutathione *in vivo*. This study, however, serves as a model to support the possibility of valproic acid increasing phenytoin toxicity by increasing the toxic arene oxide.

In a previous study of the hepatotoxicity of valproic acid, it was indicated that polytherapy is one of the major factors other than age [6]. In the study, most subjects were given phenytoin or phenobarbital. Both drugs are metabolized by aromatic ring hydroxylation. In addition to the mechanism of enzyme induction by phenobarbital or phenytoin to form 4-ene-valproate [7], it is also possible that the hepatotoxicity is due to the increase of arene oxide intermediates in the phenytoin or phenobarbital metabolism.

The clinical significance of the above-mentioned interaction mechanism needs to be investigated further. In the clinical situation, both valproic acid and phenytoin are given to the steady state and the metabolism of valproic acid is induced. The effect of phenytoin induction on the formation of fifteen valproic acid metabolites has been reported [7]. Various metabolites were increased or decreased by induction. Some of these valproic acid metabolites may be hepatotoxic by themselves. The interaction is complicated if some of metabolites are inhibitory to the epoxide hydrolase, which will increase hepatotoxicity indirectly.

In summary, phenytoin is known to be metabolized to form *p*-hydroxyphenyl-phenyl-hydantoin, possibly via the arene oxide intermediate. The reactive arene oxide may bind to macromolecules irreversibly and cause toxicity. In this study, valproic acid was found to increase the irreversible binding of phenytoin to rat liver microsomes at high concentrations. The effect was similar to that of TCPO and could be reversed by adding glutathione. Valproic acid may increase the hepatotoxicity of phenytoin.

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