Valproic Acid II: Effects on Oxidative Stress, Mitochondrial Membrane Potential, and Cytotoxicity in Glutathione-Depleted Rat Hepatocytes

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Received March 11, 2005; accepted April 20, 2005

Oxidative stress has been associated with valproic acid (VPA) treatment, and mitochondrial dysfunction has been implicated in the pathogenesis of VPA-idiosyncratic hepatotoxicity. The present study investigated the effect of VPA and the role of GSH on oxidative stress, mitochondrial membrane potential, and toxicity in freshly isolated rat hepatocytes. Hepatocytes were isolated from Sprague-Dawley rats, and total levels of glutathione (GSH) reduced by pretreatment with a combination of L-buthionine sulfoximine (2 mM) and diethylmaleate (0.5 mM) prior to VPA (0–1000 µg/ml) treatment. Oxidative stress was determined by measuring the levels of 15-F2t-isoprostane (15-F2t-IsoP) and 2',7'-dichlorofluorescein (DCF). Mitochondrial membrane potential (ΔΨm) was determined by using the dual-fluorescent dye JC-1, and cell viability was evaluated by the water-soluble tetrazolium salt WST-1 assay. Exposure of rat hepatocytes to VPA (0–1000 µg/ml) resulted in a time- and dose-dependent increase in 15-F2t-IsoP and DCF fluorescence, and these levels were further elevated in GSH-reduced hepatocytes. In control hepatocytes, VPA had no effect on cell viability; however, significant cytotoxicity was observed in the glutathione-depleted hepatocytes treated with 1000 µg/ml VPA. The ΔΨm was only reduced in glutathione-reduced hepatocytes at 500 and 1000 µg/ml VPA. Our novel findings indicate that acute treatment of freshly isolated rat hepatocytes with VPA resulted in oxidative stress, which occurred in the absence of cytotoxicity, and that glutathione confers protection to hepatocytes against mitochondrial damage by VPA.

Key Words: Valproic acid; hepatocytes; 15-F2t-isoprostane; mitochondrial membrane potential; glutathione; 2',7'-dichlorofluorescein.

Valproic acid (2-propyl-pentanoic acid, VPA), a broad-spectrum antiepileptic drug now also used for bipolar and migraine control, is associated with hepatotoxicity (Granneman et al., 1984; Kesterson et al., 1984). Although the mechanism of the liver toxicity remains elusive, oxidative stress, as a result of overproduction of reactive oxygen species (ROS) and/or compromised antioxidant capacity, has been hypothesized to play a role in the etiology of the toxicity (Farrell and Abbott, 1991; Pippenger et al., 1991). A number of studies have investigated the possibility that VPA treatment is associated with oxidative stress in patients (Cengiz et al., 2000; Graf et al., 1998; Hurd et al., 1984) and in animal models (Cotariu et al., 1990; Raza et al., 1997).

Primary cultured rat hepatocytes have been utilized to study VPA-associated hepatotoxicity. Earlier 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 C, 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 in rats led to a dose-dependent elevation in plasma and liver levels of the endogenous lipid peroxidation marker, 15-F2t-isoprostaglandin (15-F2t-IsoP) (Tong et al., 2003). High daily doses of VPA to rats for 14 consecutive days produced an elevation in 15-F2t-IsoP that preceded the onset of liver necrosis and steatosis (Tong et al., 2005). 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-F2t-IsoP levels truly reflects oxidative stress and whether this results in mitochondrial dysfunction and hepatocyte toxicity.

There is evidence that glutathione homeostasis may be altered as a consequence of reactive metabolites and/or reactive oxygen species produced during VPA treatment (Cengiz et al., 2000; Cotariu et al., 1990; Graf et al., 1998; Raza et al., 1997). Reduced glutathione (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 provides further evidence for a protective role of GSH (Farrell and Abbott, 1991).

The objective of the present study with 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-F2t-Isop assays; (2) mitochondrial membrane potential (ΔΨm), 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) and that GSH-depletion exacerbates oxidative stress, mitochondrial membrane depolarization, and cytotoxicity in rat hepatocytes treated with high concentrations of VPA.

**MATERIALS AND METHODS**

**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 Media were purchased from BD Biosciences Inc. (Baie d’Urfe, QC, Canada). Invitrogen (Burlington, ON, Canada). Percoll was purchased from Amersham Biosciences Inc. (Ann Arbor, MI). WST-1 was obtained from Roche Diagnostics (Laval, QC, Canada). JC-1 (5,5′,6.6′-tetrachloro-1,1′,3′,3′-tetrachlorobenzimidazolylcarbocyanine 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).

**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.

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**Materials and methods.** Rat hepatocytes 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 CaCl2-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 medium (1.40 g/l CaCl2, 2.38 g/l HEPES, 0.35 g/l NaHCO3, 0.05 g/l trypsin inhibitor [Type II-soybean], 0.5 g/l collagenase [Type IV, pH 7.4]). Perfusion with digest medium was subjected 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 spun (50 × g) for 3 min to pellet the hepatocytes, and the pellet was resuspended 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 3 min, and the supernatant containing the dead cells was decanted. The pellet was resuspended and washed once with Hepato-Stim, and the viability and cell concentration was determined by trypsin blue exclusion. The exclusion criterion for hepatocyte viability was >90%. Cells were diluted to 4 × 105 cells/ml in Hepato-Stim Media and seeded as a monolayer culture on Matrigel Matrix coated 24- or 96-well sterile tissue culture plates at a density of 2 × 104 or 4 × 104 cells/well, respectively. Cells were allowed to attach for 2 h in a 37°C, 5% CO2/95% air 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). 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 (Maclurano et al., 1990; Plummer et al., 1981).

**15-F2t-Isop assay.** 15-F2t-Isop was determined in rat hepatocyte cultures (2 × 107 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 Hepatocyte medium). At specific time points, 2% butylated hydroxytoluene (10 μl) was added to the culture media 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-F2t-Isop as previously described (Tong et al., 2003). The results were normalized per 106 cells and expressed as the mean ± SEM.

**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 were preloaded with 5 μM DCF-DA (dissolved in Hepatocyte medium) for 20 min (37°C, 5% CO2/95% air). The diacetate form of 2′,7′-dichlorofluorescin (DCF) diffuses across the cell membrane and is hydrolyzed by intracellular esterases to yield the nonfluorescent DCFH. DCFH, upon reacting with low molecular weight hydroperoxides (i.e., H2O2), is oxidized to its highly fluorescent, 2-electron oxidation product, DCF. Following 20 min of DCF-DA loading, cells were washed once with Hepatocyte culture medium and treated with VPA (0–1000 μg/ml dissolved in Hepatocyte 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.
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/10⁶ cells.

**Measurement of mitochondrial membrane potential (Δψ_{m})**. Δψ_{m} was measured using the mitochondrial-specific dual-fluorescence probe, JC-1, based on the method described by Reers et al. (1995). 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 (orange 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 × 10⁴ 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 to included valinomycin (50 µM), carbonyl cyanide m-chlorophenylhydrazone (mCCCP, 50 µM), and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 50 µM). After the 2-h treatment, the medium was removed and replaced with 100 µl of 10 µg/ml JC-1 in Hepatozyme media. Cells were incubated with the dye for 10 min (37°C, 5% CO₂/95% air) followed by a wash with PBS, and cells were allowed to equilibrate at room temperature in the dark for 10 min. A CytoFluor plate reader (excitation wavelength 485 nm, slit width 20 nm) was used to monitor the fluorescence intensities for the monomer and the aggregated JC-1 molecules (emission wavelengths 530 nm, slit width of 25 nm, and 580 nm, slit width of 50 nm, respectively).

**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 formazen dye. After the 2-h attachment, cells (4 × 10⁴ cells/well in 96-well plates) were treated with various compounds (using Hepatozyme medium as a vehicle) for up to 8 h (37°C, 5% CO₂/95% air) followed by the addition of 10 µl of 15-F₂-IsoP stock solution per 100 µl of medium in each well. After 30 min of incubation time (37°C, 5% CO₂/95% air), 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 formazen dye (decrease in absorbance). The positive control, 4-pentenoic acid, was used to compare the cytotoxicity of VPA.

**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. 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 for 15 min at 4°C, and the supernatant was collected and stored on ice. For the deproteinization 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 approx. 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 0.5–16 µM GSH (reduced form).

**Statistical analysis**. The results were 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-Keuls’s multiple pairwise comparison post hoc test. Groups receiving different treatment concentrations of VPA (Fig. 1) or BSO and DEM (Fig. 2) were compared to normal hepatocytes at the same time point. In addition, normal hepatocytes were compared to BSO and DEM pretreated hepatocytes with either the same VPA concentration at the same time point, or different time point at the same VPA concentration (Figs. 3–6). The level of significance was set a priori at p < 0.05 in all cases.

**RESULTS**

**VPA-Induced Oxidative Stress in Freshly Isolated Rat Hepatocytes**

VPA produced a time- and concentration-dependent increase in the lipid peroxidation biomarker, 15-F₂-IsoP, in rat hepatocyte cultures (Fig. 1A). Treatment with 1000 µg/ml VPA for 30 min increased the level of 15-F₂-IsoP compared to the media control group (199 ± 18 and 104 ± 22 pg/ml/10⁶ cells, respectively). At the 120-min time point, treatment with VPA at

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FIG. 1. Time-course and dose-response relationship for the production of (A) 15-F₂-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 × 10⁴ cells/well in a 24-well plate) from 0–180 min. The media was removed at each time point for the determination of 15-F₂-IsoP. Data are reported as the mean ± SEM (n = 5 experiments). Significant differences between 0 µg/ml versus *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 × 10⁴ 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 control group (0 µg/ml VPA) and the VPA treated groups (10, 250, and 1000 µg/ml VPA) (p < 0.05).
all concentrations (100–1000 μg/ml) tested gave significantly elevated 15-F_{2t}-IsoP levels by up to 1.9-fold compared to the 0 μg/ml control group in the hepatocytes.

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 (Fig. 1B).

### 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 (i.e., 21% of control) (Fig. 2). 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) (Fig. 3). 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 (Fig. 4A). Furthermore, BSO + DEM 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 (Fig. 4B). 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 (Fig. 4B).

**Effect of VPA on Mitochondrial Membrane Potential (Δψ_m) in Freshly Isolated Rat Hepatocytes**

To examine the effect of VPA on Δψ_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). The accumulation of dye aggregates is indicated by a fluorescence shift from green (emission 530 nm) to red (emission 590 μm). A loss in Δψ_m is indicated by a decrease in red/green fluorescence intensity ratio. VPA did not affect the Δψ_m in control rat hepatocytes with respect to both time (Fig. 5A) and concentration (Fig. 5B). A loss in Δψ_m was observed in GSH-depleted hepatocytes treated with 500 and 1000 μg/ml VPA (Fig. 5B). The positive controls, mitochondrial uncouplers FCCP and mCLCCP and the potassium ionophore valinomycin, resulted in Δψ_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.

**Effect of VPA on Cell Viability**

As measured by the conversion of the tetrazolium salt WST-1 to a water-soluble formazen 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 (Fig. 6). A significant loss in cell viability (25%
Control hepatocytes
BSO+DEM pre-treated hepatocytes

of control) was detected in GSH-depleted hepatocytes at 1000 µg/ml VPA. The hepatotoxin, 4-pentenoic acid (PA, 0–1000 µg/ml, n = 2 experiments), which was used as a positive control, resulted in a dose-dependent decrease in cell viability in normal hepatocytes (i.e., not previously GSH depleted).

DISCUSSION

Our previous study in rats showed increased levels of 15-F_{2\alpha}-IsoP after a single dose of VPA, but this did not involve cytochrome P450-mediated VPA-biotransformation, as indicated in novel 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_{2\alpha}-IsoP that preceded hepatotoxicity, as assessed by an increase in serum α-GST levels and the occurrence of necrosis and steatosis (Tong et al., 2005). 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) whether acute treatment of VPA results in oxidative stress in primary cultures of rat hepatocytes, (2) whether VPA produces oxidative stress and mitochondrial dysfunction in the absence of hepatocyte toxicity, and (3) the involvement of GSH in these effects 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_{2\alpha}-IsoP at the 30-min incubation time point (Fig. 1A), corresponding to approximately the plasma T_{max} of 15-F_{2\alpha}-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 also first observed in hepatocyte cultures at the 30-min time point following VPA treatment (Fig. 1B), coinciding with the rise in 15-F2t-IsoP. At this time, this study is the first to correlate chemically-induced oxidative stress using the biomarkers DCF and 15-F2t-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-F2t-IsoP occurred prior to signs of liver necrosis and steatosis (Tong et al., 2005).

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 was 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 significantly reduced total GSH levels (to ≈ 20% of control levels) and 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). 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 cell damage. Although this study used normal fed rats, fasted rats would have reduced hepatic GSH levels so that one would predict that the degree of oxidative stress, mitochondrial membrane potential, and hepatocyte toxicity would be more pronounced in fasted rats than in fed rats.

The hypothesis of a reactive metabolite of VPA contributing to the observed Δψm, and cytotoxicity upon reduction of cellular GSH by BSO and DEM cannot 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, glutathione and N-acetylcysteine conjugates of (E)-2,4-diene-VPA, have been identified in rats and patients (Gopaul et al., 2000; 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) 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 (Tang et al., 1996; Gopaul et al., 2000). 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). The present study also investigated the effect of VPA on the Δψm of rat hepatocytes as an indicator of mitochondrial dysfunction as determined by the fluorescent molecular probe, JC-1. Our findings indicated that VPA alone did not decrease the Δψm in control hepatocytes; however, in hepatocytes with reduced glutathione levels, the Δψm was significantly decreased at the two higher VPA concentrations tested (500 and 1000 μg/ml) (Fig. 5). The decrease in Δψm 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 uncouple 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 (Niemenen 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 acute 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. The results provide a cellular basis for future investigations on elucidating the mechanism of idiosyncratic VPA-hepatotoxicity.

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$_2$t-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 content, 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. Overall, our results demonstrate that VPA-associated oxidative stress can occur in the absence of hepatocyte toxicity, which is consistent with our in vivo findings indicating that oxidative stress precedes hepatotoxicity in rats treated with VPA (Tong et al., 2005). Furthermore, our data also show that GSH serves a protective role to mitigate the deleterious effects of high concentrations on VPA on oxidative stress, mitochondrial function, and cell viability.

**ACKNOWLEDGMENTS**

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

**REFERENCES**


