TOXICOLOGICAL SCIENCES **86(2)**, 427–435 (2005)
doi:10.1093/toxsci/kfi184
Advance Access publication April 27, 2005

Valproic Acid I: Time Course of Lipid Peroxidation Biomarkers, Liver Toxicity, and Valproic Acid Metabolite Levels in Rats

Vincent Tong, Xiao Wei Teng, Thomas K. H. Chang, and Frank S. Abbott[1]

Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Received March 11, 2005; accepted April 20, 2005

A single dose of valproic acid (VPA), which is a widely used antiepileptic drug, is associated with oxidative stress in rats, as recently demonstrated by elevated levels of 15-F_{2t}-isoprostane (15-F_{2t}-IsoP). To determine whether there was a temporal relationship between VPA-associated oxidative stress and hepatotoxicity, adult male Sprague-Dawley rats were treated ip with VPA (500 mg/kg) or 0.9% saline (vehicle) once daily for 2, 4, 7, 10, or 14 days. Oxidative stress was assessed by determining plasma and liver levels of 15-F_{2t}-IsoP, lipid hydroperoxides (LPO), and thiobarbituric acid reactive substances (TBARs). Plasma and liver 15-F_{2t}-IsoP were elevated and reached a plateau after day 2 of VPA treatment compared to control. Liver LPO levels were not elevated until day 7 of treatment (1.8-fold versus control, $p < 0.05$). Liver and plasma TBARs were not increased until 14 days (2-fold vs. control, $p < 0.05$). Liver toxicity was evaluated based on serum levels of α-glutathione S-transferase (α-GST) and by histology. Serum α-GST levels were significantly elevated by day 4, which corresponded to hepatotoxicity as shown by the increasing incidence of inflammation of the liver capsule, necrosis, and steatosis throughout the study. The liver levels of β-oxidation metabolites of VPA were decreased by day 14, while the levels of 4-ene-VPA and (*E*)-2,4-diene-VPA were not elevated throughout the study. Overall, these findings indicate that VPA treatment results in oxidative stress, as measured by levels of 15-F_{2t}-IsoP, which precedes the onset of necrosis, steatosis, and elevated levels of serum α-GST.

Key Words: **valproic acid; oxidative stress; 15-F_{2t}-isoprostane; hepatotoxicity; α-glutathione S-transferase; lipid peroxidation.**

The hepatotoxicity associated with valproic acid (2-propylpentanoic acid, VPA), a traditional antiepileptic drug, has been well documented (Eadie *et al.*, 1988; Granneman *et al.*, 1984; Jezequel *et al.*, 1984; Kesterson *et al.*, 1984; Powell-Jackson *et al.*, 1984; Sussman and McLain, 1979). The type I VPA-mediated hepatotoxicity is associated with dose-dependent changes in serum levels of liver enzymes and low plasma fibrinogen levels, which are rectified upon VPA removal. The type II VPA-mediated hepatotoxicity is considered to be a rare, but often fatal, irreversible idiosyncratic reaction characterized by microvesicular steatosis that is sometimes accompanied by necrosis. Those at primary risk of fatal hepatic dysfunction are children under the age of 2 years who are receiving multiple anticonvulsants and also have significant medical problems in addition to severe epilepsy (Dreifuss *et al.*, 1987). Although the mechanism is not fully elucidated, a large body of evidence suggests that reactive VPA metabolites (i.e., 4-ene-VPA and its subsequent metabolite, (*E*)-2,4-diene-VPA) may mediate the hepatotoxicity by inhibiting mitochondrial fatty acid β-oxidation (Gerber *et al.*, 1979; Kassahun and Abbott, 1993; Zimmerman and Ishak, 1982).

More recently, there are a number of studies suggesting that excessive generation of free-radical intermediates are associated with VPA, possibly as a consequence of VPA biotransformation (Jurima-Romet *et al.*, 1996), alterations in glutathione homeostasis (Cotariu *et al.*, 1990; Graf *et al.*, 1998; Seckin *et al.*, 1999; Tang *et al.*, 1995), and/or depletion of cofactors required for antioxidant defense (Graf *et al.*, 1998; Hurd *et al.*, 1984). A rapid *in vitro* assay was developed for the detection of metabolism-dependent cytotoxicity on isolated human lymphocytes and provided a method to study the mechanisms underlying lymphocyte toxicity exhibited by VPA (Tabatabaei *et al.*, 1999). In support of this hypothesis, exposure of VPA to human lymphocytes isolated from patients who had developed hepatotoxicity while on VPA therapy demonstrated a marked increase in lymphocyte cell death compared to lymphocytes from VPA-treated patients who did not show liver toxicity (Farrell and Abbott, 1991). Mechanistic studies were carried out to evaluate the contribution of reactive metabolite and reactive oxygen species as the mechanism of the metabolism-dependent VPA-induced lymphocyte toxicity. The data obtained indicate that the observed cytotoxicity in the lymphocyte model was the result of the microsomal metabolism-dependent generation of hydrogen peroxide in the medium that can readily cross cell membranes and subsequently interact intracellularly with iron to produce the highly reactive hydroxyl free radicals (Tabatabaei *et al.*, 1999). In those individuals who have compromised antioxidant defense mechanisms, the induction of oxidative stress by VPA

[1] To whom correspondence should be addressed at Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, BC, CANADA V6T 1Z3. Fax: (604) 822–3035. E-mail: fabbott@interchange.ubc.ca.

may be responsible for or contribute to the mechanism of idiosyncratic hepatotoxicity.

A recent study demonstrated that the administration of VPA led to a dose-dependent elevation of plasma and liver levels of the lipid peroxidation marker, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP, also called 8-isoprostane or 8-iso-prostaglandin $F_{2\alpha}$) (Tong *et al.*, 2003). While the effects of VPA on 15-F_{2t}-IsoP levels were consistent with an induction of oxidative stress, two other independent measures of oxidative stress, thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LPO), were unchanged by a single dose of VPA. However, the question remains whether the elevation in 15-F_{2t}-IsoP precedes liver toxicity.

The objective of the present study was to determine the temporal relationship between oxidative stress and hepatotoxicity in rats treated with VPA and, in doing so, to provide new insight into the potential role of oxidative stress in VPA-mediated hepatotoxicity in rats. If the hepatotoxicity were induced through the formation of oxidative stress, an expected increase or accumulation of oxidative stress markers would occur prior to hepatotoxicity. 15-F_{2t}-IsoP, TBARS, and LPO were used as markers of oxidative stress, whereas histopathological findings (necrosis and steatosis), and serum α-glutathione-S-transferase (α-GST) levels were used as markers of hepatotoxicity. VPA metabolites, in particular 4-ene-VPA and (*E*)-2,4-diene-VPA, were monitored to investigate whether the observed oxidative stress and hepatotoxic events were associated with the formation in these putative reactive metabolites. The results from the present study suggest that oxidative stress precedes hepatotoxicity in VPA-treated rats.

MATERIALS AND METHODS

Chemicals. Sodium valproate and D-saccharic acid 1,4-lactone monohydrate (D-saccharolactone) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The 15-F_{2t}-isoprostane EIA kit and lipid hydroperoxide assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI). Oxi-Tek TBARS assay kit was purchased from Zepto-Metrix Co. (Buffalo, NY). Dimethylformamide and the GC derivatizing reagents pentafluorobenzyl bromide and N-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide were purchased from Pierce Chemical Co. (Rockford, IL). *N,N*-diisopropylethylamine and *tert*-butyldimethylsilyl chloride were obtained from Aldrich (Milwaukee, WI). Ethyl acetate (HPLC grade) and n-hexanes (GC/MS resolved) were purchased from Fisher Scientific (Vancouver, BC, Canada).

Animal experiments. Adult male Sprague-Dawley rats (250–300 g) were from the University of British Columbia Animal Care Facility. They were fed with rat diet (Labdiet 5001 rodent diet, PMI Feeds Inc., Richmond, IN) and water *ad libitum* and maintained in a room on a 12 h light/12 h dark cycle at constant temperature (22°C) and humidity. The University of British Columbia Animal Care Committee approved all animal experimentation. Rats were treated with an aqueous solution of VPA (dissolved in 0.9% saline) and injected ip at a dose of 500 mg/kg once a day for 2, 4, 7, 10, or 14 consecutive days. Our previous study demonstrated that a dose of 500 mg/kg VPA produced maximal elevation in plasma and liver levels of 15-F_{2t}-IsoP (Tong *et al.*, 2003). The control group was treated with 0.9% saline solution (vehicle control, 1 ml/kg, ip) for 14 consecutive days. Based on the t_{max} of plasma 15-F_{2t}-IsoP following a single 500 mg/kg VPA dose (Tong *et al.*, 2003), rats were sacrificed 30 min following the last injection by decapitation, and trunk blood was collected in Vacutainer® blood collection tubes. Serum and plasma were immediately prepared and snap-frozen in liquid nitrogen for the analysis of VPA metabolites, oxidative stress, and toxicity biomarkers. The livers were weighed, rinsed with ice-cold phosphate buffered saline (pH 7.4), and homogenized in 50 mM phosphate buffer (pH 7.4) with 5 mM D-saccharolactone and 0.005% butylated hydroxytoluene on ice. The homogenate was snap-frozen in liquid nitrogen for the determination of VPA metabolites and oxidative stress biomarkers. All biological samples were stored at −80°C.

Determination of plasma and liver 15-F_{2t}-IsoP levels. Plasma and liver levels of 15-F_{2t}-IsoP were determined by an EIA assay (Cayman Chemical Co., Ann Arbor, MI) as previously described (Tong *et al.*, 2003). Plasma 15-F_{2t}-IsoP was measured directly by adding 50 µl of fresh rat plasma to the 96-well EIA plates. For the determination of total liver 15-F_{2t}-IsoP, liver homogenate samples (500 µl) were subjected to alkaline hydrolysis by incubation with an equal volume of 15% KOH for 1 h at 40°C, followed by protein precipitation with 12 M HCl (final sample pH of 1–2). For the determination of free liver 15-F_{2t}-IsoP, liver homogenate was subject only to protein precipitation. The samples were centrifuged (1600 × *g* for 10 min) to pellet the protein and processed by liquid-liquid extraction with ethyl acetate (5 ml) by gentle rotation for 30 min. The samples were centrifuged again (1600 × *g* for 10 min) to aid in the separation of the layers. The organic layer was dried under a stream of N_2 (30°C), reconstituted in acidified water (2 ml, pH 3), and processed by a solid-phase extraction procedure adapted from Nourooz-Zadeh *et al.* (1995). Samples were extracted on a 24-channel Vac Elut Vacuum Manifold using Waters Oasis® C18 cartridges (Waters Ltd., Mississauga, ON, Canada). The cartridges were preconditioned with 2 ml methanol and 2 ml water (pH 3). Samples were washed with 3 ml water (pH 3) followed by 3 ml acetonitrile/water (15/85, v/v) and eluted with 6 ml hexane/ethyl acetate/propan-2-ol (30/65/5, v/v/v). The eluant was evaporated under N_2, and the residue was reconstituted in 1 ml of EIA phosphate buffer (Cayman Chemical Co., Ann Arbor, MI). 15-F_{2t}-IsoP was determined spectrophotometrically at 405 nm on a multi-well plate reader.

Determination of liver LPO levels. LPO levels were determined using a commercial kit (Cayman Chemical Co., Ann Arbor, MI). Plasma and liver homogenate samples (500 µl) were deproteinated and extracted under acidic conditions with 1 ml ice-cold deoxygenated chloroform, and the chloroform extract was removed following centrifugation (1600 × *g* for 5 min at 0°C) for LPO determination. The LPO assay is based on redox reactions between hydroperoxides and ferrous ions to produce ferric ions, and the resulting ferric ions are detected using thiocyanate ion as the chromagen. Chloroform extracts (500 µl) were mixed with 50 µl chromagen reagent (2.3 mM ferrous sulfate in 0.2 M HCl and 1.5% methanolic solution of ammonium thiocyanate), and 300-µl samples were transferred to a glass 96-well plate, and absorbance was determined at 500 nm. This assay used 13-hydroperoxy-octadecadienoic acid as a lipid hydroperoxide standard to construct a standard curve (linearity from 0.5 to 5 nmol hydroperoxide).

Determination of plasma and liver TBARs. The concentration of TBARS was calculated as malondialdehyde (MDA) equivalents using a commercial kit (Oxi-Tek®, Zeptometrix Corporation, Buffalo, NY). Samples (100 µl) of plasma or supernatant from liver homogenate after 10,000 × *g* (15 min, 4°C) centrifugation were mixed with an equal volume of sodium dodecyl sulfate solution and 2.5 ml of 5% thiobarbituric acid/acetic acid reagent. Samples were incubated for 60 min at 95°C. After centrifugation at 1600 × *g,* samples were transferred into a 96-well plate, and fluorescence was monitored on a multi-well plate reader with excitation set at 508 nm (20 nm band width) and emission at 560 nm (20 nm bandwidth). This assay used an MDA standard to construct a standard curve (0.5–5 nmol/ml MDA).

Determination of serum α-GST activity. Fresh serum was prepared immediately after the rats were sacrificed, and α-GST was measured in rat serum as a marker for hepatic damage by a commercially available enzyme immunoassay method (Biotrin Rat Alpha GST EIA, Biotrin, Dublin, Ireland). α-GST, a cytosolic enzyme located predominantly in liver parenchyma, was

demonstrated to be a sensitive and specific biomarker of hepatocyte injury (Clarke *et al.*, 1997; van Wagensveld *et al.*, 1997). The quantitative immunoassay is based on the sequential addition of serum sample (1:50 dilution) and rabbit anti-rat α-GST IgG conjugated to streptavidin-peroxidase complex to micro-assay wells coated with anti-rat α-GST IgG with washing between each step. After the peroxidase substrate was added to the ELISA plate, the resultant color intensity was determined on a multi-well plate reader (absorbance determined at 450 nm) and was proportional to the amount of α-GST present in the sample. The assay was linear from 1.56 to 25 μg/ml based on α-GST standard solution.

Histopathology. Livers from all treated groups were rinsed with ice-cold saline, and a small cross-section of the liver was obtained and fixed in 10% formalin-phosphate buffered saline when the rats were sacrificed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: necrosis (the percentage of the cross section containing necrotic foci) was scored 1+ (<25%), 2+ (26 to 50%), 3+ (51 to 75%), and 4+ (>75%). Steatosis (expressed as the percentage of liver cells containing fat) was scored in a similar way as described for the determination of necrosis. At least two different sections were examined per liver sample, and the pathologist was blind to the treatment groups when assessing the histology.

Determination of VPA metabolites. Oxidative and desaturated VPA metabolites were determined by GC/MS using negative ion chemical ionization and single ion monitoring. The sample preparation and assay procedures are described in a previous study (Tong *et al.*, 2003). VPA-1-*O*-acyl glucuronide (VPA-G) levels were determined in liver homogenate by an LC/MS assay using negative electrospray ionization and single ion monitoring. The assay utilizes purified VPA-G as a standard and $^{2}H_{6}$-VPA-G as its internal standard to measure VPA-G levels directly by a validated method described elsewhere (Tong *et al.*, 2005a).

Instrumentation and analytical methods. GC/MS analysis of VPA and its metabolites was carried out using an HP 6890 gas chromatograph interfaced to an HP5973 mass selective detector (Hewlett-Packard, Avondale, PA). The gas chromatograph was equipped with a capillary splitless injector and an HP7683 autosampler. The mass spectrometric data acquisition and handling software, HP Enhanced Chemstation Software G1701BA (V B.01.00) was used to control the operation of all instruments.

LC/MS analysis of VPA-G was performed using a Fisons VG Quattro tandem mass spectrometer (Micromass, Montreal, Canada) interfaced with a Hewlett Packard (Avondale, PA, USA) 1090 II Liquid chromatograph. Instrument operation and data acquisition were controlled by MassLynx® (v3.1, Micromass) software.

Fluorescent analysis for the TBARs assay was performed on a Cytofluor® Series 4000 (Applied Biosystems, Bedford, MA) multi-well fluorescent plate reader. Spectrophotometric analysis for the 15-F_{2t}-IsoP, LPO, and α-GST assays were performed on a Labsystems Multiscan Ascent® multi-well plate reader (Thermo Electron Corp., Burlington, ON, Canada).

Statistical analysis. Statistical significance of the difference between the means of multiple groups was analyzed by one-way analysis of variance and, where appropriate, was followed by Bonferonni's multiple comparison *post hoc* test. The level of significance was set *a priori* at $p < 0.05$.

RESULTS

Time Course of Plasma and Liver Levels of 15-F_{2t}-IsoP during VPA Treatment

To characterize the time course for changes in plasma and liver 15-F_{2t}-IsoP levels following VPA treatment, rats were administered VPA (500 mg/kg, ip) once a day for 2, 4, 7, 10, or 14 consecutive days. At 0.5 h after the last dose of VPA, rats were terminated. Plasma 15-F_{2t}-IsoP levels were found to be maximal on day 2 with a ≈3-fold increase (102 ± 14 pg/ml) compared to the saline (vehicle) treated control group (30 ± 3 pg/ml), and these elevated levels were similar after 4, 7, 10, and 14 days (Fig. 1A). Free and total liver 15-F_{2t}-IsoP levels were also

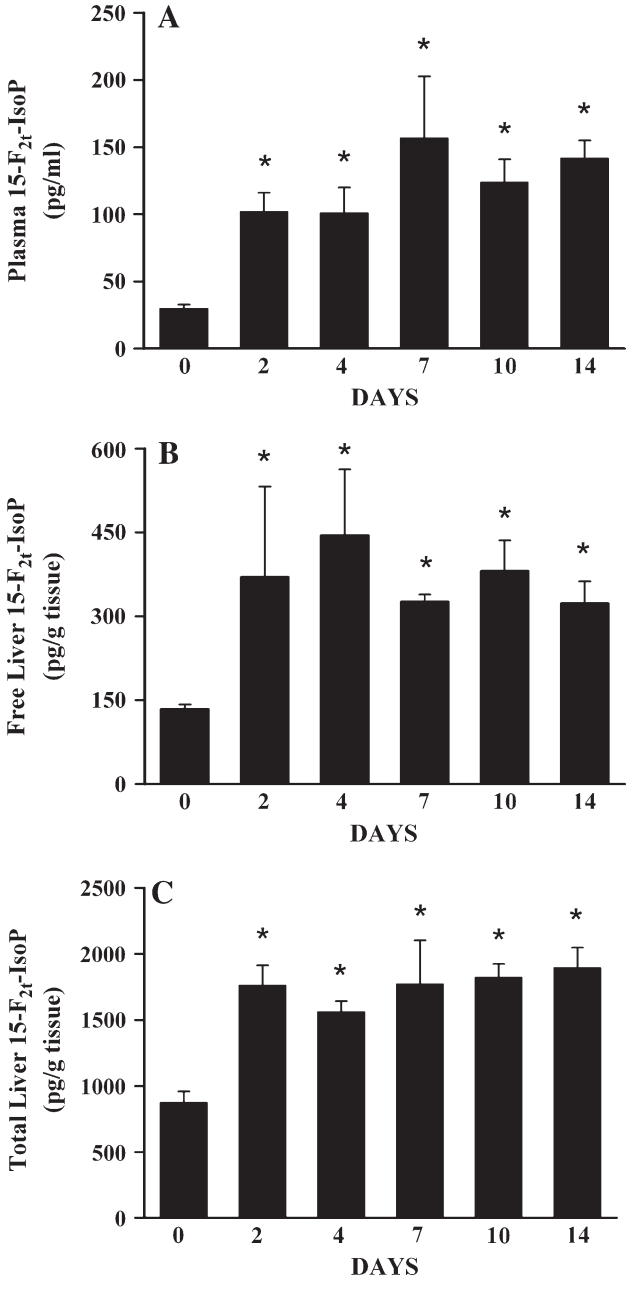

FIG. 1. Levels of (A) plasma, (B) free liver, and (C) total liver (esterified and nonesterified) 15-F_{2t}-IsoP in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, and 14 consecutive days. Control rats were treated with 0.9% saline vehicle for 14 days. Plasma and liver levels of 15-F_{2t}-IsoP were measured by an enzyme immunoassay method as described under Materials and Methods. Results are expressed as mean ± SEM, $n = 5$ individual rats per group (day 2, 4, 7, and 10 groups) and $n = 16$ rats per group (day 14 and vehicle control groups). *Significantly different compared to the saline vehicle control group ($p < 0.05$).

maximally elevated after day 2 (370 ± 160 and 1763 ± 149 pg/g tissue, respectively) compared to the control group (134 ± 8 and 877 ± 81 pg/g tissue, respectively), and these levels were similar to those seen after 4, 7, 10, and 14 days (Figs. 1B and 1C).

TBARs and LPO Levels during VPA Treatment

Liver LPO (Fig. 2) and plasma and liver TBARS (Fig. 3) were measured as other independent indicators of oxidative stress following 2, 4, 7, 10, and 14 consecutive days of VPA treatment (500 mg/kg/day, ip). Liver LPO levels were significantly elevated in the VPA-treated groups after 7 days (105 ± 5 nmol/g tissue) compared to the saline treated control group (72 ± 6 nmol/g tissue) (Fig. 2). The elevated levels of LPO were maximal after day 7 and were similar to levels observed on days 10 and 14. LPO levels in plasma were below the limit of detection of the assay in all groups (data not shown).

Plasma and liver TBARS were significantly elevated on day 14 in the VPA-treated group (2.4 ± 0.1 nmol MDA/ml plasma and 34 ± 1.7 nmol MDA/g tissue, respectively) compared to the saline-treated control group (1.6 ± 0.1 nmol MDA/ml plasma and 16.3 ± 2.1 nmol MDA/g tissue, respectively) (Fig. 3A and 3B).

Serum α-GST Levels and Histology during VPA Treatment

Serum levels of α-GST were significantly elevated after 4 days of VPA treatment (251 ± 17 μg/l) compared to basal levels determined in the control group treated with saline (57 ± 7 μg/l) (Fig. 4). The α-GST levels remained elevated to the same extent on days 7, 10, and 14.

Mortality was observed during the 14-day VPA treatment with an incidence of one out of five rats in the 4- and 7-day groups, and three out of 16 rats in the 14-day group (Table 1). In all treated groups (Figs. 5B–5F), the most common feature was scarring of the liver capsule surface (liver ''capsulitis''), and this abnormality was attended by mild inflammatory reaction localized only to the surface and usually consisted of lymphocytes and infrequent monocytes (Fig. 5C). The frequency of ''capsulitis'' increased with duration of treatment from one out of five animals affected on day 2 to almost 100% frequency from day 4 to 14, while none were reported in the saline-treated control group (Fig. 5A).

In general, massive necrosis was observed without consistent zonal pattern and involved irregular areas of hepatic tissue (Fig. 5D). All necrotic livers were subjectively scored (+4, +3, +2, or +1) to describe the severity of the cross-sectional area affected (100%, 75%, 50%, or 25%, respectively). In the VPA-treated groups, two rats in the 4-day group and one rat in the

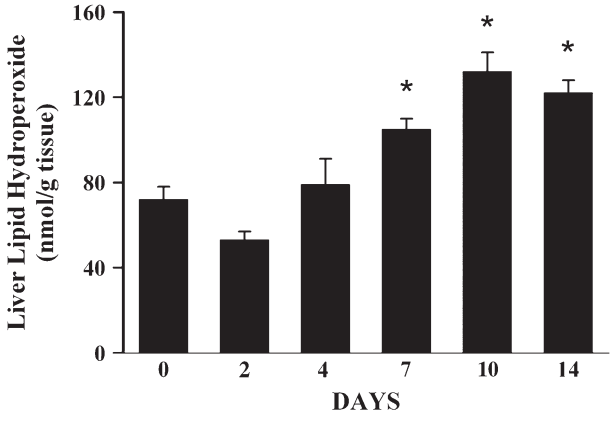

FIG. 2. Levels of liver LPO in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. The standard curve (0.5–5 nmol lipid hydroperoxide) was generated using 13-hydroperoxyoctadecadienoic acid as a lipid hydroperoxide standard. Control rats were treated with 0.9% saline (vehicle) for 14 days. Liver LPO was measured by a colorimetric assay as described under Materials and Methods. Results are expressed as mean ± SEM, $n = 5$ individual rats per group (day 2, 4, 7, and 10) and $n = 16$ individual rats per group (day 14 and vehicle control). *Significantly different compared to the saline vehicle control group ($p < 0.05$).

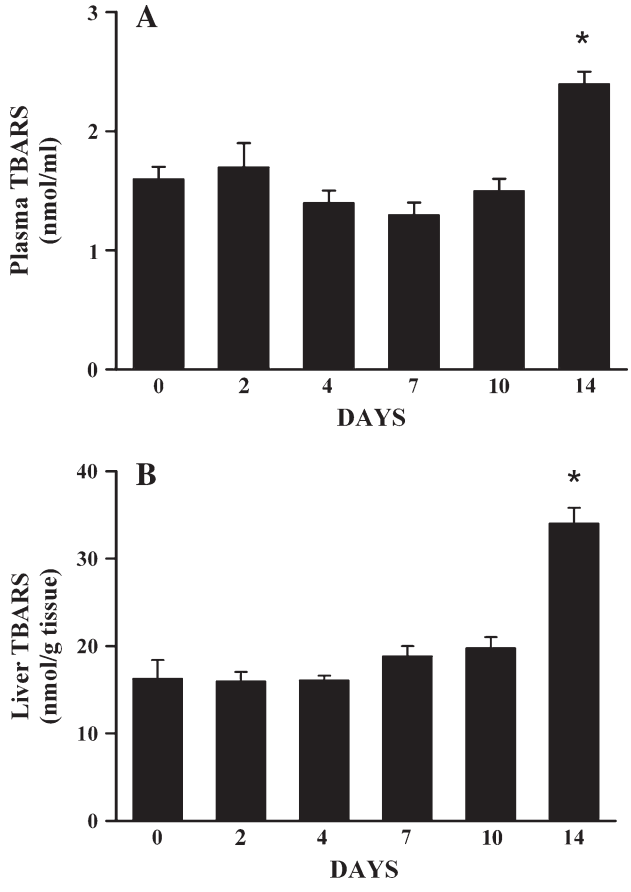

FIG. 3. Levels of (A) plasma and (B) liver TBARs in rats treated with (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. The standard curve for the TBARs assay was generated using malondialdehyde (MDA), and the results are expressed as MDA equivalents. Control rats were treated with 0.9% saline (vehicle) for 14 days. Plasma and liver TBARs were measured by a fluorometric assay as described under Materials and Methods. Results are expressed as mean ± SEM, $n = 5$ individual rats per group (day 2, 4, 7, and 10) and $n = 16$ individual rats per group (day 14 and vehicle control). *Significantly different compared to the saline-treated vehicle control group ($p < 0.05$).

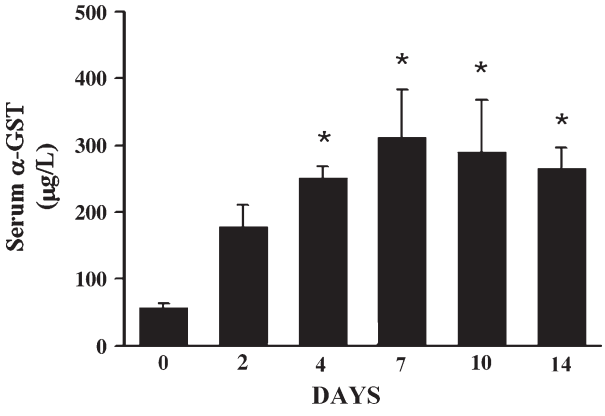

FIG. 4. Levels of serum α-GST in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. Control rats were treated with 0.9% saline vehicle for 14 days. α-GST was measured by an enzyme immunoassay method as described under Materials and Methods. Serum α-GST levels (μg/l) are expressed as mean ± SEM, $n = 5$ individual rats per group (day 2, 4, 7, and 10) and $n = 16$ individual rats per group (day 14 and vehicle control). *Significantly different compared to the saline-treated vehicle control group ($p < 0.05$).

7-day group had liver ''capsulitis'' that was accompanied with hepatocellular degeneration (necrosis score of +1) that extended locally into the parenchyma. The 14-day treatment group (Fig. 5F) had the highest incidence of massive necrosis with scores of +3 (three rats), +2 (two rats), and +1 (two rats).

Liver steatosis was observed in seven rats (Table 1), and the incidence of lipid accumulation appeared to increase with the duration of VPA treatment: one rat in the 4-day group, and two rats in the 7-day group. Four out of five animals in the 10-day group exhibited steatosis (Fig. 5E). The observed steatosis was of zonal distribution conforming to the centrilobular zones (periacinar zone 3). In all cases, steatosis was never extensive, in terms of area, and was considered mild to moderate affecting approximately 10–25% of the liver cross-section, with the exception of one animal on day 10.

Levels of VPA Metabolites during VPA Treatment

Levels of oxidative and mono- and di-unsaturated VPA metabolites were monitored in liver (Table 2) and plasma (Table 3). There was a trend of decreasing VPA metabolite levels in liver with increasing duration of VPA treatment. Significant decreases were observed with some of the liver VPA metabolites after 14 days of treatment, in particular 3-OH-VPA, 2-ene-VPA, 3-ene-VPA and (*E,E*)- and (*E,Z*)-2,3′-diene-VPA. The putative reactive VPA metabolites 4-ene-VPA and (*E*)-2,4-diene-VPA were not elevated throughout the duration of the study. Plasma VPA metabolites (Table 3) and hepatic VPA-G (Table 2) were similar in all groups treated with VPA over the duration of 14 days, with the exception of plasma (*E*)-2,4-diene-VPA, which was below the LOQ (2 ng/ml) by day 14 (Table 3).

DISCUSSION

Our previous study in rats showed increased levels of 15-F_{2t}-IsoP after a single dose of VPA, but this did not involve cytochrome P450-mediated VPA-biotransformation (Tong *et al.*, 2003). The current study investigated the levels of oxidative stress biomarkers in relation to the occurrence of hepatotoxicity over a 2-week treatment period with VPA. The results from this study demonstrated that high doses of VPA given over a time course of 14 days in rats resulted in an elevation in plasma and liver 15-F_{2t}-IsoP that preceded the occurrence of hepatotoxicity (Fig. 6).

In this study, hepatotoxicity was observed from 4 to 14 days of VPA treatment, and the liver histology was characterized predominantly by inflammation and extensive scarring of the

TABLE 1
VPA-Associated Necrosis and Steatosis in Rats Treated with VPA for 14 Days[a]

Treatment duration (days)	Total number	Number of deaths	Incidence of ''capsulitis''[b]	Incidence of necrosis[c]				Incidence of steatosis
				+1	+2	+3	+4	
0	16	0	0%	0	0	0	0	0
2	5	0	20%	0	0	0	0	0
4	5	1	100%	50%	0	0	0	25%
7	5	1	100%	25%	0	0	0	50%
10	5	0	100%	0	0	0	0	80%
14	16	3	92%	15%	15%	0	23%	0

[a]Male Sprague Dawley rats (250–300 g) treated with VPA (500 mg/kg, ip) once a day for 14 consecutive days. The 0-day treatment group received saline vehicle (1 ml/kg, ip) for 14 days. The rats were sacrificed at 0.5 h following the last dose. Liver necrosis and steatosis was determined by light microscope examination of hematoxylin-eosin stained liver samples.

[b]''Capsulitis'' is described as extensive, widespread scarring of the liver capsule.

[c]Necrosis was subjectively scored on the area (%) of liver cross section affected and is described as follows: +1 (<25%), +2 (25–50%), +3 (50–75%) and +4 (>75%).

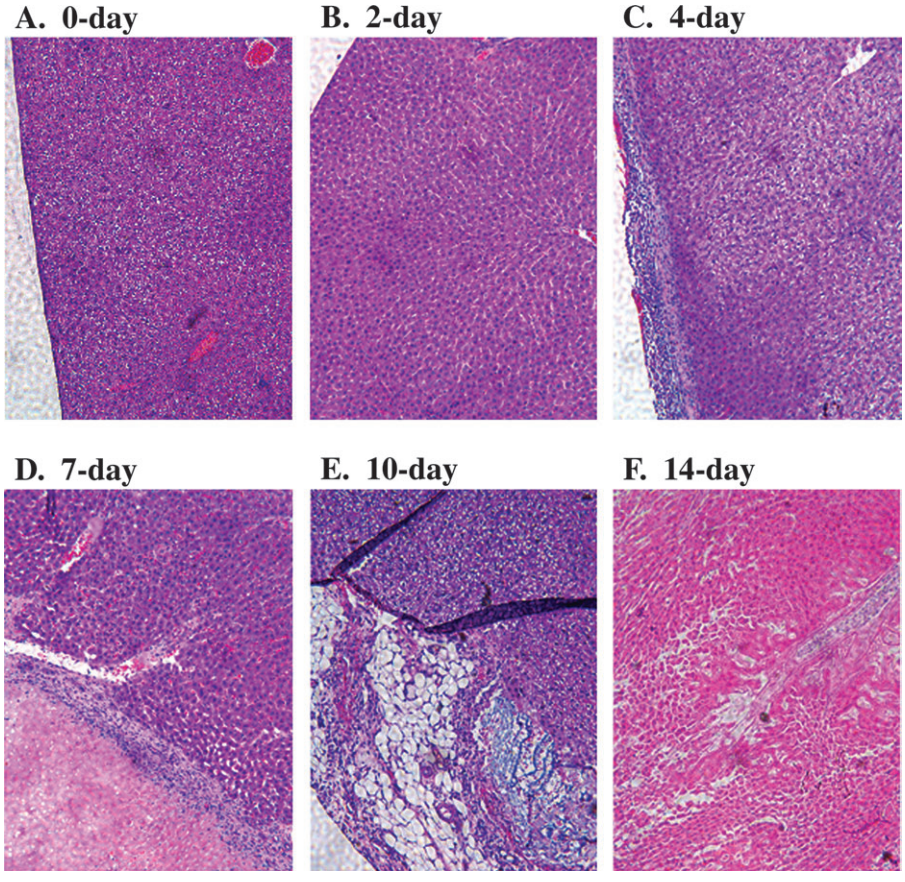

FIG. 5. Photomicrographs of liver sections from rats administered ip with VPA at 500 mg/kg once daily for up to 14 days showing progressive incidence of liver damage. The 0-day treatment group received saline for 14 days. Cross-sections (10 μm) were fixed in 10% phosphate buffered formalin followed by staining with hematoxylin and eosin. Original magnification, 40×.

liver capsule. The first incident of focal necrosis was also observed after day 4 and increased in severity to massive necrosis that appeared to have no consistent zonal pattern and involved large irregular areas of hepatic tissue. The presence of necrosis from day 4 to 14 coincided with increased levels of α-GST, which is a sensitive and specific biomarker of hepatocyte injury (Trull *et al.*, 1994). α-GST, which is found in high concentrations throughout the liver parenchyma, was

TABLE 2
Liver VPA Metabolites (μg/g Tissue) in Rats Treated with VPA for 2, 4, 7, 10, or 14 Days[a]

Liver metabolites	2 Days	4 Days	7 Days	10 Days	14 Days
4-ene	0.23 ± 0.04	0.15 ± 0.03	0.18 ± 0.03	0.14 ± 0.03	0.11 ± 0.01*
4-OH	1.13 ± 0.15	0.79 ± 0.14	0.86 ± 0.31	1.18 ± 0.28	1.09 ± 0.18
3-OH	0.59 ± 0.07	0.42 ± 0.04	0.46 ± 0.10	0.41 ± 0.09	0.31 ± 0.03*
5-OH	0.99 ± 0.11	0.68 ± 0.05	0.75 ± 0.17	0.91 ± 0.16	0.75 ± 0.08
2-ene	2.79 ± 0.17	2.23 ± 0.14	2.43 ± 0.33	2.03 ± 0.30*	1.50 ± 0.06*
3-keto	3.45 ± 0.35	2.57 ± 0.36	2.82 ± 0.32	3.05 ± 0.29	2.78 ± 0.23
4-keto	0.22 ± 0.04	0.14 ± 0.02	0.15 ± 0.04	0.19 ± 0.04	0.18 ± 0.02
3-ene	3.57 ± 0.29	2.66 ± 0.21	2.90 ± 0.39	2.43 ± 0.35	1.89 ± 0.24*
(*E,E*)-2,3′-diene	0.58 ± 0.05	0.46 ± 0.04	0.51 ± 0.09	0.53 ± 0.08	0.30 ± 0.03*
(*E,Z*)-2,3′-diene	0.12 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.12 ± 0.02	0.04 ± 0.01*
(*E*)-2,4-diene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
VPA-G	249 ± 45	224 ± 34	271 ± 38	275 ± 36	273 ± 21

[a]Male Sprague-Dawley rats (250–300 g) treated ip with VPA (500 mg/kg) or 0.9% saline (vehicle; 1 ml/kg) once a day for 14 consecutive days. The rats were sacrificed at 0.5 h following the last dose. VPA metabolite levels were determined by a GC/MS assay.

*Significantly different compared to the 2-days VPA treated group by one-way ANOVA with Bonferonni multiple comparison *post hoc* test ($p < 0.05$).

TABLE 3
Plasma VPA Metabolite Levels (μg/ml) in Rats Treated with VPA for 2, 4, 7, 10, or 14 Days[a]

Plasma metabolites	2 days	4 days	7 days	10 days	14 days
4-ene	0.14 ± 0.02	0.15 ± 0.03	0.16 ± 0.03	0.15 ± 0.04	0.16 ± 0.04
4-OH	2.07 ± 0.34	2.40 ± 0.39	2.44 ± 0.47	2.40 ± 0.65	2.03 ± 0.35
3-OH	0.93 ± 0.15	0.80 ± 0.08	0.70 ± 0.15	0.59 ± 0.14	0.49 ± 0.06
5-OH	2.32 ± 0.17	2.31 ± 0.19	2.09 ± 0.19	1.97 ± 0.29	1.71 ± 0.14
2-ene	1.75 ± 0.12	1.38 ± 0.02	1.71 ± 0.23	1.52 ± 0.21	1.39 ± 0.09
3-keto	3.36 ± 0.24	3.57 ± 0.45	3.09 ± 0.55	2.86 ± 0.31	3.30 ± 0.10
4-keto	0.59 ± 0.08	0.39 ± 0.07	0.43 ± 0.10	0.32 ± 0.07	0.30 ± 0.04
3-ene	0.52 ± 0.10	0.60 ± 0.05	0.62 ± 0.07	0.52 ± 0.06	0.60 ± 0.02
(*E,E*)-2,3′-diene	0.75 ± 0.09	0.85 ± 0.06	1.04 ± 0.16	0.92 ± 0.09	0.70 ± 0.05
(*E,Z*)-2,3′-diene	0.14 ± 0.02	0.09 ± 0.01	0.15 ± 0.02	0.14 ± 0.01	0.08 ± 0.01
(*E*)-2,4-diene	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	<LOQ

[a]Male Sprague-Dawley rats (250–300 g) treated ip with VPA (500 mg/kg) or 0.9% saline (vehicle, 1 ml/kg) once a day for 14 consecutive days. The rats were sacrificed at 0.5 h following the last dose. VPA metabolite levels were determined by a GC/MS assay.

demonstrated to be an earlier and more sensitive marker of hepatocyte injury than the release of conventionally used liver enzymes, such as the transaminases and lactate dehydrogenase (Clarke *et al.*, 1997; van Wagensveld *et al.*, 1997). In this study, alanine aminotransferase and aspartate aminotransferase were also measured in the serum by colorimetric assays; however, these results were highly variable among the animals due to the variable hemolysis observed during the sample collection (data not shown).

Fatty liver was not a predominant lesion observed in the current study. Other studies showed microvesicular steatosis as a common feature in rats at a near-lethal dose of 750 mg/kg over 48 h, but not with a lower dose of 350 mg/kg (Lewis *et al.*, 1982). Kesterson *et al.* (1984) demonstrated fatty liver in rats treated with 700 mg/kg/day for 4 days, but with a lower occurrence (two out of seven rats) at 600 mg/kg/day for 5 days. In the present study, the first incidence of fatty liver, although never extensive in area, occurred on day 4 of treatment (one out of four rats), with increasing occurrence observed after 10 days of treatment (four out of five rats) at 500 mg/kg/day. The lack of steatosis on day 14 was peculiar, and it was speculated to be related to the observation that necrosis was more severe at this time. Microvesicular steatosis was observed after a single 500-mg/kg dose of VPA in fasted rats by Olsen *et al.* (1986) and with repeated dosing also at 500 mg/kg for 7 consecutive days in rats provided with food *ad libitum* (Sugimoto *et al.*, 1987).

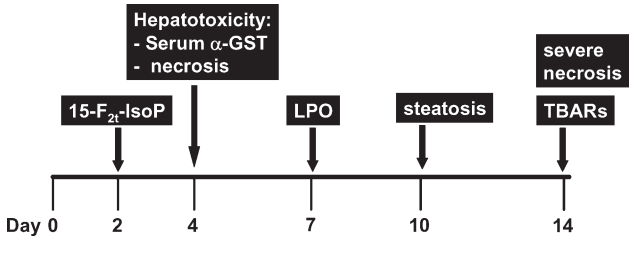

FIG. 6. Summary of results indicating the time course of hepatotoxicity and oxidative stress events. Rats were administered ip with VPA at 500 mg/kg once daily for up to 14 days. The 0-day treatment group received saline for 14 days.

The elevation in plasma and liver 15-F_{2t}-IsoP preceded the occurrence of hepatotoxicity, as determined by histological assessment and by levels of serum α-GST. After the second day of dosing, plasma and serum 15-F_{2t}-IsoP increased when compared to the saline-treated group, and these levels remained elevated to the same extent throughout the 14-day study period. A trend of increased α-GST, however, not statistically significant, was observed after the second day and was highest with the one animal that exhibited inflammation of the liver capsule. It is possible that this trend may reach statistical significance with increased sample size. According to our data, the formation of 15-F_{2t}-IsoP did not increase over time with repeated dosing. 15-F_{2t}-IsoP is a member of the F_2-isoprostanes, a series of prostaglandin $F_{2\alpha}$-isomers that is produced by a free radical-catalyzed (nonenzymatic) lipid peroxidation of arachidonic acid (Roberts and Morrow, 2000). It has been generally accepted that the cascade from arachidonic acid to 15-F_{2t}-IsoP is independent of cyclooxygenase (COX). The effects of VPA on 15-F_{2t}-IsoP are unlikely to be due to COX activity, taking into consideration the reported lack of an effect of VPA on COX expression. VPA treatment has been associated with reduced arachidonic acid turnover in rats (Chang *et al.*, 2001) and attenuation of the arachidonic acid cascade as evidenced by reduced synthesis of COX-dependent products (Kis *et al.*, 1999; Szupera *et al.*, 2000). A recent study reported that chronic administration of VPA to rats reduced protein levels of COX-1 and COX-2, total COX activity, and the metabolites of arachidonic acid produced via COX (Bosetti *et al.*, 2003). These observations are not consistent with COX-dependent formation of 15-F_{2t}-IsoP, and our interpretation is that the 15-F_{2t}-IsoP levels in this study are reflective of oxidative stress and precede the onset of hepatotoxicity in

VPA-treated rats (Fig. 6). Subsequent experiments with cultured rat hepatocytes support the evidence that oxidative stress, as measured by the 15-F_{2t}-IsoP and DCF-DA assays, could occur in the absence of hepatocyte toxicity (Tong *et al.*, 2005b).

Other biomarkers of oxidative stress, LPO and TBARs, were also examined in our study in relation to the onset of hepatotoxicity. We found that levels of liver LPO were elevated after the onset of hepatotoxicity (day 7) and remained elevated with an increasing trend to day 14. Liver and serum TBARs were not elevated until day 14, which corresponded to the time point when necrosis was most prevalent. These results are consistent with previous findings that a single dose of VPA did not alter liver and plasma TBARs and liver LPO levels (Tong *et al.*, 2003). The increase in LPO and TBARs detected at time points after the first onset of hepatic necrosis may imply that necrosis occurred first, giving rise to the elevated biomarkers of lipid peroxidation. However, it may also be argued that LPO and TBARs are less sensitive markers of lipid peroxidation and the colorimetric assays are indirect and less specific as compared to the measurement of 15-F_{2t}-IsoP by EIA. 15-F_{2t}-IsoP may represent an earlier lipid peroxidation event, while MDA, which is a degradation product of polyunsaturated fatty acid hydroperoxides detected using the TBARs assay, may simply represent later lipid peroxidation events because of the necrosis. A lack of temporal correlation among reactive oxygen species biomarkers was also reported in another study that examined pulmonary artery endothelial cells treated with H_2O_2 (Hart *et al.*, 1998). Specifically, the levels of TBARs and LPO were not significantly elevated in cells treated with H_2O_2 (50–100 μM) at a time point that produced a maximal increase in 15-F_{2t}-IsoP, however; not until the highest concentration of H_2O_2 (250 μM) were increases in levels of TBARs and LPO observed. These results were ascribed to be due to differences in biomarker sensitivity.

Our study is the first to simultaneously characterize VPA metabolite profiles, hepatotoxicity, and oxidative stress events within the same time frame of VPA treatment in the whole animal. With respect to the reactive metabolite hypothesis, there was no elevation in hepatic and plasma levels of the putative VPA reactive metabolites 4-ene-VPA and (*E*)-2,4-diene-VPA. These findings are consistent with reported studies that examined VPA metabolite profiles and hepatotoxicity in patients and showed a lack of correlation between hepatotoxicity and serum levels of 4-ene-VPA (Siemes *et al.*, 1993). A similar conclusion was also reached following comparative studies of VPA and 2-ene-VPA in rats, where the incidence of liver microvesicular steatosis was observed to be independent of plasma levels of 4-ene-VPA and (*E*)-2,4-diene-VPA (Loscher *et al.*, 1993). It was therefore suggested that these metabolites were not the decisive factors in VPA-induced hepatotoxicity, whereas more recent studies indicated that urinary N-acetylcysteine conjugates of (*E*)-2,4-diene-VPA was a better indicator of reactive metabolite exposure (Gopaul *et al.*, 2000a,b). Furthermore, the β-oxidation related metabolites 2-ene-VPA, 3-ene-VPA, 3-OH-VPA, and (*E,E*)- and (*E,Z*)-2,3′-diene-VPA were decreased in the liver by days 10–14, suggesting that inhibition of β-oxidation was not apparent until after the onset of oxidative stress and hepatotoxicity. In contrast to the P450 and β-oxidation metabolites of VPA, VPA-G levels remained unchanged throughout the study period.

In summary, VPA treatment to rats for up to 14 consecutive days initiated a sequence of events whereby increases in hepatic and plasma levels of 15-F_{2t}-IsoP occurred prior to the rise in serum α-GST levels and the onset of liver steatosis and necrosis. Consistent with the present findings in rats, treatment of primary cultures of rat hepatocytes with VPA increased the levels of the oxidative stress markers, 15-F_{2t}-IsoP and 2′,7′-dichlorofluorescein levels, under conditions where the hepatocytes were devoid of toxicity (Tong *et al.*, 2005b). Collectively, our novel findings indicate that VPA does cause oxidative stress in liver and that the oxidative stress precedes the hepatotoxicity.

ACKNOWLEDGMENTS

This research was supported by the Canadian Institutes of Health Research (CIHR Grant MOP-13744 to F.S.A and T.K.H.C.). V.T. received a CIHR Doctoral Research Award. X.W.T. received an Rx&D Health Research Foundation/CIHR Postdoctoral Fellowship and a Michael Smith Foundation for Health Research Incentive Award.

REFERENCES

Bosetti, F., Weerasinghe, G. R., Rosenberger, T. A., and Rapoport, S. I. (2003). Valproic acid down-regulates the conversion of arachidonic acid to eicosanoids via cyclooxygenase-1 and–2 in rat brain. *J. Neurochem.* **85,** 690–696.

Chang, M. C., Contreras, M. A., Rosenberger, T. A., Rintala, J. J., Bell, J. M., and Rapoport, S. I. (2001). Chronic valproate treatment decreases the *in vivo* turnover of arachidonic acid in brain phospholipids: A possible common effect of mood stabilizers. *J. Neurochem.* **77,** 796–803.

Clarke, H., Egan, D. A., Heffernan, M., Doyle, S., Byrne, C., Kilty, C., and Ryan, M. P. (1997). Alpha-glutathione S-transferase (alpha-GST) release, an early indicator of carbon tetrachloride hepatotoxicity in the rat. *Hum. Exp. Toxicol.* **16,** 154–157.

Cotariu, D., Evans, S., Zaidman, J. L., and Marcus, O. (1990). Early changes in hepatic redox homeostasis following treatment with a single dose of valproic acid. *Biochem. Pharmacol.* **40,** 589–593.

Dreifuss, F. E., Santilli, N., Langer, D. H., Sweeney, K. P., Moline, K. A., and Menander, K. B. (1987). Valproic acid hepatic fatalities: A retrospective review. *Neurology* **37,** 379–385.

Eadie, M. J., Hooper, W. D., and Dickinson, R. G. (1988). Valproate-associated hepatotoxicity and its biochemical mechanisms. *Med. Toxicol. Adverse Drug Exp.* **3,** 85–106.

Farrell, K., and Abbott, F. S. (1991). Role of N-acetylcysteine in the treatment of valproate hepatotoxicity. In *Idiosyncratic Reactions to Valproate: Clinical Risk Patterns and Mechanisms of Toxicity* (R. H. Levy and J. K. Penry, Eds.), pp. 149–153. Raven Press, New York.

Gerber, N., Dickinson, R. G., Harland, R. C., Lynn, R. K., Houghton, L. D., Antonias, J. I., and Schimschock, J. C. (1979). Reye-like syndrome associated with valproic acid therapy. *J. Pediatr.* **95,** 142–144.

Gopaul, S. V., Farrell, K., and Abbott, F. S. (2000a). Gas chromatography/negative ion chemical ionization mass spectrometry and liquid chromatography/electrospray ionization tandem mass spectrometry quantitative profiling of

N-acetylcysteine conjugates of valproic acid in urine: Application in drug metabolism studies in humans. *J. Mass Spectrom.* **35,** 698–704.

Gopaul, S. V., Farrell, K., and Abbott, F. S. (2000b). Identification and characterization of N-acetylcysteine conjugates of valproic acid in humans and animals. *Drug Metab. Dispos.* **28,** 823–832.

Graf, W. D., Oleinik, O. E., Glauser, T. A., Maertens, P., Eder, D. N., and Pippenger, C. E. (1998). Altered antioxidant enzyme activities in children with a serious adverse experience related to valproic acid therapy. *Neuropediatrics* **29,** 195–201.

Granneman, G. R., Wang, S. I., Kesterson, J. W., and Machinist, J. M. (1984). The hepatotoxicity of valproic acid and its metabolites in rats. II. Intermediary and valproic acid metabolism. *Hepatology* **4,** 1153–1158.

Hart, C. M., Karman, R. J., Blackburn, T. L., Gupta, M. P., Garcia, J. G., and Mohler, E. R., 3rd (1998). Role of 8-epi PGF2alpha, 8-isoprostane, in H_2O_2-induced derangements of pulmonary artery endothelial cell barrier function. *Prostaglandins Leukot. Essent. Fatty Acids* **58,** 9–16.

Hurd, R. W., Van Rinsvelt, H. A., Wilder, B. J., Karas, B., Maenhaut, W., and De Reu, L. (1984). Selenium, zinc, and copper changes with valproic acid: Possible relation to drug side effects. *Neurology* **34,** 1393–1395.

Jezequel, A. M., Bonazzi, P., Novelli, G., Venturini, C., and Orlandi, F. (1984). Early structural and functional changes in liver of rats treated with a single dose of valproic acid. *Hepatology* **4,** 1159–1166.

Jurima-Romet, M., Abbott, F. S., Tang, W., Huang, H. S., and Whitehouse, L. W. (1996). Cytotoxicity of unsaturated metabolites of valproic acid and protection by vitamins C and E in glutathione-depleted rat hepatocytes. *Toxicology* **112,** 69–85.

Kassahun, K., and Abbott, F. (1993). *In vivo* formation of the thiol conjugates of reactive metabolites of 4-ene VPA and its analog 4-pentenoic acid. *Drug Metab. Dispos.* **21,** 1098–1106.

Kesterson, J. W., Granneman, G. R., and Machinist, J. M. (1984). The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. *Hepatology* **4,** 1143–1152.

Kis, B., Szupera, Z., Mezei, Z., Gecse, A., Telegdy, G., and Vecsei, L. (1999). Valproate treatment and platelet function: The role of arachidonate metabolites. *Epilepsia* **40,** 307–310.

Lewis, J. H., Zimmerman, H. J., Garrett, C. T., and Rosenberg, E. (1982). Valproate-induced hepatic steatogenesis in rats. *Hepatology* **2,** 870–873.

Loscher, W., Nau, H., Wahnschaffe, U., Honack, D., Rundfeldt, C., Wittfoht, W., and Bojic, U. (1993). Effects of valproate and E-2-en-valproate on functional and morphological parameters of rat liver. II. Influence of phenobarbital comedication. *Epilepsy Res.* **15,** 113–131.

Nourooz-Zadeh, J., Gopaul, N. K., Barrow, S., Mallet, A. I., and Anggard, E. E. (1995). Analysis of F2-isoprostanes as indicators of non-enzymatic lipid peroxidation *in vivo* by gas chromatography-mass spectrometry: Development of a solid-phase extraction procedure. *J. Chromatogr. B Biomed. Appl.* **667,** 199–208.

Olson, M. J., Handler, J. A., and Thurman, R. G. (1986). Mechanism of zone-specific hepatic steatosis caused by valproate: Inhibition of ketogenesis in periportal regions of the liver lobule. *Mol. Pharmacol.* **30,** 520–525.

Powell-Jackson, P. R., Tredger, J. M., and Williams, R. (1984). Hepatotoxicity to sodium valproate: A review. *Gut* **25,** 673–681.

Roberts, L. J., and Morrow, J. D. (2000). Measurement of F(2)-isoprostanes as an index of oxidative stress *in vivo*. *Free Radic. Biol. Med.* **28,** 505–513.

Seckin, S., Basaran-Kucukgergin, C., and Uysal, M. (1999). Effect of acute and chronic administration of sodium valproate on lipid peroxidation and antioxidant system in rat liver. *Pharmacol. Toxicol.* **85,** 294–298.

Siemes, H., Nau, H., Schultze, K., Wittfoht, W., Drews, E., Penzien, J., and Seidel, U. (1993). Valproate (VPA) metabolites in various clinical conditions of probable VPA-associated hepatotoxicity. *Epilepsia* **34,** 332–346.

Sugimoto, T., Woo, M., Nishida, N., Takeuchi, T., Sakane, Y., and Kobayashi, Y. (1987). Hepatotoxicity in rat following administration of valproic acid. *Epilepsia* **28,** 142–146.

Sussman, N. M., and McLain, L. W. J. (1979). A direct hepatotoxic effect of valproic acid. *JAMA* **242,** 1173–1174.

Szupera, Z., Mezei, Z., Kis, B., Gecse, A., Vecsei, L., and Telegdy, G. (2000). The effects of valproate on the arachidonic acid metabolism of rat brain microvessels and of platelets. *Eur. J. Pharmacol.* **387,** 205–210.

Tabatabaei, A. R., Thies, R. L., and Abbott, F. S. (1999). Assessing the mechanism of metabolism-dependent valproic acid-induced *in vitro* cytotoxicity. *Chem. Res. Toxicol.* **12,** 323–330.

Tang, W., Borel, A. G., Fujimiya, T., and Abbott, F. S. (1995). Fluorinated analogues as mechanistic probes in valproic acid hepatotoxicity: Hepatic microvesicular steatosis and glutathione status. *Chem. Res. Toxicol.* **8,** 671–682.

Tong, V., Chang, T. K. H., Chen, J., and Abbott, F. S. (2003). The effect of valproic acid on hepatic and plasma levels of 15-F(2t)-isoprostane in rats. *Free Radic. Biol. Med.* **34,** 1435–1446.

Tong, V., Teng, X. W., Chang, T. K. H., and Abbott, F. S. (2005a). Valproic acid glucuronidation is associated with increases in 15-F(2t)-isoprostane in rats. *Free Radic. Biol. Med.* **38,** 1471–1483.

Tong, V., Teng, X. W., Chang, T. K. H., and Abbott, F. S. (2005b). Valproic acid II: Effects on oxidative stress, mitochondrial membrane potential, and cytotoxicity in glutathione-depleted rat hepatocytes. *Toxicol. Sci.* (accepted).

Trull, A. K., Facey, S. P., Rees, G. W., Wight, D. G., Noble-Jamieson, G., Joughin, C., Friend, P. J., and Alexander, G. J. (1994). Serum alpha-glutathione S-transferase–a sensitive marker of hepatocellular damage associated with acute liver allograft rejection. *Transplantation* **58,** 1345–1351.

van Wagensveld, B. A., Scheepers, J. J., van Gulik, T. M., Frederiks, W. M., Bleeker, W. K., Obertop, H., and Gouma, D. J. (1997). Alpha glutathione S-transferase as novel parameter for hepatocellular damage in the isolated perfused rat liver. *Transplant Proc.* **29,** 3449–3451.

Zimmerman, H. J., and Ishak, K. G. (1982). Valproate-induced hepatic injury: Analyses of 23 fatal cases. *Hepatology* **2,** 591–597.