EFFECT OF $\alpha$-FLUORINATION OF VALPROIC ACID ON VALPROYL-S-ACYL-COA FORMATION IN VIVO IN RATS

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ABSTRACT:
Studies designed to compare valproic acid (VPA) with its $\alpha$-fluorinated derivative (F-VPA) for their abilities to form acyl-CoA thioester derivatives in vivo are described. Recent studies have shown that $\alpha$-fluorination of a hepatotoxic metabolite of VPA ($\Delta^4$-VPA) resulted in a nonhepatotoxic derivative. We hypothesize that the decrease in hepatotoxicity may be related to a lack of formation of the intermediary acyl-CoA thioester. To determine the effect of $\alpha$-fluoro substitution on acyl-CoA formation, we synthesized F-VPA and compared it with VPA for its ability to form the acyl-CoA thioester derivative in vivo in rat liver. Thus, after dosing rats with VPA or F-VPA, animals were sacrificed (0.05-, 0.5-, 1-, 2-, and 5-h postadministration) for the analysis of liver tissue. High-performance liquid chromatography (HPLC) and electrospray ionization/tandem mass spectrometry analysis of liver extracts from VPA-dosed rats showed the presence of VPA-CoA that was maximal after 0.5 h (185 nmol/g of liver) and was still measurable 5-h postadministration (90 nmol/g of liver). In agreement with our hypothesis, F-VPA did not form the corresponding acyl-CoA derivative as determined by the absence of F-VPA-CoA upon HPLC analysis of liver extracts from F-VPA-dosed rats. Further examination of liver tissue for the presence of free acids revealed that the differences in acyl-CoA formation cannot be explained by differences in VPA and F-VPA free acid concentrations. From these observations and related studies showing the lack of toxicity due to $\alpha$-fluoro substitution, we propose that metabolism of VPA by acyl-CoA formation may mediate the hepatotoxicity of the drug.

The use of valproic acid (2-n-propylpentanoic acid, VPA; Fig. 1) for the treatment of seizures has been associated with an idiosyncratic hepatotoxicity that is usually characterized by hepatic microvesicular steatosis (Zimmerman and Ishak, 1982). It has been proposed that a metabolite (or metabolites) of VPA induce the associated hepatotoxicity (Lewis et al., 1982; Zimmerman and Ishak, 1982; Baillie, 1992). $\Delta^4$-VPA, an unsaturated metabolite of VPA, elicits hepatic steatosis in rats to a greater degree than VPA or other metabolites tested (Kester et al., 1991). The authors proposed that a reactive diene (2,4-unsaturated metabolite of VPA; Fig. 1) undergoes Michael addition with glutathione (GSH), which supports a proposal that the depletion of GSH by reactive metabolites of $\Delta^4$-VPA leads to a depletion of total hepatic and mitochondrial glutathione (GSH), which supports a proposal that the depletion of GSH by reactive metabolites of $\Delta^4$-VPA may lead to the associated hepatotoxicity (Kassahun et al., 1991). In agreement with this finding are observations identifying the GSH conjugate of $\Delta^2$-VPA in the bile of $\Delta^2$-VPA-dosed rats and the excretion of the corresponding N-acetylcyesteine conjugate in the urine of patients treated with VPA (Kassahun et al., 1991). The authors proposed that a reactive diene metabolite coming from the $\beta$-oxidation of $\Delta^2$-VPA-CoA, namely $\Delta^2$-VPA-CoA, represents the reactive intermediate undergoing conjugation with GSH (Fig. 2). Acyl-CoA dehydrogenase enzymes catalyze the first step in mitochondrial fatty acid $\beta$-oxidation by converting fatty acyl-CoA thioesters to their corresponding trans-2,3-
enoyl-CoA dehydrogenases (Schulz, 1990). These enzymes are believed to function by a mechanism where removal of the α-proton by an active-site base is followed by transfer of the β-hydride to a noncovalently bound flavin adenine nucleotide group (Thorpe, 1990).

The present work was prompted by an interesting study by Tang et al. (1995) on α-fluorinated analogs as mechanistic probes in VPA-induced hepatotoxicity. The substitution of a fluorine atom at the α-position to the carboxylic acid group provides a derivative that contains a chemically and enzymatically inert carbon center. The authors proposed that fluorine substitution at the α-carbon would block its metabolism to the reactive Δ^{2,4}-VPA-CoA diene metabolite, via acyl-CoA dehydrogenases, and thus markedly reduce hepatotoxicity. Results from their studies showed that the α-fluorinated analog of Δ^{2,4}-VPA (F-Δ^{2,4}-VPA) does not cause hepatic steatosis, nor does it metabolize to the glutathione conjugate of the Δ^{2,4}-VPA diene in vivo in rats. These data support the acyl-CoA dehydrogenase-mediated metabolic activation of Δ^{2,4}-VPA-CoA hypothesis. A critical part of the hypothesis that may explain the lack of hepatotoxicity induced by the fluoro derivative involves the formation of F-Δ^{2,4}-VPA-CoA (Fig. 2), although the ability of F-Δ^{2,4}-VPA to form the corresponding acyl-CoA thioester derivative has never been shown.

There have been reports indicating that α-fluorinated carboxylic acid-containing compounds do not form acyl-CoA thioester conjugates. These reports include studies with α-fluoropalmitic acid (Soltyšak et al., 1984) and perfluorooctanoic and perfluorodecanoic acids (Kuslikis et al., 1992) showing the lack of formation of their respective acyl-CoA thioester conjugates in vitro.

Hypotheses requiring the formation of the VPA-CoA thioester have been put forward to explain the mechanism of VPA-mediated hepatotoxicity and include VPA-induced CoA depletion (Thurston et al., 1985), carnitine depletion (Coulter, 1984), as well as competitive inhibition of one or several of the enzymes of fatty acid β-oxidation by VPA-CoA or VPA-CoA metabolites (Becker and Harris, 1983; Bjørge and Baillie, 1985). Therefore, we propose that an absence of formation of the acyl-CoA derivative for the α-fluoro analogs of VPA, or its hepatotoxic metabolites, could account for the associated lack of toxic effects. To test this hypothesis, the α-fluoro analog of VPA was used in in vivo metabolic studies in the rat. The studies presented here were performed to determine whether F-VPA is able to be converted to F-VPA-CoA in vivo in rat liver.

**Experimental Procedures**

**Materails.** VPA, EDTA, triethylamine, ethyl chloroformate, potassium bichromate, diisopropylamine, butyl lithium (1.6 M in hexanes), hydroxylamine hydrochloride, BSTFA, ibuprofen, and THF (anhydrous) were purchased from Aldrich Chemical (Milwaukee, WI). F-VPA, VPA-CoA, and F-VPA-CoA were synthesized as described below. CoA was purchased from Sigma (St. Louis, MO). N-Fluorobenzenesulfonimide was a gift from Allied Signal, Inc. (Buffalo, NY). All solvents used for HPLC were of chromatography grade.

**Instrumentation and Analytical Methods.** HPLC was carried out on a Shimadzu LC-600 isocratic system coupled to a Shimadzu SPD-6AV UV-Vis detector (Shimadzu, Kyoto, Japan). All HPLC analyses were performed on a reverse-phase column (Beckman C₁₈ 15 cm, 5 μ, 1 ml/min; Beckman Coulter, Inc., Fullerton, CA). Tandem liquid secondary-ion mass spectrometry was performed on a Kratos Concept IIIHH four-sector tandem mass spectrometer (Kratos Analytical Instruments, Chestnut Ridge, NY) equipped with a cesium ion source and a continuous flow liquid secondary-ion mass spectrometry probe. The solvent used contained acetonitrile (5%), thioglycerol (2%), and trifluoroacetic acid (0.1%) in water. Samples were delivered at a flow rate of 3 μl/min using a syringe pump (Applied Biosystems, Foster City, CA). ESI/MS/MS analysis was performed on a Finnigan-MAT TSQ 7000 (San Jose, CA). HPLC purified samples were introduced into the ESI source via a Harvard Apparatus syringe pump (Holliston, MA) at a flow rate of 0.3 μl/min. CID of the 12C component of the protonated molecules-of-interest was performed in the collision cell. GC/MS analysis was conducted on a HP 5890 chromatograph (Hewlett Packard, Palo Alto, CA) interfaced with a Varian 70/70 magnetic sector mass spectrometer (Varian, Palo Alto, CA). Analyses were performed in the positive ion EI mode with an emission current of 350 μA. The GC column used was a fused silica capillary column (60 m × 0.32-mm i.d., 0.25-mm film thickness) coated with a DB-1 bonded stationary phase (J&W Scientific, Rancho Cordova, CA). Samples were analyzed as their trimethylsilyl esters after derivatization with BSTFA. GC conditions were 60°C for 0.5 min, 20°C/min to a final oven temperature of 290°C. Ibuprofen was used as the internal standard for quantitation of VPA and F-VPA, and all ions monitored corresponded to the [M – CH₃]⁺ fragment. Quantitative measurements were based on the ratios of peak areas (free acid/internal standard) relative to a linear calibration curve.

1H NMR spectra were recorded (in deuterochloroform) with a General Electric QE300 spectrometer (Fairfield, CT) operating at 300 MHz. Proton chemical shifts are reported in parts per million (ppm; δ) relative to the tetramethylsilane internal standard.

**Synthesis of α-F-VPA.** F-VPA was synthesized by electrophilic α-fluorination of ethyl valproate by an analogous method to that described by Tang et al. (1995) for the synthesis of 2-fluoro-2-propyl-4-pentenoic acid. GC/MS 

![Proposed scheme for the metabolic activation of VPA.](image-url)
Synthesis of S-Acyl-CoA Thioester Derivatives of VPA and F-VPA. Synthesis of CoA thioesters was performed by conventional procedures employing ethyl chloroformate (Stadman, 1937; Grillo, 1993). Briefly, triethylamine (1.6 mmol) followed by ethyl chloroformate (1.6 mmol) was added to the free acid (1.6 mmol) dissolved in anhydrous THF (25 ml) at room temperature and while stirring. After 30 min of continued stirring, the precipitate that formed (triethylamine hydrochloride) was removed by passing through a glass funnel, fitted with a glass wool plug, and added directly into a solution containing CoA (100 mg) and KHCO₃ (1.6 mmol) in distilled water (10 ml) and THF (15 ml). The solution was stirred continuously under nitrogen gas at room temperature for 2 h, after which the reaction was terminated by the addition of concentrated HCl (8 drops). The THF was then removed by evaporation under reduced pressure, and the remaining aqueous phase was extracted with diethyl ether (4 × 50 ml). Residual diethyl ether was removed by evaporation under reduced pressure at room temperature. The solution was adjusted to pH 7.0 by the addition of NaOH (1 N). Purification of the acyl-CoA thioester standards was achieved by reverse-phase HPLC using isocratic elution with acetonitrile (20%) in 0.2 M ammonium acetate on a reversed-phase column (C18, 25 cm × 4.6 mm, 5 μm, 1 ml/min) and detected by UV absorbance (262 nm). CoA thioester-containing fractions were collected and desalted by passing through a cation exchange solid phase extraction cartridge (J. T. Baker, Phillipsburg, NJ). Desalted solutions containing CoA thioesters were then frozen (−80°C) and lyophilized to dryness. MS/MS analysis VPA-CoA (CID of MH⁺ at m/z 894, 100%; m/z 136 [adenine + H]⁺, 90%), m/z 387 [M + H – adenosine triphosphate]⁺, 12%, m/z 428 [adenosine diphosphate + 2H]⁺, 8%), and m/z 285 [M + H – 609]⁺, 6%); F-VPA-CoA (CID of MH⁺ at m/z 912, 100%; m/z 136 [adenine + H]⁺, 100%, m/z 405 [M + H – adenosine triphosphate]⁺, 100%), m/z 428 (adenosine diphosphate + 2H]⁺, 42%, m/z 508 (adenosine triphosphate + 2H]⁺, 36%, m/z 303 [M + H – 609]⁺, 14%, and m/z 565 (adenosine monophosphate]⁺, 31%).

Analysis of Acyl-CoA Metabolites. After the administration of VPA or F-VPA (0.7 mmol/kg i.p., in distilled water, pH 7.0) to male Sprague-Dawley rats (200–220 g), and at 0.05-, 0.5-, 1-, 2-, and 5-h post time points, rodents were anesthetized with diethyl ether, decapitated, and their livers immediately excised. Livers were then quickly frozen at −80°C and stored until analysis for acyl-CoA derivatives by HPLC and mass spectrometry. The preparation of liver samples for HPLC analysis was performed as follows: frozen livers were broken into small pieces (approximately 0.5 g), and 1.0-g portions (duplicate) were added to ice-cold 7% perchloric acid (2.0 ml) and centrifuged (14,000 rpm, 3 min). The resulting supernatant was transferred to a glass vial (20 ml) containing a solution of potassium phosphate buffer (0.05 M, pH 7.0, 5 ml). The pH of this solution was adjusted to 6.0 with 1 N NaOH. An aliquot of this solution (200 μl) was then immediately injected onto the HPLC (Beckman C18, 15 cm, 5 μm, 1 ml/min). The acyl-CoA derivatives were eluted by a linear gradient whereby the acetonitrile concentration of the 0.2 M ammonium acetate mobile phase was increased from 0% to 50% over 50 min. All acyl-CoA derivatives were detected by UV analysis at 262 nm. Quantitative measurements of acyl-CoA derivatives detected in liver tissue were made using a standard curve generated from absolute peak areas. Acyl-CoA metabolites formed in vivo were analyzed by tandem mass spectrometry after purification by HPLC and further processing as described above (Grillo, 1993).

Analysis of Free Acids. Liver homogenates prepared as above, 1.5 ml, duplicate) from VPA- and F-VPA-treated rats were added to 2 N NaOH (1.5 ml), to hydrolyze any acyl-CoA or acyl glucuronide esters, containing internal standard (ibuprofen, 5 μg), and the samples were left to stand at room temperature for 1 h. These solutions were then acidified (2 N HCl, 3 ml) and extracted with ethyl acetate (2 × 5 ml). The combined ethyl acetate extracts were dried (MgSO₄) and evaporated to dryness under nitrogen gas at room temperature. Diethyl ether (1 ml) was added to these dried samples, and the mixture reacted with BSTFA (300 μl) overnight at room temperature. Aliquots (10 μl) of these derivatized extracts were analyzed by GC/MS as described above.

Results

Analysis of Acyl-CoA Metabolites in Liver Tissues of VPA- and F-VPA-Treated Rats. Livers of rats treated with VPA (100 mg/kg) or F-VPA (110 mg/kg) were analyzed for their respective acyl-CoA thioester derivatives at 0.05-, 0.5-, 1-, 2-, and 5-h post i.p. administration. HPLC analysis of liver extract from a VPA-dosed rat at the 0.5-h time point revealed the presence of VPA-CoA eluting at an HPLC retention time of ~22 min (Fig. 3). No substance was detected at this retention time in control liver extracts of rats treated only with water (pH 7.0, 1 ml i.p., data not shown). The identity of this metabolite was confirmed by comparison of the HPLC and tandem mass spectrometric characteristics of the biological and corresponding synthetic standard (Figs. 3 and 4). CID of the parent MH⁺ ion (m/z 894) of the HPLC-purified biological extract provided a mass spectrum characteristic of the VPA-CoA synthetic standard, which included ions at m/z 285, m/z 387, and m/z 428 originating from the proposed cleavages (Grillo, 1993) as shown in Fig. 4. The hepatic concentration-time profile of VPA-CoA in rat liver (Fig. 6) showed that the presence of VPA-CoA was maximal in liver tissue after 0.5 h (185 nmol/g of liver) and was still measurable 5-h postadministration (90 nmol/g of liver). The concentrations of VPA-CoA at the 1- and 5-h time points are approximately equal and are consistent with the relative VPA free acid concentrations in livers 1- and 5-h post-VPA administration.
administration (Fig. 6). In addition, the nadir in hepatic VPA-CoA concentration occurring at the 2-h time point is consistent with the nadir in the hepatic VPA free acid concentration. The secondary rise in VPA and VPA-CoA concentrations at the 5-h time point is due to a reabsorption of VPA, which is released by hydrolysis of the VPA-1-O-acyl glucuronide in the large bowel (Dickinson et al., 1979). In agreement with our hypothesis, F-VPA-CoA was not detected in liver extracts from F-VPA-treated rats (Fig. 5). The small amount of VPA-CoA that is detected during the HPLC analysis of the F-VPA rat liver extract (retention time 22 min) is presumably derived from the metabolism of the 1% impurity of VPA in the F-VPA synthetic test compound.

**Discussion**

A mechanism that may explain the hepatotoxicity associated with VPA therapy involves the formation of chemically reactive metabolites of the drug (Zimmerman and Ishak, 1982; Baillie, 1992). Most attention has focused on the unsaturated metabolite of VPA, Δ⁴-VPA, because this metabolite is metabolically activated to reactive electrophilic species that may covalently bind to and injure important cellular proteins (Fig. 2; Baillie, 1992). One hepatotoxic metabolite of Δ⁴-VPA is Δ⁴,⁴-VPA, which has been shown to be reactive with nucleophiles such as glutathione (Kassahun et al., 1991). The conversion of Δ⁴-VPA to Δ⁴,⁴-VPA first involves the formation of Δ⁴-VPA-CoA thioester that is catalyzed by acyl-CoA synthetases and requires the cofactors CoA, ATP, and Mg²⁺ (Caldwell, 1984; Brass, 1994). Δ⁴-VPA-CoA then is metabolized by acyl-CoA dehydrogenases, the enzymes involved in the first step of fatty acid β-oxidation (Thorpe, 1990), to Δ⁴,⁴-VPA-CoA thioester. It has been proposed that Δ⁴,⁴-VPA-CoA reacts with and depletes mitochondrial GSH resulting in hepatocellular injury (Kassahun and Abbott, 1993; Kassahun et al., 1994). Although these data provide evidence for Δ⁴,⁴-VPA-CoA metabolic activation to reactive and potentially toxic metabolites, there are data that do not support this hypothesis. Evidence against reactive unsaturated metabolites of VPA leading to VPA-induced hepatotoxicity comes from studies in rats (Loscher et al., 1993), where Δ⁴-VPA levels did not correlate with hepatic steatosis in VPA-treated animals, and in humans (Siemes et al., 1993), where increased amounts of Δ⁴-VPA could be detected only in one of the five patients with fulminant liver failure. To clarify the role of Δ⁴-VPA, and subsequently Δ⁴,⁴-VPA, in VPA-induced hepatotoxicity, Tang et al. (1995) synthesized F-Δ⁴-VPA and used it in histopathological, biochemical, and metabolic studies in rats. Because of the chemical and metabolic stability of the carbon-fluorine bond at the α-position, the authors propose that fluorine substitution at the α-carbon would preclude its...
metabolism to the reactive diene metabolite, via acyl-CoA dehydrogenases, and thus markedly reduce hepatotoxicity. Their results, all of which strongly support the Δ^4-VPA bioactivation hypothesis, showed that F-Δ^4-VPA when given to rats resulted in no hepatic steatosis, depletion of liver mitochondrial glutathione, or formation of glutathione conjugates of the Δ^2,4-VPA metabolite (Fig. 2).

The ability of F-Δ^4-VPA to be converted to F-Δ^4-VPA-CoA thioester has not been directly determined but has been presumed to occur because the amino acid conjugate of F-Δ^4-VPA, N^2-(α-fluoro-2-propyl-pentenoyl)glutamine, has been isolated as a urinary metabolite from F-Δ^4-VPA-treated rats (Tang et al., 1997). In similar studies with mice, F-VPA was also shown to be converted extensively to the F-VPA-glutamine amide conjugate (Tang et al., 1997). Amino acid metabolites of acidic drugs are believed to occur through the intermediary of the respective acyl-CoA thioester metabolites (Caldwell et al., 1979; Caldwell, 1984), and therefore F-Δ^4-VPA is proposed to have formed the intermediate CoA thioester derivative. These results are in contrast to literature reports showing examples that α-fluorinated derivatives of carboxylic acid compounds do not form acyl-CoA thioester conjugates. These include studies with α-fluoropalmitic acid (Soltysiak et al., 1984), shown not to be incorporated into triglycerides in cultured mammalian cells, an acyl-CoA-mediated process, and in studies with perfluoro-medium chain fatty acids (Kuslikis et al., 1992), shown not to form acyl-CoA thioesters in incubations with liver microsomes and the cofactors of acyl-CoA formation (CoA, ATP, and Mg^2+). To our knowledge, it is not known whether amino acid conjugation can occur independently of the formation of reactive acyl-CoA intermediates, although results from studies with α-fluoropalmitate support involvement of CoA thioester independent steps in the modification of membrane lipids (Soltysiak et al., 1984), which are processes requiring a reactive acylating intermediate. α-Fluoropalmitic acid was found to be incorporated without modification into membrane lipids such as phosphatidylcholine, sphingomyelin, neutral glycosphingolipids, and ceramides, independent of the formation of α-fluoro-palmitoyl-CoA. It may be that the formation of the glutamine-amide conjugate of F-VPA occurs by a CoA-independent process.

Another potential mechanism for VPA-mediated hepatotoxicity comes from studies showing that acyl-CoA thioester derivatives of carboxylic acid-containing compounds are chemically reactive species that may contribute to the acylation of protein (Tishler and Goldman, 1970; Faed, 1984; Hertz and Bar-Tana, 1988; Bharadwaj and Bizzozero, 1995; Sallustio et al., 2000). In addition to the effects of VPA on CoA depletion (Thurston et al., 1985), carnitine depletion (Coulter, 1984), and competitive inhibition of fatty acid metabolism (Baillie, 1992), the chemical reactivity of VPA-CoA in transacylation-type reactions with protein nucleophiles may contribute to the covalent binding of VPA to protein in vitro and in vivo (Porubek et al.; 1989; Kassahun et al.; 1994; Bailey and Dickinson, 1996) and the associated hepatotoxicity (Nelson and Pearson, 1990; Baillie, 1992). The ability of VPA-CoA to transacylate protein nucleophiles remains to be evaluated.

We propose that the lack of hepatotoxic effects of the α-fluoro-substituted derivative of Δ^4-VPA may be due to a lack of the formation of the F-Δ^4-VPA-CoA thioester conjugate, rather than a block in the formation of the reactive diene, Δ^2,4-VPA-CoA. To test this hypothesis, we synthesized F-VPA and in the present studies tested its ability to form the F-VPA-acyl-CoA derivative in vivo in rat liver. We assumed that the effect on acyl-CoA formation that occurs for F-VPA would also occur for F-Δ^4-VPA. Analysis of liver tissue extracts by HPLC from rats treated with VPA showed the presence of VPA-CoA (Fig. 3), but no F-VPA-acyl-CoA was detected in extracts from the F-VPA-treated animals (Fig. 5). Analysis for the presence of VPA and F-VPA free acids in liver tissue showed only small differences in their concentrations, indicating that F-VPA-CoA thioester is not formed in vivo in liver even though the free acid is present (Fig. 6). Furthermore, standard curves of the acyl-CoA synthetic standards isolated from acidified liver tissue were identical for VPA-CoA and F-VPA-CoA, indicating similar extraction efficiencies for both CoA thioesters (data not shown).

The inability of the α-fluoro-substituted analogs to form acyl-CoA derivatives may result from the increased acidity of the carboxyl group (Schwartz et al., 1995; Tang et al., 1997), due to the electronegativity effects of the fluorine atom (Welch and Eswarakrishnan, 1991), which may influence the binding of the analog to the acyl-CoA synthetase enzymes (Soltysiak et al., 1984). The F-VPA analog was shown to be more acidic (pK_a = 3.55) than VPA (pK_a = 4.80), which may also help to explain the lack of the formation of F-VPA-acyl glucuronide relative to the extensive glucuronidation of VPA in vivo (Tang et al., 1997).

From these observations showing that the α-fluoro analog of VPA does not form the respective acyl-CoA derivative in vivo and that α-fluorination precludes hepatic steatosis (Tang et al., 1995), we propose that the metabolism of VPA, and unsaturated metabolites of
VPA, by acyl-CoA formation may mediate the hepatotoxicity of the drug. Ongoing studies in our laboratory are designed to evaluate the abilities of VPA and unsaturated hepatotoxic metabolites of VPA to form acyl-CoA thioesters, in vitro and in vivo, in that differences in acyl-CoA formation may be related to differences in the ability to induce hepatotoxicity.

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