Binding of a valproate metabolite to the trifunctional protein of fatty acid oxidation

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Abstract The anti-convulsant drug valproate causes hepatic failure in a small percentage of patients. We now report that the valproate metabolite 2,4-dien-valproate binds (IC_{50} = 42 μM) to the α-subunit of the trifunctional protein responsible for the second and third steps in the mitochondrial β-oxidation of fatty acids. Binding of valproate itself, or of the metabolites 2-en-valproate, 4-en-valproate or 3-hydroxy-4-en-valproate, is considerably weaker. We conclude that valproate-induced hepatotoxicity may be due in part to the reversible binding of the valproate metabolite 2,4-dien-valproate or its CoA ester to the α-subunit of the trifunctional protein with consequent inhibition of fatty acid oxidation.

Key words: Fatty acid oxidation; Gastrin-binding protein; Trifunctional protein; Valproate

1. Introduction

Valproate (2-*n*-propylpentanoate; Fig. 1) has been used for 30 years for the treatment of epilepsy. Hepatotoxicity, with initial clinical symptoms of nausea, vomiting and lethargy [1], is a rare side effect, and animal studies have implicated 4-en-valproate (2-*n*-propyl-4-pentenoate) or a derivative as the toxic metabolite [2]. The incidence of hepatotoxicity is higher in young children undergoing polytherapy [3], presumably because the second anti-convulsant (frequently phenytoin or phenobarbital) induces cytochrome P450 which in turn catalyses the formation of 4-en-valproate [4]. Toxic derivatives of 4-en-valproyl-CoA may then be generated by formation of the CoA derivative and subsequent β-oxidation within mitochondria [5].

The mitochondrial trifunctional protein (TP) catalyzes three consecutive reactions in the oxidation of long chain fatty acids. The 78 kDa α-subunit catalyses the hydration of enoyl-CoA and the dehydrogenation of 3-hydroxyacyl-CoA, and the 48–51 kDa β-subunit catalyses the cleavage of acetyl-CoA from the resultant 3-ketoacyl-CoA [6]. Binding of the peptide hormone gastrin to the α-subunit of the TP, with an IC_{50} value of 200 nM, has been demonstrated by covalent cross-linking [7,8]. Gastrin occupies both the hydratase and dehydrogenase active sites of TPα, since substrates of the enoyl-CoA hydratase activity, and products of the 3-hydroxyacyl-CoA dehydrogenase activity, both compete for gastrin binding [8]. Hence the gastrin cross-linking assay provides a convenient and sensitive assay for active site-directed inhibitors of TPα [7,8].

TPα may be the target for the anti-proliferative effects of gastrin/cholecystokinin (CCK) receptor antagonists on colon cancer cells [8]. Thus, proglumide and benzotript inhibited covalent cross-linking of iodinated gastrin to TPα, and the observed IC_{50} values correlated well with the IC_{50} values for inhibition of proliferation of several human colon carcinoma cell lines [8]. Presumably inhibition of one or both enzyme activities intrinsic to TPα by the gastrin/CCK receptor antagonists proglumide or benzotript leads to a blockade of fatty acid oxidation. The resultant reduction in energy supply might then inhibit cell proliferation.

Hereditary defects in the TP can cause death in early childhood [9–11]. A wide range of diagnostic symptoms have been reported, including vomiting, hypoketotic hypoglycemic coma, myopathy and cardiomyopathy. Pathological examination reveals widespread fat deposition in the liver, heart and kidneys. Hypothetically, blockade of fatty acid oxidation by the TP would reduce the mitochondrial output of acetyl-CoA, and hence result in hypoketotic hypoglycemia. The similarity in symptoms between patients with valproate-induced hepatotoxicity and with mutations in the gene encoding TPα suggested the hypothesis that valproate derivatives might also target TPα. We have tested the hypothesis by measuring the effect of valproate, and the valproate derivatives, 2-en-valproate, 4-en-valproate, 2,4-dien-valproate, and 3-hydroxy-4-en-valproate (Fig. 1), on the binding of gastrin to TPα. We report here that 2,4-dien-valproate binds to TPα with an affinity 50-fold greater than any other valproate derivative.

2. Materials and methods

2.1. Synthesis of valproate derivatives

Valproic acid was obtained from Sigma (St. Louis, MO). 2-en-valproic acid was obtained from Desitin (Hamburg, Germany). 4-en-valproate, 2,4-dien-valproate, and 3-hydroxy-4-en-valproate were synthesized as described previously [12].

2.2. Gastrin cross-linking assay

The TP was partially purified from detergent extracts of porcine gastric mucosal membranes by lectin and ion-exchange chromatography [7,13]. The following additional protease inhibitors were included in all buffers to prevent proteolysis: pepstatin, 1 μM; benzamidine, 1 mM; hexamethylphosphoramide, 0.1% (w/v); aprotinin, 500 U/ml. Crosslinking of ^{125}I-Nle^{15}-gastrin$_{2-17}$ to TPα with disuccinimidylsuberate was measured as described previously [7,8] in the presence of increasing concentrations of valproate or valproate metabolites. The products of the cross-linking reaction were separated by polyacrylamide gel electrophoresis, and radioactivity associated with TPα was detected and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Initial estimates of IC_{50} values, and of the levels of ^{125}I-gastrin$_{2-17}$ bound in the absence of competitor, were

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Abbreviations: CCK, cholecystokinin; TP, trifunctional protein

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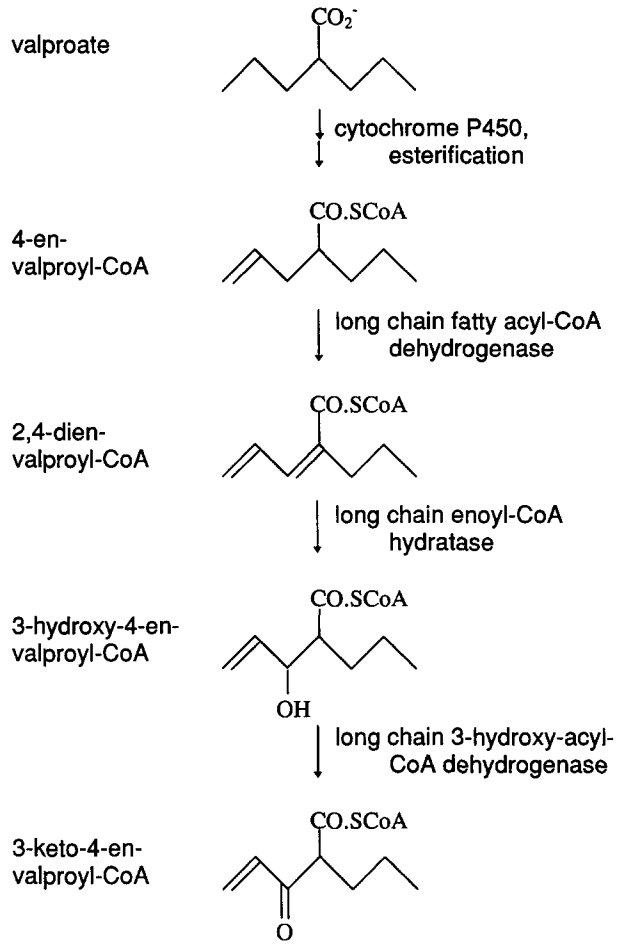

Fig. 1. Structures of valproate metabolites. Enzymes likely to be involved in the formation and further metabolism of the inhibitory valproate derivative 2,4-dien-valproyl CoA are shown. 3-Keto-4-en-valproyl-CoA is probably unstable.

obtained by fitting the data with the program EBDA, and were refined with the program LIGAND as described previously [14].

3. Results and discussion

The valproate derivative 2,4-dien-valproate inhibits the binding of iodinated gastrin to TPα (Fig. 2). The IC_{50} of 42 ± 3 μM compares favourably with the value observed for the gastrin/CCK receptor antagonist benzotript (200 μM [7]). Inhibition by valproate and other valproate derivatives was considerably weaker (Table 1). The IC_{50} values measured for 3-hydroxy-4-en-valproate and valproate itself were 2.3 and 16 mM, respectively. Although IC_{50} values could not be measured accurately for the valproate derivatives 2-en-valproate and 4-en-valproate because inhibition did not reach 50% at a concentration of 10 mM, estimates of 21 and 28 mM, respectively, were obtained. Since the natural substrates of TPα are CoA esters of long chain fatty acid derivatives, the CoA esters of all of the above compounds would presumably bind more tightly than the carboxylates.

There is no evidence at present that valproate or its metabolites bind covalently to TPα. For example, preincubation of TPα with valproate metabolites for 2 h at either 4 or 25°C does not result in a major reduction in levels of subsequent labelling with iodinated gastrin (Fig. 3). The absence of labelling is not surprising when it is considered that reaction between 2,4-dien-valproate and glutathione is slow, but that reaction between the methyl ester of 2,4-dien-valproate and glutathione is fast [12]. By analogy activation of 2,4-dien-valproate by formation of its CoA ester may be necessary for covalent labelling of TPα to occur.

The possibility was also considered that 3-hydroxy-4-en-valproate might act as a suicide substrate for TPα. An unidentified membrane-bound mitochondrial dehydrogenase resembling the TP has been shown to catalyse the NAD^+-dependent conversion of 3-hydroxy-valproyl-CoA to 3-keto-valproyl-CoA [5]. If the TP was also able to catalyse the corresponding reaction for 3-hydroxy-4-en-valproate, then the instability of the 3-keto-4-en-valproate formed might result in the permanent modification and hence inactivation of the enzyme. However addition of NAD^+ to mixtures of TPα and 3-hydroxy-4-en-valproate did not result in any further

Table 1
Binding of valproate metabolites to the trifunctional protein α-subunit

Compound	IC_{50} (μM)
Valproate	16 000 ± 11 000
2-en-valproate	21 000 ± 11 000
4-en-valproate	28 000 ± 9 000
2,4-dien-valproate	42 ± 3
3-Hydroxy-4-en-valproate	2 300 ± 1 800

Binding of valproate metabolites to the α-subunit of the trifunctional protein was measured by competition for binding of ^{125}I-[Nle^{15}]-gastrin$_{2-17}$ as described in the legend to Fig. 2. IC_{50} values (mean ± S.D. of at least two experiments) were obtained by computer fitting as described previously [14].

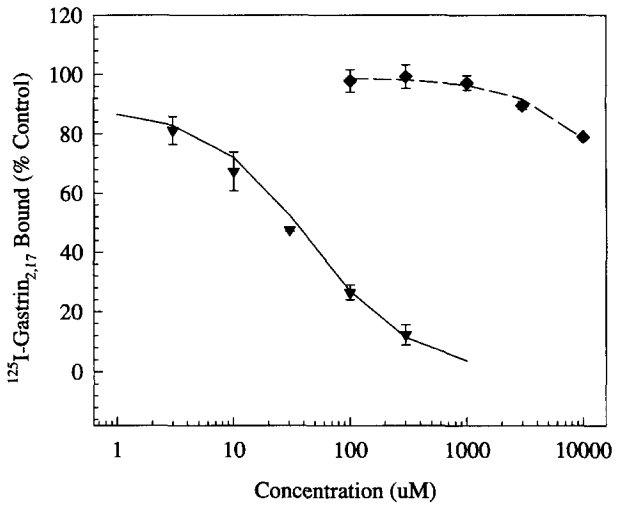

Fig. 2. Binding of valproate metabolites to the trifunctional protein α-subunit. Cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2-17}$ to TPα was measured as described in section 2 in the presence of increasing concentrations of valproate metabolites. Duplicate samples were subjected to electrophoresis on $NaDodSO_4$-polyacrylamide gels and the radioactivity associated with TPα was quantitated by phosphorimager scanning, and expressed as a percentage of the value obtained in the absence of competitor. The following values for IC_{50} and for the predicted ordinate intercept were obtained by computer fitting as described previously [14], and used to construct the indicated lines of best fit: (♦) 4-en-valproate (38 mM, 99.0%); (▼) 2,4-dien-valproate (43.9 μM, 88.6%). These values were averaged with the results of at least one other similar experiment to obtain the mean values presented in the text.

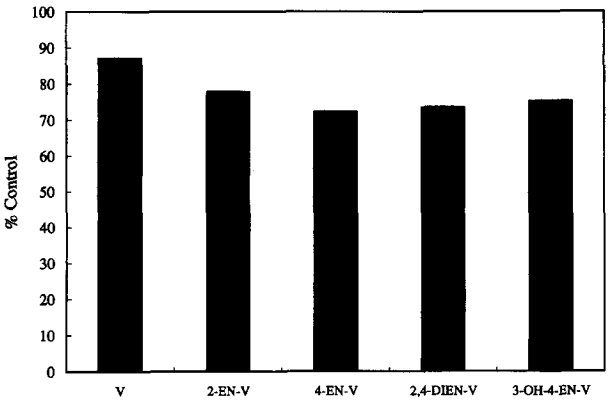

Fig. 3. Valproate metabolites do not bind irreversibly to the trifunctional protein α-subunit. Samples of TPα were preincubated with or without valproate metabolites in 50 mM HEPES, 0.1% Triton X-100, pH 7.6, for 2 h at 4°C. Cross-linking of ^{125}I-[Nle^{15}]-gastrin$_{2-17}$ to TPα was then measured at 4°C as described in section 2, and expressed as a percentage of the value obtained in the absence of valproate metabolites. The concentrations of valproate (V), 2-en-valproate (2-EN-V), 4-en-valproate (4-EN-V), or 3-hydroxy-4-en-valproate (3-OH-4-EN-VAL) were 1 mM, and of 2,4-dien-valproate (2,4-DIEN-V) 10 μM. Similar results were obtained when the preincubation was at 25°C.

reduction in levels of subsequent labelling with iodinated gastrin (data not shown).

Does 2,4-dien-valproate inhibit either the long chain enoyl-CoA hydratase or the long chain 3-hydroxyacyl-CoA dehydrogenase activity intrinsic to TPα? It has generally not been possible to compare the IC_{50} values for inhibitors of gastrin binding to TPα with the IC_{50} values for the same compounds as inhibitors of the enzyme activities of TPα, since the compounds often absorb strongly in the wavelength range used for the spectrophotometric assays. However, benzotript inhibits both the enoyl-CoA hydratase and the 3-hydroxyacyl-CoA dehydrogenase activities of the TP (Hashimoto and Baldwin, unpublished data). It therefore seems likely that the valproate derivative 2,4-dien-valproate will also inhibit both hydratase and dehydrogenase activities.

The following hypothetical mechanism (Fig. 1) is therefore proposed for valproate-induced hepatotoxicity. Valproate is oxidized to 4-en-valproate by cytochrome P450, the levels of which may have been elevated by simultaneous treatment with a second enzyme-inducing anti-convulsant. 4-en-valproate is then converted to its CoA derivative, and oxidized to 2,4-dien-valproyl-CoA by long chain fatty acyl-CoA dehydrogenase. 2,4-dien-valproyl-CoA binds reversibly to TPα, and competitively inhibits one or both of the long chain enoyl-CoA hydratase and the long chain 3-hydroxyacyl-CoA dehydrogenase activity intrinsic to TPα. The possibility that covalent labelling of TPα by 2,4-dien-valproyl-CoA might lead to irreversible inactivation of the enzyme remains to be investigated. In either case blockade of TPα presumably leads to the inhibition of mitochondrial long chain fatty acid oxidation observed by Kesterson and coworkers [2], with resultant hypoketotic hypoglycemia.

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