INVESTIGATION OF VALPROIC ACID-ASSOCIATED OXIDATIVE STRESS AND HEPATOTOXICITY

by

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ABSTRACT

A serious adverse reaction of valproic acid (VPA), a widely used anti-epileptic, is a rare but potentially fatal hepatotoxicity affecting mostly children under 2 years of age with developmental delay and who are on VPA polytherapy. The mechanism of the hepatotoxicity remains unknown. This thesis describes *in vivo* and *in vitro* studies to examine the hypothesis that VPA increases oxidative stress in rats that in turn is associated with liver toxicity. 15-F_{2t}-Isoprostaglandin (15-F_{2t}-IsoP), a free-radical catalyzed, lipid peroxidation breakdown product of arachidonic acid, was measured as an indicator of oxidative stress. Our findings showed a dose-dependent elevation in plasma and liver 15-F_{2t}-IsoP with plasma concentration-time profiles similar to that of the drug in rats given a single high dose of VPA. This increase in 15-F_{2t}-IsoP did not involve cytochrome P450-mediated biotransformation even though rats treated with both phenobarbital (PB) and VPA resulted in greater levels of 15-F_{2t}-IsoP compared to rats treated with only VPA. These results prompted further mechanistic studies in rats to establish an association between VPA glucuronidation and 15-F_{2t}-IsoP levels. VPA glucuronidation, the major VPA biotransformation pathway, correlated with 15-F_{2t}-IsoP formation. Levels of both 15-F_{2t}-IsoP and VPA-1-*O*-acyl glucuronide (VPA-G) were elevated by PB and reduced by inhibitors of VPA-glucuronidation in rats after a single dose of VPA. The fluorinated analogue of VPA (α-fluoro-VPA), which was a poor substrate for glucuronidation, did not elevate 15-F_{2t}-IsoP levels.

To determine whether there was a temporal relationship between VPA-associated oxidative stress and hepatotoxicity, rats were given high daily doses of VPA for 14 days. VPA elevated levels of 15-F_{2t}-IsoP prior to the onset of hepatic necrosis and steatosis. An *in vitro* model using primary cultured rat hepatocytes also demonstrated that VPA induces oxidative stress as measured by elevated levels of 15-F_{2t}-IsoP and 2′,7′-dichlorofluorescein (DCF). In hepatocytes with reduced levels of glutathione, these oxidative stress biomarkers were further elevated, and

were accompanied by mitochondrial dysfunction and hepatocyte toxicity. The work presented is significant in that it supports the hypothesis that VPA-associated oxidative stress occurs prior to hepatotoxicity and the link of acyl glucuronidation of VPA to the production of reactive oxygen species is unique.

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ABBREVIATIONS AND SYMBOLS

$\Delta\Psi_m$	Mitochondrial membrane potential
°C	Degrees celsius
(*E*)-2,4-diene VPA	(*E*)-2-propyl-2,4-pentadienoic acid
(*E*)-2-ene VPA	(*E*)-2-propyl-2-pentenoic acid
(*E,E*)-2,3′-diene VPA	(*E,E*)-2-propyl-2,3′-pentadienoic acid
(*E,Z*)-2,3′-diene VPA	(*E,Z*)-2-propyl-2,3′-pentadienoic acid
α-F-4-ene-VPA	2-fluoro-2-propyl-4-pentenoic acid
α-fluoro-VPA (α-F-VPA)	2-fluoro-2-propylpentanoic acid
μl	Microliter
15-F_{2t}-IsoP	15-F_{2t}-Isoprostane
2,3-DHBA	2,3-dihydroxybenzoic acid
2-PGA	2-propylglutaric acid
2-PSA	2-propylsuccinic acid
3-ene-VPA	2-propyl-3-pentenoic acid
3-keto-VPA	2-propyl-3-oxopentanoic acid
3-OH-VPA	2-propyl-3-hydroxypentanoic acid
4-ene-VPA	2-propyl-4-pentenoic acid
4-keto-VPA	2-propyl-4-oxopentanoic acid
4-OH-VPA	2-propyl-4-hydroxypentanoic acid
5-OH-VPA	2-propyl-5-hydroxypentanoic acid
ABT	1-Aminobenzotriazole
amu	Atomic mass unit
ANOVA	Analysis of variance
BSO	Buthionine sulfoximine
CAT	Catalase
CCl_4	Carbon tetrachloride
C_{max}	Maximal plasma concentration
CoA	Coenzyme-A
CV	Coefficient of variation
DCF	2′,7′-Dichlorofluorescein
DCF-DA	2′,7′-Dichlorofluorescin diacetate

DCFH	2′,7′-Dichlorofluorescin
DEM	Diethylmaleate
DPPD	N,N′-diphenyl-p-phenylenediamine
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme linked immunoassay
ESP+	Positive electrospray
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
g	Gram
GABA	γ-aminobutyric acid
GC	Gas chromatography
GC/MS	Gas chromatography-mass spectrometry
GSH	Glutathione, reduced
GSH-reductase	Glutathione reductase
GSSG	Glutathione, oxidized
GST	Glutathione S-transferases
GST-Px	Glutathione peroxidase
H&E	Hematoxylin-eosin
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
$HO^{\cdot}$	Hydroxyl radical
HPLC	High pressure liquid chromatography
I.D.	Internal diameter
ip	Intraperitoneal
kg	Kilogram
kV	Kilovolts
LC/MS	Liquid chromatography-mass spectrometry
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LOQ	Limit of quantitation
LPO	Lipid hydroperoxides
mCLCCP	carbonyl cyanide m-chlorophenylhydrazone
MDA	Malondialdehyde
mg	Milligram

min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
MTBSTFA	N-(*tert.*-butyldimethylsilyl)-N-methyltrifluoroacetamide
NADPH	Nicotine adenine dinucleotide
NaOH	Sodium hydroxide
ND	Not detected
ng	Nanogram
NCI	Negative ion chemical ionization
nmol	Nanomole
NMR	Nuclear magnetic resonance
P450	Cytochrome P-450
PA	4-pentenoic acid
PB	Phenobarbital, sodium
PBS	Phosphate-buffered saline
PFBBr	Pentafluorylbenzyl bromide
pg	Picogram
PSI	Pounds per square inch
QC	Quality control
r^2	Coefficient of determination
ROS	Reactive oxygen species
SEM	Standard error of means
SIM	Single ion monitoring
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBDMS	*Tert.*-butyl-dimethylsilyl chloride
TMA	Trimethylacetic acid
t_{max}	Time of occurrence of maximal plasma concentration
UDPGA	UDP-Glucuronic acid
UGT	UDP-Glucuronosyltransferase
V	Volts
VPA	Valproic acid
VPA-G	Valproyl-1-O-acyl glucuronide

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DEDICATION

This thesis is dedicated to my family:
Mom, Dad, Vivian, Gloria, Chuck, and Baba.

1 *Introduction*

1.1 VALPROIC ACID

Valproic acid (2-n-propyl-pentanoic acid, VPA, Figure 1-1) is an 8 carbon, short-chain, branched fatty acid that was serendipitously discovered to demonstrate anticonvulsant properties when it was used as a drug vehicle for other anticonvulsant test compounds (Meunier *et al.* 1963). Subsequently, valproic acid (VPA) was introduced into clinical studies in France in 1967 and was approved by the Food and Drug Administration in 1978 (Loscher 1999a). VPA is available for use as the free acid, its sodium salt, and as a therapeutic combination of the parent compound and its sodium salt in a 2:1 molar ratio (divalproex sodium) (Davis *et al.* 1994).

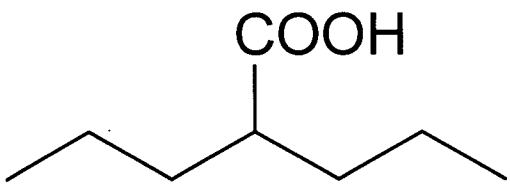

Figure 1-1: Structure of VPA.

1.2 THERAPEUTIC USE AND MECHANISM OF ACTION

Valproic acid is a broad-spectrum antiepileptic drug requiring relatively high-doses (25-60 mg/kg/day) for the treatment of several epileptic seizure types including absence, myoclonic, and partial and generalized tonic-clonic seizures (Loscher 1999b; Sarisjulius and Dulac 1999; Willmore 1999). More recently, the use of VPA has been therapeutically extended for the management of bipolar disorder (Lennkh and Simhandl 2000; Post *et al.* 1996) and migraine (Silberstein and Wilmore 1996). The mechanism of the antiepileptic action of VPA is not well understood; however, it is most likely related to its ability to potentiate the effects of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), by either increasing its production or inhibiting its degradation. VPA has been implicated in the activation of glutamic acid decarboxylase, an enzyme involved in the activation of glutamate which is required for GABA synthesis (Loscher 1989; Loscher and Schmidt 1981). In addition, it appears to inhibit GABA-transaminase and succinic semialdehyde dehydrogenase, two enzymes involved in the successive degradation of GABA (Loscher *et al.* 1985; van der Laan *et al.* 1979; Zeise *et al.* 1991). In another proposed mechanism, VPA appears to have a non-specific membrane stabilizing effect by reducing high frequency repetitive firing of neurons through action at sodium and/or potassium channels (McLean and Macdonald 1986; Slater and Johnston 1978). None of the mechanisms alone can adequately explain VPA's broad-spectrum anticonvulsant effects; thus, it is likely that multiple mechanisms may be involved.

1.3 PHARMACOKINETICS

The gastrointestinal absorption of VPA is rapid and complete in humans with > 90% bioavailability from all available formulations (Shen 1999). The high bioavailability stems from the fact that it readily crosses the intestinal mucosa with no site specificity (Levy and Shen 1995). Plasma peak concentrations are dependent on formulation and are achieved within 1-3

h for immediate release preparations and 3-8 h for sustained-release formulations (Davis *et al.* 1994). VPA is characterized by a relatively low volume of distribution that ranges from (0.1-0.2 L/kg in adults, and 0.15-0.4 L/kg in neonates, infants, and children (Cloyd *et al.* 1993; Gugler and von Unruh 1980; Hall *et al.* 1983; Herngren *et al.* 1991; Herngren and Nergardh 1988; Irvine-Meek *et al.* 1982). The low volume of distribution is attributed to the compound's extensive and saturable (nonlinear) plasma protein binding (~90% at therapeutic concentrations) and its high degree of ionization at physiological pH (pKa 4.8), which act to confine VPA largely to the vascular space and extracellular fluids (Levy and Shen 1995).

VPA is not subject to significant first pass effect and is a low clearance compound (unbound plasma clearance ranging from 1-3 ml/min/kg) with elimination half-lives ranging from 9-18 h (Davis *et al.* 1994; Rettenmeier *et al.* 1987; Zaccara *et al.* 1988). The clearance is dose-dependent with nonlinearities resulting from saturable protein binding and/or saturable metabolism (Bowdle *et al.* 1980). Elimination of VPA is primarily via hepatic metabolism with only 1 to 3% of the dose excreted as the parent compound in urine (Bowdle *et al.* 1980; Dickinson *et al.* 1989; Gugler and von Unruh 1980).

1.4 METABOLISM

Although the structure of VPA is relatively simple, its metabolic fate is extremely complex with approximately 50 metabolites identified, of which 16 are consistently observed in humans (Figure 1-2) (Abbott and Anari 1999; Baillie and Sheffels 1995). VPA is primarily conjugated to glucuronic acid with approximately 10-70% of the dose recovered in human urine (Dickinson *et al.* 1989; Levy *et al.* 1990) as the 1-*O*-acyl-β-D-ester linked glucuronide (Dickinson *et al.* 1979). VPA also competes with endogenous fatty acids for mitochondrial and peroxisomal β-oxidation resulting in the formation of the metabolites (*E*)-2-propyl-2-pentenoic acid ((*E*)-2-ene-VPA), 2-

propyl-3-pentenoic acid (3-ene-VPA), 2-propyl-3-oxopentanoic acid (3-keto-VPA) (Abbott and Anari 1999; Baillie and Sheffels 1995; Bjorge and Baillie 1991; Granneman *et al.* 1984b; Ponchaut *et al.* 1992). To a lesser extent, VPA undergoes P450-dependent ω and ω-1 oxidation to form 2-propyl-5-hydroxypentanoic acid (5-OH-VPA) and 2-propyl-4-hydroxypentanoic acid (4-OH-VPA), respectively (Rettenmeier *et al.* 1987). The formation of 3-OH-VPA is also formed via direct cytochrome P-450-mediated microsomal hydroxylation of the parent compound (Rettenmeier *et al.* 1987). Further oxidation of 5-OH-VPA results in the dicarboxylic acid, 2-propylglutaric acid (2-PGA). Oxidation of 4-OH-VPA leads to the formation of 2-propyl-4-oxopentanoic acid (4-keto-VPA) and 2-propylsuccinic acid (2-PSA) (Granneman *et al.* 1984b). The mono–unsaturated metabolite 2-propyl-4-pentenoic acid (4-ene-VPA) is catalyzed by P450 enzymes from a distinct carbon-centre radical after hydrogen abstraction (Rettie *et al.* 1987). Subsequent β-oxidation of 4-ene-VPA results in the di-unsaturated (*E*)-2,4-diene-VPA, both which have been implicated in the pathogenesis of hepatotoxicity by VPA (Gerber *et al.* 1979; Kesterson *et al.* 1984; Lewis *et al.* 1982; Zimmerman and Ishak 1982).

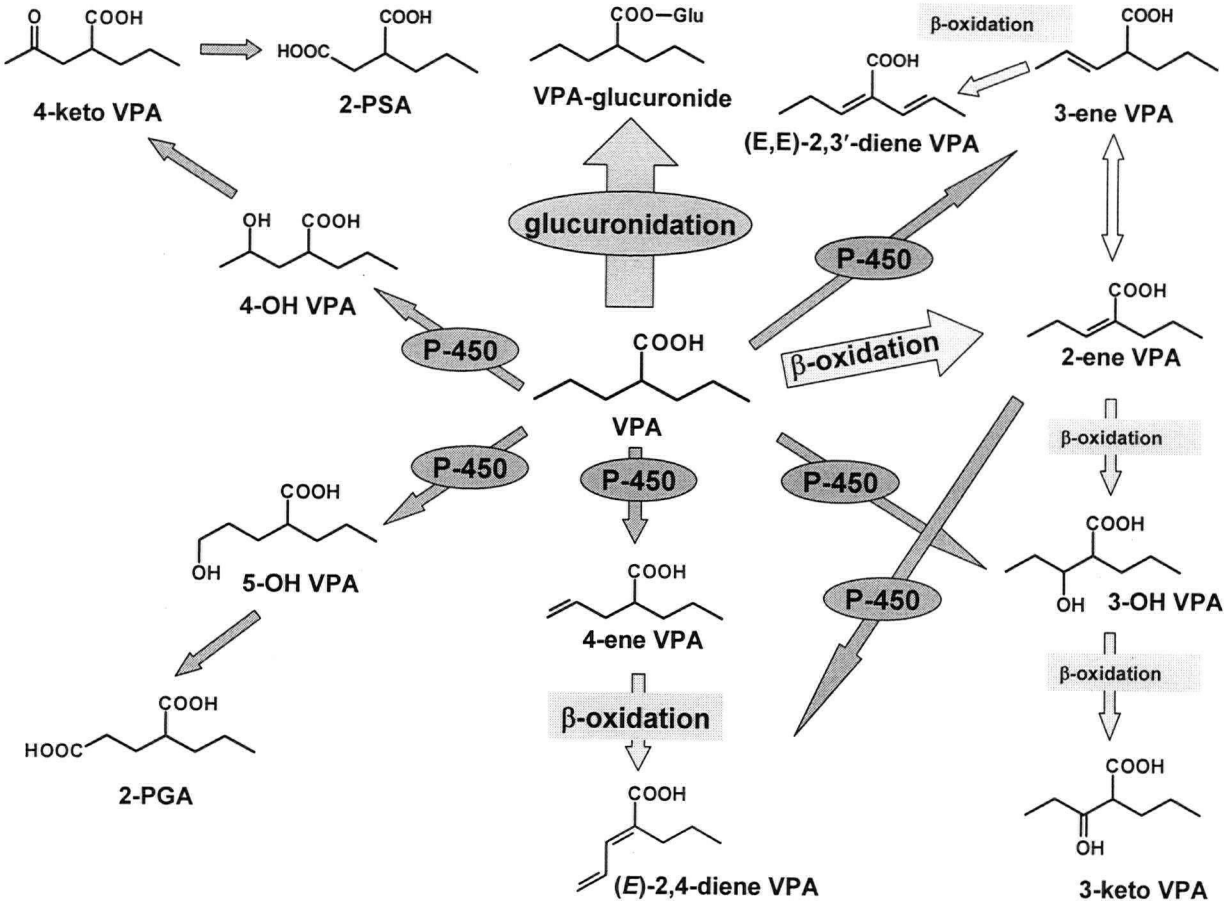

Figure 1-2: Summary of the metabolic pathways (P450, β-oxidation, and glucuronidation) for frequently observed VPA metabolites in human (Abbott and Anari 1999).

1.5 VPA HEPATOTOXICITY

The hepatotoxicity induced by VPA is considered to fall into two categories (Dreifuss *et al.* 1989; Dreifuss *et al.* 1987). One is the reversible hepatic dysfunction associated with VPA therapy (Itoh *et al.* 1982; Mathis *et al.* 1979; Powell-Jackson *et al.* 1984; Thygesen and Boesen 1982; Ware and Millward-Sadler 1980; Willmore *et al.* 1978) that is characterized by low plasma fibrinogen concentrations and dose-related elevation in liver enzymes in blood (Sussman and McLain 1979). In most cases, the above abnormalities are reversed upon dosage reduction or discontinuation of VPA therapy. The most serious VPA side effect reported in man is irreversible and potentially fatal hepatic damage that is described as being unpredictable, rare, and dose-independent. The first cases of fatal hepatotoxicity were reported in 1979 (Gerber *et al.* 1979; Mathis *et al.* 1979; Suchy *et al.* 1979); since then, over 100 cases have been reported (Bryant and Dreifuss 1996; Dreifuss *et al.* 1989; Dreifuss *et al.* 1987; Itoh *et al.* 1982; Scheffner *et al.* 1988; Zimmerman and Ishak 1982). The primary risk factors of fatal hepatotoxicity associated with VPA therapy were found to be co-administration of other anti-epileptics such as phenytoin or phenobarbital, as well as young age (children less than 2 years old) (Dreifuss *et al.* 1987). Between the years 1978-1984, the hepatic fatality rate of patients on VPA monotherapy under 2 years of age was 1/7000 (Dreifuss *et al.* 1987). This rate decreased to 1/45000 in patients that were older than 2 years of age (Dreifuss *et al.* 1987). With polytherapy, the fatality rate increased to 1/500 for patients less than 2 years old and 1/12000 for those older than 2 years (Dreifuss *et al.* 1987). Patients in the highest-risk group typically had severe epilepsy and other medical conditions including mental retardation, developmental delay, congenital anomalies, and other neurological diseases (Appleton *et al.* 1990; Dreifuss *et al.* 1987). The prescribing patterns for VPA have changed since the high risk population was identified and a follow-up retrospective study between the years 1985-1986 reported a decreased incidence (≈

5- fold) of hepatic fatalities despite a significant increase in the overall use of VPA (Dreifuss *et al.* 1989).

The common clinical symptoms associated with VPA hepatotoxicity are nausea, vomiting, anorexia, lethargy, edema, fever, hypoglycemia, hemorrhage, jaundice, and finally hyperammonemic coma and death (Cotariu and Zaidman 1988). Jeavons reviewed 67 cases of VPA-associated hepatotoxicity and reported the occurrence of hepatotoxicity within 3 months of the onset of VPA treatment in 75% of the cases (Jeavons 1984). Liver biopsy revealed microvesicular steatosis, a condition characterized by the accumulation of numerous small lipid vesicles in the hepatocyte (Fromenty *et al.* 1997), and often accompanied by centrizonal necrosis (Jeavons 1984; Zimmerman and Ishak 1982). In the 67 cases reviewed, microvesicular steatosis accompanied by necrosis was found in 22 cases, necrosis alone in 20 cases, and steatosis alone in 8 cases (Jeavons 1984). Electron-microscopy of liver biopsies revealed a pattern of primary parenchymal-organelle injury characterized by the following ultrastuctural changes: enlarged and densely packed mitochondria (megamitochondria), large mitochondrial granules in the matrix, altered cristae with crystalline inclusions, and disorganized rough endoplasmic reticulum (Itoh *et al.* 1982; Mathis *et al.* 1979). In rats, high doses of VPA were shown to cause hepatic steatosis (Kesterson *et al.* 1984; Lewis *et al.* 1982; Sobaniec-Lotowska *et al.* 1993) and to result in swollen and ruptured mitochondria (Jezequel *et al.* 1984).

1.5.1 VPA-induced metabolic and biochemical disturbances

The pathogenesis of severe VPA hepatotoxicity is not clear, but the observation of microvesicular steatosis is consistent with a disturbance in mitochondrial function and/ or fatty acid metabolism (Fromenty *et al.* 1997; Fromenty and Pessayre 1997; Powell-Jackson *et al.* 1984). Investigations have centred on mitochondrial dysfunction as a likely explanation for valproate-induced toxicity. The histological data appear to suggest that the fatal liver failure due

to VPA resembled Reye's syndrome, a condition for which a mitochondrial origin had been established (Mitchell *et al.* 1980; Partin *et al.* 1971), and is associated with inhibition of the mitochondrial β-oxidation system (Jezequel *et al.* 1984; Mortensen 1980). This was evident in early metabolic studies of VPA in humans in which the urinary excretion of C6-C10 dicarboxylic acids, indicative of impaired hepatic fatty acid β-oxidation, was significantly increased after a dose of VPA (Mortensen *et al.* 1980). The lipid content of liver increased within 2-4 h after VPA administration to fasted rats, mainly in the periportal lobular regions (Jezequel *et al.* 1984; Olson *et al.* 1986). VPA also inhibited ketogenesis in the same regions of the lobule in the perfused rat liver (Olson *et al.* 1986) and in isolated rat hepatocytes (Coude *et al.* 1983). As a further indication of impaired fatty acid metabolism, the β-oxidation of palmitic acid (a long chain fatty acid) in isolated rat hepatocytes (Coude *et al.* 1983) and of decanoic acid (a medium chain fatty acid) in rat liver homogenate (Bjorge and Baillie 1985) were inhibited by VPA. Several major hypotheses have developed to rationalize the observed inhibition of mitochondrial β-oxidation by VPA and the resultant hepatotoxicity. Some of these include: I) drug – associated coenzyme-A deficiency; II) carnitine deficiency; III) reactive toxic metabolites of VPA, and IV) a combination of increased oxidative stress and reduced free radical scavenger activity.

1.5.2 Coenzyme A depletion hypothesis

A major hypothesis developed to explain the inhibition of mitochondrial β-oxidation and the associated hepatotoxicity was the sequestering of coenzyme A (CoA) by VPA as valproyl-CoA (Kesterson *et al.* 1984; Thurston *et al.* 1983). The transport and subsequent β-oxidation of fatty acids require prior activation to CoA thioesters, which is catalyzed by the ATP-dependent acyl CoA synthetase located on the outer mitochondrial membrane. Thus, at the high dose of VPA employed, depletion of the intracellular free CoA pool due to the conversion of VPA to its acyl-CoA thioester may impair mitochondrial β-oxidation (Brass 1994). In rat mitochondrial

incubations, VPA was converted to the corresponding CoA thioester, and in addition, the following CoA esters of the β-oxidation metabolites were identified: 2-ene-VPA, 3-OH-VPA, and 3-keto-VPA (Li *et al.* 1991). Valproyl-CoA was poorly hydrolyzed in liver preparations (Moore *et al.* 1988) and 3-keto-VPA-CoA appeared to resist cleavage by 3-ketoacyl-CoA thiolase (Li *et al.* 1991), resulting in the sequestering of CoA. The resulting depletion of the free CoA pool in the mitochondrial matrix was considered a major contributing factor for VPA hepatotoxicity (Harris *et al.* 1991). In support of this hypothesis, incubation of VPA with isolated rat hepatocytes resulted in dose-dependent decreases in free concentrations of CoA, and this was accompanied by an inhibition of the oxidation of long- and medium-chain fatty acids (Becker and Harris 1983; Turnbull *et al.* 1983). However, it may be argued that the depletion of CoA should be VPA dose-dependent, and yet the hepatotoxicity appears to be idiosyncratic in nature (dose-independent and rare).

1.5.3 Carnitine deficiency hypothesis

Carnitine deficiency has been associated with Reye's syndrome (Glasgow *et al.* 1980) and was postulated to be involved in VPA-associated impairment of fatty acid metabolism (Coulter 1984). L-carnitine (L-β-hydroxy-gamma-nitro-aminobutyric acid) is an essential cofactor in the β-oxidation pathway by facilitating transport of fatty acyl-CoA from the mitochondrial intermembrane space across the inner mitochondrial membrane to the matrix (Coulter 1991). Prior to transport across the inner mitochondrial membrane, fatty acyl-CoA is esterified into fatty acyl-carnitine via carnitine palmitoyltransferase I. The transported fatty acyl-carnitine is subsequently converted back to the fatty acyl-CoA by carnitine palmitoyltransferase II (located on the matrix side of the inner mitochondrial membrane) for subsequent β-oxidation reactions in the matrix. Treatment of VPA has been associated with decreased levels of serum carnitine (Bohles *et al.* 1982; Laub *et al.* 1986; Murphy *et al.* 1985; Ohtani *et al.* 1982). Another study

revealed an increased acylcarnitine/ free carnitine ratio that was accompanied by detectable levels of acetylcarnitine and valproylcarnitine in the urine of patients on VPA therapy (Millington *et al.* 1985). Thus, patients on VPA therapy with inborn errors of carnitine metabolism would theoretically be predisposed to idiosyncratic VPA-hepatotoxicity. Although carnitine supplementation has been recommended in the pediatric population on VPA therapy (Raskind and El-Chaar 2000), the overall significance of VPA-induced carnitine deficiency in causing impairment of β-oxidation of fatty acids is not clear.

1.5.4 Reactive metabolite hypothesis

It has been postulated that VPA hepatotoxicity is mediated by one or more of its metabolites (Kesterson *et al.* 1984; Lewis *et al.* 1982; Zimmerman and Ishak 1982). The metabolic pathways of VPA leading to reactive metabolites and their glutathione conjugates are outlined in Figure 1-3. The similarity of VPA-induced hepatotoxicity to Reye's syndrome and Jamaican Vomiting Sickness (microvesicular steatosis due to inhibition of β-oxidation, hypoglycemia due to impaired gluconeogenesis, hyperammonemia due to interference with the urea cycle) led researchers to postulate that 4-ene-VPA was responsible for the liver injury because the metabolite was structurally similar to two known hepatotoxicants, i.e. methylene-cyclopropylacetic acid (a metabolite of hypoglycin) and 4-pentenoic acid (Gerber *et al.* 1979; Zimmerman and Ishak 1982). VPA undergoes desaturation to 4-ene-VPA in rat liver microsomes catalyzed by P450 (Rettie *et al.* 1988; Rettie *et al.* 1987), and more specifically by the recombinant human P450's CYP2A6, CYP2C9 (Ho *et al.* 2003; Sadeque *et al.* 1997) and CYP2B6 (Anari *et al.* 2000). Overall, the pathway is relatively minor, but it has been shown that the metabolic flux through the 4-ene-VPA pathway is elevated by co-administration of P450 inducers (phenytoin, PB, or carbamazepine), a known risk factor for VPA-induced hepatotoxicity (Cotariu and Zaidman 1988; Dreifuss *et al.* 1987; Levy *et al.* 1990; Lewis *et al.* 1982;

Zimmerman and Ishak 1982). Further investigations demonstrated that microvesicular steatosis could be reproduced by chronic administration of 4-ene-VPA and (*E*)-2,4-diene-VPA, an intermediate in the β-oxidation pathway of 4-ene-VPA, to rats (100 mg/kg for 5 days) (Granneman *et al.* 1984a; Kesterson *et al.* 1984; Lewis *et al.* 1982). When tested *in vitro*, 4-ene-VPA demonstrated the greatest toxicity to rat hepatocyte cultures compared to 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, and 2-PGA (Kingsley *et al.* 1983; Kingsley *et al.* 1980) and was an effective inhibitor of mitochondrial β-oxidation in rat liver preparations (Bjorge and Baillie 1985; Ponchaut *et al.* 1992). Radiolabelled 4-ene-VPA was seen to bind covalently to rat liver proteins (Porubek *et al.* 1989) and P450 enzymes (Prickett and Baillie 1986). A proposed mechanism of 4-ene-VPA hepatotoxicity suggests that the mitochondrial biotransformation of 4-ene-VPA to (*E*)-2,4-diene-VPA will lead to the formation of 3-keto-4-ene-VPA (Baillie 1988). Based on the evidence that the β-oxidation of 4-pentenoic acid produces the highly reactive electrophile, 3-keto-4-pentenoic acid, that irreversibly inhibits 3-ketoacyl-CoA thiolase (Schulz 1983), 3-keto-4-ene-VPA was suspected to alkylate and thereby irreversibly inhibit β-oxidative enzyme(s) in a similar manner (Baillie 1988). Although 3-keto-4-ene-VPA was not directly identified, there exist several lines of indirect evidence for the existence of this putative metabolite. Consistent with this mechanism, the activity of acetoacetyl-CoA thiolase was observed to be irreversibly inhibited in rat hepatocyte cultures treated with 4-ene-VPA (Porubek *et al.* 1991). Two precursors of 3-keto-4-ene-VPA, i.e. (*E*)-2,4-diene-VPA and 3-OH-4-ene-VPA were identified using perfused rat livers (Rettenmeier *et al.* 1985) and 3-keto-4-ene-VPA was indirectly identified in very low amounts as its GSH conjugate, 5-GS-3-keto-VPA (Kassahun *et al.* 1994).

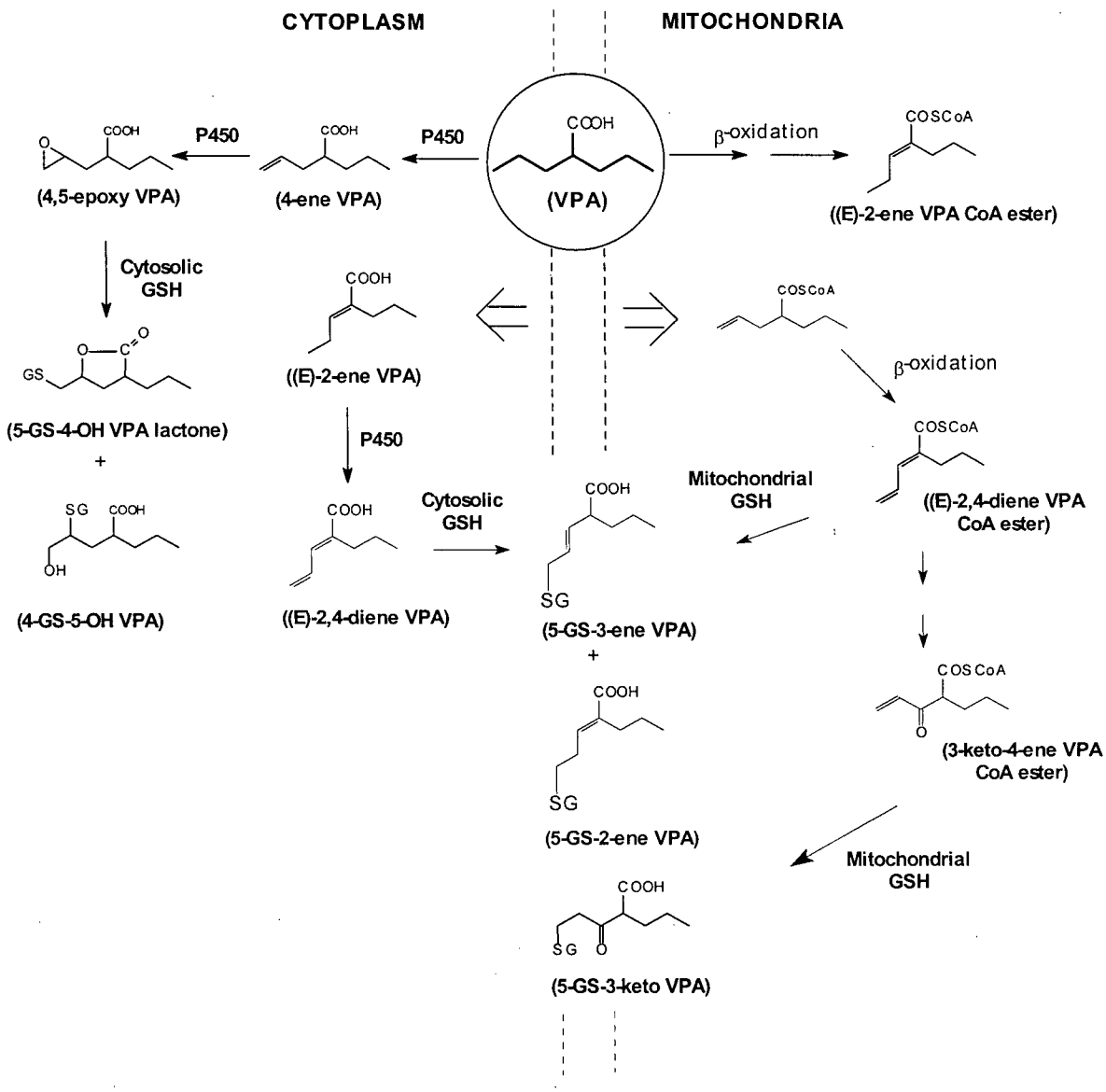

Figure 1-3: Metabolic pathways of VPA leading to reactive metabolites and their GSH conjugates (Tang *et al.* 1995). Subsequently, the GSH conjugates undergo further mercapturic acid metabolism to produce the respective N-acetylcysteine (NAC) conjugates. The NAC conjugates of 3-heptanone (the suspected decarboxylated product of 5-GS-3-keto-VPA), 5-GS-4-OH-VPA γ-lactone and 4-GS-5-OH-VPA have been identified in rat urine of 4-ene-VPA treated rats only, and not in 4-ene-VPA treated guinea pigs nor in patients on VPA therapy (Gopaul *et al.* 2000b).

Further evidence of chemically reactive intermediates of 4-ene-VPA was provided by the detection of GSH and N-acetylcysteine conjugates of (*E*)-2,4-diene-VPA in the bile and urine, respectively, of rats dosed with 4-ene-VPA or (*E*)-2,4-diene-VPA (Kassahun *et al.* 1991). The major metabolite of 4-ene-VPA was determined to be (*E*)-2,4-diene-VPA and the GSH conjugation of this reactive metabolite was believed to occur in the mitochondria (Kassahun *et al.* 1991; Kassahun *et al.* 1994). A number of other GSH conjugates were identified in the bile of rats treated with 4-ene-VPA: 5-GS-3-keto-VPA from the conjugation of GSH with 3-keto-4-ene-VPA; 5-GS-3-ene-VPA from the conjugation of GSH with (*E*)-2,4-diene (Kassahun *et al.* 1991; Kassahun *et al.* 1994); and 5-GS-4-OH-VPA lactone from the conjugation of GSH with 4,5-dihydroxy-VPA-γ-lactone (Prickett and Baillie 1986). The latter is produced from the spontaneous hydrolysis of 4,5-epoxy-VPA, a P450-catalyzed reactive metabolite identified in the bile of 4-ene-VPA treated rats (Kassahun *et al.* 1994). The VPA metabolite, (*E*)-2,4-diene-VPA, which is produced from either microsomal P450-catalyzed dehydrogenation of (*E*)-2-ene-VPA and/or the mitochondrial β-oxidation of 4-ene-VPA, was shown to be hepatotoxic when administered to rats (Kesterson *et al.* 1984). Two novel GSH conjugates, CoA-activated and glucuronide-activated GSH conjugates of (*E*)-2,4-diene-VPA, have been identified in the mitochondria and cytoplasm of rat liver homogenate, respectively, in (*E*)-2,4-diene-VPA treated rats (Tang and Abbott 1996). These findings suggest that glucuronide formation activates (*E*)-2,4-diene-VPA to further conjugate with GSH via a Michael addition reaction. Based on the principle that the role of GSH may serve as a trap for reactive species, the conjugation reactions may produce a localized depletion of the finite pool of mitochondrial GSH that would result in oxidative stress, covalent binding and subsequent inactivation of enzymes with accompanying hepatocellular damage (Kassahun *et al.* 1991; Kassahun *et al.* 1994).

To reiterate, VPA-mediated hepatotoxicity is presumed to result from mitochondrial β-oxidation of the P450-dependent VPA metabolite, 4-ene-VPA, to (*E*)-2, 4-diene-VPA, which in the CoA

thioester form either depletes GSH and/or produces a putative inhibitor of β-oxidation enzymes. Acyl-CoA dehydrogenase enzymes catalyze the initial step in mitochondrial fatty acid β-oxidation by converting fatty acyl-CoA thioesters to their corresponding trans-2,3-enoyl-CoA derivatives (Schulz 1991). The enzymes are believed to function by a mechanism involving α-proton abstraction by an active site base followed by transfer of the β-hydride (Thorpe and Kim 1995). To further investigate VPA-induced hepatotoxicity, a mechanistic study was conducted in which the α-fluorinated 4-ene-VPA analogue (α-F-4-ene-VPA) was synthesized (Tang *et al.* 1995) with the rationale that 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 centre. This analogue is expected to be inert to β-oxidation to its reactive (*E*)-2,4-diene-VPA metabolite via acyl-CoA dehydrogenases, and thus reduce/prevent hepatotoxicity. Administration of α-F-4-ene-VPA (113 mg/kg x 5 days, ip) to rats resulted in the absence of hepatic microvesicular steatosis, while 4-ene-VPA (100 mg/kg x 5 days, ip) induced severe hepatic microvesicular steatosis and mitochondrial alterations (Tang *et al.* 1995). Consistent with these findings, the major metabolites of 4-ene-VPA, (*E*)-2,4-diene-VPA and its NAC conjugate, were not detected in rats administered α-F-4-ene-VPA (Tang *et al.* 1995). Mitochondrial GSH levels were also reduced to 68% of control in the rats administered 4-ene-VPA. This observed decrease in hepatotoxicity was further investigated and results indicated that α-F-4-ene-VPA did not form the corresponding acyl-CoA derivative in liver extracts of α-F-4-ene-VPA-treated rats (Grillo *et al.* 2001). The data suggest that VPA-associated hepatotoxicity involves a sequence of events: (1) P450-dependent formation of 4-ene-VPA, (2) 4-ene-VPA activated to its 4-ene-VPA-CoA thioester, and (3) its subsequent β-oxidation to the electrophilic metabolite, (*E*)-2,4-diene-VPA-CoA that potentially depletes mitochondrial GSH. This localized depletion of GSH would make the cell more susceptible to oxidative stress, reactive metabolite covalent binding, and ultimately to mitochondrial dysfunction.

In support of a reactive metabolite mechanism, the N-acetylcysteine conjugate of (*E*)-2,4-diene-VPA, was identified in the urine of patients receiving VPA therapy, with the levels being 3-4 times higher in those who had developed VPA-related hepatotoxicity (Kassahun *et al.* 1991). Furthermore, treatment with oral N-acetylcysteine was associated with clinical and biochemical recovery and survival of a small number of patients diagnosed with severe hepatotoxicity (i.e. microvesicular steatosis) while on VPA therapy (Farrell and Abbott 1991; Farrell *et al.* 1989a). It appeared that serum concentrations of 4-ene-VPA were higher in pediatric patients than in older patient groups (Abbott *et al.* 1986; Tatsuhara *et al.* 1987), the former suggested to be at most risk to VPA hepatotoxicity (Dreifuss *et al.* 1987). Furthermore, patients who had taken VPA co-administered with the P450 enzyme inducers, phenytoin, PB, or carbamazepine, were more susceptible to VPA-induced liver injury than those under VPA monotherapy. On the other hand, there is evidence suggesting that the VPA-mediated hepatotoxicity does not correlate with plasma levels of 4-ene-VPA or its derivatives. VPA metabolite profiles of patients were determined and the hepatotoxicity appeared to be independent of 4-ene-VPA formation (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. It was therefore suggested that these metabolites were not the decisive factors in VPA-induced hepatotoxicity (Loscher 1993). However, it remains questionable whether or not levels of 4-ene-VPA or (*E*)-2,4-diene-VPA are delineating factors for VPA-mediated hepatotoxicity. Recent studies in our laboratory demonstrated the applicability of measuring N-acetylcysteine conjugates arising from the reactive (*E*)-2,4-diene-VPA in patient urines as an indicator of reactive metabolite exposure (Gopaul *et al.* 2000a, b; Tang *et al.* 1995) Furthermore it was demonstrated that higher doses of VPA associated with younger patients and those on VPA polytherapy resulted in a greater exposure of these patients to reactive metabolites of VPA (Gopaul *et al.* 2003).

1.6 OXIDATIVE STRESS HYPOTHESIS

1.6.1 Oxidative stress

Cellular generation of oxygen radicals and peroxides (reactive oxygen species, ROS) is a continuous and physiological event upon oxygen consumption (Chance *et al.* 1979; Sies and Moss 1978). Free radical intermediates (e.g. superoxide anion, $O_2^{\bullet-}$) are formed through the univalent reduction of molecular oxygen, and are highly toxic to cellular components. The superoxide radical undergoes dismutation to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) enzymatically via superoxide dismutase (SOD). Hydrogen peroxide can undergo an iron-catalyzed conversion to hydroxyl radical ($OH^{\bullet}$) (Reed 1995). The hydroxyl radical is one of the most chemically reactive species known and it can react at the site of formation with neighbouring tissue macromolecules. Oxidative stress is a term used to describe a condition of excessive production of ROS and/or decrease in antioxidant levels (see section 1.6.3) (Sies 1991). This pro-oxidant and anti-oxidant imbalance towards the former (Figure 1-4) can result in damage to tissue macromolecules such as lipids, proteins, and DNA.

1.6.2 Lipid peroxidation and biomarkers

Lipid peroxidation can be defined as the oxidative deterioration of polyunsaturated fatty acids (PUFA), which are abundantly located in phospholipid cell membranes (Halliwell and Gutteridge 1999). Lipid peroxidation is initiated by hydrogen abstraction on the phospholipid side chain (i.e. arachidonate, Figure 1-5) by free radical species (e.g. the hydroxyl radical). The resultant reactive carbon-centre radical reacts with O_2 to form peroxyl radicals ($ROO^{\bullet}$), which can propagate the lipid peroxidation chain reaction by removing another hydrogen atom from a neighbouring PUFA side chain generating more lipid hydroperoxides (Halliwell and Gutteridge 1999). Malondialdehyde (MDA, Figure 1-5) is a low molecular weight, reactive aldehyde, formed via the decomposition of certain primary and secondary lipid peroxidation products (e.g.

cyclic endoperoxides) (Esterbauer *et al.* 1991). MDA is commonly detected by the thiobarbituric acid reactive substances (TBARS) assay by forming a 1:2 adduct (MDA:thiobarbituric acid) which can be measured by fluorometry (Janero 1990). The isoprostanes are prostaglandin analogs formed during the free radical-catalyzed lipid peroxidation of arachidonic acid (Figure 1-5 and Figure 1-6) (Morrow *et al.* 1990b). F_2-Isoprostranes are also used as biomarkers of lipid peroxidation, and in particular, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP, also known as 8-isoprostane) has been shown to be elevated in animal models of oxidative stress and also in humans (Gopaul *et al.* 1995; Morrow *et al.* 1995; Morrow *et al.* 1990a; Morrow *et al.* 1990b; Morrow *et al.* 1994). Recently, 15-F_{2t}-IsoP has become a commonly used marker of oxidative stress that can be determined either by GC/MS or an enzyme-linked immunoassay (EIA) methods (Proudfoot *et al.* 1999).

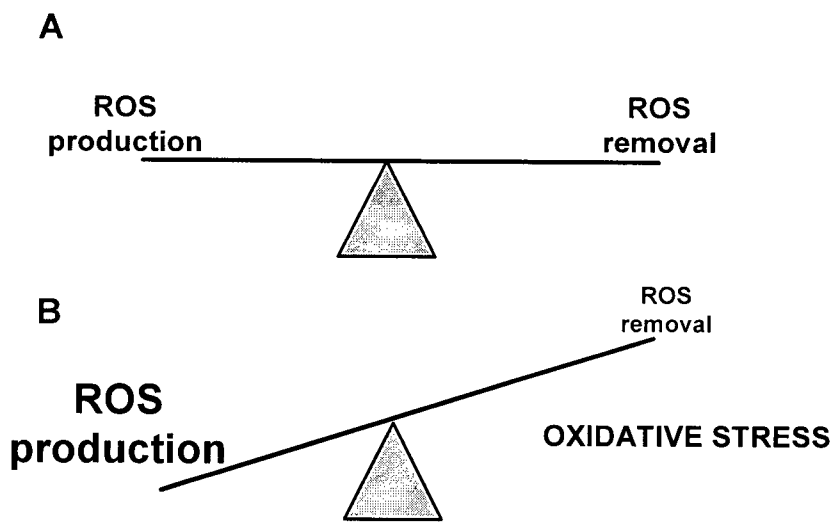

Figure 1-4: Schematic describing the concept of oxidative stress as a balance between ROS production and ROS removal. (A) A balance exists between ROS production through normal cellular respiration and ROS removal by antioxidants. (B) Oxidative stress is a term that describes an imbalance between ROS production and ROS removal.

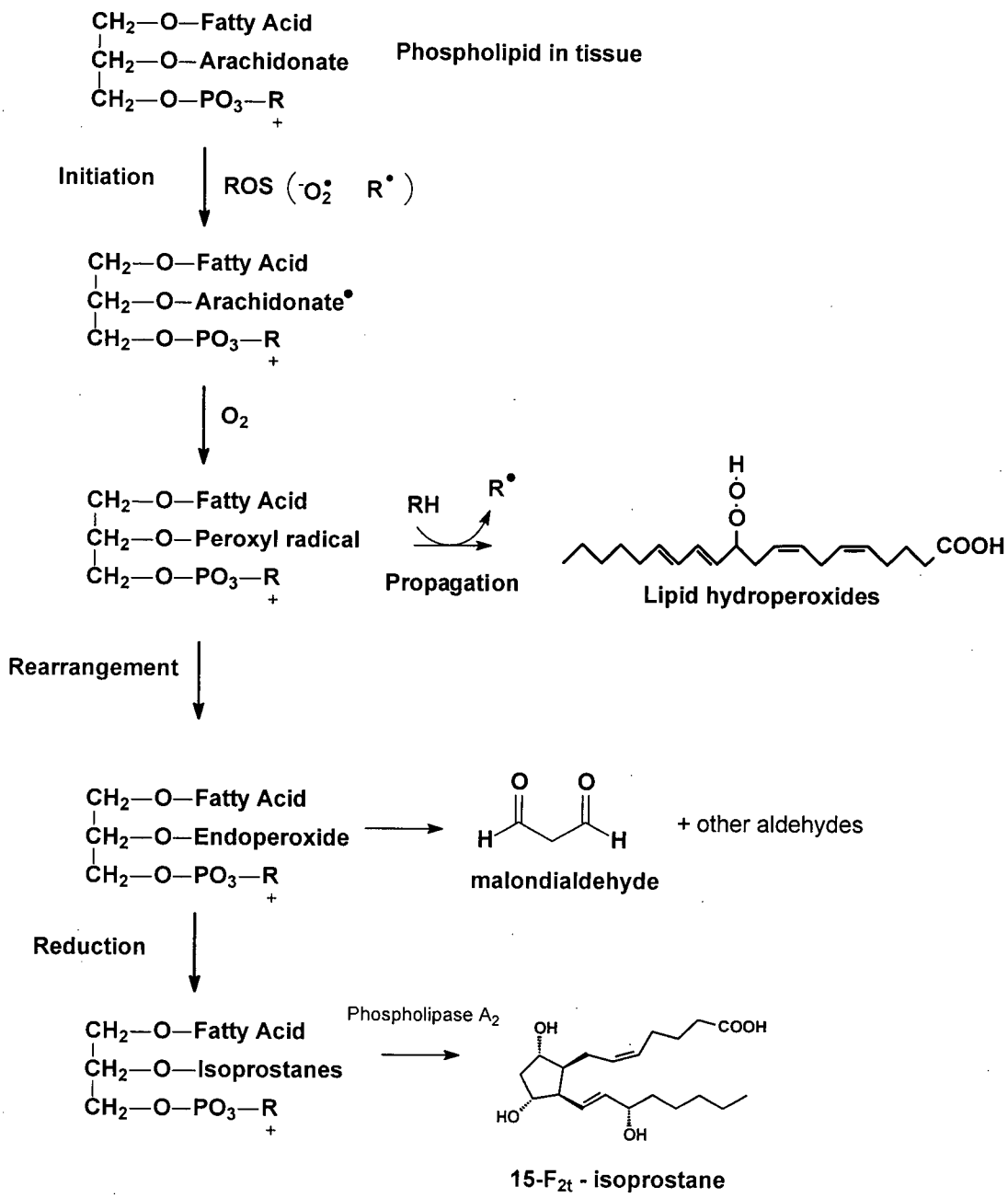

Figure 1-5: Schematic describing the formation of lipid peroxidation biomarkers: lipid hydroperoxides, malondialdehyde, and 15-F_{2t}-isoprostane (modified from Cayman Chemical Co., 15-F_{2t}-Isoprostane assay kit booklet).

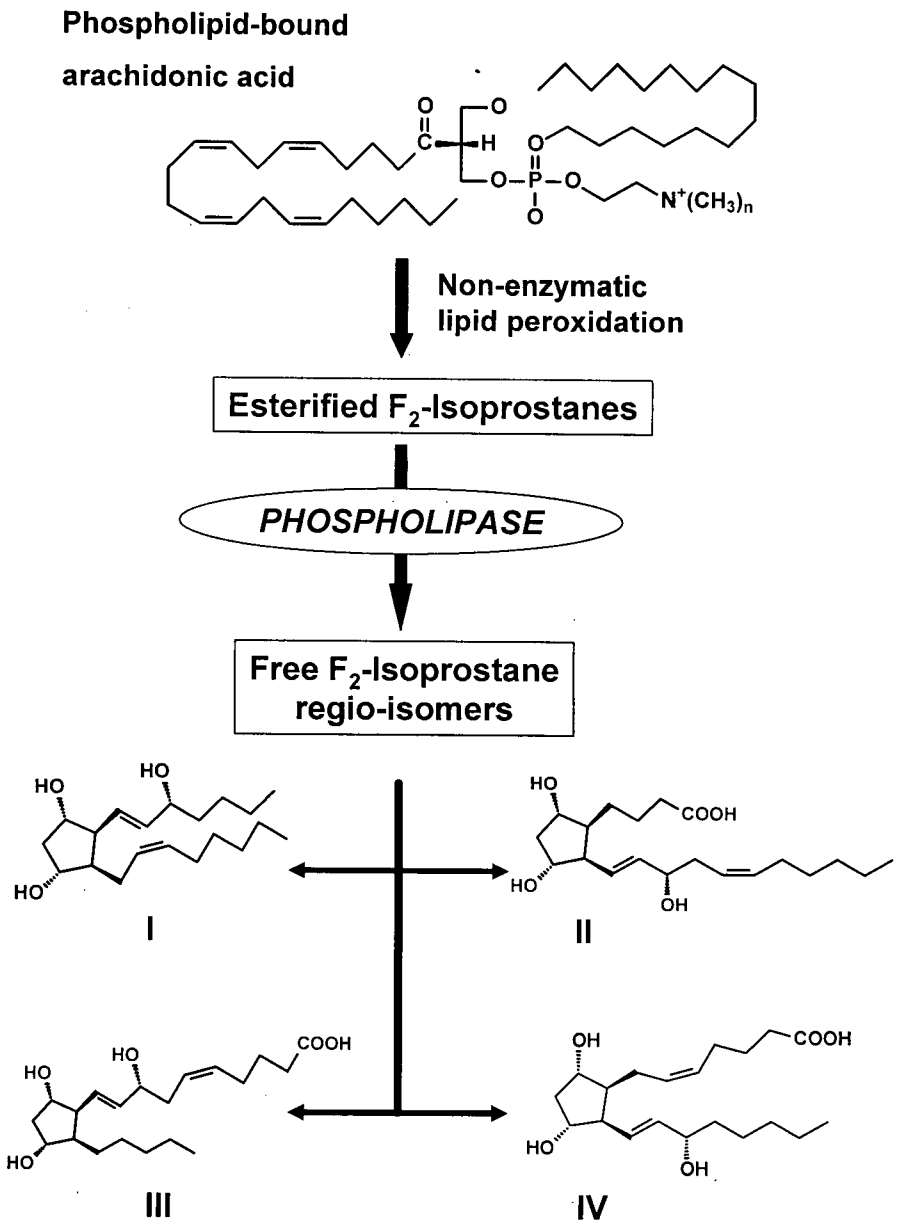

Figure 1-6: Formation of F_2-isoprostane regio-isomers during non-enzymatic peroxidation of arachidonic acid. Regio-isomer IV represents 15-F_{2t}-IsoP (Gopaul *et al.* 1995).

1.6.3 Antioxidant defense

To regulate the free radical reactions, a defense system exists which includes enzymes (SOD, catalase (CAT), glutathione peroxidase (GSH-Px) and small molecules (Vitamins C and E, uric acid, GSH, albumin or bilirubin), and repair systems that prevent the accumulation of oxidatively damaged molecules (Pincemail 1995). Superoxide dismutase (SOD), a cytosolic and mitochondrial enzyme, serves as the first line of defense against the accumulation of toxic superoxide free radicals by catalyzing their dismutation to form hydrogen peroxide. Catalase, a cytoplasmic heme-enzyme found in high concentrations in peroxisomes, catalyzes the divalent reduction of hydrogen peroxide to water.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is a ubiquitous tripeptide thiol synthesized in the cytosol by the consecutive actions of γ-glutamylcysteine synthetase and glutathione synthetase reactions (Meister 1995a) and is transported into the mitochondria (Griffith and Meister 1985; Meister 1995b). GSH serves not only as an important biomolecule for the conjugation of reactive metabolites, but also as an antioxidant to protect cells against ROS (Reed 1990). GSH is directly involved in the enzymatic reduction of ROS via the actions of the glutathione peroxidase – glutathione reductase system (Figure 1-7). GSH-peroxidase biotransforms hydrogen peroxide to water through the oxidation of GSH. The process represents detoxification when levels of electrophile are low, but toxic consequences may occur if cellular GSH is depleted (Reed 1990). Based on the principle that GSH may serve as a trap for reactive VPA metabolites and/or reactive oxygen species (i.e. lipid hydroperoxides) formed in the mitochondria, its conjugation reaction may result in a localized depletion of mitochondrial GSH that would result in oxidative stress, mitochondrial dysfunction, and ultimately lead to hepatocellular damage.

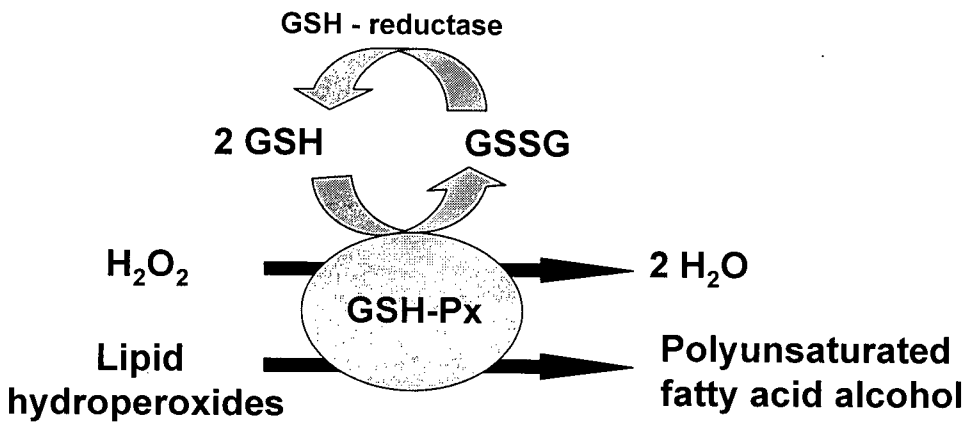

Figure 1-7: A schematic describing the glutathione peroxidase (GSH-Px) – glutathione reductase (GSH-reductase) antioxidant system.

1.6.4 Valproic acid and oxidative stress

There is also a growing body of evidence suggesting that idiosyncratic hepatic toxicity may be mediated, at least in part, by the excessive generation of free radical intermediates as a consequence of xenobiotic bioactivation. Adverse effects during VPA therapy in patients has been associated with alterations in antioxidant enzymes characterized with reduced GSH-peroxidase and elevated GSH reductase activities (Graf *et al.* 1998). In addition, VPA therapy produced significant depletion of zinc and selenium in plasma of rats and humans, trace elements required for antioxidant enzyme activity (Graf *et al.* 1998; Hurd *et al.* 1984). Both total hepatic and hepatic mitochondrial GSH levels have been depleted with VPA (Cotariu *et al.* 1990; Seckin *et al.* 1999) and 4-ene-VPA (Tang *et al.* 1995) exposure in rats. GSH-reductase and GSH-peroxidase activities were reported to be reduced, parallel to the depletion of GSH and NADPH after a single dose of VPA in rats (Cotariu *et al.* 1990). The cytotoxicity of 4-ene-VPA in GSH-depleted rat hepatocytes was attenuated with vitamin C and E, while α-F-4-ene-VPA did not elicit toxicity under similar conditions (Jurima-Romet *et al.* 1996). These findings

are consistent with increased levels of ROS after VPA exposure. VPA treatment of human lymphocytes from patients who had developed hepatotoxicity while on VPA therapy demonstrated marked increases in cell death compared to lymphocytes from VPA-treated patients that did not show liver toxicity (Farrell and Abbott 1991; Farrell *et al.* 1989a). This concentration-dependent toxicity could be inhibited by inactivation of the microsomal metabolizing system in the incubation (Farrell *et al.* 1988, 1989b). A further study demonstrated that the lymphocyte toxicity induced by VPA could also be inhibited by the addition of GSH or N-acetylcysteine. This result prompted the use of N-acetylcysteine in the successful treatment of three children who developed severe hepatotoxicity while receiving VPA. 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.* 1997). Mechanistic studies were carried out to evaluate the contribution of reactive metabolite and reactive oxygen species in the mechanism of the metabolism-dependent VPA-induced lymphocyte toxicity (Tabatabaei *et al.* 1999). The addition of catalase or 10-phenanthroline (a cell permeable iron chelator) to the incubation mixture resulted in significant reduction in cytotoxicity without altering VPA metabolite levels. However, the cell impermeable iron chelator, desferroximine, did not reduce cytotoxicity. The data support the hypothesis 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 with intracellular iron to produce the highly reactive hydroxyl free radicals (Tabatabaei *et al.* 1999). Further evidence implicating the involvement of oxidative stress with VPA treatment was demonstrated with the use of salicylate (2-hydroxybenzoic acid) as a specific hydroxyl radical ($OH^{\bullet}$) trap. This produces the stable hydroxylation product, 2,3-dihydroxybenzoic acid (2,3-DHBA) that can be specifically identified and quantified by LC/MS (Tabatabaei and Abbott 1999). VPA (100 mg/kg) treatment in rats resulted in increased formation of 2,3-DHBA with a mean maximal plasma level 2.5-fold greater

than the control group. In a separate study, vitamin E (α-tocopherol) and N,N′-diphenyl-p-phenylenediamine (DPPD) protected rat hepatocyte cultures against VPA-induced toxicity (Buchi *et al.* 1984). In a latter study, a protective effect by vitamin C and E against 4-ene-VPA-induced cytotoxicity in hepatocytes was reported (Jurima-Romet *et al.* 1996). Based on the available evidence, the induction of oxidative stress by VPA might be responsible or contribute to the mechanism of hepatotoxicity, and the idiosyncratic nature of this toxicity may occur in those individuals who have compromised anti-oxidant defenses.

1.7 RESEARCH RATIONALE & HYPOTHESIS

VPA is a widely used antiepileptic compound having desirable broad-spectrum activity. Although VPA is generally considered safe, it is limited by a rare but potentially fatal hepatotoxicity that was associated with the deaths of over one hundred children in the early to mid 1980's. The mechanism of fatal hepatotoxicity is unknown, and this has stimulated extensive research to investigate possible mechanisms. Traditionally, our laboratory has taken the approach to investigate the reactive metabolite theory by identifying reactive metabolites and examining patient risk factors that would help explain the idiosyncratic nature of the toxicity. Recently, we have found evidence for the generation of reactive oxygen species with VPA treatment in an *in vitro* lymphocyte model and an *in vivo* rat model as a possible mechanism of VPA-associated hepatotoxicity.

Based on the available evidence, the hypothesis to be tested in this thesis is that VPA is associated with oxidative stress through the formation of reactive oxygen species and the depletion of GSH as a consequence of VPA biotransformation, and that these are key factors contributing to the hepatotoxicity. Therefore, evidence for oxidative stress associated with VPA treatment is expected to occur prior to hepatotoxicity.

1.8 RESEARCH OBJECTIVES

The objectives of the proposed research are as follows:

1. To determine if VPA is associated with oxidative stress in rats as determined by *in vivo* biomarkers of lipid peroxidation.

2. To investigate the effect of modulating the biotransformation pathways of VPA (P450, glucuronidation, and β-oxidation) on oxidative stress in rats.

3. To examine the effects of multiple VPA dosing on the temporal relationship between oxidative stress and hepatotoxicity.

4. To develop an *in vitro* model to investigate the relationship between oxidative stress, mitochondrial dysfunction, and toxicity by VPA in primary cultured rat hepatocytes.

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2 *The Effect of VPA on 15-F_{2t}-Isoprostane Levels in Rats*

2.1 INTRODUCTION

Recently, an *in vitro* assay consisting of an incubation system with PB-induced rabbit microsomes and human lymphocytes was developed as a model to investigate the involvement of reactive oxygen species and the role of biotransformation in VPA-induced cytotoxicity (Tabatabaei *et al.* 1997). The removal of NADPH from the system or the addition of catalase or 1,10-phenantholine (a cell permeable iron chelator) to the incubation mixture resulted in significant reduction in lymphocyte cytotoxicity (Tabatabaei *et al.* 1999). These results support the hypothesis that the observed cytotoxicity was the result of the generation of hydrogen peroxide by a microsomal-dependent pathway, and that the produced hydrogen peroxide can readily cross cell membranes and subsequently react with intracellular iron to produce highly reactive hydroxyl free radicals.

Another method for the measurement of oxidative stress was demonstrated using salicylate as a specific hydroxyl radical ($HO^{\bullet}$) trap that produces stable hydroxylation products (Halliwell *et al.* 1991). Our laboratory has developed an LC/MS assay to quantify 2, 3-dihydroxybenzoic acid (2, 3-DHBA) as a specific, non-enzymatic hydroxylation product of salicylate (Tabatabaei and Abbott 1999). *In vivo* studies with rats demonstrated that plasma levels of 2,3-DHBA were elevated 2.5- and 6.5-fold following exposure to VPA (100 mg/kg) and 1,1,1-trichloroethane (700 mg/kg), respectively, compared to saline control (Tabatabaei 1998). The $HO^{\bullet}$ trap approach is not without limitations because it involves the administration of salicylate, which

may influence the biotransformation (i.e. P450, β-oxidation, glutathione conjugation) of the test compound that may possibly confound the study.

In this study, we have focused on the measurement of a 15-series F_2-isoprostane (15-F_{2t}-IsoP), formerly known as 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) (Cracowski *et al.* 2002) as a direct, *in vivo* index of oxidative stress. The endogenous formation of 15-F_{2t}-IsoP is by a non-cyclooxygenase-dependent mechanism involving the free radical-catalyzed breakdown product of arachidonic acid (Morrow *et al.* 1990). This marker is useful because of its potential application in pediatric patients on VPA therapy.

The objectives of the present study were to investigate the effect of VPA in rats on the levels of 15-F_{2t}-IsoP and to determine if modulation of the P450-dependent VPA biotransformation pathways influences the plasma and liver levels of 15-F_{2t}-IsoP. For comparison, we also measured thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxide levels, even though they are generally considered to be less sensitive and less specific than 15-F_{2t}-IsoP as markers for oxidative stress.

2.2 MATERIALS & METHODS

2.2.1 Reagents

VPA (sodium salt), SKF-525A (proadifen), 1-aminobenzotriazole (ABT), octanoic acid, and carbon tetrachloride (CCl_4) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium phenobarbital (PB) and anhydrous sodium sulphate were purchased from BDH Chemicals Inc., (Toronto, ON, Canada). The 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) EIA kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Dimethylformamide and the gas chromatography derivatizing reagents pentafluorobenzyl bromide (PFBBr) and N-(tert-

butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) were purchased from Pierce Chemical Co. (Rockford, IL). N,N-diisopropylethylamine and *tert.*-butyldimethylsilyl chloride were obtained from Aldrich (Milwaukee, WI). HPLC grade ethyl acetate, acetonitrile, methanol, chloroform, and n-hexanes (GC/MS resolved) were purchased from Fisher Scientific (Vancouver, BC, Canada).

2.2.2 Animals

Adult male Sprague-Dawley rats (225–250 g) were obtained 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. All animal experiments were approved by the University of British Columbia Animal Care Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.3 Treatment of animals and sample collection

Rats were administered with VPA, octanoic acid, or saline at the doses and duration indicated in each figure legend. To modulate P450 enzyme activity, rats were injected ip with SKF-525A (80 mg/kg, single dose), ABT (100 mg/kg, single dose), or PB (80 mg/kg once daily for 4 consecutive days) prior to VPA administration (500 mg/kg, ip). At a predetermined time after VPA treatment, as indicated in each figure legend, rats were sacrificed by decapitation, trunk blood was collected, and plasma was prepared. An aliquot of the plasma was used immediately for the determination of 15-F_{2t}-IsoP levels and the remainder was stored at –80°C for the subsequent analysis of VPA, its metabolites, TBARS, and lipid hydroperoxide levels. Livers were weighed and homogenized in 20 ml of 50 mM phosphate buffer (pH 7.4) under cold conditions. The homogenate was processed immediately for the analysis of ROS biomarkers

(described below). Aliquots were stored at -70°C for the later determination of VPA metabolite levels. As a positive control, carbon tetrachloride (CCl_4, 2 ml/kg, ip) or corn oil (vehicle control) was given to Sprague-Dawley rats and animals were sacrificed 4 h later, the time point when plasma 15-F_{2t}-IsoP levels were found to be maximal at the given CCl_4 dose (Morrow *et al.* 1992).

2.2.4 15-F_{2t}-IsoP enzyme immunoassay (EIA)

Plasma and liver levels of 15-F_{2t}-IsoP were determined by an EIA assay according to the manufacturer's protocol. The assay is based on competition between unknown amounts of free 15-F_{2t}-IsoP present in the samples and fixed amounts of 15-F_{2t}-IsoP-acetylcholinesterase conjugate (15-F_{2t}-IsoP-tracer) for 15-F_{2t}-IsoP-specific rabbit antiserum. The 15-F_{2t}-IsoP and 15-F_{2t}-IsoP tracer complex with rabbit antiserum and bind to the mouse monoclonal anti-rabbit IgG antibody coated on a 96-well plate. After an incubation period of 20 h, the plates were washed and treated with Ellman's reagent (Cayman Chemical Co., Ann Arbor, MI), which contains a mixture of acetylthiocholine iodide (5%) and 5,5′-Dithiobis-(2-nitrobenzoic acid) (10%), to produce an enzymatic product that was determined spectrophotometrically at 405 nm on a Labsystems Multiscan Ascent multi-well plate reader (Thermo Electron Corp., Burlington, ON, Canada). The intensity of color is proportional to the amount of 15-F_{2t}-IsoP tracer bound to the well, which is inversely proportional to the amount of free 15-F_{2t}-IsoP present in the sample. Free plasma 15-F_{2t}-IsoP was measured directly by adding 50 μl of fresh rat plasma to 96-well plates coated with mouse monoclonal anti-rabbit IgG. For the determination of total 15-F_{2t}-IsoP in plasma (100 μl) and liver (500 μl), samples were subject 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 1-2). For the determination of free liver 15-F_{2t}-IsoP, liver homogenate was subjected only to protein precipitation. The samples were centrifuged (1600 x 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 x 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 (Varian Inc., Lexington, MA) using Waters Oasis® C18 cartridges (Waters Ltd., Mississauga, ON, Canada). The cartridges were pre-conditioned 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). The eluant was evaporated under N_2 and the residue reconstituted in 1 ml of EIA phosphate buffer (Cayman Chemical Co., Ann Arbor, MI) for 15-F_{2t}-IsoP determination. Solid phase extraction recovery was performed by comparing extracted 15-F_{2t}-IsoP quality control (QC) samples spiked with blank plasma and liver matrices to non-extracted QC samples.

2.2.5 Validation of the 15-F_{2t}-IsoP EIA

The 15-F_{2t}-IsoP EIA was validated over a concentration range of 4-150 pg/ml in EIA buffer, plasma and liver matrices. Method validation was performed by determining inter-assay accuracy (% bias) and precision (% CV) using quality control samples at low (10 pg/ml), mid (50 pg/ml), and high (125 pg/ml) concentrations. This was accomplished by analyzing calibration curves and QC samples on separate days in EIA buffer (n = 6), plasma (n = 6), and liver (n = 3) matrices. Quantitation of QC samples was performed by analyzing the calibration curve standards and back calculating the concentration of each QC sample from the obtained slope and intercept. A bias and precision of ≤ 20% at the low QC and ≤ 15 % for all other QC samples was considered to be acceptable.

2.2.6 *Thiobarbituric acid reactive substances (TBARS) assay*

The concentration of TBARS was calculated as malondialdehyde (MDA) equivalents (Figure 2-1) using a commercial kit (Oxi-Tek®, Zeptometrix Corporation, Buffalo, NY). Samples (100 μl) of plasma or supernatant from 10,000 x g, 15 min centrifugation of liver homogenate 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 x g, samples were transferred into a 96-well plate and fluorescence was monitored on a Cytofluor® Series 4000 multi-well plate reader (Applied Biosystems, Bedford, MA) 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).

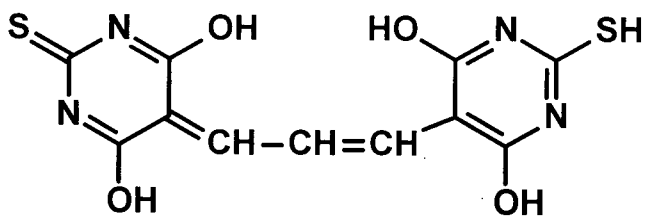

Figure 2-1: Structure of MDA-TBARS adduct. MDA forms a 1:2 adduct with thiobarbituric acid and is detected by fluorometry (ZeptoMetrix Co., TBARS assay kit booklet).

2.2.7 *Lipid hydroperoxide (LPO) assay*

Hydroperoxide levels were determined using a commercial kit (Cayman Chemical Co., Ann Arbor, MI). Plasma and liver homogenate samples (500 μl) were de-proteinated and extracted under acidic conditions with 1 ml of ice-cold de-oxygenated chloroform. The chloroform extract was removed following centrifugation (1600 x g for 5 min at 0°C) for hydroperoxide determination. The LPO assay is based on redox reactions with hydroperoxides and ferrous ions to produce ferric ions and the resulting ferric ions are detected using thiocyanate ion as the chromagen (Figure 2-2). 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-hydroperoxyoctadecadienoic acid as a lipid hydroperoxide standard to construct standard curves (0.5 - 5 nmol lipid hydroperoxide) against which unknown samples were plotted.

$$ROOH + Fe^{2+} \longrightarrow RO^{\bullet} + Fe^{3+}$$

$$RO^{\bullet} + Fe^{2+} + H^{+} \longrightarrow ROH + Fe^{3+}$$

$$Fe^{3+} + 5SCN^{-} \longrightarrow Fe(SCN)_5^{2-}$$

λ_{max}: 500 nm

Figure 2-2: Reduction/oxidation reaction scheme involved in the LPO assay (Cayman Chemical Co., Lipid hydroperoxide assay booklet).

2.2.8 VPA and metabolite assay

The assay for VPA and its metabolites was from a modified method (Anari *et al.* 2000). Rat plasma (10-100 μl) or liver (50 μl) samples, and 50 μl of an internal standard mixture containing 2 μg/ml of each deuterated metabolite standard were added to borosilicate glass screw top culture tubes. The mixture was acidified to pH 3.5 and made up to 1 ml with 1 M KH_2PO_4 buffer (pH 3.5) and extracted with 6 ml of ethyl acetate by gentle rotation of the phases for 30 min. The organic extract was transferred to another borosilicate glass screw top test tube containing anhydrous sodium sulfate, vortex mixed, and centrifuged at 1600 x g for 10 min. The ethyl acetate was dried to approximately 50-100 μl under a gentle stream of N_2 (0.5 PSI at 30°C) using a Zymark Turbo-Vap® LV Evaporator (Zymark Co., Hopkinton, MA). *N,N*-diisopropylethylamine (30 μl) and pentafluorobenzyl bromide (10 μl) were added to the mixture and heated at 40°C for 1 h for the pentafluorobenzyl ester derivatization of the carboxylic acid groups. The mixture was cooled at room temperature and treated with 20 μl of dimethylformamide and 30 μl of the mixture of 2% *tert.*-butyldimethylsilyl chloride in MTBSTFA, and heated at 60°C for 2 h for the second step *tert.*-butyldimethylsilylation of hydroxyl groups. The derivatized sample was cleaned up to remove non-volatiles by drying it down to a residue under a stream of N_2 and reconstituting it in 150 μl of hexane by vortex mixing. The mixture was centrifuged (1600 x g, 10 min) and the hexane layer was transferred to an autosample vial for injection onto the gas chromatograph (GC).

2.2.9 Gas chromatography-mass spectrometry (GC/MS) instrumentation

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

were used to control the operation of all instruments. Metabolite separation was carried out on a fused-silica narrow bore GC column (60 m x 0.25 mm I.D., 0.25 μm film thickness) coated with the non-polar stationary phase SolGel 1-ms (SGE Inc., Austin, TX) in combination with a fused-silica 5-meter GC Z-guard column (Phenomenex, Torrance, CA). The carrier gas was helium with a constant column flow rate of 0.5 ml/min, a column head pressure of 18.5 PSI, and a septum purge flow rate of 23 ml/min. Samples (1 μl) were injected in the splitless mode (injector temperature, 250°C) and cold-trapped at 40°C. The column oven temperature was raised rapidly to 140°C and programmed linearly as follows: 1°C/min to 160°C, then 10°C/min to 270°C and held at 270°C for 5 min. Solvent delay was set at 17 min and the total run time was 37.8 min. The mass spectrometer was operated in negative chemical ionization (NCI) mode with single ion monitoring and a fixed filament emission current and electron energy of 50 μA and 150 eV, respectively. The ion source temperature and quadrupole temperatures were set at 200°C and 106°C, respectively. The GC interface was held at 270°C. Methane was used as a reagent gas for NCI operation with the reagent gas pressure set to 0.18 mTorr.

Synthesized standards and deuterated internal standards were monitored at the specified m/z and retention time as shown in Table 2-1. The assay was based on negative ion selected ion monitoring of the $(M-181)^-$ anions of the double derivatized (PFB and TBDMS) analytes that corresponded to the loss of the PFB moiety at the carboxylate group (Anari *et al.* 2000). A standard curve was constructed for each metabolite over the concentration range of 2 to 500 ng/ml.

Table 2-1: Diagnostic ions and retention times of PFB/TBDMS derivatives of VPA, VPA metabolites and deuterated analogues from standard reference samples in rat plasma.

Compound	Ion monitored	Retention time (min)
[$^2H_7$] 4-ene VPA	148	18.67
4-ene VPA	141	18.81
[$^2H_7$] VPA	150	18.88
VPA	143	19.12
3-ene VPA	141	19.32
(*E*)-2-ene-[$^2H_7$] VPA	148	22.38
(*E*)-2-ene VPA	141	22.59
(*E,Z*)-2,3′-diene VPA	139	23.20
(*E*)-2,4-diene VPA	139	23.45
(*E,E*)-2,3′-diene VPA	139	24.52
4-keto-[$^2H_7$] VPA	164	25.57
4-keto VPA	157	25.70
4-OH-[$^2H_7$] VPA	280	31.17, 31.45
4-OH VPA	273	31.22, 31.50
3-OH-[$^2H_7$] VPA	280	31.28
3-OH VPA	273	31.34
3-keto-[$^2H_7$] VPA	278	32.44
3-keto VPA	271	32.49
5-OH-[$^2H_7$] VPA	280	32.54
5-OH VPA	273	32.59
2-PSA	339	34.02
2-PGA	353	35.08

2.2.10 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 the Student Newman-Keul's multiple range test. The level of significance was set a priori at $p < 0.05$.

2.3 RESULTS

2.3.1 15-F_{2t}-IsoP EIA

Calibration curves of 15-F_{2t}-IsoP demonstrated linearity over the range 4 - 150 pg/ml with linear regression coefficients > 0.99 (r^2). The inter-assay variability (% CV) and precision (% bias) based on QC samples in buffer, plasma, and liver matrices were ≤ 15% for the high and mid concentrations, and ≤ 20% at the low concentration (Table 2-2). The mean analytical recovery of 15-F_{2t}-IsoP from the liquid-liquid/solid phase extraction procedure was performed by comparing extracted QC samples from non extracted QC samples. All QC samples spiked with liver homogenate demonstrated approximately 90% recovery, while QC samples spiked with plasma demonstrated approximately 90% recovery at the high and mid concentration QC samples and 80% recovery at low concentration QC samples.

2.3.2 Dose-dependent effect of VPA on plasma levels of free 15-F_{2t}-IsoP

To determine the effect of VPA on the plasma levels of 15-F_{2t}-IsoP, adult male rats were injected with a single ip dose (50, 100, 250, or 500 mg/kg body weight) of VPA or an equivalent volume of saline (vehicle control). As shown in Figure 2-3, VPA at doses of 250 and 500 mg/kg increased plasma levels of 15-F_{2t}-IsoP approximately 2.2-fold, when compared to the control group. In contrast, the lower doses had no effect. The positive control CCl_4 (2 ml/kg, ip), resulted in a 7-fold increase in plasma 15-F_{2t}-IsoP levels compared to the corn oil vehicle control group with values of 221 ± 20 and 32 ± 6 pg/ml, respectively (n=6, mean ± SEM, Figure 2-4).

Table 2-2: Inter-assay variation of the 15-F_{2t}-IsoP enzyme immunoassay.[a]

Matrix	Parameter	QC low[b]	QC mid[c]	QC high[d]
EIA Buffer	% CV	5	13	4
(n=6)	% Bias	-4	8	2
Plasma	% CV	5	12	4
(n=6)	% Bias	-4	8	3
Liver	% CV	7	3	7
(n=3)	% Bias	-3	-4	6

[a]Method validation was performed by evaluating inter-assay accuracy (% bias) and precision (coefficient of variation, % CV) of the low, mid, and high QC concentrations in EIA buffer, blank rat plasma, and blank rat liver on separate days. Quantitation of QC samples was performed by analyzing the calibration curve standards and back calculating the concentration of each QC sample from the obtained slope and intercept. [b]QC low (10 pg/ml). [c]QC mid (50 pg/ml). [d]QC high (125 pg/ml).

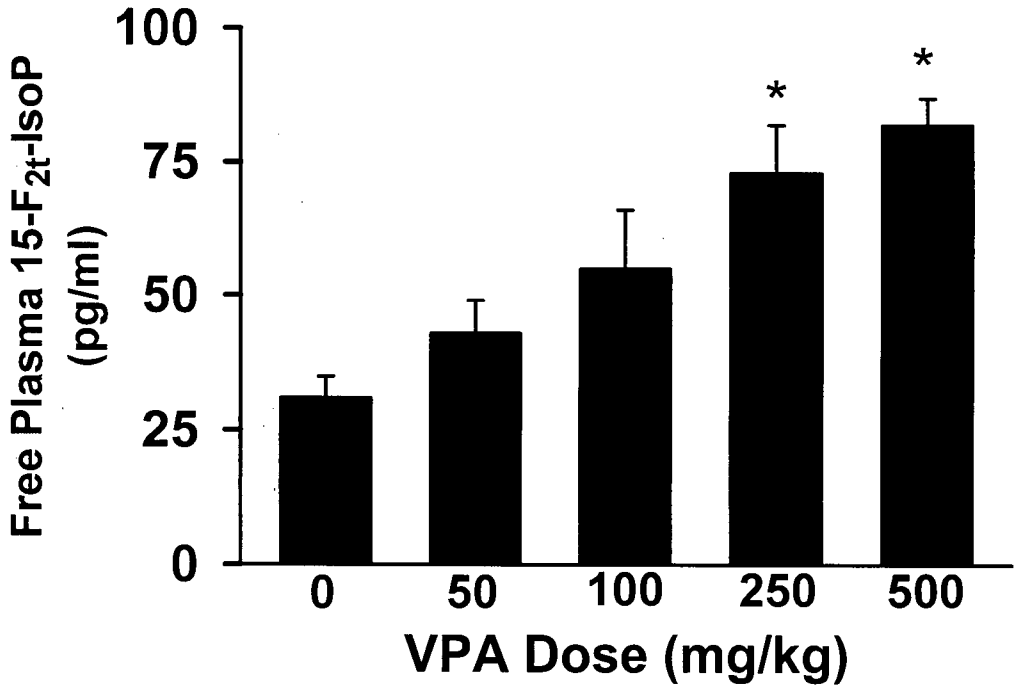

Figure 2-3: Influence of single dose VPA on free plasma 15-F_{2t}-IsoP levels. Adult male Sprague-Dawley rats (n = 4/group) were injected with a single ip dose of VPA (50, 100, 250 or 500 mg/kg). Control rats received an equivalent volume (1 ml/kg) of the vehicle (0.9% NaCl). At 0.5 h after VPA treatment rats were sacrificed and blood was collected. Values are means ± SEM. *Significantly different from control, $p < 0.05$.

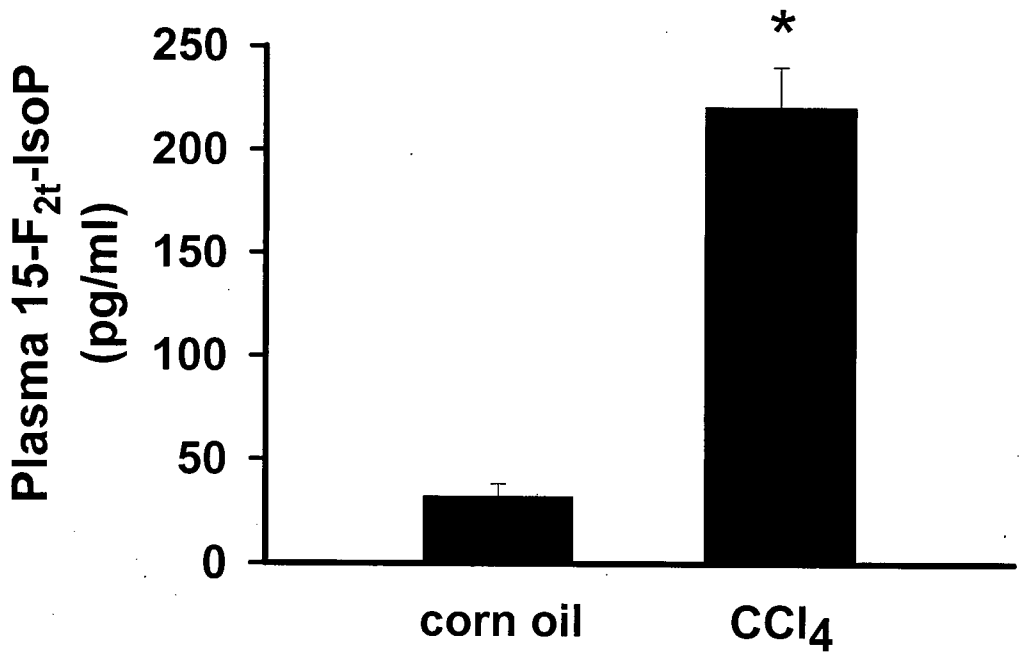

Figure 2-4: The effect of CCl_4 treatment on levels of 15-F_{2t}-IsoP. Male Sprague-Dawley rats were treated with a single ip dose of corn oil (vehicle control) or CCl_4 (2 ml/kg). At 4 h after treatment, rats were sacrificed and blood was collected. *Significantly different from control, $p < 0.05$.

2.3.3 Time-dependent effect of VPA on plasma levels of free 15-F_{2t}-IsoP

To characterize the time-course of the elevation of plasma levels of 15-F_{2t}-IsoP by VPA, rats were administered a single ip dose of VPA (500 mg/kg) and blood samples were collected at 0, 0.5, 1, 1.5, 2, 4 and 8 h thereafter. As shown in Figure 2-5, the plasma concentration of 15-F_{2t}-IsoP was the greatest at 0.5 h after VPA administration and returned to basal levels by 4 h post-dosing. Interestingly, the temporal profile of plasma 15-F_{2t}-IsoP (t_{max} = 0.5 h and C_{max} = 46 ± 9 pg/ml, mean + SEM, n = 4 rats per group) was similar to that of plasma VPA (t_{max} = 1 h and C_{max} = 578 ± 49 pg/ml). Overall, based on the dose-response and time-course experiments, the greatest increase in plasma 15-F_{2t}-IsoP levels after a single dose of VPA was 2.3-fold, when compared with the saline-treated control group.

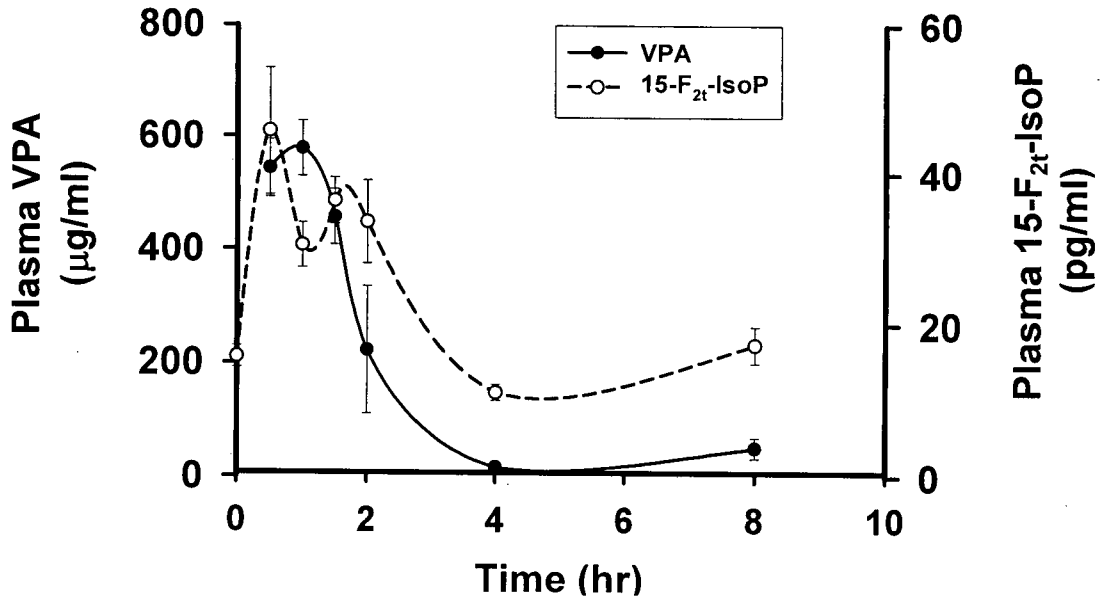

Figure 2-5: Time-course of plasma VPA and 15-F_{2t}-IsoP. Adult male Sprague-Dawley rats (250-300g) were injected with a single ip dose of VPA (500 mg/kg). Control rats received an equivalent volume (1 ml/kg) of the vehicle (0.9% NaCl). At various times after VPA treatment (0.5, 1, 1.5, 2, 4, and 8 h), rats were sacrificed and blood was collected. Values are means ± SEM (n = 4/time point).

2.3.4 *Effect of PB on plasma and liver levels of free 15-F_{2t}-IsoP, TBARS, LPO, and VPA metabolites in rats treated with VPA*

To determine the effect of inducing P450-mediated biotransformation of VPA on the elevation of plasma 15-F_{2t}-IsoP levels by this drug, rats were pretreated with PB (80 mg/kg, ip) or an equal volume of saline (vehicle control) once daily for four consecutive days. Animals were treated with saline vehicle or a single dose (500 mg/kg) of VPA on the following day and sacrificed 0.5 h after VPA administration. PB pretreatment and VPA exposure (500 mg/kg) resulted in a 2-fold increase of free plasma 15-F_{2t}-IsoP compared to VPA treatment alone, and a 5-fold increase compared to PB pretreatment and saline vehicle controls (Figure 2-6A). A similar trend in the elevation of free liver 15-F_{2t}-IsoP levels was determined in liver samples of the treatment groups (Figure 2-6B). Plasma and liver levels of TBARS and hydroperoxides did not differ among the treatment groups, including the CCl_4-treated rats (Table 2-3).

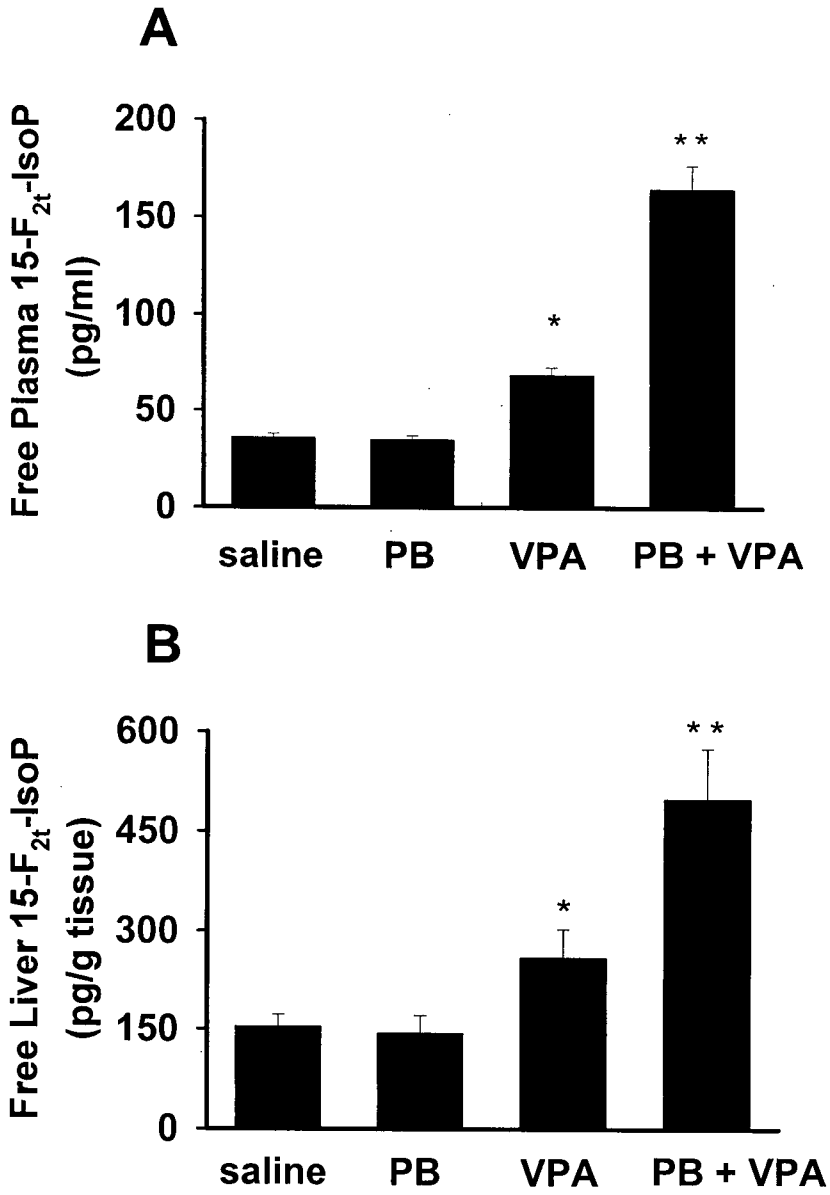

Figure 2-6: Effect of PB pretreatment on levels of 15-F_{2t}-isoP measured as the free form in the plasma and liver of VPA-treated rats. Adult male Sprague-Dawley rats were pretreated with sodium phenobarbital (PB, 80 mg/kg, ip, once daily for 4 days) or saline (vehicle control). Animals were treated with a single dose of VPA (500 mg/kg, ip) or saline on the following day. At 0.5 h after treatment, rats were sacrificed, and blood and liver were collected. Values are means ± SEM (*n* = 6/group). *Significantly different from control, $p<0.05$; **Significantly different from the VPA-treated group, $p < 0.05$.

Table 2-3: TBARS and lipid hydroperoxide levels detected in plasma and liver of VPA treated rats and pretreated with PB.[a]

	Treatment (n=6/group)	Plasma (nmol/ml)	Liver (nmol/g tissue)
TBARS	Saline	1.0 ± 0.1	11.9 ± 0.2
(MDA equivalents)	PB [a]	1.1 ± 0.1	12.8 ± 0.7
	VPA [b]	1.2 ± 0.1	12.2 ± 0.6
	PB+VPA [c]	1.1 ± 0.1	13.8 ± 0.6

	Treatment (n=6/group)	Plasma (nmol/ml)	Liver (nmol/ g tissue)
Lipid hydroperoxides	Saline	ND	72 ± 7
	PB [a]	ND	64 ± 5
	VPA [b]	ND	61 ± 5
	PB+VPA [c]	ND	62 ± 6

[a]Values represent the mean ± SEM (n=6). One-way analysis of variance using Newman-Keuls test was performed on the data. [b]Rats were pretreated with saline vehicle followed by VPA (500 mg/kg, ip). [c]Rats were pretreated with PB (80 mg/kg/day for 4 days) and by VPA (500 mg/kg, ip). ND, not detected.

PB pretreatment and VPA exposure was associated with increased levels of 4-ene VPA, (*E*)-2, 4-diene VPA, 4-OH-VPA, 4-keto-VPA, 3-OH-VPA and 5-OH-VPA compared to VPA treated rats (Table 2-4). Liver VPA metabolite levels showed a similar pattern with the exception that 5-OH-VPA was not different. The β-oxidation metabolites (2-ene-VPA, 3-keto-VPA, 3-ene-VPA, (*E,E*)-2,3′-diene-VPA) and the dicarboxylic acids (2-PSA and 2-PGA) were not different between the two groups (data not shown).

Table 2-4: Effect of PB pretreatment on plasma and liver levels of VPA metabolites in rats treated with VPA.[a]

	Plasma (x 10^2 ng/ml)		Liver (x 10^2 ng/g tissue)	
	Saline[b]	PB [c]	Saline [b]	PB [c]
4-ene VPA	1.3 ± 0.17	6.5 ± 1.3 *	0.96 ± 0.05	3.9 ± 0.10 #
(*E*)-2,4-diene VPA	< LOQ	0.73 ± 0.08 *	< LOQ	0.15 ± 0 #
3-OH VPA	3.9 ± 0.32	19 ± 3.7 *	3.7 ± 0.08	12 ± 0.40 #
4-OH VPA	18 ± 2.6	121 ± 19 *	8.8 ± 0.42	69 ± 2.4 #
5-OH VPA	30 ± 2.6	52 ± 6.4 *	40 ± 1.4	36 ± 1.2
4-keto VPA	4.0 ± 0.44	45 ± 5.5 *	1.3 ± 0.07	17 ± 0.48 #

[a]Values represent the mean ± SEM (n=6). One-way analysis of variance using Newman-Keuls test was performed on the data. [b]Rats were pretreated with saline vehicle followed by VPA (500 mg/kg, ip). [c]Rats were pretreated with PB (80 mg/kg/day for 4 days, ip), followed by VPA (500 mg/kg, ip) on the next day, and sacrificed 0.5 h later. *Indicates a statistically significant difference in the plasma compared to the saline pretreatment group. #Indicates a statistically significant difference in the liver compared to the saline pretreatment group. < LOQ, below the limit of quantitation (2 ng/ml) for the GC/MS assay of VPA metabolites.

2.3.5 Lack of an effect by SKF-525A on VPA-induced increase in plasma 15-F_{2t}-IsoP levels.

To determine the effect of inhibition of P450-mediated biotransformation of VPA on the elevation of plasma 15-F_{2t}-IsoP levels by this drug, rats were pretreated with SKF-525A (80 mg/kg, ip) or saline (vehicle control) 30 min prior to the administration of VPA. SKF-525A is known to inhibit multiple rat hepatic P450 enzymes (Buening and Franklin 1976; Murray 1988). As shown in Figure 2-7, SKF-525A exposure resulted in similar levels of 15-F_{2t}-IsoP compared to the saline control, and SKF-525A pretreatment did not influence the elevated levels of 15-F_{2t}-IsoP observed in VPA exposed rats.

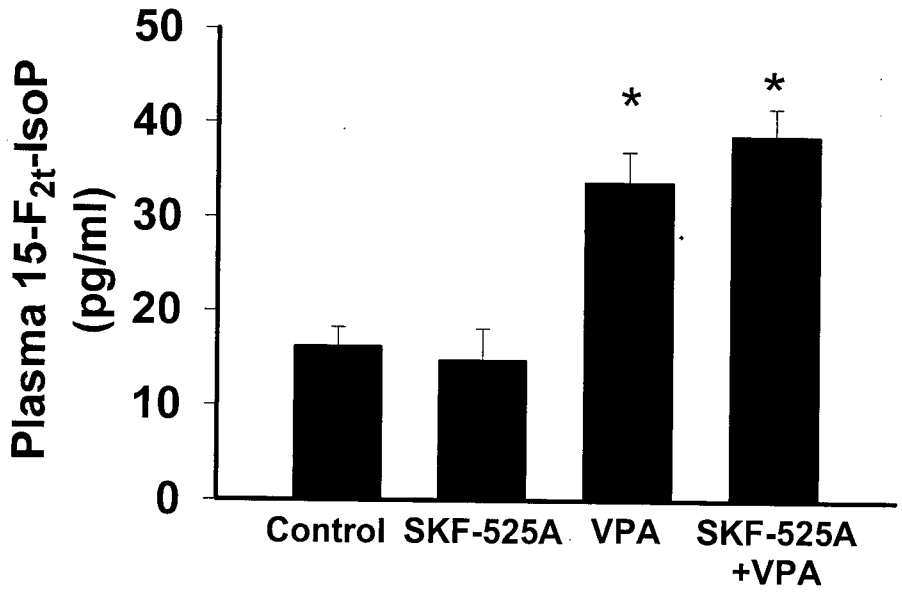

Figure 2-7: SKF-525A pretreatment on 15-F_{2t}-IsoP levels in VPA-treated rats. Adult male Sprague-Dawley rats were pretreated with a single ip dose of SKF-525A (80 mg/kg) or saline (vehicle control). A single ip dose of VPA (500 mg/kg) or saline (vehicle control) was administered 0.5 h after the pretreatment. At 0.5 h after VPA treatment rats were sacrificed and blood was collected. *Significantly different from control, $p < 0.05$.

2.3.6 Effect of ABT on plasma levels of free 15-F_{2t}-IsoP in VPA-treated rats

ABT is a mechanism-based inactivator of multiple constitutive P450 enzymes (Ortiz de Montellano and Mathews 1981). To determine the effect of inhibition of P450-mediated biotransformation of VPA on the elevation of plasma 15-F_{2t}-IsoP levels by this drug, ABT (100 mg/kg, ip) was administered to rats 2 h prior to VPA treatment (250 mg/kg). This regimen of ABT treatment is known to produce maximal inhibition (Mugford *et al.* 1992). ABT had no effect on the elevation of plasma 15-F_{2t}-IsoP levels by VPA (Figure 2-8), even though treatment with this compound resulted in undetectable levels of plasma 4-ene-, 3-OH-, 4-OH-, 5-OH-, and 4-keto-VPA concentrations (Table 2-5).

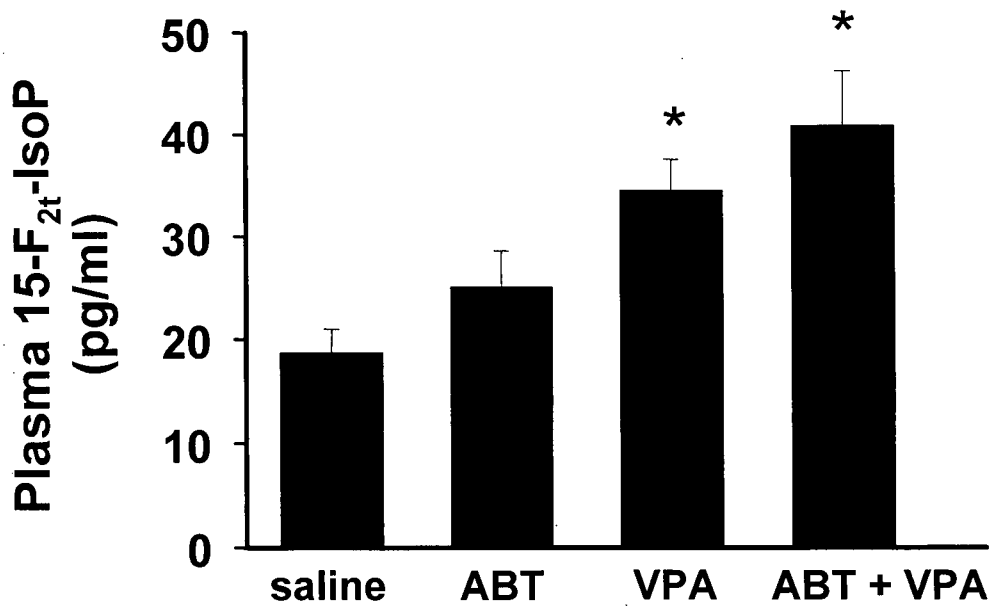

Figure 2-8: Effect of ABT pretreatment on free plasma 15-F_{2t}-IsoP levels in VPA-treated rats. Adult male Sprague-Dawley rats were pretreated with a single ip dose of 1-aminobenzotriazole (ABT, 100 mg/kg) or saline. A single ip dose of VPA (250 mg/kg) or saline (vehicle control) was administered 2 h after the pretreatment. At 0.5 h after VPA treatment rats were sacrificed and blood was collected. Values are means ± SEM (*n* = 6/group). *Significantly different from control, $p < 0.05$.

Table 2-5: Effect of 1-aminobenzotriazole on plasma levels of VPA metabolites.[a]

	Saline [b] (x 10^2 ng/ml)	ABT [c] (x 10^2 ng/ml)
4-ene VPA	1.2 ± 0.07	< LOQ
(*E*)-2,4-diene VPA	< LOQ	< LOQ
3-OH VPA	2.6 ± 0.37	< LOQ
4-OH VPA	11 ± 0.67	< LOQ
5-OH VPA	27 ± 1.6	< LOQ
4-keto VPA	4.2 ± 0.22	< LOQ

[a]Values represent the mean ± SEM (n=4). [b]Rats were pretreated with saline vehicle (ip), followed by VPA (250 mg/kg) 2 h after. [c]Rats were pretreated with ABT (100 mg/kg, ip) followed by VPA (250 mg/kg) 2 h after. ND, not detected.

2.3.7 Effect of SKF-525A on the enhancement of plasma levels of free 15-F_{2t}-IsoP by PB in rats treated with VPA

SKF-525A is an effective inhibitor of PB-inducible P450 enzymes in rat liver (Buening and Franklin 1976; Murray 1988). To determine the effect of inhibiting VPA biotransformation by PB-inducible P450 enzymes on plasma levels of 15-F_{2t}-IsoP, PB-pretreated (80 mg/kg/day, ip, for 4 days) rats were administered SKF-525A (80 mg/kg, ip) or saline at 0.5 h prior to VPA administration (500 mg/kg, ip). SKF-525A pretreatment did not alter the elevated levels of 15-F_{2t}-IsoP by PB in the VPA-treated rats (Figure 2-9). According to GC/MS analysis, SKF-525A administration reduced the levels of 4-ene-VPA, (*E*)-2,4-diene-VPA, 4-OH-VPA, 4-keto-VPA, 3-OH-VPA, and 5-OH-VPA in rats treated with the combination of PB and VPA (Table 2-6).

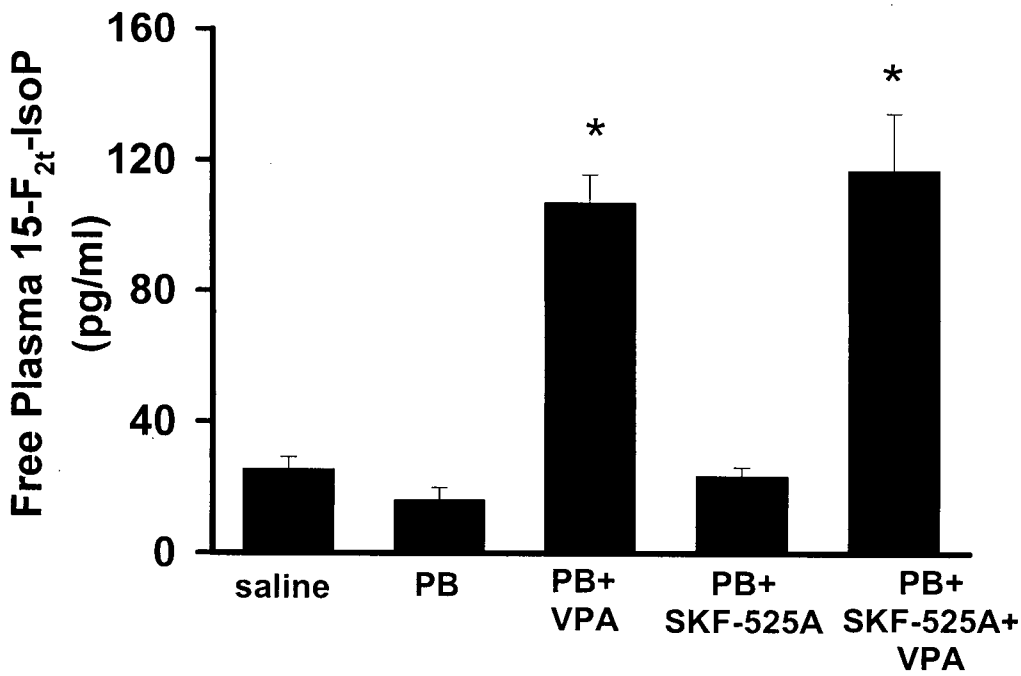

Figure 2-9: PB and SKF-525A pretreatment on plasma 15-F_{2t}-IsoP levels in VPA-exposed rats. Adult male Sprague-Dawley rats were pretreated with PB (80 mg/kg/day, ip) for 4 consecutive days. A single dose of SKF-525A (80 mg/kg, ip) or saline (vehicle control) was given on the following day. At 0.5 h following SKF-525A administration, rats were treated with VPA (500 mg/kg) or saline. At 0.5 h after VPA treatment rats were sacrificed and blood was collected. Values are means ± SEM (*n* = 6/group). *Significantly different from control, $p < 0.05$.

Table 2-6: Effect of SKF-525A on plasma levels of VPA metabolites in rats treated with both PB and VPA.[a]

	Saline [b] (x 10^2 ng/ml)	SKF-525A [c] (x 10^2 ng/ml)
4-ene VPA	11 ± 1.5	1.8 ± 0.28 *
(*E*)-2,4-diene VPA	0.60 ± 0.08	< LOQ
3-OH VPA	17 ± 2.8	7.3 ± 1.1 *
4-OH VPA	260 ± 4.0	49 ± 5.8 *
5-OH VPA	82 ± 1.2	46 ± 3.5 *
4-keto VPA	120 ± 2.5	15 ± 3.0 *

[a] Values represent the mean ± SEM (n=6). One-way analysis of variance using Newman-Keuls test was performed on the data. [b] Rats were pretreated with PB (80 mg/kg/day for 4 days), followed by saline on the next day and VPA (500 mg/kg, ip) 0.5 h later. [c] Rats were pretreated with PB (80 mg/kg/day for 4 days), followed by SKF-525A (80 mg/kg, ip) on the next day and VPA (500 mg/kg, ip) 0.5 h later. Rats were sacrificed 0.5 h after VPA treatment. *Indicates a statistically significant difference from the saline control group. < LOQ, below the limit of quantitation (2 ng/ml) for the GC/MS assay of VPA metabolites.

2.3.8 Lack of an effect on plasma 15-F_{2t}-IsoP levels by octanoic acid.

Octanoic acid (Figure 2-10), an 8-carbon straight chain fatty acid, was compared with VPA to determine if the elevation of plasma 15-F_{2t}-IsoP levels by VPA occurs with other fatty acids of similar structure. Rats were administered ip with octanoic acid at an equivalent dose (500 mg/kg) of VPA that resulted in maximal elevation of plasma 15-F_{2t}-IsoP levels or 0.9% saline (vehicle control). There was no significant difference between the plasma levels of 15-F_{2t}-IsoP in the octanoic acid-treated group (24 ± 5 pg/ml) and the control group (22 ± 3 pg/ml).

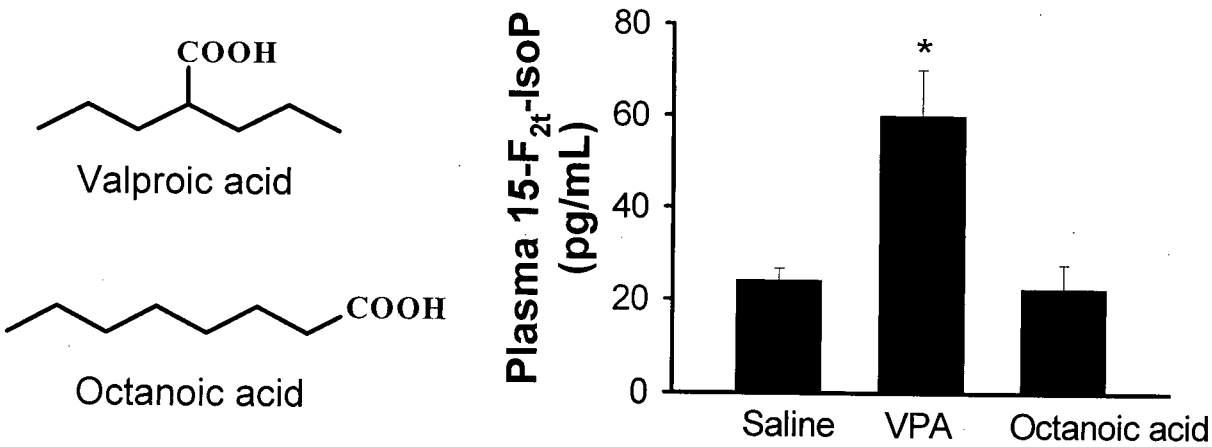

Figure 2-10: Comparison between VPA and octanoic acid on the effect of plasma 15-F_{2t}-IsoP levels in rats. Adult male Sprague-Dawley rats were treated with a single dose of VPA or octanoic acid (each at 500 mg/kg, ip). The control group was treated with saline vehicle. At 0.5 h after treatment, rats were sacrificed, and blood was collected for the immediate preparation of plasma. Values are means ± SEM (*n* = 4/group). *Significantly different from the saline-treated control group, $p < 0.05$.

2.4 DISCUSSION

It has been hypothesized that the responsible mediator of VPA-induced hepatotoxicity is produced by the P450-dependent formation of reactive metabolites, such as 4-ene-VPA and its subsequent mitochondrial β-oxidation metabolite, (*E*)-2,4-diene-VPA (Baillie 1992; Granneman *et al.* 1984; Kassahun *et al.* 1991; Kassahun *et al.* 1994; Kesterson *et al.* 1984; Porubek *et al.* 1989; Tang and Abbott 1996; Tang *et al.* 1995). This hypothesis was based on earlier observations that 4-ene-VPA was structurally similar to the known hepatotoxins 4-pentenoic acid and methylenecyclopropylacetic acid (Gerber *et al.* 1979), and that patients on VPA antiepileptic polytherapy with other antiepileptic compounds were at increased risk of VPA-induced idiosyncratic hepatotoxicity (Bryant and Dreifuss 1996; Dreifuss *et al.* 1989; Dreifuss *et al.* 1987). Another hypothesis involves the generation of ROS upon VPA exposure (Pippenger *et al.* 1991; Wilder and Hurd 1991), which may or may not be dependent on VPA biotransformation. In the present study in rats, experiments were performed to determine the effect of VPA on plasma and hepatic levels of 15-F_{2t}-IsoP, a sensitive marker of ROS.

The application of 15-F_{2t}-IsoP as a stable, *in vivo* marker for oxidative stress was demonstrated in animal models of free radical injury and lipid peroxidation (Morrow *et al.* 1990), and is increasingly being used as an *in vivo* index of lipid peroxidation. The formation of F_2-isoprostanes has been shown to increase significantly in rats exposed to CCl_4 and diquat (Morrow *et al.* 1992; Morrow *et al.* 1990) and in heavy smokers (Morrow *et al.* 1995). Quantitation of F_2-isoprostanes has been traditionally carried out by GC/MS (Morrow and Roberts 2002) and more recently by EIA methods. However, studies comparing both methods have demonstrated a lack of correlation due to inadequate separation and antibody cross-reactivity of closely related isoprostanes using GC/MS and immunoassay, respectively (Bessard *et al.* 2001; Mori *et al.* 1999; Proudfoot *et al.* 1999). The current study uses a commercially

available immunoassay that was validated in our laboratory and was shown to detect low concentrations (pg/ml levels) of 15-F_{2t}-IsoP in a small volume of sample (50 μl of plasma). In our study, saline-treated rats (control) had basal free plasma levels of 15-F_{2t}-IsoP in the range of 10-35 pg/ml. These levels of 15-F_{2t}-IsoP increased in a dose-dependent manner following single exposures of VPA, and the temporal profile of 15-F_{2t}-IsoP concentration in plasma followed that of VPA. These findings indicate that VPA treatment results in an increase in lipid peroxidation, and that the liver may be the source of the lipid peroxidation products.

Conflicting data exist in the literature with respect to the effect of VPA on lipid peroxidation. In a 2-year prospective study, epileptic children on VPA therapy demonstrated an elevation in the extent of lipid peroxidation as measured by the TBARS assay as compared to healthy controls, with a concomitant reduction in glutathione peroxidase levels and an increase in superoxide dismutase levels (Yuksel *et al.* 2001). Also, increased lipid peroxidation was observed in mice chronically treated with VPA, as demonstrated by increased TBARS (malondialdehyde equivalents) in the liver (Raza *et al.* 1997). However, other studies investigating the effects of VPA did not show significant differences in lipid peroxidation using the same biomarker for oxidative stress. For instance, rats treated with single doses of 500 or 1000 mg/kg VPA did not result in changes in TBARS levels in both liver homogenates and hepatic mitochondrial fractions (Seckin *et al.* 1999). A similar lack of increase in TBARS level by VPA treatment (1 mM) was also demonstrated in a rat hepatocyte model (Klee *et al.* 2000). However, in another study, the free radical scavengers vitamin E and *N,N'*-diphenyl-p-phenylenediamine protected against VPA toxicity in a rat hepatocyte model, suggesting that VPA hepatotoxicity is associated with lipid peroxidation (Buchi *et al.* 1984). In our results, although levels of TBARS and lipid hydroperoxides in plasma and liver were not affected by single doses of VPA, 15-F_{2t}-IsoP was found to be elevated suggesting that this is a more sensitive biomarker of oxidative stress. To further verify this difference in response of biomarkers, a 7-fold increase in plasma 15-F_{2t}-IsoP

was observed with carbon tetrachloride (data not shown) with no elevation in plasma or liver TBARS. This result was consistent with other studies that show that an elevation of 15-F_{2t}-IsoP in CCl_4-treated rats is not accompanied by an increase in TBARS levels (Basu 1999; Sodergren *et al.* 2001).

In the present study, rats pretreated with PB prior to VPA administration generated marked increases in levels of free 15-F_{2t}-IsoP in plasma and liver compared to the saline control and VPA-treated groups. Levels of total 15-F_{2t}-IsoP (sum of free and esterified) were also measured in plasma and liver. A single large dose of VPA, regardless of PB pretreatment, did not result in changes in total 15-F_{2t}-IsoP even though free 15-F_{2t}-IsoP levels in plasma and liver were elevated (data not shown). A possible explanation of this result is the combination of PB pretreatment followed by VPA treatment enhances the transition of the esterified form of 15-F_{2t}-IsoP to the free form. Thus the basal ratio of total/free 15-F_{2t}-IsoP in plasma and liver decreases from 3.3 and 4.6, respectively, after VPA to approximately 1.5 after PB/VPA treatment. This transition from esterified to free 15-F_{2t}-IsoP may also be inferred from a study that investigated the effect of vitamin E on liver levels of 15-F_{2t}-IsoP, where free 15-F_{2t}-IsoP was attenuated while total 15-F_{2t}-IsoP remained unchanged (Sodergren *et al.* 2001). However, in our study, this phenomenon was not observed in animals pretreated with PB alone, suggesting that PB does not simply enhance the release of the esterified form of 15-F_{2t}-IsoP. Although levels of total 15-F_{2t}-IsoP were not altered after a single dose of VPA, regardless of PB pretreatment, total levels of liver 15-F_{2t}-IsoP were elevated after chronic treatment of VPA for 2 weeks, suggesting that multiple doses of VPA are required to observe an overall increase in total lipid peroxidation in the liver.

The elevation of free 15-F_{2t}-IsoP in PB/VPA treated rats corresponded with increased plasma levels of 4-ene-VPA and (*E*)-2, 4-diene-VPA among other P450-dependent VPA metabolites.

These findings are consistent with the reactive metabolite hypothesis that suggests that an increase in the metabolic flux through the PB-inducible P450 pathway leads to higher levels of 4-ene-VPA and (*E*)-2,4-diene-VPA, which may be responsible for the marked elevation in plasma 15-F_{2t}-IsoP levels. However, SKF-525A, which attenuated P450-derived VPA metabolite levels, did not influence the PB-induced elevation in plasma levels of 15-F_{2t}-IsoP. This finding suggests that the mechanism by which PB increases the elevation of 15-F_{2t}-IsoP in VPA-treated rats does not involve VPA metabolites formed by pathways inhibited by SKF-525A.

P450 systems have been demonstrated to contribute to the *in vitro* formation of ROS as monitored by the formation of 2′,7′-dichlorofluorescin (DCF) by an NADPH-dependent process, which can be blocked by SKF-525A (Bondy and Naderi 1994). *In vitro* induction of P450 by dexamethasone and PB resulted in marked production of 2, 3-DHBA as an index of hydroxyl radical production, which was attenuated by SKF-525A (Strolin-Benedetti *et al.* 1999). Therefore, if the pathway leading to the P450-dependent formation of 4-ene-VPA, and thus (*E*)-2,4-diene-VPA, contributes directly or indirectly to the production of ROS, it should then be possible to influence 15-F_{2t}-IsoP production with the use of P450 inhibitors. Our study, however, demonstrated the lack of effect of P450 inhibitors (ABT and SKF-525A) on 15-F_{2t}-IsoP plasma levels. Based on the results obtained with P450 inhibitors, there appears to be a discrepancy between the *in vitro* studies using human lymphocytes (Tabatabaei *et al.* 1999) and our *in vivo* studies regarding the importance of P450-dependent VPA biotransformation on ROS generation. The discontinuity may reflect differences in biotransformation between models (microsomes vs. whole animal), the effector cells (lymphocytes vs. whole animal), and the ROS biomarker measured (H_2O_2 vs. 15-F_{2t}-IsoP). To help clarify the *in vivo* and *in vitro* discrepancy, research is currently underway to evaluate *in vitro* data seen with VPA using primary cultures of rat hepatocytes.

A possible explanation for the influence of PB on the VPA-dependent generation of ROS may involve the transactivation of the constitutive androstane receptor (CAR) by PB. This nuclear receptor has been shown to mediate the expression of multiple enzymes, including CYP2B (Honkakoski *et al.* 1998), glutathione S-transferase (GST) Pi (Zhang *et al.* 2002), UDP-glucuronosyltransferase (UGT) 1A1 (Sugatani *et al.* 2001), and a peroxisomal β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (Kassam *et al.* 2000). In a recent study, known CAR activators (e.g. PB and 1,4-bis[2-(3,5-dichloropyridyl-oxy)]benzene) in combination with acetaminophen treatment resulted in hepatotoxicity in wild-type but not in CAR-null mice (Zhang *et al.* 2002). Conceivably, CAR may also play a role in the observed enhancement of ROS levels by PB in rats administered VPA. Studies are now in progress to elucidate the cellular and molecular mechanisms by which VPA produces oxidative stress, how PB is able to modulate this effect, and the impact of VPA-dependent ROS generation on mitochondrial dysfunction and hepatotoxicity. The *in vivo* studies described in the manuscript do raise issues regarding the importance of oxidative stress and/or the possibility of covalent binding, the latter not being ruled out, in the proposed mechanism of VPA-associated hepatotoxicity.

In summary, our data demonstrate that VPA results in a dose- and time-dependent increase in free plasma levels of 15-F_{2t}-IsoP that follow the plasma profile of VPA in rats. PB-pretreatment significantly enhanced free plasma and liver levels of 15-F_{2t}-IsoP by a mechanism that remains unclear; however, this was not due to enhanced production of P450-dependent metabolites such as 4-ene-VPA.

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3 *Valproic Acid Glucuronidation is Associated with Increases in 15-F_{2t}-Isoprostane Levels in Rats*

3.1 INTRODUCTION

F_2-isoprostanes, a series of prostaglandin $F_{2\alpha}$-isomers, are produced by a free-radical-catalyzed (cyclooxygenase-independent) lipid peroxidation of arachidonic acid. Among the F_2-isoprostanes, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) is used as a marker for lipid peroxidation (Morrow *et al.* 1992; Morrow *et al.* 1990a; Morrow *et al.* 1990b; Morrow *et al.* 1994). Recently, we reported that VPA was associated with an increase in oxidative stress as measured by serum and liver levels of 15-F_{2t}-IsoP in rats (Tong *et al.* 2003). The increase in 15-F_{2t}-IsoP was dose-dependent and its plasma concentration-time profile was similar to that of VPA. Interestingly, this increase in 15-F_{2t}-IsoP by VPA was further enhanced by pretreatment with PB. However, the elevation in 15-F_{2t}-IsoP was not associated with P450-dependent VPA-biotransformation because pretreatment with P450 inhibitors did not influence the levels of 15-F_{2t}-IsoP, even though there was a dramatic decrease in the levels of VPA oxidative and desaturated metabolites (Tong *et al.* 2003).

The effect of PB on the VPA-associated increase in 15-F_{2t}-IsoP was an interesting observation and prompted us to investigate the role of VPA glucuronidation, a major VPA biotransformation pathway, on 15-F_{2t}-IsoP levels. VPA forms the 1-*O*-acyl glucuronide conjugate (VPA-G) and this is catalyzed by UDP-glucuronosyltransferase (UGT) enzymes, which transfer the glucuronide moiety from UDP-glucuronic acid (UDPGA) to the carboxylate group of VPA. PB induces specific UGT enzymes and elevates levels of hepatic UDPGA (Granneman *et al.* 1984;

Heinemeyer *et al.* 1985; Watkins and Klaassen 1982). Specifically, rat UGT2B1 and human UGT2B7 (both are PB-inducible), UGT1A3, UGT1A6, and UGT1A9 have all been reported to catalyze VPA glucuronidation (Ethell *et al.* 2003; Ritter 2000; Sakaguchi *et al.* 2004). VPA-G undergoes extensive enterohepatic recycling in the rat (Dickinson *et al.* 1979; Pollack and Brouwer 1991) and it was demonstrated that greater than 90% of the VPA in rat bile was in the form of the glucuronide conjugate (Watkins and Klaassen 1981). Glucuronidation is dependent upon intracellular UGT activity as well as UDPGA concentration (Boelsterli 2002).

The present study in rats investigated the influence of modulating VPA glucuronidation on oxidative stress, as measured by plasma and hepatic levels of 15-F_{2t}-IsoP. For comparison, we also determined plasma and hepatic levels of 15-F_{2t}-IsoP in rats treated with α-fluoro-VPA (α-F-VPA), which is an analogue of VPA demonstrated to be a poor substrate for glucuronidation (Tang *et al.* 1997). A novel and intriguing finding in our study is that VPA-G formation is associated with oxidative stress. This report also describes a novel assay for the direct measurement of VPA-G by an LC/MS method.

3.2 MATERIALS & METHODS

3.2.1 Materials

2-Propyl-pentanoic acid (sodium salt, VPA), (-)-borneol, salicylamide, D-saccharic acid 1,4-lactone (D-saccharolactone), and 1-chlorobutane were purchased from Sigma Chemicals Co. (Oakville, ON, Canada). 2-(Propyl-3,3,3-d_3) pentanoic-5,5,5-d_3 acid ([$^2H_6$]-VPA, 99.5 atom % D) was obtained from CDN Isotopes (Pointe-Claire, QC, Canada). Sodium phenobarbital (PB) was purchased from BDH Chemicals Inc. (Toronto, ON, Canada). The 15-F_{2t}-isoprostane (8-isoprostane) EIA kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Ethyl acetate, acetonitrile (both HPLC grade), N-hexanes (GC/MS resolved), and diethyl ether were purchased

from Fisher Scientific (Vancouver, BC, Canada). α-F-VPA was synthesized in our laboratory by a previously described method (Tang *et al.* 1997).

3.2.2 Instrumentation and analytical methods

^{1}H-NMR spectra were obtained on a Bruker (Silberstreifen, Germany) Avance 300 Spectrometer in the Department of Chemistry at the University of British Columbia. Chemical shifts are expressed relative to tetramethylsilane.

LC/MS analysis of VPA-1-*O*-acyl glucuronide 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, data acquisition and integration were controlled by MassLynx® (v3.1, Micromass, Montreal, Canada) software. Negative electrospray was used as the means of ionization. The collision energy was 15 eV, source temperature 140°C, capillary voltage 3 kV, and cone voltage 15 V with skimmer offset by 5 V. The multipliers 1 and 2 were set at 650 V. The low mass and high mass resolutions were set at 5.0 for MS1. To record the full daughter ion spectra of VPA-G, mass resolutions were set at 12.5 (MS1) and 5.0 (MS2), MS/MS dwell times were adjusted to provide a scan rate of 1 sec/100 amu, and collision-induced dissociation used argon gas at a pressure of 3.0×10^{-4} mbar. The HPLC was fitted with a Phenomenex Columbus C18 column (150 × 2 mm, 5 μm, Torrance, CA). The HPLC autoinjector syringe and sample loop volumes were 25 μl and 250 μl, respectively. The mobile phase consisted of a mixture of acetonitrile/water with 10 mM ammonium acetate and delivered at 0.2 ml/min at room temperature. Linear gradient conditions were as follows: 20% to 80% acetonitrile from 0 to 5 min and a return to 20% acetonitrile at 5.5 min and held at 20% acetonitrile for 3.5 min for equilibration. Total run time was 9 min.

3.2.3 Animals

Adult male Sprague-Dawley rats (250–300 g) were obtained 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 hr light/12 hr dark cycle at constant temperature (22°C) and humidity. All animal experiments were approved by the University of British Columbia Animal Care Committee.

3.2.4 Isolation of VPA-1-O-acyl glucuronide (VPA-G) and [$^2H_6$]-VPA-1-O-acyl glucuronide ([$^2H_6$]-VPA-G)

Rats were anesthetized with urethane (1.2 g/kg ip) in an aqueous solution (0.4 g/ml) and their bile ducts cannulated with PE-10 tubing. Control bile was collected for 20 min. Rats (3 individual rats per group) were administered either VPA (100 mg/ml) in saline) or [$^2H_6$]-VPA (100 mg/ml) prepared with an equimolar amount of 2 mmol NaOH and 0.9% saline) at 100 mg/kg every 2 h (4 doses in total) by ip injection, and bile was collected on ice for a total of 8 hours. The purification of VPA-G and [$^2H_6$]-VPA-G was performed using the method by Cannell *et al.* (Cannell *et al.* 2002) Briefly, aliquots of rat bile (4 ml) were acidified with 5 M phosphoric acid to pH 2 and centrifuged (3000 × *g,* 10 min, room temperature). The supernatant (4 ml) was extracted with 1-chlorobutane (3 × 12 ml) to remove free VPA or [$^2H_6$]-VPA, and the aqueous fraction was extracted with diethyl ether (3 × 12 ml). The diethyl ether extracts were evaporated *in vacuo* to dryness to give a dark-green residue. The residue was reconstituted in mobile phase (15% acetonitrile/85% water in 10 mM ammonium acetate) and subjected to further purification by HPLC. Bile extracts were injected on a Zorbax 300SB-C8 semi-preparative column (9.4 mm I.D. × 250 mm) and eluted at a flow rate of 3 ml/min with a linear gradient of 15% to 33% acetonitrile in water containing 10 mM ammonium acetate over a run time of 12 min. HPLC-purified fractions were collected, pooled, and subjected to lyophilization.

LC/MS/MS mass spectrum of 2-propyl-pentanoyl-1-*O*-acyl glucuronide (VPA-G, Figure 3-1A), *m/z* (%): 319 ($[M-H]^-$, 30), 175 (G -2H, 50), 143 (VPA-H, 50), 113 (G -$HOCH_2CH_2OH$, 100), 103 (11), 59 (8). ^1H NMR (D_20) (Figure 3-2A): δ 0.75 (t, 6H, J_{HH} = 7.2 Hz, 5,5′), 1.21-1.14 (m, 4H, 4,4′), 1.51-1.38 (m, 4H, 3,3′), 2.44 (m, 1H, 2), 3.42 (m, 3H, G, 2″,3″,4″), 3.75 (d, 1H, J_{HH} = 9 Hz, G, 5″), 5.42 (d, 1H, J_{HH} = 7.8 Hz, G, 1″). The ^1H NMR spectrum for VPA-G was in agreement with the spectrum obtained by Azaroual *et al.* (2000).

LC/MS/MS mass spectrum of 2-(propyl-3,3,3-d_3) pentanoyl-5,5,5-d_3-1-*O*-acyl glucuronide ($[^2H_6]$-VPA-G, Figure 3-1B), *m/z* (%): 325 ($[M-H]^-$, 35), 258 (9), 199 (5), 175 (G -2H, 50), 149 (d_6-VPA -H, 45), 113 (G -$HOCH_2CH_2OH$, 100), 103 (12), 59 (12). ^1H NMR (D_20) (Figure 3-2B): δ 1.16 (t, 4H, J_{HH} = 7.1 Hz, 4, 4′), 1.45 (m, 4H, 3, 3′), 2.44 (m, 1H, 2′), 3.43 (m, 3H, G, 2″,3″,4″), 3.79 (d, 1H, J_{HH} = 7.9 Hz, G, 5′), 5.43 (d, 1H, J_{HH} = 7.8 Hz, G, 1″).

3.2.5 Treatment of animals and sample collection

Rats were administered with VPA, α-F-VPA, or 0.9% saline (vehicle control) at the doses and duration indicated in each figure legend. To modulate VPA glucuronidation, rats were injected ip with PB (80 mg/kg/day for 4 consecutive days in 0.9% saline), (-)-borneol (320 mg/kg in corn oil), salicylamide (1 mmol/kg in 50% glycerol), or combination of PB for 4 days and (-)-borneol treatment prior to VPA dosing (500 mg/kg, ip) at a time point indicated in each figure legend. At 0.5 h after VPA administration, rats were sacrificed by decapitation, trunk blood was collected, and plasma was prepared. An aliquot of the plasma was used immediately for the determination of 15-F_{2t}-IsoP levels. Livers were weighed and homogenized in 30 ml of 50 mM phosphate buffer (pH 7.4) with 5 mM D-saccharolactone under ice-cold conditions. The homogenate was processed immediately for the analysis of 15-F_{2t}-IsoP levels. Aliquots of liver homogenate were stored at -80°C for subsequent determination of VPA-G.

3.2.6 Sample preparation for VPA-G assay by LC/MS

Working stock solutions of 1 mg/ml VPA-G and 0.1 mg/ml [$^2H_6$]-VPA-G were prepared in distilled water, aliquots stored at -80°C, and were used for all calibration curve standards. Calibration standards (at concentrations of 0.5, 1, 5, 10, 25 and 50 μg/ml) were prepared in triplicate by adding appropriate volumes of the working stock solution in water to a final volume of 1 ml. Rat liver homogenate samples (0.5 ml) were diluted with water (0.5 ml). Samples were acidified by the addition of 1 ml of 1 M KH_2PO_4 (pH 2.5). [$^2H_6$]-VPA-G (50 μl of a 0.1 mg/ml solution) served as the internal standard and was added to each sample. Liquid-liquid extraction was performed twice with methyl *tert*-butyl ether (5 ml for each extraction) and the extracts were pooled and evaporated to dryness under nitrogen. The residue was reconstituted in water and filtered using Acrodisk® (0.2 micron, Gelman Sciences) syringe filters directly into autosampler vials. An aliquot (10 μl) was injected on the HPLC. In α-F-VPA-treated rats, liver samples were processed as described above and the α-F-VPA-G levels were determined indirectly by LC/MS using the same response factor as the VPA-G standard since no synthetic standard for α-F-VPA-G was available.

3.2.7 Validation of the LC/MS VPA-G assay

Assay validation was performed by evaluating inter-assay and intra-assay accuracy (% bias) and precision (coefficient of variation, %CV) of the low (0.5 μg/ml), mid (8 μg/ml), and high (40 μg/ml) concentration quality control (QC) samples. The QC samples were prepared in blank liver homogenate containing 5 mM D-saccharolactone, flash-frozen in liquid nitrogen, stored at –80°C, and aliquots thawed for daily use. This was accomplished by analyzing 5 sets of calibration curves (triplicate readings) and QC samples (6 replicates) on 5 separate days (inter-assay) and on the same day (intra-assay). Quantification of QC samples was performed by

analyzing the calibration curve standards and back calculating the concentration of each QC sample from the obtained slope, intercept, and the peak area ratios.

The accuracy of the assay was assessed as the % bias of the nominal concentration observed for the spiked QCs and a bias of < 15% at each concentration was considered to be acceptable accuracy. The precision of the assay (% CV) was determined from the variance observed for the mean of replicate QCs of low, mid and high concentrations. Precision values of < 15% CV at the mid and high QC concentrations and < 20% at the low QC concentration were considered to meet the validation acceptance criteria (Karnes *et al.* 1991).

The mean percent recovery of VPA-G spiked in blank rat liver homogenate was determined at concentrations (0.5, 1, 5, 10, 25, and 50 μg/ml) representing the entire range of the calibration curve.

3.2.8 15-F_{2t}-IsoP Assay

Plasma and hepatic levels of 15-F_{2t}-IsoP were determined using an EIA assay kit (Cayman Chemical Co., Ann Arbor, MI) as described previously in section 2.2.4.

3.2.9 Statistical analysis

Statistical significance of the difference between the means of multiple groups was analyzed by one-way analysis of variance and was followed by the Student-Newman-Keuls multiple range *post hoc* test. Comparison between pretreated and treated groups (2 × 2 format) was analyzed by two-way analysis of variance and was followed by the Student Newman-Keuls multiple range *post hoc* test. The level of significance was set *a priori* at $p < 0.05$.

3.3 RESULTS

3.3.1 Biosynthesis and identification of VPA-glucuronide

VPA-G and its deuterated internal standard, [$^2H_6$]-VPA-G, were isolated from rat bile for the purpose of developing and validating an LC/MS assay. The isolation and purification of the glucuronides from the bile of rats treated with VPA or [$^2H_6$]-VPA were achieved by extraction with 1-chlorobutane and diethyl ether followed by purification by HPLC. The glucuronide conjugates were identified by LC/MS (Figure 3-1) and by ^{1}H-NMR (Figure 3-2). Under LC/MS using multiple reaction monitoring mode, VPA-G and [$^2H_6$]-VPA-G resulted in glucuronide fragments: transitions *m/z* 319 $\rightarrow$175 and *m/z* 325 $\rightarrow$175, respectively, and in diagnostic fragments: transitions *m/z* 319 $\rightarrow$143 and 325 $\rightarrow$149, corresponding to the respective VPA and [$^2H_6$]-VPA moieties (Figure 3-1). The analysis of VPA-G from liver homogenates of rats treated with VPA was performed by LC/MS using reverse phase HPLC with gradient elution and single ion monitoring (*m/z* 319 for VPA-G and *m/z* 325 for [$^2H_6$]-VPA-G). Results show that both VPA-G and its internal standard, [$^2H_6$]-VPA-G were detected in whole liver homogenate having retention times of 4.9 min, with the internal standard eluted approximately 0.04 min earlier (Figure 3-3A). The VPA-G standard isolated from the bile of rats treated with VPA was shown to co-elute with the analyte detected in the liver homogenates of VPA-treated rats (Figure 3-3C). No peak-elution for VPA-G was detected in liver homogenates of control rats treated with 0.9% saline (Figure 3-3B).

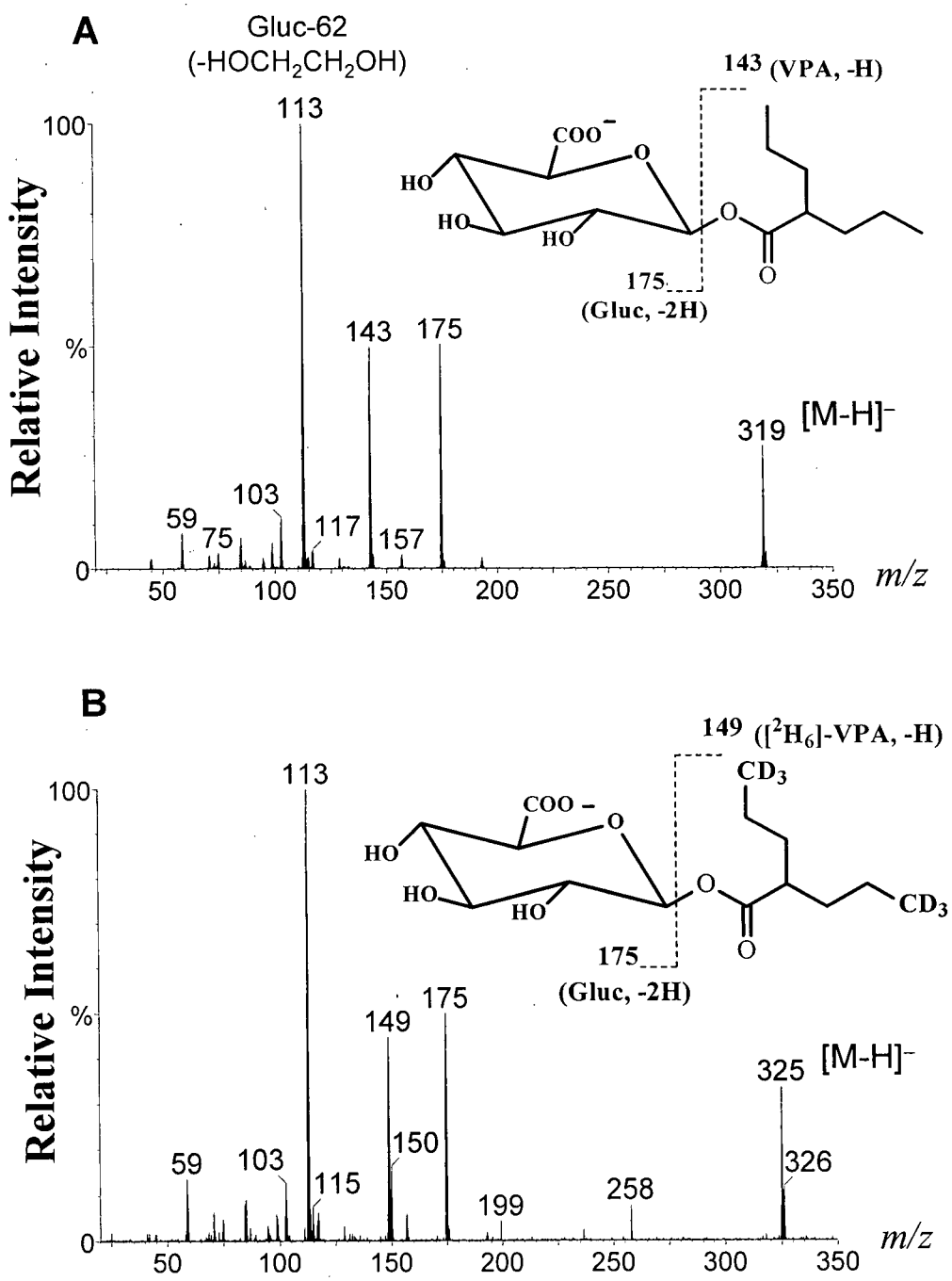

Figure 3-1: Collision-induced dissociation mass spectra of (A) VPA-1-*O*-acyl glucuronide (VPA-G) and (B) [$^2H_6$]-VPA-1-*O*-acyl glucuronide ([$^2H_6$]-VPA-G) purified from rat bile obtained by LC/MS/MS with negative ion electropspray ionization. Gluc = glucuronide.

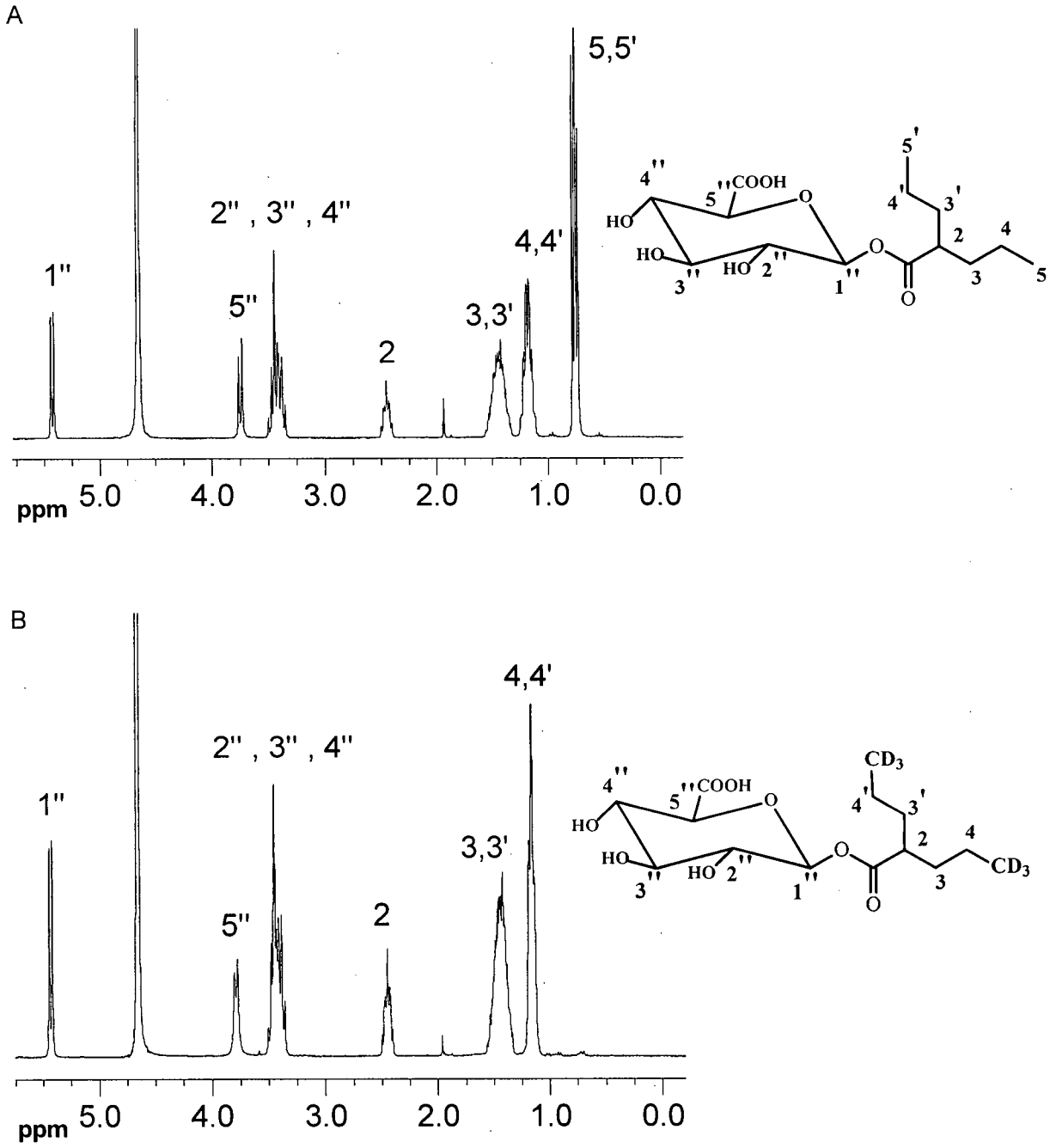

Figure 3-2: ^{1}H-NMR spectra (D_2O) of biosynthesized (A) 2-propyl-pentanoyl-1-*O*-acyl glucuronide (VPA-G) and (B) 2-(propyl-3,3,3-d_3) pentanoyl-5,5,5-d_3-1-*O*-acyl glucuronide ([$^2H_6$]-VPA-G) purified from rat bile.

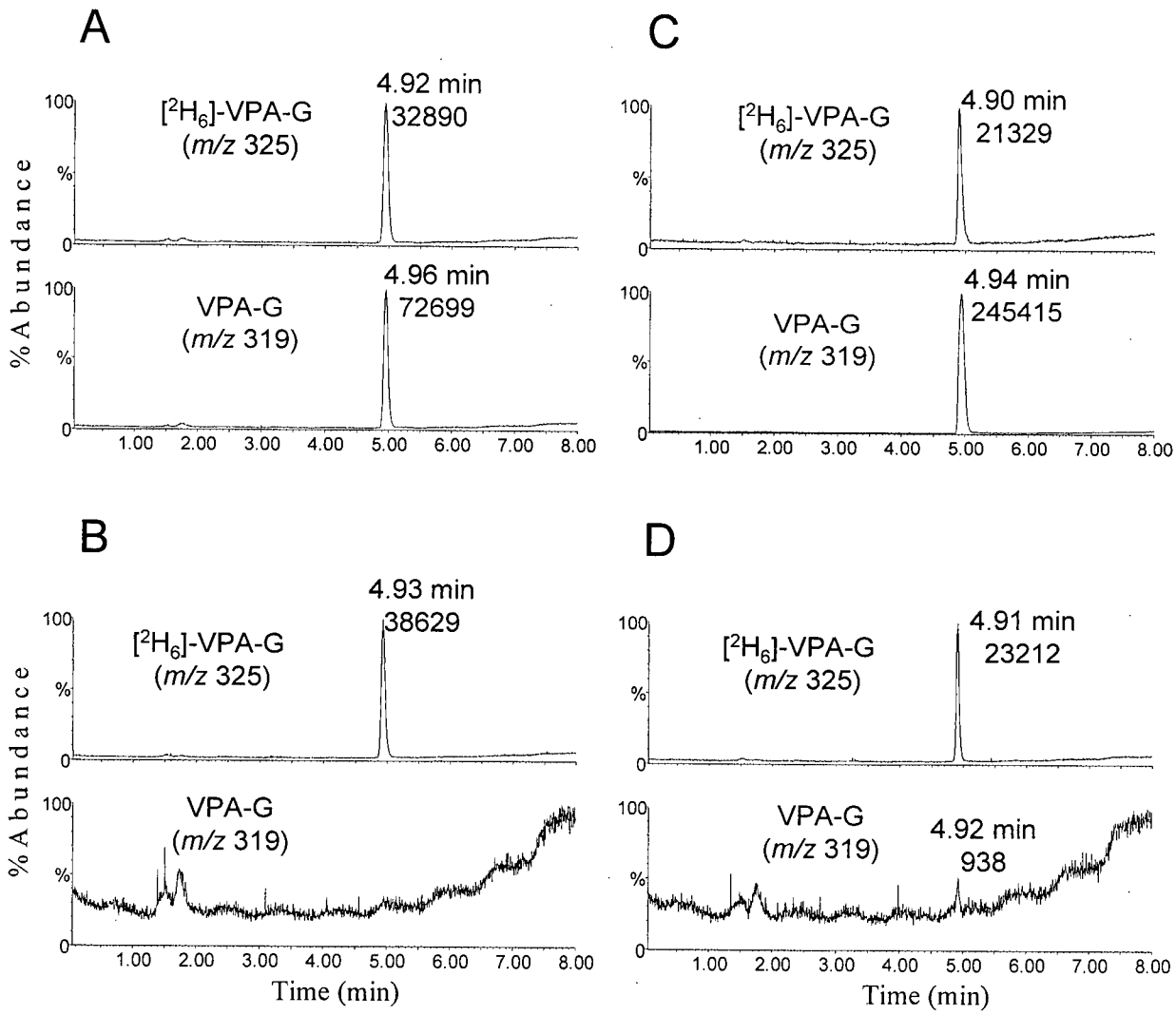

Figure 3-3: Representative LC/MS chromatograms of VPA-G (*m/z* 319) (A) as biosynthesized standard spiked in blank liver homogenate, and from liver homogenates from rats treated ip with (B) 0.9% saline, (C) VPA (500 mg/kg), or (D) (-)-borneol (320 mg/kg) followed by VPA (500 mg/kg) 0.5 hr later. [$^2H_6$]-VPA-G (*m/z* 325), the internal standard, was spiked into each sample.

3.3.2 Validation of LC/MS VPA-G assay

A quantitative assay for VPA-G in liver homogenate was developed using liquid-liquid extraction with methyl *tert.*-butyl ether as the extraction solvent after samples were acidified with 1M KH_2PO_4 (1 ml, pH 2.5). The mean percent recovery of VPA-G spiked in blank rat liver homogenate was determined at concentrations (0.5, 1, 5, 10, 25, and 50 μg/ml) representing the entire range of the calibration curve and was found to be approximately 78%. Calibration curves were prepared in triplicate in water and demonstrated linearity over the range 0.5-50 μg/ml ($r^2 > 0.99$) with mean slope and intercept values of 0.277 ± 0.004 and –0.008 ± 0.007, respectively (n = 5 validation days). The inter-assay and intra-assay variability and precision based on quality control (QC) samples of VPA-G at high (40 μg/ml), mid (8 μg/ml) and low (0.5 μg/ml, representing the LOQ) concentrations in blank liver homogenate were all less than 15% CV and less than ± 15% bias (Table 3-1). The LOQ for the VPA-G assay was estimated to be 0.5 μg/ml, based on the validation results in accordance of having a CV of < 20% (Karnes *et al.* 1991) and a signal to noise ratio of greater than 5 to 1. Although no matrix interferences were observed in the liver homogenate samples, we observed a trend of negative bias in both inter- and intra-assay determination which increased at the high QC sample, suggesting sample degradation. However, the sample degradation observed was minimal as evidenced by the bias values shown in Table 3-1, which in all cases fell within the acceptance criteria. When D-saccharolactone was not included in the homogenization buffer, marked sample degradation in the liver homogenate was noted with extraction recoveries of < 20% (data not shown). To minimize the potential degradation of VPA-G during processing, samples were thawed quickly, immediately placed on ice, and acidified with cold phosphate buffer prior to extraction. Samples were stable at –80°C as demonstrated by a lack of increasing negative assay bias in the QC samples with time over the 5 validation days and in subsequent analysis runs post-validation.

3.3.3 Dose-dependent increases in plasma and hepatic 15-F_{2t}-IsoP and VPA-G levels in VPA-treated rats

A dose-dependent increase in plasma 15-F_{2t}-IsoP levels was observed following VPA treatment with marked elevation at 250 mg/kg (73 ± 9 pg/ml) and 500 mg/kg doses (82 ± 5 pg/ml) (Figure 3-4A) compared to the saline-treated control group (31 ± 4 pg/ml). However, liver (free and total) 15-F_{2t}-IsoP levels were only significantly elevated after 500 mg/kg doses (Figure 3-4C and D, respectively). Hepatic VPA-G levels increased over the dose range and reached a maximum at doses of 250 and 500 mg/kg (Figure 3-4B).

Table 3-1: Inter- and intra-assay variation of the VPA-G assay by LC/MS.[a]

Inter-assay validation

Day	QC low	QC mid	QC high
1	0.52	8.37	37.7
2	0.57	8.38	37.6
3	0.51	7.43	36.8
4	0.43	7.72	35.8
5	0.42	7.71	36.2
Nominal conc. (mg/ml)	0.50	8.00	40.0
Mean conc. (mg/ml)	0.49	7.92	36.8
SD	0.06	0.43	0.85
C.V. (%)	13.1	5.4	2.3
BIAS (%)	-2.21	-0.96	-7.91

Intra-assay validation

Replicate #	QC low	QC mid	QC high
1	0.61	7.21	36.7
2	0.45	7.52	37.1
3	0.44	7.52	35.8
4	0.54	7.47	36.8
5	0.51	7.53	36.9
6	0.49	7.33	37.5
Nominal conc. (mg/ml)	0.50	8.00	40.0
Mean conc. (mg/ml)	0.51	7.43	36.8
SD	0.06	0.13	0.57
C.V. (%)	12.5	1.8	1.5
BIAS (%)	1.10	-7.10	-7.94

[a] Method validation was performed by evaluating inter-assay accuracy (% bias) and precision (coefficient of variation, % CV) of the low, mid, and high QC concentrations in blank rat liver homogenate on separate days. Quantitation of QC samples was performed by analyzing the calibration curve standards and back calculating the concentration of each QC sample from the obtained slope and intercept.

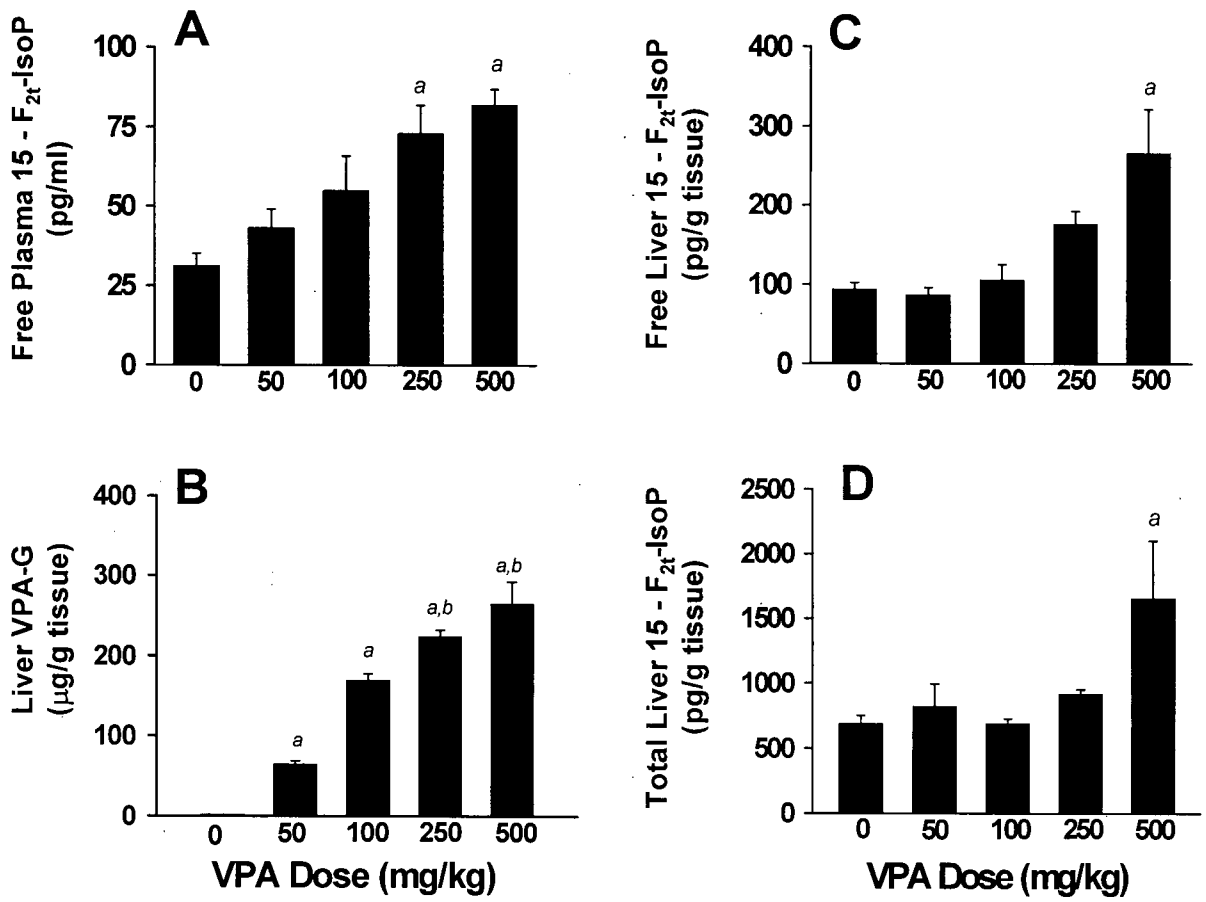

Figure 3-4: Dose-dependent effect of VPA on (A) plasma, (B) free and (D) total liver levels of 15-F_{2t}-IsoP, and (C) liver VPA-G. Rats were injected with a single ip dose of VPA (50, 100, 250 or 500 mg/kg). Control rats received an equivalent volume (1 ml/kg) of saline vehicle. Results are shown as mean ± SEM for 4 individual rats per treatment group. [a]Significantly different from control, $p < 0.05$; [b]Not significantly different from the 250 mg/kg group, $p > 0.05$.

3.3.4 Effect of (-)-borneol on VPA-glucuronide and 15-F_{2t}-IsoP levels in rats treated with VPA

To determine the effect of UDPGA depletion on VPA-glucuronidation and on the plasma and liver levels of 15-F_{2t}-IsoP, rats (n = 8 individual rats per group) were pretreated with a single dose of (-)-borneol (320 mg/kg, ip) or an equal volume of corn oil (vehicle control). At 0.5 h after (-)-borneol pretreatment, rats were treated with saline vehicle or a single dose of VPA (500 mg/kg, ip). This regimen of (-)-borneol treatment is a modification of described procedures (Watkins and Klaassen 1982) and demonstrated maximal inhibition of the glucuronidation of 2-phenylpropionic acid, a carboxylic acid similar in structure to VPA (Li *et al.* 2003). (-)-Borneol inhibited VPA-G formation by approximately 97% compared to animals treated with VPA alone (Table 3-2 and Figure 3-3D). The concentration of VPA-G detected in VPA-treated rats pretreated with (-)-borneol was 9 ± 3 μg/g tissue, which was significantly lower than that determined in livers of rats treated with only VPA (315 ± 26 μg/g tissue). Concomitant with this decrease in VPA-G, the apparent level of 15-F_{2t}-IsoP detected in plasma of rats treated with (-)-borneol and VPA (60 ± 4 pg/ml) was significantly lower than the levels seen in VPA-treated rats (93 ± 4 pg/ml), but still greater than levels seen in control rats (35 ± 4 pg/ml) (Figure 3-5A). A similar trend was also seen in liver; however, the levels of free and total 15-F_{2t}-IsoP were decreased in the (-)-borneol/VPA group to levels seen in the vehicle control group (Figure 3-5B and Figure 3-5C). (-)-Borneol alone did not have an effect on plasma or liver 15-F_{2t}-IsoP levels compared to vehicle control rats.

3.3.5 Effect of salicylamide on VPA-glucuronide and 15-F_{2t}-IsoP levels in rats treated with VPA

Salicylamide pretreatment, at a dose (2 mmol/kg) and a time point (0.5 h prior to VPA) shown to maximally deplete UDPGA (Howell *et al.* 1986; Kamisako *et al.* 1990), produced similar results

as observed with (-)-borneol with respect to VPA-G and 15-F_{2t}-IsoP levels. Liver VPA-G levels significantly decreased from 247 ± 54 μg/g tissue in VPA-treated animals to 60 ± 19 μg/g in salicylamide/VPA-treated animals. Furthermore, in the salicylamide-pretreated rats, 15-F_{2t}-IsoP levels in plasma and liver were significantly decreased after VPA dosing compared to VPA-treated animals without pretreatment with salicylamide (Figure 3-6A-C).

3.3.6 Effect of PB on VPA-glucuronide and 15-F_{2t}-IsoP levels in rats treated with VPA

When rats were pretreated with PB prior to VPA dosing, there was a further 2.2-fold increase in plasma 15-F_{2t}-IsoP levels (149 ± 18 pg/ml) compared to the VPA-treated group (68 ± 5 pg/ml) (Figure 3-7A). Free and total liver 15-F_{2t}-IsoP levels were also elevated in PB-pretreated animals (369 ± 37 and 3194 ± 325 pg/g tissue, respectively) compared to rats treated with VPA only (237 ± 29 and 2664 ± 182 pg/g tissue, respectively) (Figure 3-7C and D). Associated with the increase in 15-F_{2t}-IsoP levels in PB/VPA treated rats, was a marked elevation in VPA-G levels (356 ± 11 μg/g tissue) compared to the VPA group (266 ± 17 μg/g tissue) (Table 3-2).

3.3.7 Effect of (-)-borneol on PB-induced increases in VPA-glucuronide and 15-F_{2t}-IsoP levels in rats treated with VPA

When PB-pretreated rats were treated with a single dose of (-)-borneol prior to VPA treatment, the levels of plasma 15-F_{2t}-IsoP were reduced from 149 ± 18 (PB/VPA group) to 109 ± 12 pg/ml (Figure 3-7A-C). In the same treatment group, the free liver and total liver 15-F_{2t}-IsoP were reduced to vehicle control levels. Associated with the observed decrease in 15-F_{2t}-IsoP in the PB/borneol/VPA group was a significant decrease in VPA-G levels (129 ± 12 μg/g tissue) compared to both PB/VPA (356 ± 11 μg/g tissue) and VPA groups (266 ± 17 μg/g tissue) (Table 3-2).

Table 3-2: The effects of pre-treatment with PB, (-)-borneol, and salicylamide on VPA-G levels measured in liver homogenate.[a]

Pretreatment	Treatment	Sample size	VPA-G (μg/g tissue)
Corn oil [b]	VPA	n = 8	259 ± 20
(-)-Borneol	VPA	n = 8	12 ± 4*
Glycerol [c]	VPA	n = 4	247 ± 54
Salicylamide	VPA	n = 4	60 ± 19*
Saline (4d)[d]/ corn oil	VPA	n = 8	266 ± 17
PB (4d)[d]/ corn oil	VPA	n = 8	356 ± 11*
PB (4d)[e]/ (-)-Borneol	VPA	n = 8	129 ± 12**

[a]Values represent the mean ± SEM. Rats were pretreated with [b]corn oil vehicle or (-)-borneol (320 mg/kg, ip), [c]glycerol vehicle (50%) or salicylamide (1 mmol/kg, ip), [d]saline vehicle or PB (80 mg/kg/day, 4 consecutive days, ip) followed by VPA-treatment (500 mg/kg, ip) 0.5 h later. [e]Rats were pretreated with PB (80 mg/kg/day, 4 consecutive days, ip), followed by (-)-borneol on day 5 at 0.5 h prior to VPA treatment (500 mg/kg, ip). *Significantly different from the vehicle pretreated + VPA group ($p < 0.05$). **Significantly different from all other groups ($p < 0.05$).

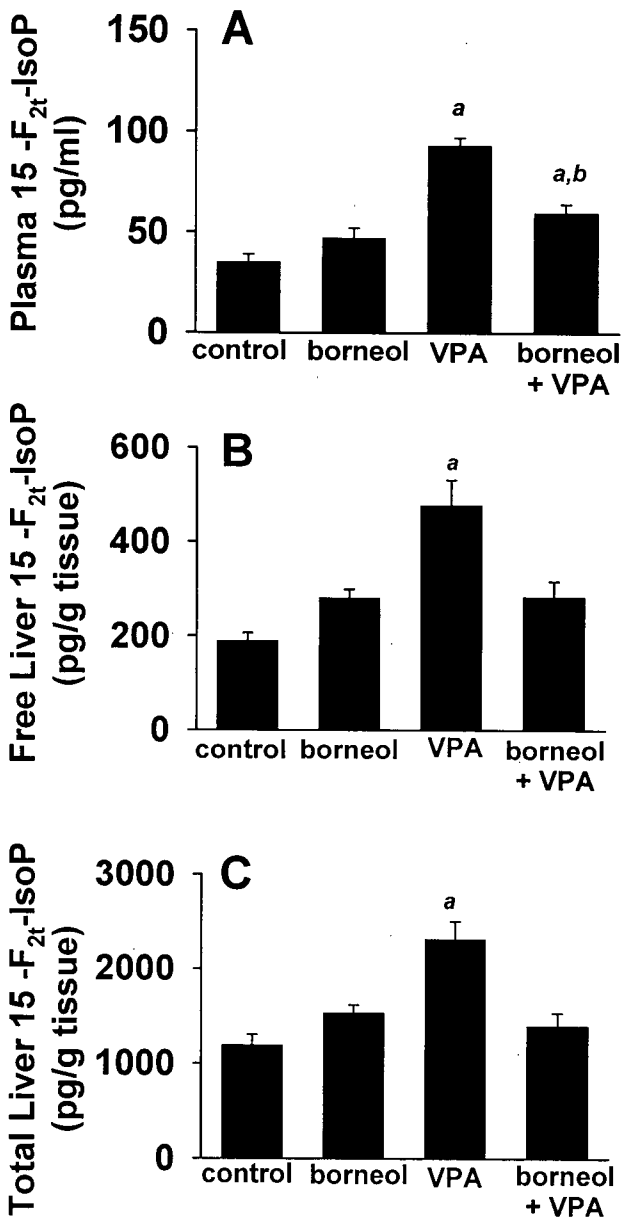

Figure 3-5: Effect of (-)-borneol pretreatment on levels of (A) plasma 15-F_{2t}-IsoP, (B) free liver 15-F_{2t}-IsoP, and (C) total liver 15-F_{2t}-IsoP in VPA-treated rats. Sprague-Dawley rats were pretreated ip with (-)-borneol (320 mg/kg) or corn oil (vehicle). At 0.5 h later, the rats were treated ip with a single dose of VPA (500 mg/kg) or 0.9% saline (vehicle). At 0.5 h after VPA treatment, rats were sacrificed, and blood and liver were collected. Results are shown as mean ± SEM for 8 individual rats per treatment group. [a]Significantly different from the control group, $p<0.05$; [b]Significantly different from the VPA group, $p<0.05$.

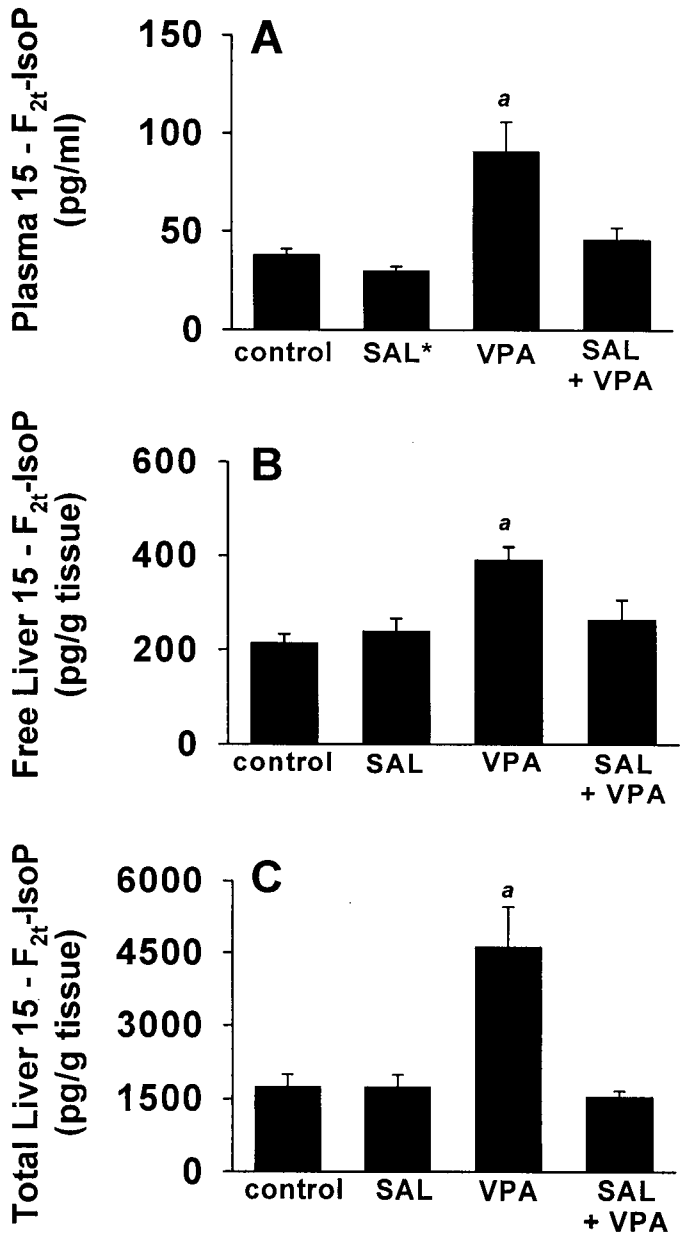

Figure 3-6: Effect of salicylamide (SAL) pretreatment on levels of (A) plasma 15-F_{2t}-isoP, (B) free liver 15-F_{2t}-isoP, and (C) total liver 15-F_{2t}-isoP in VPA-treated rats. Adult male Sprague-Dawley rats were pretreated ip with salicylamide (1 mmol/kg) or 50% glycerol (vehicle). At 0.5 h later, the rats were treated ip with a single dose of VPA (500 mg/kg) or 0.9% saline (vehicle). At 0.5 h after VPA treatment, rats were sacrificed, and blood and liver were collected. Results are shown as mean ± SEM for 4 individual rats per treatment group. [a]Significantly different from all other groups, $p < 0.05$.

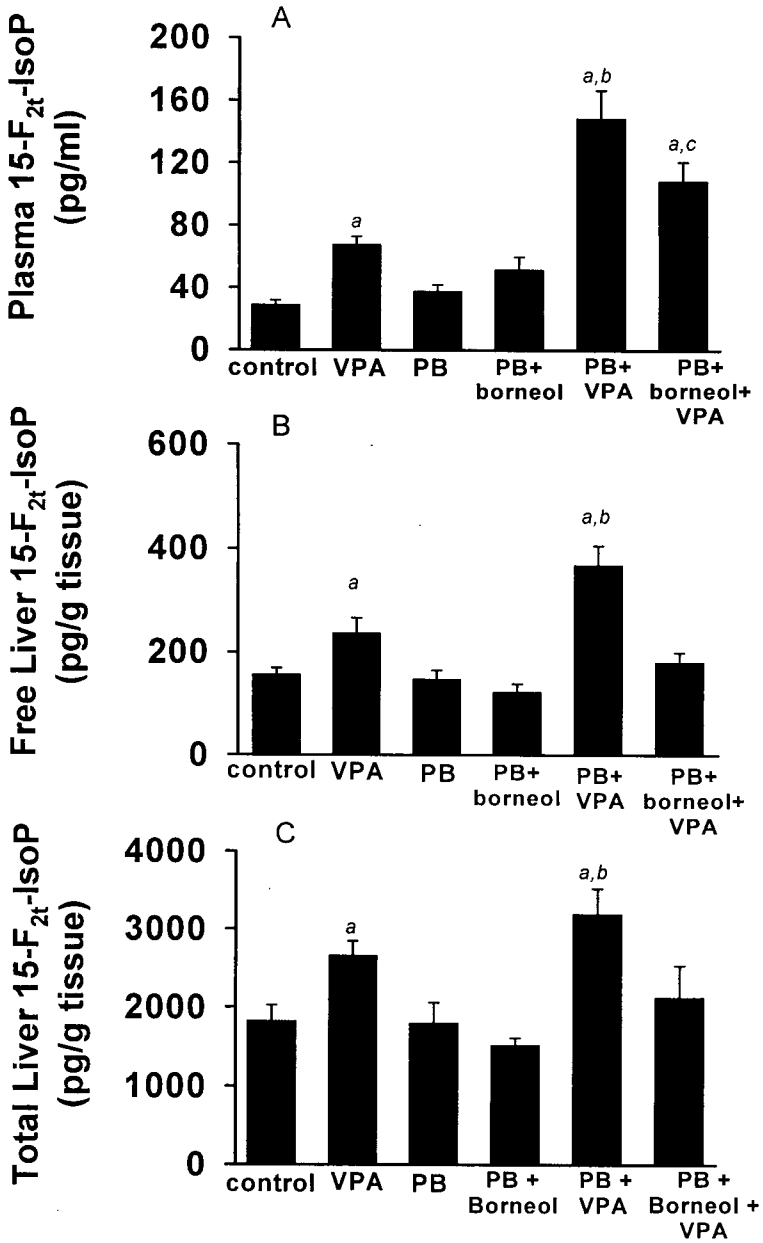

Figure 3-7: Effect of (-)-borneol on the PB-induced levels of (A) plasma, (B) free and (C) total liver 15-F_{2t}-IsoP in VPA-treated rats. Rats were pretreated ip with PB (80 mg/kg) or 0.9% saline (vehicle) once daily for 4 days. On day 5, rats were treated ip with (-)-borneol (320 mg/kg) or corn oil (vehicle) at 0.5 h prior to VPA administration (500 mg/kg in 0.9% saline vehicle). At 0.5 h after VPA treatment, rats were sacrificed, and blood and liver were collected. Results are shown as mean ± SEM for 8 individual rats per treatment group. [a]Significantly different from the control group, $p < 0.05$; [b]Significantly different from the VPA group, $p < 0.05$; [c]Significantly different from the VPA and PB/borneol/VPA groups, $p < 0.05$.

3.3.8 *Effect of α-F-VPA on α-F-VPA-G and 15-F_{2t}-IsoP levels*

α-F-VPA was selected for comparison with VPA for the effect on 15-F_{2t}-IsoP because α-F-VPA was demonstrated to form the glucuronide conjugate in only minor amounts (Tang *et al.* 1997). Rats were treated with a single dose of 3.47 mmol/kg VPA (equivalent to 500 mg/kg) or α-F-VPA (3.47 mmol/kg) and sacrificed at 0.5 h later. Plasma levels of 15-F_{2t}-IsoP following α-F-VPA treatment (44 ± 2 pg/ml) were similar to those of saline-treated vehicle control rats (36 ± 2 pg/ml), but significantly less than that in the VPA-treated group (83 ± 3 pg/ml) (Figure 3-8A-C).

3.3.9 *Effect of PB on α-F-VPA-G and 15-F_{2t}-IsoP levels in rats treated with α-F-VPA*

α-F-VPA was further investigated in rats pretreated with PB to see if PB could elevate the levels of 15-F_{2t}-IsoP as observed in rats treated with VPA. However, when α-F-VPA was administered to PB-pretreated rats, levels of plasma (66 ± 17 pg/ml) and free liver (110 ± 17 pg/g tissue) 15-F_{2t}-IsoP were not elevated and were similar to those of the saline control group. By comparison, PB-pretreated rats demonstrated significant elevation in plasma (169 ± 20 pg/ml) and free liver (197 ± 28 pg/g tissue) 15-F_{2t}-IsoP in the VPA-treated group compared to the saline-treated group (68 ± 6 pg/ml and 118 ± 6 pg/g tissue) (Figure 3-8E-G).

Following a single dose of α-F-VPA (3.47 mmol/kg), α-F-VPA-G levels were 44 ± 6 μg/g tissue and 36 ± 7 μg/g tissue in control rats (Figure 3-8D) and PB-pretreated rats (Figure 3-8H), respectively. These values are approximately 19% and 11% of the amount of VPA-G seen following an equimolar dose of VPA to control rats (Figure 3-8D) and PB-pretreated rats (Figure 3-8H), respectively.

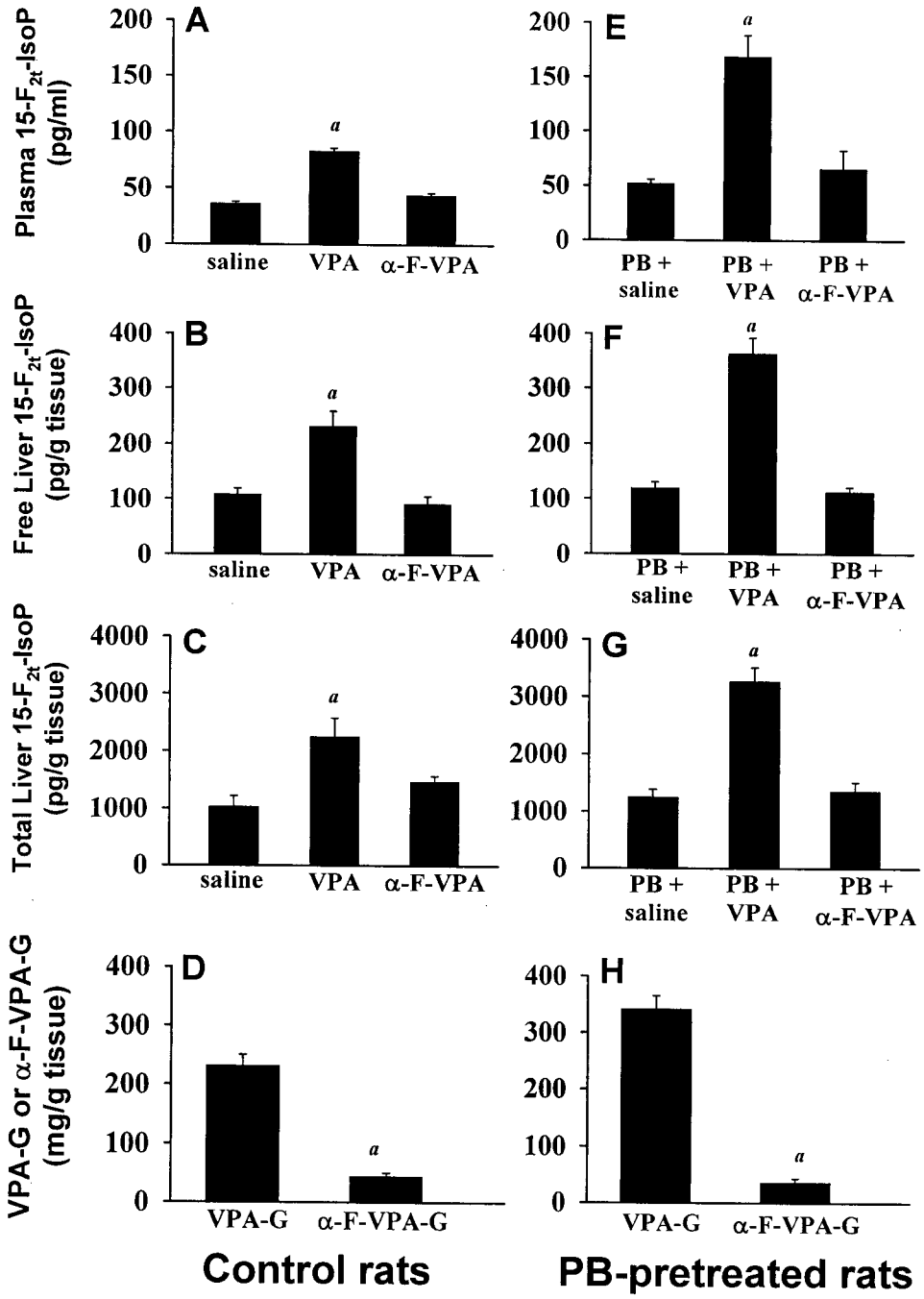

Figure 3-8: Comparison of VPA and α-F-VPA in producing 15-F_{2t}-IsoP and VPA-G or α-F-VPA-G, respectively, in control rats and in PB-pretreated rats. Rats were treated with a single dose of 0.9% saline (vehicle), VPA, or α-F-VPA, both at 3.47 mmol/kg, and sacrificed at 0.5 h later (A-D). In some groups, rats were pretreated ip with PB at 80 mg/kg once daily for 4 days (E-H). Results are shown as mean ± SEM for 4 individual rats per treatment group. [a] Significantly different from all other groups, $p < 0.05$.

3.4 DISCUSSION

Our recent study indicated that the administration of VPA (250 mg/kg and 500 mg/kg) increased plasma and hepatic levels of 15-F_{2t}-IsoP, a marker of oxidative stress (Tong *et al.* 2003). This finding was confirmed in the present study. A novel and intriguing finding from the present study is the association between the levels of 15-F_{2t}-IsoP and VPA-G in rats treated with VPA, as shown in a series of *in vivo* studies that modulated the acyl-glucuronidation of VPA. Another novel aspect of this study is our new method for the direct LC/MS quantification of VPA-G in rat liver homogenate. The method is advantageous because levels of VPA-G are determined directly, as opposed to indirect determination that requires chemical hydrolysis with sodium hydroxide and/or enzymatic hydrolysis with β-glucuronidase. Furthermore, the assay is relatively rapid and provides highly specific detection.

In the present study, VPA at the maximal dose of 500 mg/kg consistently elevated 15-F_{2t}-IsoP levels and PB pre-treatment had an enhanced effect with VPA resulting in an even greater elevation of 15-F_{2t}-IsoP. These results were consistent with previous findings that also demonstrated the effect of PB on VPA-induced 15-F_{2t}-IsoP (Tong *et al.* 2003). This increase in plasma and liver 15-F_{2t}-IsoP by PB-pretreatment coincided with a 40% increase in liver VPA-G compared to saline-pretreated rats administered VPA. This 40% increase in liver VPA-G levels was similar to the magnitude of increase of VPA-G observed in PB-pretreated animals seen in other studies (Booth *et al.* 1996; Watkins and Klaassen 1982). Studies investigating the effects of PB on VPA-associated hepatotoxicity showed that lower doses of VPA (300 mg/kg) are required to produce the same hepatotoxic events in PB-pretreated rats as compared to rats given a single large dose of VPA (700 mg/kg) (Kesterson *et al.* 1984; Sugimoto *et al.* 1987). This enhancement in VPA hepatotoxicity by PB has been associated with elevated levels of potentially reactive 4-ene-VPA and (*E*)-2, 4-diene-VPA via induction of P-450 enzymes (Cotariu

and Zaidman 1988; Dreifuss *et al.* 1987; Levy *et al.* 1990; Zimmerman and Ishak 1982). However, our recent study indicated that the PB-induced increase in hepatic and plasma levels of 15-F_{2t}-IsoP in VPA-treated rats was not associated with P450-mediated biotransformation (Tong *et al.* 2003).

PB also induces specific UGT enzymes that catalyze the glucuronidation of VPA, such as UGT2B1 in rat and UGT2B7 in humans (Ritter 2000), and increases the levels of hepatic UDPGA by 1.3-fold (Watkins and Klaassen 1982). As shown in the present study, (-)-borneol and salicylamide lowered liver VPA-G to approximately 10% and 25%, respectively, of the levels seen in control rats (Table 2). (-)-Borneol and salicylamide are compounds that deplete UDPGA (Howell *et al.* 1986; Kamisako *et al.* 1990; Watkins and Klaassen 1982) and were previously used to inhibit the acyl glucuronidation of VPA (Watkins and Klaassen 1982) and 2-phenylpropionic acid (Li *et al.* 2003). The inhibition of VPA-G by both (-)-borneol and salicylamide decreased plasma and liver 15-F_{2t}-IsoP levels and our results suggest a positive correlation between VPA-G and 15-F_{2t}-IsoP levels. In a subsequent experiment, the combination of (-)-borneol and VPA in PB-pretreated rats was used to examine if the elevation in 15-F_{2t}-IsoP due to PB-pretreatment was associated with the increased VPA-G formation. The results demonstrated that the VPA-associated increase in 15-F_{2t}-IsoP levels was manipulated by chemically modulating the VPA-glucuronidation pathway in either direction. The liver VPA-G levels observed in the PB + (-)-borneol + VPA (500 mg/kg) group were attenuated to equivalent levels seen following a single low dose of VPA (50-100 mg/kg) that was not associated with significant increases in 15-F_{2t}-IsoP levels (Fig. 4).

Our study also used α-F-VPA as a mechanistic probe to gain insight into the relationship between VPA-acyl glucuronidation and 15-F_{2t}-IsoP levels. Previous studies that used fluorinated analogues of VPA to investigate the reactive metabolite hypothesis of VPA-

associated hepatotoxicity showed that 4-ene-VPA, but not the α-fluorinated analogue (α-F-4-ene-VPA), produced microvesicular steatosis and GSH (total and mitochondrial) depletion in rats (Tang *et al.* 1995). *In vitro* models of hepatotoxicity also demonstrated greater cytotoxicity and GSH depletion with VPA and 4-ene-VPA when compared to their respective α-fluorinated analogues (Jurima-Romet *et al.* 1996; Neuman *et al.* 2001). In contrast to VPA, α-F-VPA undergoes acyl-glucuronidation to only a minor extent (< 5% of the dose) and is not a substrate for fatty acid β-oxidation in rats (Grillo *et al.* 2001; Tang *et al.* 1997). Based on these observations, α-F-VPA was examined in rats for its ability to affect the levels of 15-F_{2t}-IsoP. As shown in this study, the rats treated with α-F-VPA produced significantly less plasma and liver 15-F_{2t}-IsoP compared to the VPA group at equimolar doses. Consistent with our hypothesis that VPA acyl-glucuronidation is associated with oxidative stress, there was significantly less α-F-VPA-G than VPA-G observed in the liver. Furthermore, in PB-pretreated rats, levels of α-F-VPA-G and 15-F_{2t}-IsoP were similar to the levels seen in the saline control group, whereas both VPA-G and 15-F_{2t}-IsoP were further elevated after an equivalent dose of VPA. In an earlier pharmacokinetic study using mice, both VPA and α-F-VPA (0.83 mmol/kg) exhibited similar serum t_{max} of 15-30 min; however, α-F-VPA was characterized with higher peak serum concentrations (4.39 μmol/ml *vs.* 2.88 μmol/ml) and a lower elimination rate constant (1.50×10^{-3}/min *vs.* 2.02×10^{-2}/min) than those of VPA, respectively (Tang *et al.* 1997). The relatively lower clearance of α-F-VPA compared to VPA was ascribed to the absence of β-oxidation and the markedly reduced glucuronidation of α-F-VPA. The fluorine substituent increases the acidity of the carboxylate group (pKa = 3.55) compared to VPA (pKa = 4.80) (Tang *et al.* 1997), which may influence its binding to UGT and subsequent conjugation to the glucuronide moiety. Increasing the degree of substitution at the α-carbon position with the fluorine group may also decrease the reactivity of the glucuronide conjugate since there appears to be an inverse relationship between the degree of substitution at the alpha-carbon to the carboxylic acid and its

covalent binding to protein (Benet *et al.* 1993). It would be reasonable to assume that the distinction in the ability of VPA and α-F-VPA to produce 15-F_{2t}-IsoP may be due to the inherent differences in their metabolism to their respective glucuronide conjugates rather than their peak serum concentrations and their rate of elimination in serum.

Acyl glucuronide conjugates are electrophilic intermediates with the intrinsic potential to be reactive. The reactivity of acyl-glucuronides manifests themselves via potential pathways such as hydrolysis (resulting in the regeneration of parent compound), rearrangement (isomerization via intramolecular acyl migration), and covalent adduct formation with neighbouring macromolecules (Bailey and Dickinson 2003; Benet *et al.* 1993; Boelsterli 2002). Although VPA-G undergoes non-specific enzymatic hydrolysis in the plasma and liver, and β-glucuronidase-dependent hydrolysis in the intestine, it is generally considered to be an unreactive metabolite based on *in vitro* incubation studies (Williams *et al.* 1992). However, VPA-G has been demonstrated to undergo non-enzymatic, intramolecular rearrangement into various β-glucuronidase-resistant isomers in slightly alkaline environments, such as bile (Dickinson *et al.* 1984). The effect of PB-pretreatment is non-specific and capable of inducing not only UGTs, but other drug-metabolizing enzymes such as P450 enzymes involved in VPA biotransformation. In PB-pretreated animals, significant elevations in levels of P450-dependent oxidative and desaturated VPA metabolites were observed (Tong *et al.* 2003), all of which could be subsequently glucuronide conjugated. One possibility is that the acyl-glucuronide of P450-dependent VPA metabolites (i.e. 3-OH-, 4-OH-, 5-OH-, 4-ene-VPA) may be directly involved in the production of 15-F_{2t}-IsoP. The reactive nature of these secondary acyl glucuronide metabolites and their ability to undergo rearrangement via intramolecular acyl migration to yield potentially reactive, β-glucuronidase-resistant, positional isomers has not been investigated.

A possible explanation for the association between oxidative stress and the formation of VPA-G may involve the enterohepatic cycling of VPA. VPA-G, which is extensively hydrolyzed in the liver, intestine, and to a minor extent in whole blood, is subject to recirculation and theoretically should generate glucuronic acid in the process. Given the large VPA doses employed, this systemic cycling of VPA to VPA-G and back to VPA may lead to a substantial release of free glucuronic acid. In a recent study, glucuronic acid was shown to induce oxidative stress *in vitro*, as measured by the fluorescence marker 2′,7′-dichloro-dihydrofluorescein diacetate (Kim *et al.* 2004). The effects of hepatic and systemic glucuronic acid exposure on oxidative stress, as well as the determination of glucuronic acid levels during VPA-treatment will need to be examined to test the possibility that glucuronic acid plays a role in the elevation of 15-F_{2t}-IsoP during VPA-treatment. The importance of hepato-biliary recirculation as a mechanism of methapyrilene hepatotoxicity was illustrated when cannulation and exteriorization of the bile duct to interrupt enterohepatic recirculation resulted in a reduction in necrosis (Ratra *et al.* 2000). Thus, similar bile duct exteriorization to divert bile during VPA administration may be an informative study to determine whether or not enterohepatic recirculation plays a role in VPA-associated 15-F_{2t}-IsoP elevation.

An explanation for the elevation in oxidative stress in VPA-treated rats following PB pretreatment is the effect of PB on the biliary excretion of VPA. VPA has a choleretic effect (Dickinson *et al.* 1979; Watkins and Klaassen 1981), which was ascribed to the osmotic effect driven by the excretion of VPA conjugates across the canalicular membrane into bile. PB pretreatment prior to VPA dosing decreases bile flow and subsequent biliary excretion of VPA-G (Booth *et al.* 1996; Watkins *et al.* 1982). The canalicular egress of glucuronide conjugates has been shown to involve the saturable ATP-dependent MRP-2 organic anion transport protein (Wright and Dickinson 2004). Since PB-pretreatment decreases bile flow, elevates liver VPA-G formation, and because biliary excretion is saturable at the high doses of VPA used (Watkins

and Klaassen 1982), there may be a shift to higher VPA-G levels in the liver and increased egress of VPA-G from the hepatocyte into blood.

In summary, our novel data demonstrate that VPA-G formation is associated with increases in hepatic and plasma levels of 15-F_{2t}-IsoP in rats. The mechanism by which 15-F_{2t}-IsoP is elevated by VPA-glucuronidation remains unclear. Studies are currently underway to determine whether formation of VPA-G itself or a by-product(s) of VPA glucuronidation is responsible for the elevation in 15-F_{2t}-IsoP.

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4 *Time-course of oxidative stress biomarkers and liver toxicity in rats treated with valproic acid*

4.1 INTRODUCTION

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}-isoprostaglandin (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. Based on this finding, 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, and histopathological findings (necrosis and steatosis), as well as 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 also monitored to investigate whether the observed oxidative stress and hepatotoxic events were associated with increases in these putative reactive metabolites. This novel study establishes the time-course for hepatotoxicity and oxidative stress in VPA-treated rats.

4.2 METHODS & MATERIALS

4.2.1 Chemicals

2-Propyl-pentanoic acid (sodium valproate, VPA), D-saccharic 1,4-lactone monohydrate (D-saccharolactone), and 10% phosphate-buffered formalin 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). The 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).

4.2.2 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 the dose of 500 mg/kg VPA produced maximum elevation in plasma and liver levels of 15-F_{2t}-IsoP (see Figure 2-3). 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} (30 min, see Figure 2-5) of plasma 15-F_{2t}-IsoP following a single 500 mg/kg VPA dose, rats were sacrificed 30 min following the last injection by

decapitation and trunk blood 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.

4.2.3 Determination of oxidative stress biomarkers

Plasma and liver levels of 15-F_{2t}-IsoP were determined by an EIA assay as described in *2.2.4*. The concentration of TBARS in plasma and liver were calculated as malondialdehyde (MDA) equivalents using a fluorescent assay as described in *2.2.6*. Lipid hydroperoxide levels were determined spectrophotometrically using a commercial kit as described in 2.2.7.

4.2.4 Determination of serum alpha glutathione S-transferase (α-GST) as a liver toxicity marker

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 in-between each step. After the peroxidase substrate was added to the sandwich ELISA, the resultant color intensity was determined on a

multi-well plate reader (absorbance at 450 nm) and is proportional to the amount of α-GST present in the sample. The assay was linear from 1.56-25 μg/ml based on α-GST standard solution.

4.2.5 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 as 1+ with < 25% of the area containing necrotic cells; 2+, with 26 to 50% of the area containing necrotic cells; 3+, with 51 to 75% of the area containing necrotic cells; and 4+, with > 75% of the area containing necrotic cells. 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 2 different sections were examined per liver sample and the pathologist was blind to the treatment groups when assessing the histology.

4.2.6 Determination of VPA metabolites

Oxidative and de-saturated 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 [$^2H_6$]-VPA-G as its internal standard to measure VPA-G levels directly by a validated method described elsewhere (section 3.2.6).

4.2.7 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-1-*O*-acyl glucuronide 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 analyses 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).

4.2.8 Statistical Analysis

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

4.3 RESULTS

4.3.1 Time-course for 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 and plasma and liver 15-F_{2t}-IsoP levels were determined (Figure 4-1A-C). 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 (Figure 4-1A). Free and total liver 15-F_{2t}-IsoP levels were also 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 (Figure 4-1B and 1C).

4.3.2 TBARS and LPO levels during VPA treatment

Liver LPO (Figure 4-2) and plasma and liver TBARS (Figure 4-3A and B) 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) (Figure 4-2). 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 after 14 days 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) (Figure 4-3A and B).

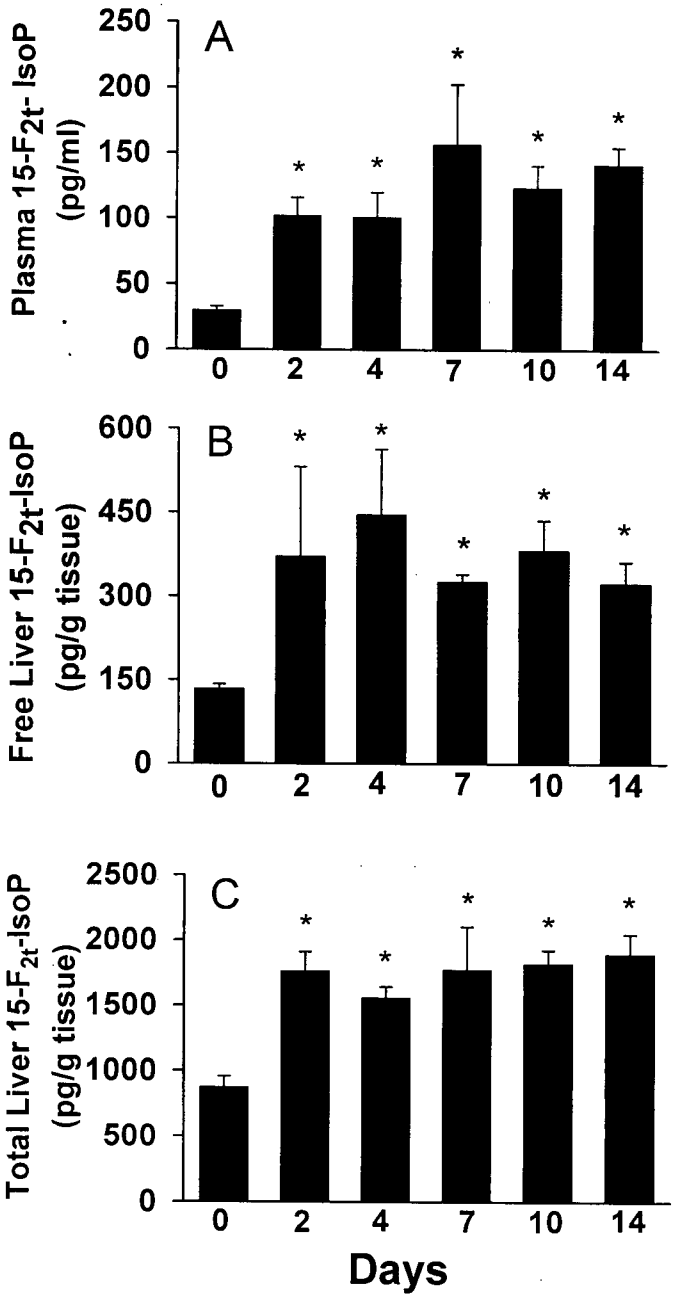

Figure 4-1: Levels of (A) plasma, (B) free liver and (C) total liver (esterified and non-esterified) 15-F_{2t}-IsoP in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. Control rats were treated with 0.9% saline vehicle for 14 days. Plasma and liver levels of 15-F_{2t}-IsoP 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 ($p<0.05$).

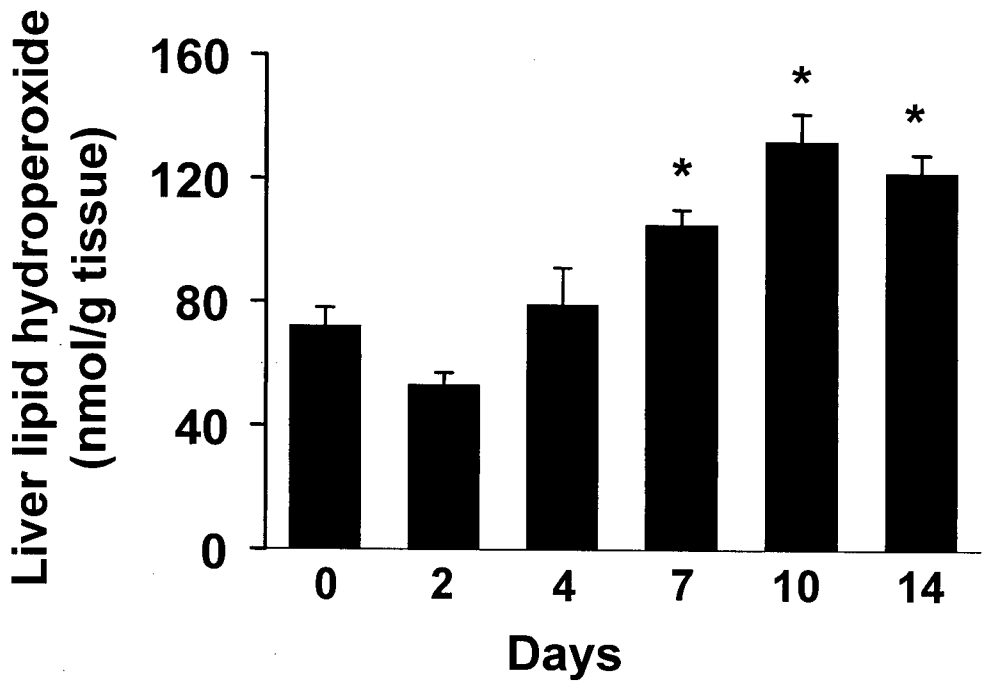

Figure 4-2: Levels of liver LPO in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. The standard curve (0-5 nmol lipid hydroperoxide) was generated using 13-hydroperoxyoctadecadienoic acid as a lipid hydroperoxide standard. Control rats were treated with 0.9% saline (vehicle) for 14 days. Liver LPO was measured by a colormetric 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 ($p<0.05$).

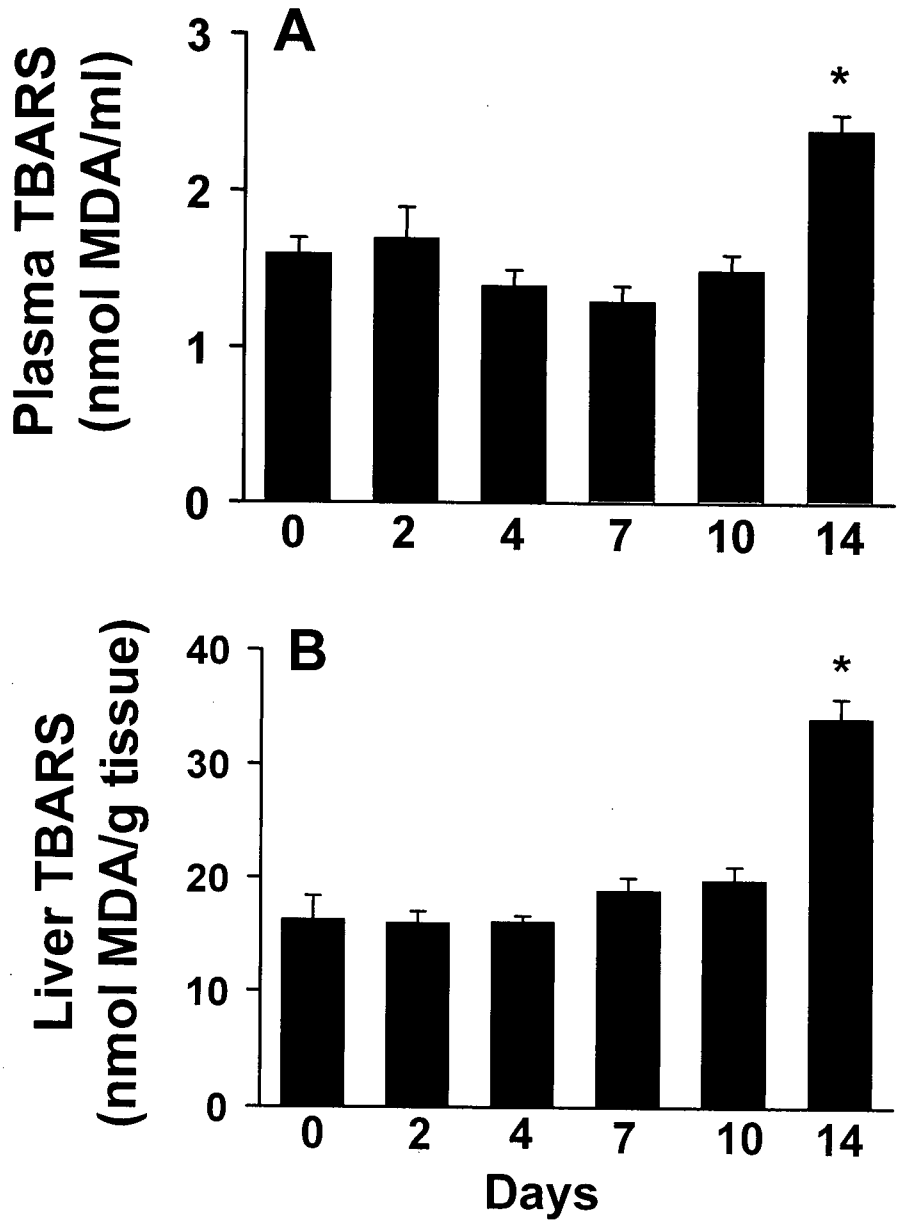

Figure 4-3: Levels of (A) plasma and (B) liver TBARS in rats treated with (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. The standard curve for the TBARS assay was generated using malondialdehyde (MDA) and the results are expressed as MDA equivalents. Control rats were treated with 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 ($p < 0.05$).

4.3.3 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 for 14 days (57 ± 7 μg/L) (Figure 4-4). The α-GST levels remained elevated to the same extent on days 7, 10 and 14.

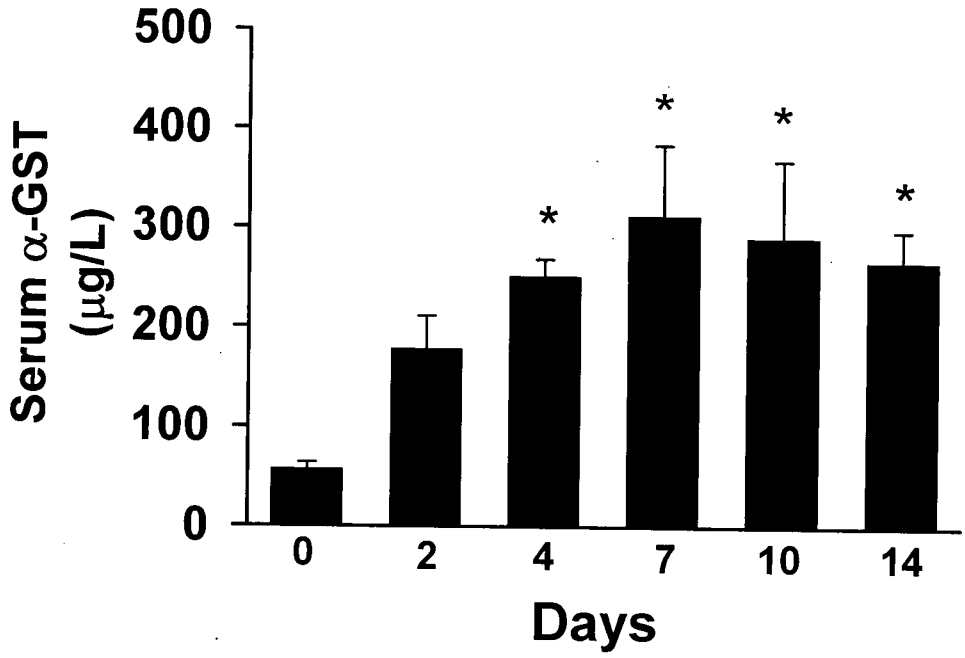

Figure 4-4: Levels of serum α-GST in rats treated with VPA (500 mg/kg once daily, ip) for 2, 4, 7, 10, or 14 consecutive days. Control rats were treated with 0.9% saline vehicle for 14 days. α-GST was measured by an enzyme immunoassay method as described under *Materials and Methods*. Serum α-GST levels (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 ($p < 0.05$).

Mortality was observed during the 14-day VPA treatment with an incidence of 1 out of 5 rats in the 4- and 7-day groups, and 3 out of 16 rats in the 14-day group (Table 4-1). In all treated groups, the most common feature was scarring of the liver capsule surface (liver "capsulitis") and this abnormality was attended by a mild inflammatory reaction localized only to the surface and usually consisted of lymphocytes and infrequent monocytes (Figure 4-5C). The frequency of "capsulitis" increased with duration of treatment from 1 out of 5 animals affected on day 2 to almost 100% frequency from day 4 to 14, while none were seen in the saline-treated control group.

Liver necrosis was always associated with "capsulitis" but not vice versa, and without any signs of inflammation such as cellular infiltration into the parenchyma. In general, massive necrosis was observed without a consistent zonal pattern and involved irregular areas of hepatic tissue. All necrotic livers were subjectively scored (+4, +3, +2, or +1) to describe the severity of the cross-sectional area affected (>75%, 50-75%, 25-49%, or <25%, respectively). The 14-day treatment group (Figure 4-5F) had the highest incidence of massive necrosis with scores of +3 (3 rats), +2 (2 rats), and +1 (2 rats). In other treatment groups, 2 rats in the 4-day group and 1 rat in the 7-day group, widespread liver "capsulitis" was observed and this was accompanied with hepatocellular degeneration (necrosis score of +1) that extended locally into the parenchyma.

Liver steatosis was observed in 7 rats (Table 4-1) and the incidence of lipid accumulation appeared to increase with the duration of VPA treatment: 1 rat in the 4-day group, and 2 rats in the 7-day group. Four out of five animals in the 10-day group exhibited steatosis (Figure 4-5E). The observed steatosis was of zonal distribution conforming to the centrilobular zones (periacinar zone 3). In all cases, steatosis was never extensive in area, and considered mild to

moderate affecting approximately 10-25% of the liver cross-section, with the exception of one animal on day 10.

Table 4-1. VPA-associated necrosis and steatosis in rats treated with VPA for 14 consecutive days.[a]

Treatment Duration (days)	Total number	Number of deaths	Incidence of "capsulitis"	Incidence of necrosis +1	+2	+3	+4	Incidence of steatosis
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 were 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 are described as follows: +1 (< 25%), +2 (26-50%), +3 (51-75%) and +4 (> 75%).

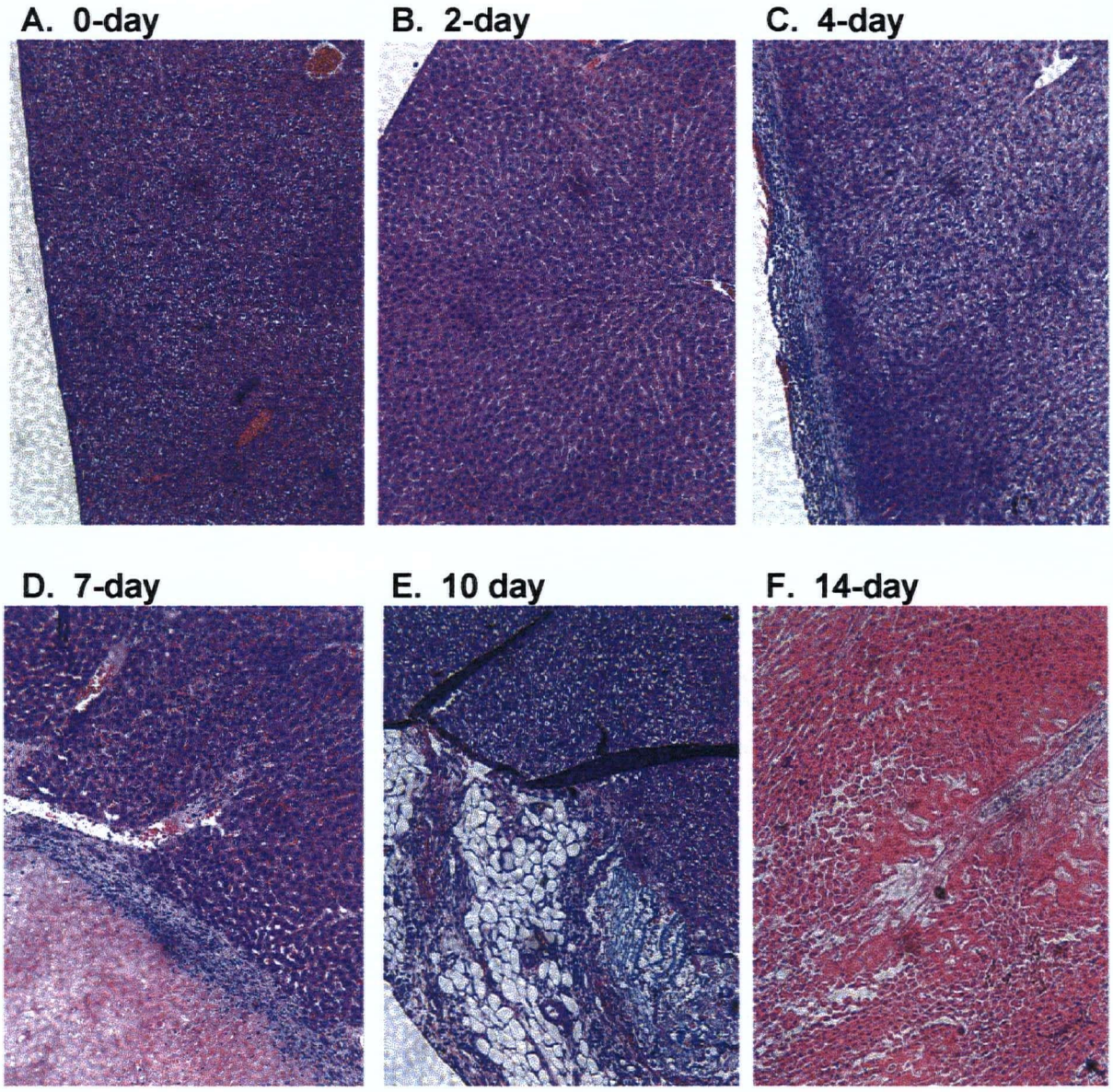

Figure 4-5: Photomicrographs of liver sections from rats administered ip with VPA at 500 mg/kg once daily for up to 14 days showing progressive incidence of liver damage. The 0-day treatment group received saline for 14 days. Livers were fixed in 10% phosphate buffered formalin and cross-sections (10 μm) were stained with hematoxylin and eosin. Original magnification, 40X.

4.3.4 Levels of VPA metabolites during VPA treatment

Levels of oxidative and mono- and di-desaturated VPA metabolites were monitored in liver (Table 4-2) and plasma (Table 4-3). 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 4-3) and hepatic VPA-G (Table 4-2) 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 4-2: Liver VPA metabolite levels (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 metabolites were determined by a GC/MS assay.

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

Table 4-3: 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
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 metabolites were determined by a GC/MS assay.

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

4.4 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 P450-mediated VPA-biotransformation, as indicated in subsequent mechanistic experiments (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 (Figure 4-6).

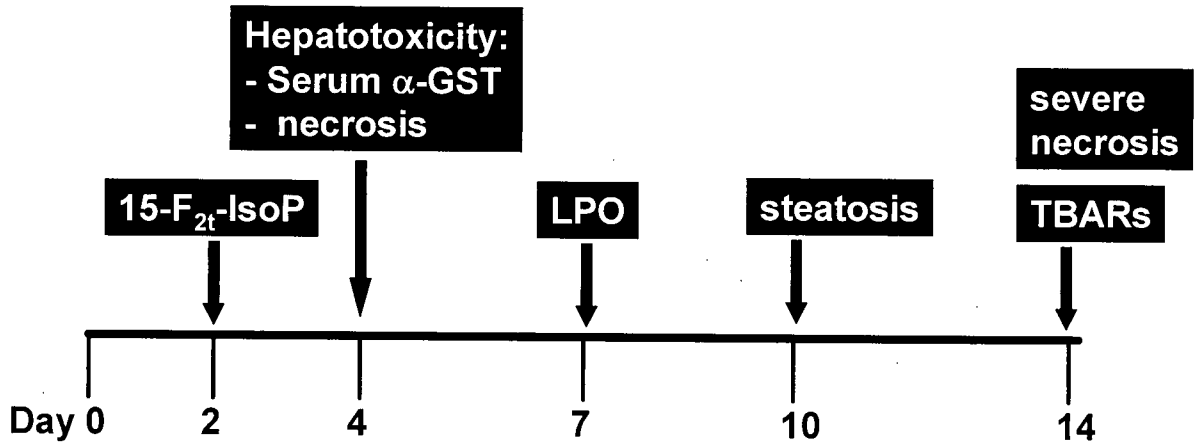

Figure 4-6: Summary of results indicating the time-course of hepatotoxicity and oxidative stress events. Rats were administered 500 mg/kg VPA ip once daily for up to 14 days. The 0-day treatment group received saline for 14 days.

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 liver capsule. The first incidence 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 days 4 to 14 coincided with increased serum 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 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 amino transferase were also measured in the serum by colormetric assays (procedure not described). However, these results were highly variable among 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. (Kesterson *et al.* 1984) demonstrated fatty liver in rats treated with 700 mg/kg/day for 4 days, but with a lower occurrence (2 out of 7 rats) at 600 mg/kg/day for 5 days. In another study, light microscopy did not reveal steatosis following the 200 or 600 mg/kg doses in 24 h; however, an increase in liver lipid and triglyceride levels as observed by 3 h at the higher dose (Jezequel *et al.* 1984). In the above studies, electron microscopy revealed numerous lipid vacuoles and ultrastructural changes to the mitochondria believed to be a result of mitochondrial dysfunction that is consistent with inhibition of β-oxidation. In the present study, the first incidence of fatty liver, although never extensive in area, occurred on day 4 of treatment (1 out of 4 rats) with

increasing occurrence observed after 10 days of treatment (4 out of 5 rats) at the dose of 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. In other studies that used the same 500 mg/kg dose of VPA, microvesicular steatosis was observed after a single dose in fasted rats by osmium tetroxide staining and biochemical analysis (Olson *et al.* 1986) and with repeated dosing also at 500 mg/kg for 7 consecutive days by electron microscopy in rats provided with food *ad libitum* (Sugimoto *et al.* 1987). Although steatosis was first observed on day 4 under light microscopy, the extent of steatosis may be underestimated in our study since electron microscopy and staining with oil-red O or osmium tetroxide was not performed. The reported studies suggest that VPA induces steatosis in a dose- and time-dependent manner in rats.

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 compared to the saline-treated group, and these levels remained elevated to the same extent throughout the 14-day study period. According to our data, the formation of 15-F_{2t}-IsoP did not increase over time with repeated dosing. The question of whether or not the observed increase in 15-F_{2t}-IsoP following VPA administration is reflective of oxidative stress still remains to be determined. 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 (non-enzymatic) 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). However, in extrahepatic tissues, evidence exists for a COX-dependent formation of 15-F_{2t}-IsoP involving contribution from constitutive COX-1 and/or inducible COX-2 isoforms in a kidney model for ischemia-reperfusion injury (Favreau *et al.* 2004), an isolated rat kidney glomeruli model (Klein *et al.* 2001) and in thrombin or arachidonic

acid activated platelets (Klein *et al.* 1997; Pratico *et al.* 1995). In these reported studies, the non-selective COX inhibitors indomethacin and diclofenac were able to suppress the formation of 15-F_{2t}-IsoP. This suggests that 15-F_{2t}-IsoP may have a dual origin such that both free radicals and COX enzymes may contribute to their formation. However, the effects of VPA on 15-F_{2t}-IsoP are unlikely to be due to COX activity. 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). While taking into consideration of the reported lack of an effect of VPA on COX expression, our interpretation is that 15-F_{2t}-IsoP levels are indeed reflective of oxidative stress and precede the onset of hepatotoxicity in VPA-treated rats (Figure 4-6). My subsequent experiments with cultured rat hepatocytes indicate that oxidative stress, as measured by the 15-F_{2t}-IsoP and DCF-DA assays, can occur in the absence of hepatocyte toxicity (See Chapter 5, Figure 5-3 and Figure 5-8).

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 colormetric assays to be less specific and indirect as compared to the

measurement of 15-F_{2t}-IsoP by EIA. On the other hand, 15-F_{2t}-IsoP may represent an earlier lipid peroxidation event, while MDA, a degradation product of polyunsaturated fatty acid hydroperoxides detected using the TBARS assay, may simply represent later lipid peroxidation events as a consequence of the necrosis. A lack of temporal correlation among ROS 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, only at the highest concentration of H_2O_2 (250 μM) were increases in levels of TBARS and LPO observed. These results were ascribed 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, which suggests that these metabolites are not responsible for the oxidative stress or hepatotoxicity observed in the present study. 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 were 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 did not occur until after the onset of oxidative stress and hepatotoxicity. An interesting observation is that VPA-G levels in the liver were unchanged throughout the study period. Our previous results (see Chapter 3) linked VPA-G and 15-F_{2t}-IsoP levels in the liver following a single large dose of VPA to rats. Since the liver VPA-G levels remained similar and did not decrease throughout the study, this finding is consistent with the observation that VPA-G levels correlated with the elevated levels of 15-F_{2t}-IsoP seen from day 2.

In conclusion, VPA treatment to rats for up to 14 consecutive days initiated a sequence of hepatotoxic and oxidative stress events and these findings provide evidence that VPA treatment is associated with oxidative stress that precedes its hepatotoxicity. Studies are ongoing to measure the ratio of oxidized and reduced GSH in the liver cytosolic and mitochondrial fractions of rats treated with VPA to obtain another independent indicator of oxidative stress.

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5 *Investigation of VPA-Associated Oxidative Stress in Primary Cultured Rat Hepatocytes*

5.1 INTRODUCTION

Primary cultured rat hepatocytes have been well utilized to study VPA-associated hepatotoxicity. Early studies demonstrated that VPA caused dose-dependent toxicity to rat hepatocytes (Kingsley *et al.* 1983). It was also demonstrated that VPA was associated with biochemical disturbances such as inhibition of fatty acid oxidation, gluconeogenesis, ketogenesis, urea synthesis, and reduced levels of acetyl-CoA in rat hepatocyte cultures (Becker and Harris 1983; Coude *et al.* 1983; Turnbull *et al.* 1983). In support of the oxidative stress hypothesis of VPA hepatotoxicity, it was first suggested that lipid peroxidation was involved in VPA hepatotoxicity when the antioxidants, vitamin E, α-tocopherol, and *N*, *N'*-diphenyl-p-phenylenediamine, conferred protection against VPA toxicity in rat hepatocyte cultures (Buchi *et al.* 1984; Jurima-Romet *et al.* 1996). Our previous work demonstrated that a single dose of VPA administered in rats led to a dose-dependent elevation in plasma and liver levels of the endogenous lipid peroxidation marker, 15-F_{2t}-isoprostaglandin (15-F_{2t}-IsoP) (Tong *et al.* 2003). High daily doses of VPA to rats for 14 consecutive days produced an elevation in 15-F_{2t}-IsoP that preceded the onset of liver necrosis and steatosis (Tong *et al.* 2005, manuscript submitted). In the same study, two other independent measures of oxidative stress, thiobarbituric acid reactive substances and lipid hydroperoxides, were elevated at later time points. These findings are consistent with the hypothesis that VPA is associated with oxidative stress; however, the question remains whether the elevation in 15-F_{2t}-IsoP levels truly reflects oxidative stress and whether this results in mitochondrial dysfunction and hepatocyte toxicity.

There is evidence that GSH homeostasis may be altered, either as a consequence of reactive metabolites and/or reactive oxygen species produced during VPA treatment (Cotariu *et al.* 1990; Graf *et al.* 1998; Olson *et al.* 1986; Raza *et al.* 1997; Yuksel *et al.* 2000). Reduced GSH is an important cell protecting biomolecule against chemical-induced cytotoxicity by direct or enzymatic (glutathione-S-transferase) conjugation with electrophilic compounds (Reed 1990). GSH is also an important cellular antioxidant that is capable of direct or enzymatic (glutathione peroxidase) conjugation with ROS such as lipid hydroperoxides and hydrogen peroxide (Meister 1983). A useful approach to understanding the role of GSH in chemical toxicity is to determine the consequences of reducing or depleting cellular GSH. This was demonstrated when GSH-depleted rat hepatocytes exhibited greater toxicity to 4-ene-VPA than normal control hepatocytes, and the toxicity was attenuated with the addition of antioxidants, vitamin C and vitamin E (Jurima-Romet *et al.* 1996). The successful treatment of severe VPA hepatotoxicity with N-acetylcysteine in a small number of pediatric epileptic patients provided further evidence for a protective role of GSH (Farrell and Abbott 1991).

The objective of the present study using freshly isolated rat hepatocytes was to determine the acute effects of VPA on: (1) oxidative stress, as measured by the 5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate (DCF-DA) and 15-F_{2t}-IsoP assays; (2) mitochondrial membrane potential ($\Delta\Psi_m$), as determined by the JC-1 assay; and (3) hepatocyte toxicity, as determined by the WST-1 assay. Furthermore, the role of GSH was investigated by comparing the effects of VPA on the above endpoints in GSH-reduced hepatocytes and in normal control hepatocytes. Findings from the present study support the hypothesis that VPA produces oxidative stress prior to hepatotoxicity, which is consistent with our previous *in vivo* findings (Tong *et al.* 2005, manuscript submitted), and that GSH-depletion exacerbates oxidative stress, mitochondrial membrane depolarization, and cytotoxicity in rat hepatocytes treated with high concentrations of VPA.

5.2 METHODS & MATERIALS

5.2.1 Chemicals

2-Propyl pentanoic acid (sodium valproate, VPA), DL-buthionine-[S,R]- sulfoximine (BSO), diethylmaleate (DEM), butylated hydroxytoluene, trypsin inhibitor (Type II-soybean) and collagenase (Type IV) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Matrigel basement membrane matrix and Hepato-Stim medium were purchased from BD Biosciences (Mississauga, ON, Canada). The 15-F_{2t}-isoprostane EIA kit and the glutathione assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI). WST-1 was obtained from Hoffman-La Roche (Mississauga, ON, Canada). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and DCF-DA were purchased from Molecular Probes (Eugene, OR). Liver Perfusion® medium, Hepatocyte Wash® medium, Hepatozyme, Hank's Balanced Salt Solution (HBSS) and phosphate buffered saline (PBS) were purchased from Invitrogen (Burlington, ON, Canada). Percoll was purchased from Amersham Biosciences Inc. (Baie d'Urfe, QC, Canada).

5.2.2 Animals

Adult male Sprague-Dawley rats (250-300 g) were obtained 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. All animal experiments were approved by the University of British Columbia Animal Care Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

5.2.3 Rat hepatocyte isolation and culture

Rats were anesthetized with sodium pentobarbital (60 mg/kg, ip) and the abdomen was opened by a midline incision. The liver, inferior vena cava and the hepatic portal vein were exposed. Silk sutures were tied loosely around the inferior vena cava, the superior vena cava, and the hepatic portal vein. The hepatic portal vein was cannulated with a 21 gauge Teflon catheter, the needle removed, and the catheter secured. A two-step, collagenase perfusion technique first involved perfusing the liver with Ca^{2+}-free Liver Perfusion medium at a rate of 25 ml/min for 6 to 8 min using a peristaltic pump. The inferior vena cava was severed immediately to allow for the efflux of the perfusate. The perfusion solution was changed to a HBSS digest media (1.40 g/L $CaCl_2$, 2.38 g/L HEPES, 0.35 g/L $NaHCO_3$, 0.05 g/L trypsin inhibitor (Type II-soybean), 0.5 g/L collagenase (Type IV, pH 7.4). Perfusion with digest media was subjectively determined to be complete (approximately 6-8 min). All perfusion media were warmed to 37°C. Upon digestion, the liver was excised and placed in a sterile petri-dish containing Hepatocyte Wash medium. Cells were mechanically dispersed using a blunt glass rod. The cell suspension was filtered through sterile 60 μm Nytex® mesh cloth into 50 ml Falcon® centrifuge tubes on ice. The suspension was centrifuged (50 × g) for 3 min to pellet the hepatocytes and the pellet re-suspended in fresh wash medium. A Percoll solution (26.1 ml sterile Percoll and 3.9 ml of 10 × HBSS) was mixed with 20 ml of the cell suspension by inversion of the tube. The mixture was centrifuged at 4°C at 50 × g for 5 min and the supernatant containing the dead cells was decanted. The pellet was resuspended and washed once with Hepato-Stim medium and the viability and cell concentration was determined by trypan blue exclusion. The exclusion criterion for hepatocyte viability was > 90%. Cells were diluted to 4×10^5 cells/ml in Hepato-Stim medium and seeded as a monolayer culture on Matrigel matrix coated 24- or 96-well sterile tissue culture plates at a density of 2×10^5 or 4×10^4 cells/well, respectively. Cells were

allowed to attach for 2 h in a 37°C, 5% CO_2 incubator prior to experimental treatment. Attachment efficiency was determined to be approximately 95%.

In experiments involving hepatocytes depleted of GSH, hepatocytes were pretreated for 2 h prior to VPA treatment with a combination of DL-buthionine-[S,R]- sulfoximine (BSO) and diethylmaleate (DEM). BSO is a potent and specific transition state inhibitor of γ-glutamylcysteine synthetase that depletes cellular GSH pools by blocking its synthesis (Griffith 1981). Diethylmaleate (DEM), an electrophilic compound, was also used to deplete cellular GSH directly by undergoing extensive conjugation with GSH in a reaction catalyzed by glutathione-S-transferases (Maellaro *et al.* 1990; Meredith and Reed 1982; Miccadei *et al.* 1988; Plummer *et al.* 1981).

5.2.4 15-F_{2t}-IsoP Assay

15-F_{2t}-IsoP was determined in rat hepatocyte cultures (2×10^5 cells/well in 24-well plates) as an index of oxidative stress. After 2-h cell attachment, the hepatocytes were treated with VPA (0-1000 μg/ml dissolved in Hepatozyme medium). At specific time points, 2% butylated hydroxytoluene (10 μl) was added to each culture to prevent sample auto-oxidation during storage and processing and the culture medium (1 ml) including the cell monolayer was transferred to polypropylene Eppendorf™ tubes and immediately snap-frozen in liquid nitrogen. All samples were stored at –80°C and processed the next day for the determination of 15-F_{2t}-IsoP as previously described (Tong *et al.* 2003). The results were normalized per 10^6 cells and expressed as the mean ± SEM.

5.2.5 DCF-DA assay

Production of intracellular ROS was also monitored by the fluorescence emission of 2′,7′-dichlorofluorescein (DCF). After 2-h cell attachment, the hepatocytes (4×10^4 cells/well in a 96-well plate) were preloaded with 5 μM DCF-DA (dissolved in Hepatozyme medium) for 20 min (37°C, 5% CO_2). The diacetate form of 2′,7′-dichlorofluorescin (DCFH) diffuses across the cell membrane and is hydrolyzed by intracellular esterases to yield the non-fluorescent DCFH. DCFH, upon reacting with low molecular weight hydroperoxides (i.e. H_2O_2), is oxidized to its highly fluorescent, 2-electron oxidation product, 2′,7′-dichlorofluorescein (DCF) (Figure 5-1). Following 20 min of DCF-DA preloading, cells were washed once with Hepatozyme medium and treated with VPA (0-1000 μg/mL, dissolved in Hepatozyme medium). Fluorescence was determined immediately after addition of VPA or its vehicle, and at 5, 15, 30, 45, 60, 90, and 120 min on a Cytofluor® Series 4000 (Applied Biosystems, Bedford, MA) multi-well fluorescent plate reader (excitation: 485 nm with slit width of 25 nm; emission: 530 nm with a slit width of 20 nm). The concentration of DCF was determined from calibration curves prepared from DCF (Polysciences Inc., Warrington, PA) standard and the results were reported as pmol DCF/10^6 cells.

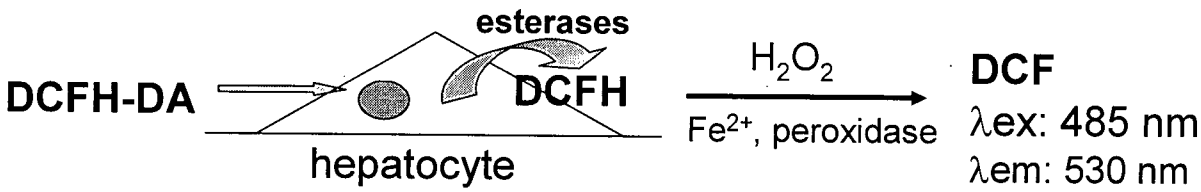

Figure 5-1: Schematic describing the mechanism of the DCF-DA assay. DCF-DA is taken up into cells, where intracellular esterases cleave the diacetate groups to produce the non-fluorescent DCFH. DCFH serves as a substrate for the iron-catalyzed oxidation by H_2O_2 to yield the fluorescent DCF as a indirect measure of oxidative stress.

5.2.6 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was measured using the mitochondrial specific dual-fluorescence probe, JC-1, based on modified methods (Reers *et al.* 1995; Reers *et al.* 1991). JC-1 is a ratiometric dye that is internalized as a monomer dye (green fluorescence, emission wavelength: 530 nm) and is concentrated by respiring mitochondria with negative inner membrane potential into J-aggregate dye (red fluorescence, emission wavelength: 590 nm) (Smiley *et al.* 1991). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Cells in culture (4×10^4 cells/well in a 96-well plate) were treated with various compounds up to 2 h. Positive controls to which VPA (0 - 1000 μg/ml, dissolved in Hepatozyme medium) were compared included valinomycin (50 μM), a potassium ionophore, and carbonyl cyanide m-chlorophenylhydrazone (mCLCCP, 50 μM) and carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP, 50 μM), both uncouplers of oxidative phosphorylation. After the 2-h treatment, the medium was removed and replaced with 100 μl of 10 μg/ml JC-1 in Hepatozyme medium. Cells were incubated with the dye for 10 min (37°C, 5% CO_2/95% air) followed by a wash with PBS, and cells were allowed to equilibrate at room temperature in the dark for 10 min. A CytoFluor® Series 4000 plate reader (Applied Biosystems, Bedford, MA) was set to an excitation wavelength of 485 nm (slit width: 20 nm) to monitor the fluorescence intensities for the monomer and the aggregated JC-1 molecules (emission wavelengths: 530 nm with a slit width of 25 nm, and 590 nm with a slit width of 5 nm, respectively).

5.2.7 Cell viability

Cell viability was measured by the WST-1 method in 96-well plates. This method is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells to a water-soluble formazan dye. After the 2-h attachment, cells (4×10^4 cells/well in 96-well plates) were treated with VPA (using Hepatozyme medium as a vehicle) for 8 h (37°C, 5% CO_2)

followed by the addition of 10 μl WST-1 stock solution per 100 μl of medium in each well. After 30 min of incubation (37°C, 5% CO_2), absorbance was determined at 450 nm on a Labsystems Multiskan Ascent® multi-well plate reader (Thermo Electron Corp., Burlington, ON, Canada). A decrease in cell viability was indicated by a decrease in the amount of formazan dye (decrease in absorbance). The positive control, 4-pentenoic acid, was used to compare the cytotoxicity of VPA.

5.2.8 Glutathione (GSH) assay

GSH levels in hepatocytes were determined using a commercially available kit (Cayman Chemical Co., Ann Arbor, MI) that is based on an enzymatic recycling method, using glutathione reductase, for the quantitation of total GSH levels (Figure 5-2). The rat hepatocytes were collected after pretreatment with BSO and DEM for 2 h and homogenized in 0.5 ml of cold 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 10,000 × g (15 min at 4°C) and the supernatant was collected and stored on ice. For the deproteination procedure, the samples were acidified with an equal volume of meta-phosphoric acid (10% solution in water), mixed on a vortex mixer, and allowed to stand at room temperature for 5 min. Samples were centrifuged (2000 × g at room temperature) to pellet the protein, and the supernatant (0.5 ml) collected. The pH of the sample was increased to approximately 8 with the addition of 25 μl of a 4 M solution of triethanolamine, prior to GSH determination. Total GSH levels were determined with a standard curve over a concentration range of 0 - 16 μM GSH (reduced form).

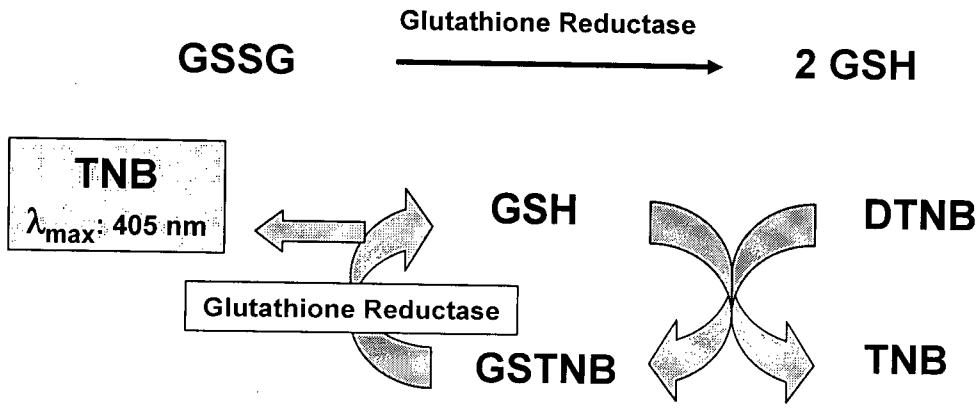

Figure 5-2: GSH recycling in the presence of glutathione reductase and Ellman's Reagent (DTNB, 5,5′-dithio*bis*-2-nitrobenzoic acid). TNB (5-thio-2-nitrobenzoic acid) is monitored spectrophotmetrically (λ_{max} = 405 nm) as an indirect measure of GSH levels. (Cayman Chemical Co., GSH assay kit booklet).

5.2.9 Statistical analysis

The results are reported as means ± SEM. Statistical significance of the difference between the means of groups were analyzed by one-way ANOVA and followed by the Student-Newman-Keul's multiple pairwise comparison *post hoc* test. Groups receiving different treatment concentrations of VPA (Figure 5-3) or BSO and DEM (Figure 5-4) were compared to normal hepatocytes at the same time point. In addition, either normal hepatocytes were compared to BSO and DEM-pretreated hepatocytes with different VPA concentrations at the same time point, or different time points at the same VPA treatment (Figure 5-5, Figure 5-6, and Figure 5-7). The level of significance was set a priori at $p < 0.05$.

5.3 RESULTS

5.3.1 VPA-induced oxidative stress in primary culture rat hepatocytes

VPA produced a time- and concentration-dependent increase in the lipid peroxidation biomarker, 15-F_{2t}-IsoP, in rat hepatocyte cultures (Figure 5-3A). Treatment with 1000 μg/ml VPA for 30 min increased the level of 15-F_{2t}-IsoP compared to the control group (199 ± 18 and 104 ± 22 pg/ml/10^6 cells, respectively). At the 120 min time point, treatment with VPA at all concentrations (100 - 1000 μg/ml) resulted in significantly elevated 15-F_{2t}-IsoP levels by up to 1.9-fold compared to the 0 μg/ml control group.

To verify the increase in oxidative stress by VPA, an independent indicator of intracellular oxidative stress was measured with DCF fluorescence intensity in VPA-treated hepatocytes. Oxidation of DCFH to DCF was elevated in a time- and concentration-dependent manner, with significant increases in DCF detected in the 10, 250 and 1000 μg/ml VPA-treated cells at time points from 30 to 120 min of incubation (Figure 5-3B).

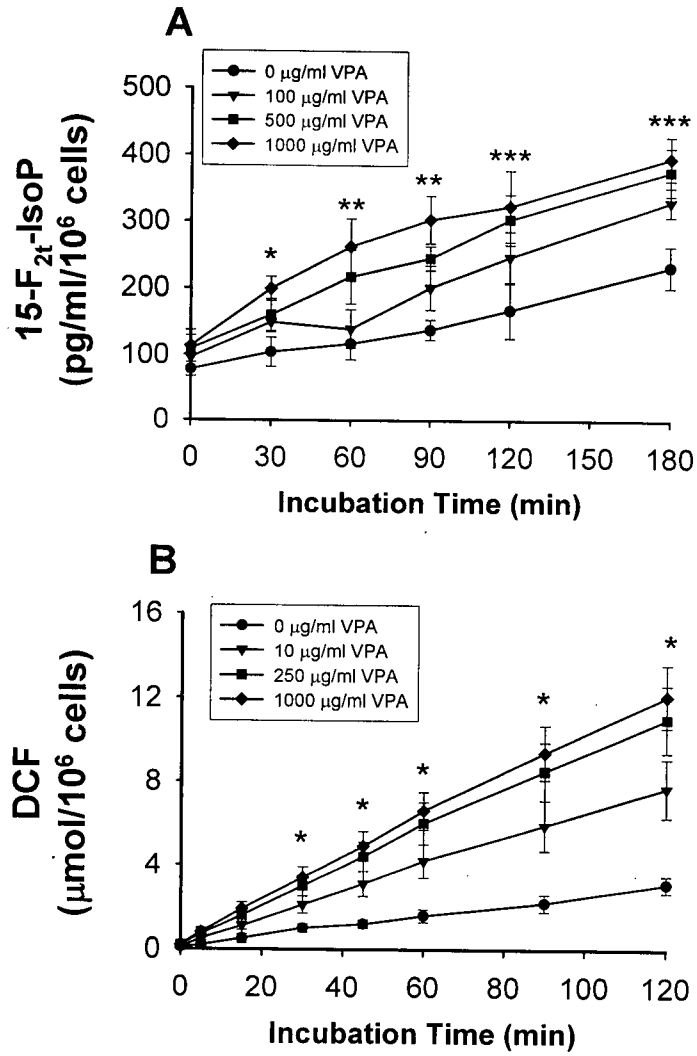

Figure 5-3: Time-course and dose-response relationship for the production of (A) 15-F_{2t}-IsoP and (B) DCF in freshly isolated rat hepatocytes treated with VPA. (A) Freshly isolated rat hepatocytes were incubated with 0, 100, 500, and 1000 μg/ml VPA (4 × 10^5 cells/well in a 24-well plate) from 0 - 120 min, at 30 min intervals. The medium was removed at each time point for the determination of 15-F_{2t}-IsoP. Data are reported as the mean ± SEM (n = 5 experiments). Significant differences between 0 μg/ml vs. *1000 μg/ml, **1000 and 500 μg/ml, ***1000, 500, and 100 μg/ml groups at each time point ($p < 0.05$). (B) Hepatocytes (4 × 10^4 cells/well in 96-well plates) were preloaded with 5 μM DCF-DA for 20 min and treated with VPA (0 - 1000 μg/ml). Intracellular oxidative stress was measured with DCF fluorescence intensity (Ex: 485, Em: 530 nm). Data are reported as the mean ± SEM (n = 8 experiments). *Significantly different between the 10, 250, and 1000 μg/ml VPA groups compared to the 0 μg/ml VPA (control) group ($p<0.05$).

5.3.2 *VPA-induced oxidative stress in GSH-depleted rat hepatocytes*

To verify the effects of BSO and DEM pretreatment, total cellular GSH levels were measured in hepatocytes treated with varying concentrations of these two chemicals, either individually or in combination. Total intracellular GSH levels were decreased to a maximum of 50% with 8 mM BSO and 37% with 2 mM DEM compared to control levels (data not shown). To enhance GSH depletion, the combination of 2 mM BSO and 0.5 mM DEM resulted in maximal reduction of GSH (21% of control) (Figure 5-4). In all subsequent experiments, hepatocytes were pretreated with the combination of 2 mM BSO and 0.5 mM DEM.

Reduction of GSH in hepatocytes resulted in greater increases of 15-F_{2t}-IsoP when compared to the non-GSH reduced control group at the concentrations of VPA tested: 1000 μg/ml (1.7-fold), 500 μg/ml (1.4-fold increase), and 100 μg/ml (1.4-fold increase) (Figure 5-5). The time-dependent and dose-dependent formation of DCF was compared between control and BSO + DEM-pretreated hepatocytes treated with VPA. DCF levels were significantly elevated in BSO + DEM-pretreated hepatocytes compared to control hepatocytes treated with 1000 μg/ml VPA over the time-course from 45 to 120 min (Figure 5-6A). Furthermore, BSO + DEM-pretreated hepatocytes produced a 1.3 to 1.5-fold increase in DCF levels compared to control hepatocytes treated with 250, 500 and 1000 μg/ml VPA at the fixed incubation time of 120 min (Figure 5-6B). Strictly within the GSH-reduced hepatocytes, VPA (1000 μg/ml) produced a maximum 4.9-fold increase in DCF levels compared to the control group (0 μg/ml VPA) at a fixed incubation time of 2 h (Figure 5-6B).

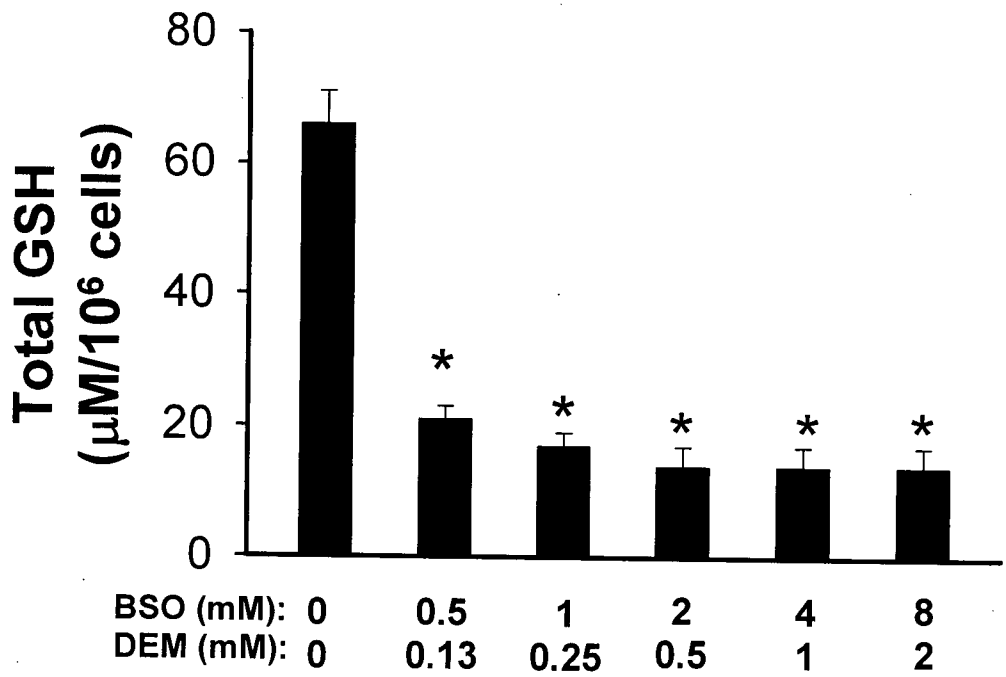

Figure 5-4: Total GSH levels determined in freshly isolated rat hepatocytes pretreated with a combination of DL-buthionine-[S,R]-sulfoximine (BSO) and diethylmaleate (DEM). Rat hepatocytes were pretreated with serial dilutions of a mixture of BSO (8 mM) and DEM (2 mM) for 2 h. Results are normalized per 10^6 cells and expressed as mean ± SEM (n = 4 experiments). *Significantly different compared with the control group ($p < 0.05$).

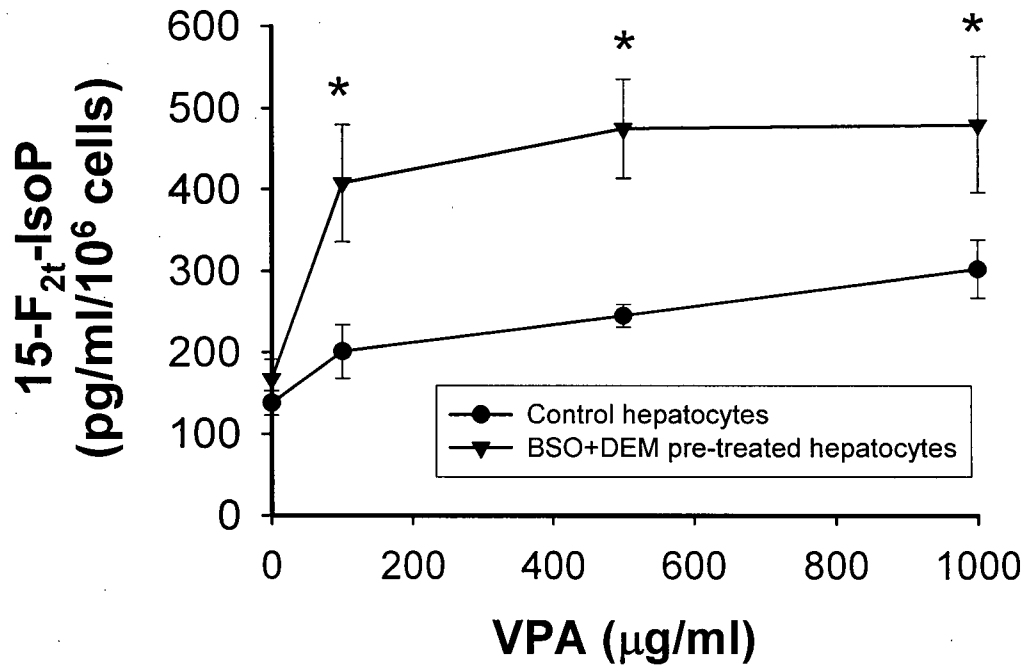

Figure 5-5: Dose-dependent changes in 15-F_{2t}-IsoP by VPA in freshly isolated rat hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats and were pretreated with a combination of BSO (2 mM) and DEM (0.5 mM) for 2 h. Hepatocytes were treated with VPA (0 - 1000 μg/mL) for an additional 2 h. Data are reported as the mean ± SEM (n = 5 experiments) *Significant differences between control hepatocytes vs. BSO and DEM pretreated hepatocytes ($p < 0.05$).

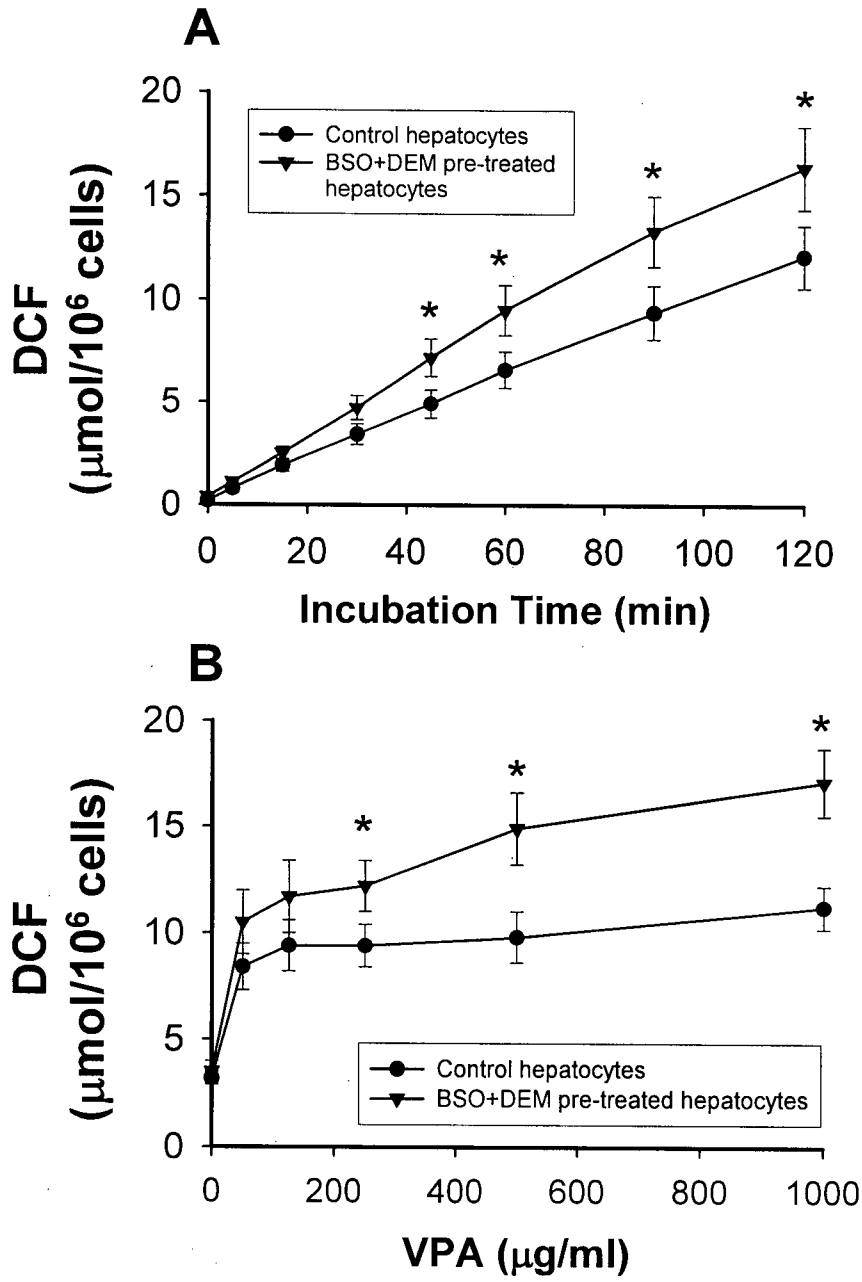

Figure 5-6: Comparison of the time- and concentration-dependent effect of VPA on the production of DCF in control, and BSO and DEM-pretreated rat hepatocytes. (A) Hepatocytes were treated with 1000 μg/ml VPA and DCF levels measured over time. (B) Hepatocytes were treated with VPA (0 - 1000 μg/ml) and DCF levels were measured at a fixed incubation time of 2 h. Data are reported as the mean ± SEM (n = 8 experiments). *Significantly different between BSO + DEM pretreated hepatocytes vs. control hepatocytes at each time point ($p < 0.05$).

5.3.3 Effect of VPA on mitochondrial membrane potential ($\Delta\Psi_m$) in primary culture rat hepatocytes

To examine the effect of VPA on $\Delta\Psi_m$, the hepatocytes were treated with JC-1, a cationic, lipophilic dual fluorescence dye that exhibits potential-dependent accumulation in mitochondria (Reers *et al.* 1995; Reers *et al.* 1991). The accumulation of dye aggregates is indicated by a fluorescence shift from green (emission: 530 nm) to red (emission: 590 m). A loss in $\Delta\Psi_m$ is indicated by a decrease in red/green fluorescence intensity ratio. VPA did not affect the $\Delta\Psi_m$ in control rat hepatocytes with respect to both time (Figure 5-7A) and concentration (Figure 5-7B). A loss in $\Delta\Psi_m$ was observed in GSH-depleted hepatocytes treated with 500 and 1000 μg/mL VPA (Figure 5-7B). The positive controls, mitochondrial un-couplers FCCP, mCLCCP and the potassium ionophore, valinomycin, resulted in $\Delta\Psi_m$ depolarization as indicated by a 50 - 60% decrease in the fluorescence ratio intensities by 15 min of treatment compared to their respective 0 min controls.

5.3.4 Effect of VPA on cell viability

As measured by the conversion of the tetrazolium salt WST-1 to a water-soluble formazan dye (absorbance at 450 nm) in rat hepatocytes, cell viability was not affected by VPA treatment (0 - 1000 μg/ml) for 8 h of incubation in normal control hepatocytes (Figure 5-8). A significant loss in cell viability (25% of control) was detected in GSH-depleted hepatocytes at 1000 μg/ml VPA. The hepatotoxin, 4-pentenoic acid (4-PA, 0 - 1000 μg/ml, n = 2 experiments) which was used as a positive control, resulted in a dose-dependent decrease in cell viability.

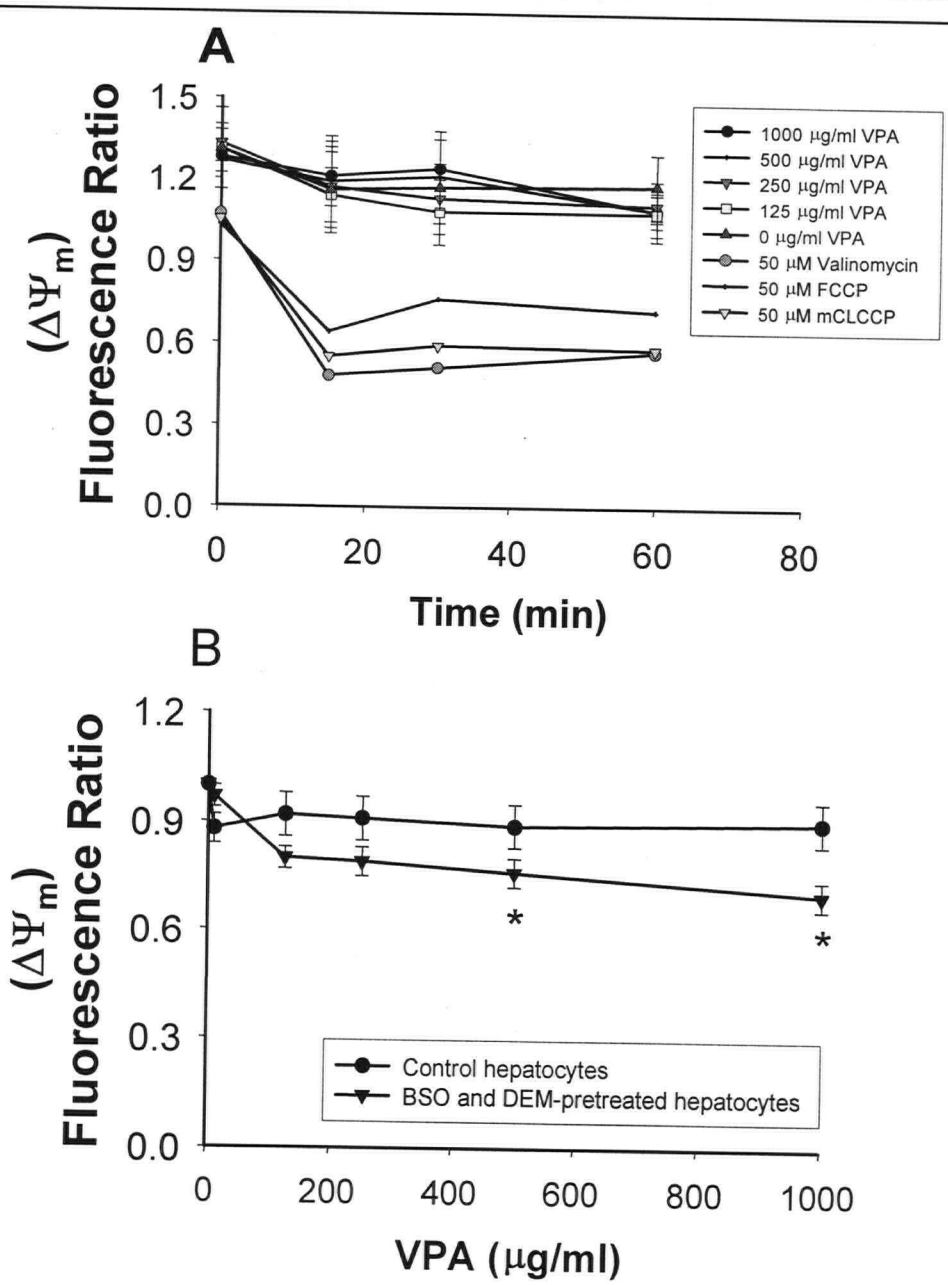

Figure 5-7: The time-course and concentration-dependent effects of VPA on mitochondrial membrane potential ($\Delta\Psi_m$) assessed by the JC-1 fluorescent probe. (A) Rat hepatocytes (4×10^4 cells/well in 96-well plates) were treated with VPA (0 - 1000 µg/ml, n = 3 experiments) or positive control compounds valinomycin, FCCP, mCLCCP (50 µM each, n=2 experiments) for up to 60 min. (B) Control and BSO+DEM-pretreated rat hepatocytes were treated with VPA (0-1000 µg/ml) for 2 h. Values are expressed as mean ± SEM (n = 5 experiments). *Significantly different between the control vs. BSO+DEM pretreated hepatocytes ($p < 0.05$).

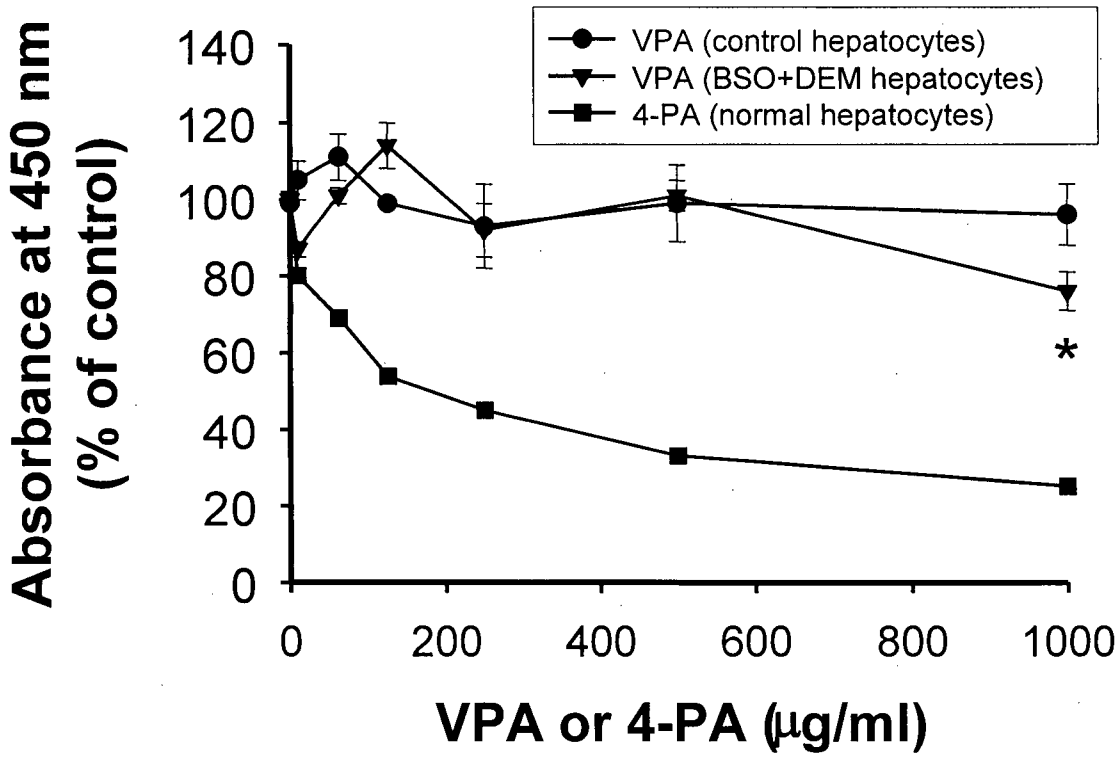

Figure 5-8: The effect of VPA on cell viability as measured by WST-1 assay. Control and BSO + DEM-pretreated rat hepatocytes (4×10^4 cells/well in 96-well plates) were treated with VPA (0-1000 µg/mL) for 8 h. Absorbance values are expressed as percentage of control (0 µg/ml VPA) and represent mean ± SEM (n = 5 experiments). The positive control was 4-pentenoic acid (4-PA, n = 2 experiments). *Significantly different between the control vs. BSO + DEM pretreated hepatocyte ($p<0.05$).

5.4 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 P450-mediated VPA-biotransformation, as indicated in previous mechanistic experiments (Tong *et al.* 2003). In a subsequent study, daily doses of VPA for 14 days in rats resulted in an elevation in plasma and liver 15-F_{2t}-IsoP that preceded the occurrence of necrosis and steatosis (Tong *et al.* 2005, manuscript submitted). In the same study, other biomarkers of oxidative stress, thiobarbituric acid reactive substances and lipid hydroperoxides, were measured and were found to be elevated after the initial occurrence of hepatotoxicity. Emanating from these findings, the current study is an *in vitro* approach to address: (1) if the rise in 15-F_{2t}-IsoP by VPA is reflective of oxidative stress, (2) whether or not the oxidative stress precedes hepatotoxicity, and (3) the involvement of GSH in these effects by VPA.

The overall objective of this study was to investigate the relationship between oxidative stress, mitochondrial membrane potential, and toxicity in freshly isolated rat hepatocytes treated with VPA. The experiments were designed to test the hypothesis that VPA treatment is associated with oxidative stress and mitochondrial dysfunction and that depletion of cellular GSH augments these responses by VPA. The first part of the study was to determine if oxidative stress could be detected following acute exposure to VPA in normal hepatocytes. The results indicated that cultured rat hepatocytes treated with VPA showed early increases in 15-F_{2t}-IsoP at the 30 min incubation time point (Figure 5-3A), corresponding to approximately the plasma t_{max} of 15-F_{2t}-IsoP observed in a previous study following a single dose of VPA (500 mg/kg, ip) to rats (Tong *et al.* 2003). In addition, intracellular oxidative stress was confirmed by monitoring the oxidation of DCFH to the fluorescent DCF as an independent indicator of oxidative stress. Significant elevation in DCF was first observed in hepatocyte cultures also at the 30 min time point

following VPA treatment (Figure 5-3B), coinciding with the rise in 15-F_{2t}-IsoP. At this time, this study is the first to correlate chemically-induced oxidative stress using the biomarkers DCF and 15-F_{2t}-IsoP in the same study. Another important finding was that the elevation in oxidative stress was not accompanied with hepatocyte toxicity during acute exposure to VPA. This *in vitro* observation is in agreement with previous *in vivo* findings that VPA elevated levels of 15-F_{2t}-IsoP occurred prior to signs of liver necrosis and steatosis (Tong *et al.* 2005, manuscript submitted).

Another objective of this study was to investigate the role of GSH in VPA-mediated oxidative stress in the hepatocyte model. The hypothesis to be tested is that cells with reduced levels of GSH have compromised antioxidant capabilities, and as a consequence, are more susceptible to VPA-induced oxidative stress. Rat hepatocytes pretreated with the combination of BSO and DEM had significantly reduced total GSH levels (to ≈ 20% of control levels), and as a consequence, resulted in significantly elevated oxidative stress compared to control hepatocytes over the same range of VPA concentrations. This suggests that GSH does play a protective role as an antioxidant against VPA-mediated oxidative stress. VPA treatment in rats and patients has also been linked to a decrease in GSH levels and alterations in glutathione peroxidase, a key intracellular antioxidant enzyme (Cengiz *et al.* 2000; Cotariu *et al.* 1990; Graf *et al.* 1998; Raza *et al.* 1997; Yuksel *et al.* 2000). Individuals deficient in GSH, either due to inborn errors (Bruggemann *et al.* 2004), malnutrition (Bray and Taylor 1993), or disease states such as those associated with hepatitis, hepatic cirrhosis, or HIV (White *et al.* 1994) may be theoretically more susceptible to xenobiotic-induced oxidative stress. Based on the available evidence, VPA may mediate its toxicity by a mechanism that involves the production of ROS in combination with decreased antioxidant capabilities (decreased levels of GSH) that ultimately leads to oxidative stress.

The hypothesis of a reactive metabolite of VPA contributing to the observed $\Delta\Psi_m$ and cytotoxicity upon reduction of cellular GSH by BSO and DEM can not be ruled out. Of the many metabolites, only 4-ene-VPA and (*E*)-2,4-diene-VPA have been shown to cause hepatic steatosis in the rat (Kesterson *et al.* 1984). Furthermore, indicators of reactive metabolite exposure, GSH and N-acetylcysteine conjugates of (*E*)-2,4-diene-VPA, have been identified in rats and patients (Gopaul *et al.* 2000b; Kassahun *et al.* 1991). The formation of (*E*)-2,4-diene-VPA and the subsequent depletion of GSH in the mitochondria provided a rationale for a reactive metabolite mechanism for mitochondrial dysfunction and microvesicular steatosis (Tang *et al.* 1995). However, in a recent study that involved high daily doses of VPA for 14 days to rats (Tong *et al.* 2005, manuscript submitted), there was no detectable increase in the measured VPA metabolites that accompanied the observed oxidative stress and hepatotoxicity. It may be argued that metabolite levels, in particular 4-ene-VPA and (*E*)-2,4-diene-VPA, may not be the delineating factor for VPA-mediated hepatotoxicity (Loscher *et al.* 1993; Siemes *et al.* 1993). More recent studies indicated that GSH or N-acetylcysteine conjugates of (*E*)-2,4-diene-VPA would be a better indicator of reactive metabolite exposure (Gopaul *et al.* 2000a, b; Tang *et al.* 1996). To establish a link between reactive metabolite exposure and oxidative stress, it would be useful in future studies to measure the levels of GSH conjugates of VPA metabolites in isolated hepatocytes treated with VPA.

The pathogenesis of severe VPA hepatotoxicity is not clear, but the observation of microvesicular steatosis is consistent with a disturbance in mitochondrial function and/ or fatty acid metabolism (Fromenty and Pessayre 1995; Powell-Jackson *et al.* 1984). The present study also investigates the effect of VPA on the $\Delta\Psi_m$ of rat hepatocytes as an indicator of mitochondrial dysfunction as determined by the fluorescent molecular probe, JC-1. Our findings indicate that VPA alone does not decrease the $\Delta\Psi_m$ in control hepatocytes; however, in hepatocytes with reduced GSH levels, the $\Delta\Psi_m$ was significantly decreased at the highest VPA

concentration tested (1000 μg/ml) (Figure 5-7). The decrease in $\Delta\Psi_m$ was also associated with significant cytotoxicity in GSH-reduced hepatocytes. These findings suggest that the added stress of GSH removal during VPA treatment is associated with increased oxidative stress levels, mitochondrial dysfunction, and cytotoxicity. Recently, similar studies with acetaminophen (Masubuchi *et al.* 2005; Reid *et al.* 2005), salicylate (Trost and Lemasters 1997), and clofibrate (Qu *et al.* 2001) established an association between elevated oxidative stress and the loss in $\Delta\Psi_m$ by induction of the mitochondrial membrane transition, both occurring before the onset of cell toxicity. Other factors known to decrease $\Delta\Psi_m$ include K^+ and Ca^{2+} ionophores, cyanide compounds known to un-couple oxidative phosphorylation such as FCCP, pH-dependent ischemia-reperfusion, and oxidative stress (Lemasters *et al.* 1998). *tert*-Butylhydroperoxide, a short chain lipid hydroperoxide analog, was demonstrated to generate ROS within the mitochondria that induced the mitochondrial membrane transition, and resulted in the loss in $\Delta\Psi_m$ and ATP depletion prior to hepatocyte toxicity (Imberti *et al.* 1993; Nieminen *et al.* 1997). In many models of oxidative stress, there is a general agreement that GSH must be depleted below a certain critical threshold, before the extent of cytotoxicity correlates with the magnitude of GSH depletion (Kaplowitz and Tsukamoto 1996). Our study is consistent with a mechanism of VPA-hepatotoxicity that involves oxidative stress through the combination of ROS production and GSH depletion, resulting in the subsequent loss in $\Delta\Psi_m$, and ultimately leading to cytotoxicity. Future studies would be required to establish a causal link between VPA-induced oxidative stress, mitochondrial depolarization, and cytotoxicity. One approach would be to block the mitochondrial membrane transition with cyclosporine A or trifluoperazine (Lemasters *et al.* 1998) to see if VPA-induced cytotoxicity can be prevented in GSH-depleted hepatocytes.

In summary, we demonstrated that VPA is capable of producing oxidative stress in freshly isolated rat hepatocytes as measured by elevated levels of 15-F_{2t}-IsoP and DCF. However, the

oxidative stress did not result in mitochondrial dysfunction or hepatocyte toxicity. By comparison, in hepatocytes pretreated with BSO and DEM to reduce total GSH, the levels of the oxidative stress biomarkers were further elevated, and this was accompanied by mitochondrial dysfunction (as detected by a decrease in $\Delta\Psi_m$) and cytotoxicity in hepatocytes treated with high concentrations of VPA. These results are significant because they support the hypothesis that VPA-associated oxidative stress occurs prior to hepatotoxicity, and indicate that GSH serves a protective role to mitigate the deleterious effects of high concentrations on VPA on mitochondrial function and cell viability.

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6 *Global Summary, Future Studies, Conclusions*

6.1 SUMMARY OF FINDINGS AND FUTURE STUDIES

In vivo and *in vitro* studies were conducted with VPA, a broad-spectrum anticonvulsant, in order to investigate whether oxidative stress is associated with VPA-hepatotoxicity. As there is limited and conflicting evidence for the involvement of oxidative stress during VPA treatment, investigational toxicity studies with emphasis on probing the metabolic pathways of VPA while measuring oxidative stress biomarkers will provide a better understanding of the role of VPA biotransformation (i.e. cytochrome P450, UDPGT) and its association with oxidative stress.

Our initial investigation measured levels of 15-F_{2t}-IsoP as a biomarker for lipid peroxidation after a single high dose of VPA (500 mg/kg, ip) to rats (Tong *et al.* 2003). Dose-dependent elevations in plasma and liver 15-F_{2t}-IsoP levels were observed with a plasma-concentration time profile similar to that of VPA (t_{max} of approximately 30 min).

Other independent measures of oxidative stress, thiobarbituric reactive acid substances and lipid hydroperoxides, were not elevated and raised the question if the increased levels of 15-F_{2t}-IsoP were truly reflective of oxidative stress. The formation of 15-F_{2t}-IsoP is assumed to involve a free-radical, non-cyclooxygenase-dependent mechanism; however, literature evidence exists that cyclooxygenase (COX-1 and COX-2) isoforms may contribute to its production (Pratico *et al.* 1995). Future studies in our laboratory would be to examine the effects of indomethacin, a non-selective COX inhibitor, on the VPA-induced 15-F_{2t}-IsoP levels observed in rats. However, in our favour, VPA was demonstrated to down-regulate both COX-1 and COX-2 protein levels

and total COX activity (Szupera *et al.* 2000), making it unlikely that the increase in 15-F_{2t}-IsoP by VPA was COX-dependent.

In support of our *in vivo* findings, VPA elevated levels of 15-F_{2t}-IsoP and DCF in rat hepatocyte cultures. With the available evidence, the overall interpretation at the present time is that the increases in 15-F_{2t}-IsoP do reflect oxidative stress.

Further mechanistic experiments examined the effects of chemical inducers (PB) and inhibitors (ABT and SKF-525A) of cytochrome-P450 on 15-F_{2t}-IsoP levels (Tong *et al.* 2003). Our results demonstrated a lack of involvement of cytochrome-P450 biotransformation on the elevated 15-F_{2t}-IsoP levels. However, an interesting finding was that rats treated with both PB and VPA showed levels of 15-F_{2t}-IsoP greater than those seen with rats treated with VPA alone, whereas PB itself had no effect on 15-F_{2t}-IsoP levels.

The results with the PB- and VPA-treated animals prompted further studies examining the role of VPA-glucuronidation, the major VPA biotransformation pathway that is also inducible by PB, on oxidative stress (Tong *et al.* 2004, manuscript submitted). Mechanistic experiments used chemical inducers (PB) and inhibitors ((-)-borneol and salicylamide) to modulate levels of liver VPA-G. An intriguing finding was the association between the levels of VPA-G and 15-F_{2t}-IsoP in rats treated with a single dose of VPA. This is the first study to associate VPA-glucuronidation, which is normally considered a detoxification pathway, with oxidative stress. This novel finding prompts further studies to address the mechanism of how VPA-glucuronidation can give rise to oxidative stress. One possible mechanism may be a consequence of the extensive hydrolysis of VPA-G in the liver compartment at the site of formation and in the intestinal compartment as a result extensive enterohepatic recirculation. As a result, the systemic cycling of VPA to VPA-G and back to VPA may lead not only to

potentially reactive β-glucuronidase-resistant isomers of VPA-G, but to theoretically high concentrations of free glucuronic acid due to the large doses of VPA employed. Glucuronic acid has recently been associated with oxidative stress as measured by DCF formation (Kim *et al.* 2004).

Future studies are required to investigate the possibility that the enterohepatic recirculation of VPA-G and the glucuronic acid generated in the process are involved in the formation of 15-F_{2t}-IsoP, and several approaches can be taken to investigate this hypothesis. The enterohepatic recirculation of VPA-G would be abolished in bile duct-cannulated rats dosed with VPA, and because the bile is exteriorized, lower levels of 15-F_{2t}-IsoP would be expected. The multi-drug resistance protein-2 (MRP-2) has been identified to transport VPA-G across the canalicular membrane into bile; therefore, MRP-2 knockout rats dosed with VPA would be expected to have decreased levels of VPA-G excreted in the bile, decreased enterohepatic recirculation, and less VPA-G hydrolysed, that would ultimately lead to lower levels of free glucuronic acid, and theoretically lower levels of 15-F_{2t}-IsoP. Another approach would be to inhibit β-glucuronidase activity, the enzyme predominantly responsible for the hydrolysis of VPA-G, and this should result in elevated systemic VPA-G levels and sequestering of glucuronic acid. Direct measurement of plasma and liver levels of free glucuronic acid would be required for the correlation with oxidative stress. The question remains whether the enzymatic formation of VPA-G via UDP-glucuronosyl transferases or the product itself (VPA-G) is responsible for the rise in 15-F_{2t}-IsoP. Another question, one perhaps of clinical importance, emanating from these findings is whether or not other compounds (i.e. diclofenac, acetaminophen, lamotrigine) that are subject to glucuronidation can also generate oxidative stress as measured by 15-F_{2t}-IsoP.

In support of the hypothesis that VPA-glucuronidation is associated with oxidative stress, a comparative study with VPA and its α-fluorinated analog, α-fluoro-VPA, was investigated in rats

with regard to their inherent ability to generate 15-F_{2t}-IsoP (Tong *et al.* 2004, manuscript submitted). α-Fluoro-VPA, a poor substrate for glucuronidation, did not elevate levels of 15-F_{2t}-IsoP. However, the possibility that VPA-induced oxidative stress may have involved mitochondrial fatty acid β-oxidation, the second largest metabolic pathway for VPA, cannot be ruled out. α-Fluoro-VPA is also inert to β-oxidation, and based on the lack of induction on 15-F_{2t}-IsoP levels, the results also support the hypothesis that the source of oxidative stress may originate from the mitochondria and involve fatty acid oxidation of VPA.

Octanoic acid, an 8-carbon linear chain analog of VPA that undergoes extensive β-oxidation, was examined to see whether another fatty acid of similar structure is capable of elevating 15-F_{2t}-IsoP levels. Our findings showed that octanoic acid did not elevate 15-F_{2t}-IsoP, which suggests that the increase in 15-F_{2t}-IsoP was specific to VPA.

As a future study to further investigate the role of β-oxidation on VPA-associated oxidative stress, the use of trimethylacetic acid (TMA) will be used as a chemical inhibitor of β-oxidation by depleting acetyl coenzyme-A, a cofactor required for fatty acid transport. In a preliminary study in rats pre-treated with a single dose of TMA (500 mg/kg) prior to VPA dosing, the results indicated that the 15-F_{2t}-IsoP levels remained elevated compared to VPA-treated rats (Tong *et al.*, unpublished data). However, TMA pre-treatment was only capable of reducing β-oxidation metabolites of VPA by ≈ 30-40%, and future studies are required to optimize the TMA dose to maximally inhibit β-oxidation and to measure the effects on the associated oxidative stress.

More detailed *in vivo* toxicity studies investigated whether the VPA-induced oxidative stress could be associated with hepatotoxicity in rats (Tong *et al.* 2005a, manuscript submitted). Our findings indicated that high daily doses of VPA given for 14 days elevated 15-F_{2t}-IsoP levels prior to hepatotoxicity. Liver histology revealed that inflammation of the liver capsule was the

most common feature, and this was accompanied by increasing severity and frequency of necrosis by day 14. Hepatic steatosis increased in frequency from days 4-10 and was never considered severe. Levels of TBARS and LPO, which were not affected after a single dose of VPA, were found to be elevated after multiple doses of VPA and after the initial onset of hepatotoxicity. The question remains whether there is a causal relationship between oxidative stress and hepatotoxicity, and this may be addressed with studies that involve supplementing antioxidants (i.e. vitamin C, vitamin E) and/or N-acetylcysteine to rats given VPA for the same 2-week period. The levels of VPA metabolites, in particular the putative reactive metabolites 4-ene-VPA and (*E*)-2,4-diene-VPA, were not elevated throughout the study and may also be inadequate indicators of reactive metabolite exposure. GSH and N-acetylcysteine conjugates of VPA metabolites may be more informative indicators of reactive metabolite exposure (Gopaul *et al.* 2000a, b; Tang *et al.* 1996) and these conjugates should be determined in the 14-day toxicity study in rats. The β-oxidation metabolites of VPA in the liver decreased in the late stages of the toxicity study (between days 10 to 14) which suggests that inhibition of β-oxidation in the liver occurred after the onset of hepatotoxicity. To strengthen the evidence that oxidative stress occurs prior to VPA-hepatotoxicity, a second indicator of oxidative stress should be monitored in VPA-treated rats. The ratio of oxidized to reduced glutathione (GSSG/GSH) levels would be another independent indicator to monitor changes in oxidant status during VPA treatment. Our laboratory is currently developing an assay for the direct and simultaneous determination of GSH and GSSG by LC/MS/MS.

In vitro studies using primary cultured rat hepatocytes provide more evidence that oxidative stress occurs prior to VPA-hepatotoxicity (Tong *et al.* 2005b, manuscript submitted). Levels of 15-F_{2t}-IsoP and DCF were both elevated following VPA treatment at a time point similar to the t_{max} seen *in vivo*, without evidence of hepatocyte toxicity. GSH plays a protective role in VPA toxicity since GSH-depleted hepatocytes exhibited greater levels of oxidative stress at high

levels of VPA and this was accompanied by the loss of mitochondrial membrane potential and cytotoxicity. This *in vitro* model provides a platform that allows a similar investigation for structure-activity relationships using a variety of VPA analogs (i.e. α-F-VPA) with the ultimate goal of developing a new antiepileptic compound similar in efficacy to VPA, but devoid of undesirable properties such as oxidative stress, mitochondrial dysfunction, and cytotoxicity.

6.2 CONCLUSION

This data presented in this thesis provide strong evidence that VPA is associated with oxidative stress that precedes hepatotoxicity. The finding that VPA exacerbates oxidative stress, mitochondrial dysfunction, and toxicity in GSH-reduced hepatocytes indicates that the mechanism of hepatotoxicity may be multi-factorial, such that the combination of high doses of VPA, and oxidative stress in the form of insufficient levels of antioxidants (e.g. GSH) and elevation in lipid peroxidation may make an individual more susceptible to hepatotoxicity. This thesis also makes a significant overall contribution to the field of VPA research, as it is the first *in vivo* study to examine VPA-associated oxidative stress and hepatotoxicity within the same study period. In addition, this study describes a novel quantitative assay for VPA-G by LC/MS and is the first report implicating VPA-G to be associated with oxidative stress.

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