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Valproic Acid Increases Conservative Homologous Recombination Frequency and Reactive Oxygen Species Formation: A Potential Mechanism for Valproic Acid-Induced Neural Tube Defects

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ABSTRACT

Valproic acid, a commonly used antiepileptic agent, is associated with a 1 to 2% incidence of neural tube defects when taken during pregnancy; however, the molecular mechanism by which this occurs has not been elucidated. Previous research suggests that valproic acid exposure leads to an increase in reactive oxygen species (ROS). DNA damage due to ROS can result in DNA double-strand breaks, which can be repaired through homologous recombination (HR), a process that is not error-free and can result in detrimental genetic changes. Because the developing embryo requires tight regulation of gene expression to develop properly, we propose that the loss or dysfunction of genes involved in embryonic development through aberrant HR may ultimately cause neural tube defects. To determine whether valproic acid induces HR, Chinese hamster ovary 3-6 cells, containing a neomycin direct repeat recombination substrate, were exposed to valproic acid for 4 or 24 h. A significant increase in HR after exposure to valproic acid (5 and 10 mM) for 24 h was observed, which seems to occur through a conservative HR mechanism. We also demonstrated that exposure to valproic acid (5 and 10 mM) significantly increased intracellular ROS levels, which were attenuated by preincubation with polyethylene glycol-conjugated (PEG)-catalase. A significant change in the ratio of 8-hydroxy-2′-deoxyguanosine/2′-de-oxyguanosine, a measure of DNA oxidation, was not observed after valproic acid exposure; however, preincubation with PEG-catalase significantly blocked the increase in HR. These data demonstrate that valproic acid increases HR frequency and provides a possible mechanism for valproic acid-induced neural tube defects.

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Valproic acid is a commonly prescribed antiepileptic agent used to treat a wide variety of seizure disorders (Chapman et al., 1982). Furthermore, valproic acid has also proven to be effective in the treatment of bipolar disorder (Bowden, 2003), migraine prophylaxis (Freitag, 2003), and neuropathic pain (Covington, 1998). Despite its effectiveness and widespread use, valproic acid is teratogenic in both animals (Brown et al., 1980) and humans (Robert and Rosa, 1983). Of particular concern is the 1 to 2% risk of neural tube defects, most of which are spina bifida, with the use of valproic acid during the first trimester of pregnancy (Bjerkedal et al., 1982). This is 10 to 20 times the prevalence rate for neural tube defects in the general population (Frey and Hauser, 2003). However, the molecular mechanism by which valproic acid causes neural tube defects has not been elucidated.

The teratogenicity of many drugs and chemicals is believed to be initiated through the bioactivation of the parent com-

ABBREVIATIONS: ROS, reactive oxygen species; 8-OH-2′-dG, 8-hydroxy-2′-deoxyguanosine; 2′-dG 2′-deoxyguanosine; CHO, Chinese hamster ovary; DCF, dichlorodihydrofluorescein; CM-H_2DCF, 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate; HR, homologous recombination; LOH, loss of heterozygosity; PEG, polyethylene glycol; kb, kilobase(s).

pound to form a reactive intermediate, followed by the formation of ROS (Wells and Winn, 1996; Wells et al., 1997). The developing embryo is particularly sensitive to increases in ROS formation because enzymes in embryonic and fetal tissue that detoxify ROS generally tend to have low activities (Wells et al., 1997). If ROS levels exceed the cellular detoxifying capabilities and a state of oxidative stress ensues, detrimental consequences to the embryo can result and may ultimately lead to teratogenesis (Wells et al., 1997).

Several studies suggest indirectly that valproic acid-initiated teratogenicity may be caused by oxidative stress. For example, previous research has shown that catalase, which detoxifies hydrogen peroxide, prevented valproic acid-induced lymphocyte toxicity and that 1,10-phenanthroline, an iron chelator, also decreased valproic acid-mediated cytotoxicity in vitro (Tabatabaei and Abbott, 1999). These results suggest that the production of hydrogen peroxide and the succeeding iron-catalyzed formation of hydroxyl radicals may be the specific ROS that mediates valproic acid-induced toxicity (Tabatabaei and Abbott, 1999). In addition, valproic acid has been shown to inhibit cardiomyocyte differentiation of embryoid bodies derived from murine pluripotent embryonic stem cells through an increase in ROS (Na et al., 2003). Alternatively, the antioxidant vitamin E has been shown to decrease the frequency of valproic acid-induced neural tube defects in mice, suggesting that ROS may play a role in the failure of the neural tube to develop properly (Al Deeb et al., 2000).

One of the consequences of increased ROS production is oxidative DNA damage (Klaunig and Kamendulis, 2004). Previous research suggests that the mechanism of teratogenesis for both thalidomide and phenytoin may be mediated through an increase in oxidative DNA damage (Winn and Wells, 1995; Parman et al., 1999). Oxidized bases can be repaired by base excision repair and nucleotide excision repair; however, during the repair process, DNA double-strand breaks can be generated (Lindahl and Wood, 1999). For example, when oxidized bases located close together and on opposite strands are being repaired through base excision repair and nucleotide excision repair, the simultaneous excision of the damaged bases can result in a double-strand break (Pfeiffer et al., 2000). Alternatively, single-strand breaks that are generated during the repair of oxidative DNA damage can be converted to double-strand breaks during replication (van den Bosch et al., 2002). In addition, double-strand breaks can be caused by free radicals themselves (van Gent et al., 2001).

DNA double-strand breaks are repaired through two mechanisms: nonhomologous end-joining, and HR (van den Bosch et al., 2002). Although HR often repairs DNA double-strand breaks with a high degree of fidelity, it has been shown that HR is not always an error-free process and can contribute to genomic instability through the production of genetic changes, including the loss of heterozygosity (LOH) and gene deletions and duplications (Bishop and Schiestl, 2003). Because of its potential to contribute to genetic changes, HR is believed to play a major role in the development of some diseases, including cancer (Bishop and Schiestl, 2003). Because proper development of the embryo requires highly regulated gene expression, HR that results in genetic changes has the potential to disrupt these tightly coordinated processes and may underlie the mechanism of valproic acid-induced neural tube defects.

This study was conducted to determine whether valproic acid increases HR through an increase in ROS formation and subsequent oxidative DNA damage in the Chinese hamster ovary 3-6 (CHO 3-6) recombination reporter cell line. In addition, PEG-catalase was used to determine whether antioxidant administration could decrease valproic acid-induced HR. The results of this study show that valproic acid does increase ROS levels and HR frequency but not DNA oxidation. These data support our hypothesis that HR may be the underlying mechanism mediating valproic acid-induced teratogenesis.

Materials and Methods

Cell Culture. The CHO 3-6 cell line, obtained from Jac A. Nickoloff (Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, NM), was used to assess HR frequency. This cell line contains a single, stably integrated direct repeat neomycin recombination substrate (Fig. 1). The neomycin gene located at the 5′-end of the substrate is regulated by the mouse mammary tumor virus promoter, but it is inactive because of the insertion of a HindIII recognition sequence causing a frameshift mutation. Located at the 3′-end of the substrate is a wild-type neomycin gene that is inactive because it lacks a promoter. This neomycin gene serves as a template to repair double-stand breaks within the 5′ neomycin gene by HR. Therefore, only those cells that have undergone HR and have a functional neomycin gene will be resistant to the antibiotic G418 (Geneticin; Gibco Life Technologies, Burlington, ON, Canada). CHO 3-6 cells were maintained in α-minimum essential media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco Life Technologies) at 37°C in 5% CO_2.

Recombination Frequency Assays. CHO 3-6 cells were plated at a density of 1×10^6/10-cm culture dish (Corning Inc., Corning, NY). After a 4-h incubation to allow the cells to adhere to the plates, the cells were treated with valproic acid (0.5, 1, 5, and 10 mM; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) or the vehicle control (media) for either 4 or 24 h. The doses of valproic acid were

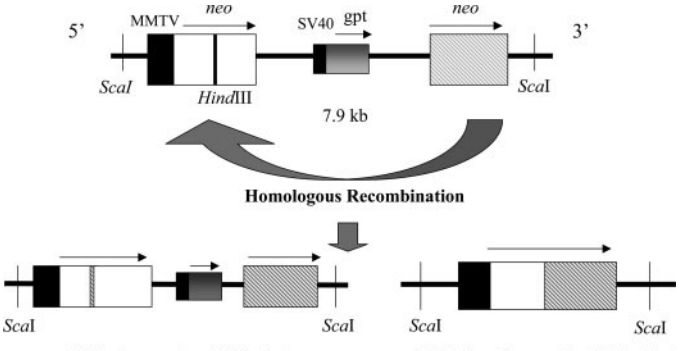

Fig. 1. Structure of the direct repeat neomycin recombination substrate in CHO 3-6 cells. The *neomycin* (*neo*) gene located at the 5′-end of the substrate is regulated by the mouse mammary tumor virus (MMTV) promoter but is inactive because of the insertion of a HindIII recognition sequence, causing a frameshift mutation. The downstream wild-type *neo* is inactive because of the absence of a promoter but serves as a template to repair damage within by the 5′-*neo*. The *neo* genes flank a simian virus 40 promoter-driven *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene. HR by gene conversion without an associated crossover event results in a 7.9-kb product and the loss of the HindIII recognition sequence. Crossovers, unequal sister chromatid exchanges, and single-strand annealing deletes one copy of the neo and simian virus gpt, resulting in a 2.6-kb product. All of these events result in the loss of the HindIII site and confer G418 resistance (Nickoloff, 1992).

chosen based on evidence showing that the plasma concentration of valproic acid in pregnant women can reach 1.162 mM (Thisted and Ebbesen, 1993). The cells were then washed twice with phosphate-buffered saline, and fresh media containing G418 (500 μg/ml) were added to the plates. The cells were incubated for 2 weeks at 37°C and then stained with 1% crystal violet in methanol. For plating efficiency studies to assess cell death, the above protocol was used, except that 300 cells/10-cm culture dish were plated, G418 was not added, and the cells were grown for 1 week. To determine HR frequency, the number of G418-resistant colonies per live cells plated was calculated and expressed as a -fold increase from the respective control group.

For HR studies using PEG-catalase, a similar protocol was used as described above, except PEG-catalase (200 and 400 U/ml) was administered immediately after cell plating, and the cells were incubated for 24 h before being dosed with valproic acid (10 mM) for 24 h (Ceolotto et al., 2004).

Southern Hybridization. To determine the types of HR events induced by valproic acid, CHO 3-6 cells were treated with valproic acid (5 and 10 mM) or vehicle control (media) for 24 h and then treated with G418 as described above. Ten G418-resistant colonies from each treatment group consisting of 20 cells or more were identified under a light microscope, isolated, and grown to confluence in six-well plates (Corning Inc.). The DNA was then extracted using the QIAGEN DNeasy Tissue Kit (QIAGEN, Mississauga, ON, Canada). Genomic DNA was digested with ScaI and HindIII restriction endonucleases (New England BioLabs Inc., Mississauaga, ON, Canada), and Southern hybridization was conducted using ^{32}P-labeled neomycin cDNA as the probe to determine the size of the HR products as described previously (Nickoloff, 1992). A 7.9-kb band represents a conservative HR event, whereas a 2.6-kb band represents a nonconservative HR event.

ROS Studies. CHO 3-6 cells were plated at a density of 1×10^6 cells/10-cm culture dish and were allowed to adhere for 4 h. Cells were preincubated for 30 min with 10 μM concentration of the ROS-sensitive dye 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF) or vehicle (dimethyl sulfoxide) before dosing with valproic acid (5 or 10 mM) for 0.5, 1, 2, or 4 h. The interaction of CM-H_2DCF with ROS results in intracellular dichlorofluorescein derivatives, which are highly fluorescent (Li et al., 2002). Therefore, dichlorofluorescein fluorescence was measured by flow cytometry as an indication of the relative amount of intracellular ROS. For the ROS studies using PEG-catalase, a similar protocol as described above was used except that PEG-catalase was added to the plates when the cells were initially plated for 24 h before treatment with CM-H_2DCF, and the cells were exposed to valproic acid (10 mM) for 1 h.

DNA Oxidation. CHO 3-6 cells were treated as described for the recombination frequency assays, except that DNA was immediately isolated after a 1-, 4-, or 24-h exposure to valproic acid using the QIAGEN DNeasy Tissue Kit (QIAGEN). The DNA was then processed to individual nucleosides using a modified method as described previously (Huang et al., 2001). 8-OH-2′-dG and 2′-dG were separated and quantified using high-pressure liquid chromatography with electrochemical detection by using a YMCbasic 150 × 4.6 mm column (YMC, Inc., Milford, MA) under isocratic conditions consisting of a mobile phase of 5% methanol and 95% 100 mM sodium acetate buffer, pH 5.2. The separated nucleosides were detected using a CoulArray Electrochemical Detector (ESA, Inc., Chelmsford, MA).

Statistical Analysis. Results were analyzed using a standard computerized statistical program (GraphPad Prism 3.0; GraphPad Software Inc., San Diego, CA). Groups were compared using a two-way analysis of variance if required and/or a one-way analysis of variance. The Neumann-Keuls multiple comparison test was used for post hoc analysis. The minimum level of significance used throughout was $p < 0.05$.

Results

Valproic Acid-Initiated Cell Death. Cell survival experiments were conducted to determine the cytotoxic effects of valproic acid on CHO 3-6 cells and these values were used to subsequently determine HR frequency. Exposure to valproic acid for 4 h did not cause a significant change in cell survival across all treatment groups (Fig. 2). However, exposure to valproic acid (10 mM) for 24 h caused a significant decrease in cell survival compared with the control group ($p < 0.001$, Fig. 2).

Valproic Acid-Induced Homologous Recombination. After a 4-h exposure to valproic acid, no significant change in HR frequency was observed across all valproic acid-treated groups compared with the control treatment group (Fig. 3). However, after treatment of CHO 3-6 cells with valproic acid for 24 h, a 3-fold increase in HR frequency was observed in cells exposed to 5 mM valproic acid, and a 3.5-fold increase in HR frequency was observed after a 10 mM valproic acid exposure ($p < 0.05$, Fig. 3).

Characterization of Homologous Recombination Events. To elucidate the mechanism by which valproic acid initiates HR, we characterized the HR events after exposure to valproic acid. The CHO 3-6 cell line produces two distinct HR products: a 7.9-kb product as a result of gene conversion without an associated crossover event, and a 2.6-kb product as a result of deletion associated with repair. When Southern hybridization was carried out on 10 colonies from each treatment group (0, 5, and 10 mM valproic acid), all colonies analyzed had the 7.9-kb HR product, indicating that gene conversion without an associated crossover event is the predominant HR mechanism that repairs valproic acid-induced DNA damage (Fig. 4).

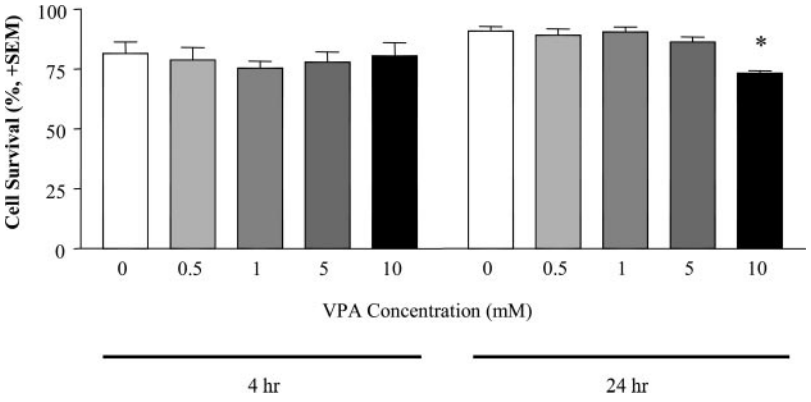

Fig. 2. Valproic acid-initiated cell death. The percentage of CHO 3-6 cells that survived after a 4- or 24-h exposure to valproic acid (0, 0.5, 1, 5, and 10 mM). Cell survival was determined by calculating the number of colonies formed after 1 week divided by the number of cells plated; $n = 3$ for the 4-h treatment group, and $n = 5$ for the 24 h treatment group. *, significant difference from the 0 mM treatment group ($p < 0.001$).

Valproic Acid-Induced ROS Formation. To determine whether the increase in HR frequency after exposure to valproic acid was due to oxidative stress, intracellular ROS levels were evaluated in CHO 3-6 cells exposed to valproic acid using the ROS-sensitive dye CM-H_2DCF. Exposure to valproic acid (10 mM) for 1 or 2 h caused a significant increase in ROS levels compared with the control group (Fig. 5), whereas exposure to 5 mM valproic acid caused an increase in ROS after 1 h of incubation. When PEG-catalase was administered 24 h before a 10 mM valproic acid treatment for 1 h, a significant decrease in ROS formation was observed compared with the 10 mM valproic acid treatment group (Fig. 6).

DNA Oxidation as a Result of Valproic Acid Exposure. To determine whether the ROS generated after valproic acid exposure caused oxidative DNA damage, CHO 3-6 cells were exposed to valproic acid (10 mM) for 1, 4, or 24 h, and the ratio of 8-OH-2′-dG/2′-dG, which is a measure of DNA oxidation, was calculated. No significant differences in the ratio of 8-OH-2′-dG/2′-dG were observed in cells treated with valproic acid compared with control (Fig. 7).

Protection against Valproic Acid-Induced Homologous Recombination with PEG-Catalase. Cell survival experiments were carried out to determine the cytotoxic effects of PEG-catalase and valproic acid in CHO 3-6 cells. Exposure to valproic acid (10 mM) for 24 h caused a significant decrease in cell survival compared with the control treatment group ($p < 0.05$, Fig. 8). Alternatively, dosing with PEG-catalase (200 and 400 U/ml) for 24 h before dosing with valproic acid for 24 h did not cause a significant change in cell survival across all treatment groups (Fig. 8). Preincubation with PEG-catalase (400 U/ml) 24 h before valproic acid exposure completely blocked valproic-acid induced HR ($p < 0.05$, Fig. 9).

Discussion

Valproic acid is a frontline antiepileptic drug used to treat a variety of seizure disorders and is also used in the treatment of bipolar disorders, migraine prophylaxis, and neuropathic pain (Chapman et al., 1982; Covington, 1998; Bowden, 2003; Freitag, 2003). However, in utero exposure to valproic acid during the first trimester of pregnancy is associated with a 1 to 2% risk of neural tube defects (Bjerkedal et al., 1982). Given the teratogenic potential of valproic acid and the expanding use of this drug clinically, elucidating the mechanism of valproic acid-induced neural tube defects is critical. In the present study, we investigated the novel idea that HR, a DNA double-strand break repair mechanism, may mediate valproic acid-induced teratogenesis by analyzing HR frequency and the types of HR events that occur after valproic acid exposure. We also investigated the role of valproic acid-mediated ROS production in initiating HR. Our results show that valproic acid exposure generates ROS and increases HR frequency in CHO 3-6 cells, suggesting a new and novel mechanism by which valproic acid may mediate neural tube defects.

Both carcinogenic and teratogenic agents have been shown to induce HR. For example, the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and metabolites of benzene metabolism, which are carcinogens, and the teratogen phenytoin have been shown to induce HR in the CHO 3-6 cell line (Winn, 2003; Winn et al., 2003; Chan et al., 2004). In the present study, we demonstrated that exposure to 5 and 10 mM valproic acid for 24 h significantly increased HR frequency in CHO 3-6 cells. Because DNA damage induces HR, our results suggest that valproic acid is capable of being genotoxic. Previous studies that examined the genotoxicity of valproic acid by analyzing sister chromatid exchanges in peripheral lymphocytes from patients treated with valproic acid have shown contradictory results. Sister chromatid exchanges occur via the HR repair pathway and are used as an indicator of chromosomal stability after exposure to a potentially mutagenic agent. A significant increase in sister chromatid exchanges was observed in peripheral lymphocytes from epileptic children treated with valproic acid (Hu et al., 1990). However, another study demonstrated that valproic acid treatment did not induce sister chromatid exchanges in peripheral lymphocytes in adult male patients (Schaumann et al., 1989). Further evidence indicates that valproic acid may contribute to genomic instability, because a growing body of evidence demonstrates that valproic acid alters chromatin structure because it can function as a histone deacetylase inhibitor, leading to the acetylation of histone tails (Marchion et al., 2005). This change in the chromatin structure relaxes the conformation of DNA, making it more susceptible to DNA damage, including double-strand breaks (Coyle et al., 2005). Valproic acid's histone deacetylase activity has been attributed to certain pathologies, including valproic acid-induced teratogenesis and valproic acid-associated acute leu-

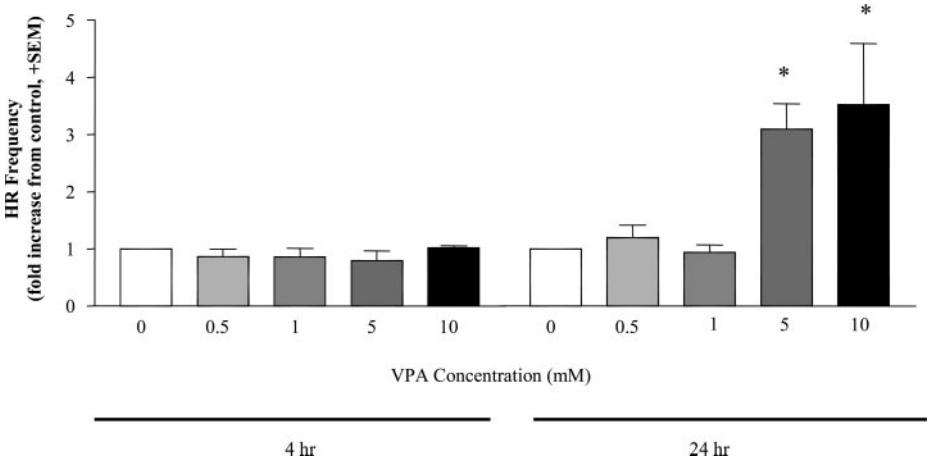

Fig. 3. Valproic acid-induced homologous recombination. The frequency of HR after treatment of CHO 3-6 cells to valproic acid (0, 0.5, 1, 5, and 10 mM). HR frequencies were determined by calculating the number of G418-resistant colonies formed after 2 weeks divided by the number of live cells plated and expressed as a fold increase from control; $n = 3$ for the 4-h treatment group, and $n = 5$ for the 24-h treatment group. *, significant difference from the control treatment group ($p < 0.05$).

kemia, and is the basis behind the current testing of the effectiveness of valproic acid in cancer therapy (Phiel et al., 2001; Camphausen et al., 2005; Coyle et al., 2005).

Although HR is a DNA repair process, it is not error-free and can contribute to genetic instability through the formation of gene deletions, duplications, translocations, and LOH (Bishop and Schiestl, 2003). Because HR can ultimately lead to genomic instability, considerable evidence associating HR with carcinogenesis has been documented (Bishop and Schiestl, 2003). Through Southern blot analysis, we show that valproic acid induces HR between direct repeats through gene conversion without associated crossovers. A gene conversion event can lead to a genetic mutation if a mutated allele is copied via HR to repair the double-strand break (Bishop and Schiestl, 2003). Therefore, the significance of gene conversion is that it can result in an LOH. This type of event is important in the initiation of some cancers, because tumor suppressor genes may be deleted by this mechanism, or mutated alleles can be used as a template during HR (Bishop and Schiestl, 2003). We propose that similar to the progression of carcinogenesis, teratogenesis can occur in this manner, because genes critical to the developing embryo may be altered through an LOH. We postulate that the fidelity of double-strand break repair via HR is especially important in the embryo, in which both the rapid replication and differentiation of cells are necessarily linked to the tight regulation of numerous developmental genes. Increases in HR and lack of HR fidelity would increase the chances of critical genes either being turned off (because of the loss of a gene) or turned on (multiplications) at inappropriate times during embryonic development.

Previous research suggests that genetic instability can result from oxidative stress (Limoli et al., 2003). To determine whether valproic acid induces a state of oxidative stress in the CHO 3-6 cell line, ROS levels were measured after exposure to valproic acid using the ROS-sensitive dye CM-H_2DCF. Our results show that valproic acid does, in fact,

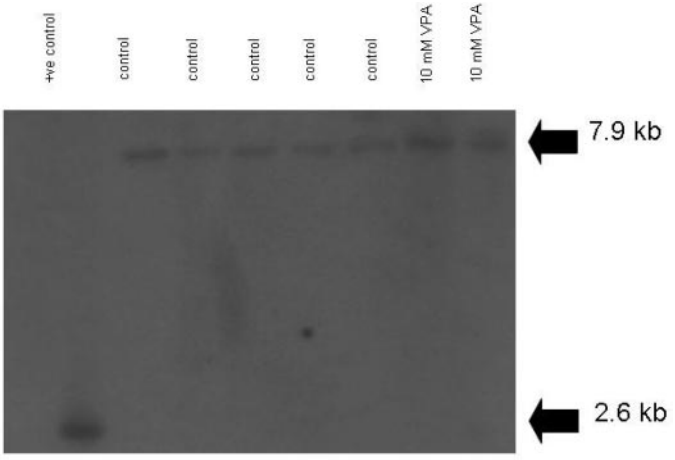

Fig. 4. Characterization of homologous recombination events as a result of valproic acid exposure. Representative result indicating that valproic acid-induced HR occurs through a conservative mechanism (no loss of DNA), resulting in a 7.9-kb product as demonstrated by Southern hybridization. Lane 1, positive control (neo cDNA); lanes 2 to 6, 0 mM valproic acid; lanes 7 and 8, 10 mM valproic acid.

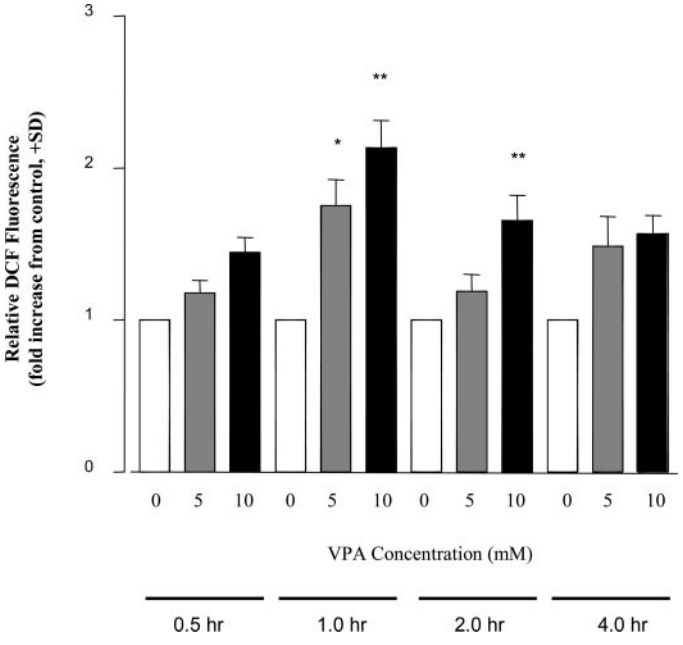

Fig. 5. Valproic acid-induced ROS formation. The relative increase in DCF fluorescence as a measure of changes in ROS formation in CHO 3-6 cells after treatment with valproic acid (0, 5, and 10 mM) for 0.5, 1, 2, and 4 h. Asterisks indicate a significant difference from the respective control group for each time point (*, $p < 0.01$; **, $p < 0.001$; $n = 3$).

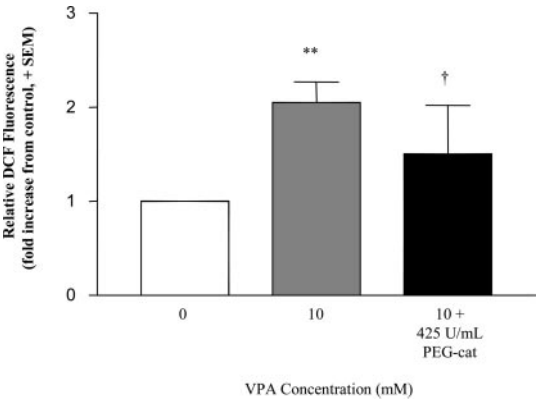

Fig. 6. Protection against valproic acid-induced ROS formation by PEG-catalase. The relative change in DCF fluorescence as an indirect measure of ROS formation in CHO 3-6 cells pretreated with PEG-catalase (425 units/ml) before exposure to 10 mM valproic acid for 1 h. **, significant difference from the control group; †, significant difference from the 10 mM valproic acid treatment group (**, $p < 0.01$, †, $p < 0.05$; $n = 6$).

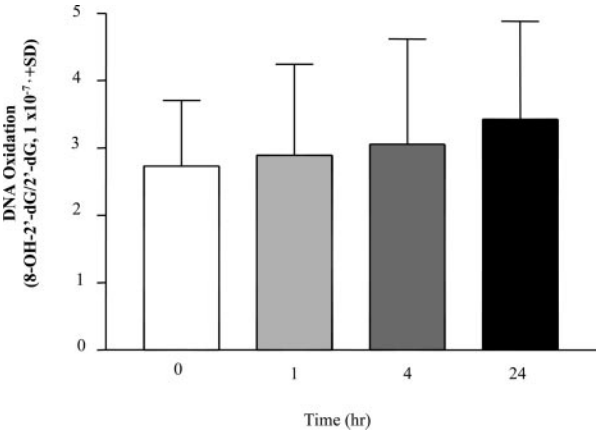

Fig. 7. DNA oxidation as a result of valproic acid exposure. The ratio of 8-OH-2′-dG/2′-dG in CHO 3-6 cells after exposure to valproic acid (0, 0.5, 1, 5, and 10 mM) for 4 and 24 h ($n = 3$, $p < 0.05$).

increase the generation of ROS in the CHO 3-6 cell line. These results are consistent with a previous study which showed that valproic acid causes an increase in ROS formation in embryoid bodies derived from murine pluripotent embryonic stem cells (Na et al., 2003). In the present study, PEG-catalase was administered to determine whether antioxidants could protect against valproic acid-induced ROS formation. Catalase protects cells from hydrogen peroxide by the enzymatic conversion of hydrogen peroxide to water and molecular oxygen (Mates et al., 1999). Although hydrogen peroxide is a mild oxidant itself, it can be converted to the highly toxic and short-lived hydroxyl radical via the Fenton reaction (Mates et al., 1999). Hydroxyl radicals have been implicated in the formation of DNA damage, including the formation of 8-OH-2′-dG (Klaunig and Kamendulis, 2004). Our results demonstrate that preincubation with PEG-catalase before the administration of valproic acid significantly decreased the production of ROS, although ROS levels did not decrease to baseline levels. In a previous study using in vitro human lymphocyte preparations, catalase significantly protected against valproic acid-induced cytotoxicity; however, similar to findings in the present study, cell death levels were not completely attenuated to baseline levels (Tabatabaei and Abbott, 1999). Given that increased DCF fluorescence can result because of exposure to various ROS and reactive nitrogen species, DCF is considered a general marker for these species (Li et al., 2002). This suggests that valproic acid exposure not only leads to increased hydrogen peroxide production but to other ROS as well. Currently, the source or the mechanism of valproic acid-mediated ROS production is not known. Therefore, it is possible that ROS, such as the superoxide radical, are generated upon exposure to valproic acid, which catalase does not detoxify.

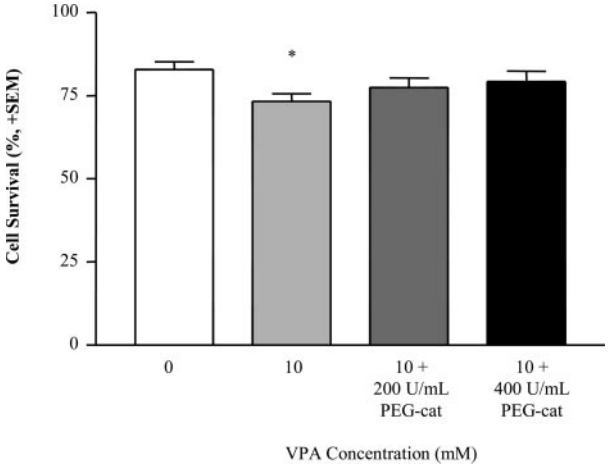

Fig. 8. Valproic acid and PEG-catalase initiated cell death. The percentage of CHO 3-6 cells that survived after a 24-h exposure to PEG-catalase followed by a 24-h exposure to valproic acid. Cell survival was determined by calculating the number of colonies formed after 1 week divided by the number of cells plated; $n = 15$ for the control and 10 mM valproic acid treatment groups, and $n = 8$ for the valproic acid plus 200 U/ml PEG-catalase treatment group and $n = 4$ for the valproic acid plus 400 U/ml treatment group. *, significant difference from the 0 mM treatment group ($p < 0.05$).

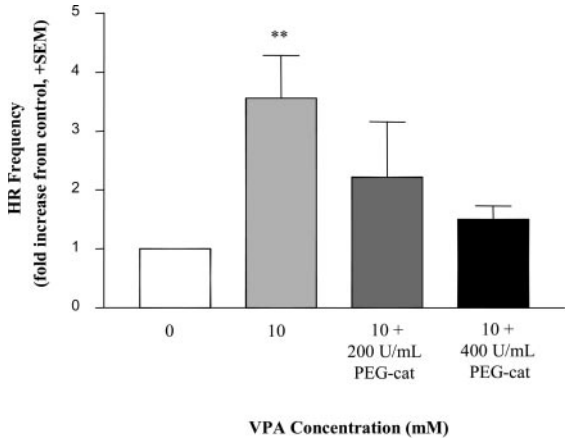

Fig. 9. Protection by PEG-catalase from valproic acid-induced homologous recombination. The frequency of HR after treatment of CHO 3-6 cells to PEG-catalase (200 and 400 U/ml) for 24 h followed by valproic acid (10 mM) for 24 h. HR frequencies were determined by calculating the number of G418-resistant colonies formed after 2 weeks divided by the number of live cells plated and expressed as a -fold increase from control; $n = 15$ for the control and 10 mM valproic acid treatment groups; $n = 8$ for the valproic acid plus 200 U/ml PEG-catalase treatment group; and $n = 4$ for the valproic acid plus 400 U/ml treatment group. **, significant difference from the 0 mM treatment group ($p < 0.01$).

The formation of ROS can lead to DNA damage, including base and sugar modifications, DNA cross-links, and single- and double-strand breaks (Klaunig and Kamendulis, 2004). One of the major base modifications caused by ROS is the formation of 8-OH-2′-dG, which results from the hydroxylation of the C-8 position of guanine (Barnes and Lindahl, 2004). 8-OH-2′-dG is commonly used as a biomarker of oxidative DNA damage, and increased levels of 8-OH-2′-dG have been associated with both cancer promotion and teratogenesis (Winn and Wells, 1995; Barnes and Lindahl, 2004). Our results show that valproic acid exposure did not cause an increase in the formation of 8-OH-2′-dG. Likewise, in a previous study, ROS levels were significantly increased in chromosomally unstable cells compared with chromosomally stable cells; however, an increase in 8-OH-2′-dG formation was not apparent in those cells that possessed elevated ROS levels (Limoli et al., 2003). In addition, it has been demonstrated that administration of 4-ene valproic acid, a potentially hepatotoxic and teratogenic metabolite of valproic acid, in rats resulted in a depletion of the mitochondrial glutathione pool (Tang et al., 1995). Therefore, if valproic acid-induced ROS production is due to the depletion of antioxidant defense mechanisms in the mitochondria, the excess ROS may not be in a close enough proximity to the nucleus to cause oxidative DNA damage. It is also possible that some of the oxidative damage could have been repaired before the direct measurement of 8-OH-2′-dG.

To determine whether the increased production of ROS after exposure to valproic acid was associated with the increase in HR, HR studies were conducted using PEG-catalase. Our results show that pretreatment with the antioxidative enzyme PEG-catalase completely blocked the observed increase in HR initiated by exposure to valproic acid in CHO 3-6 cells. These results support our hypothesis that valproic acid causes oxidative stress, which then increases HR. The protective effects of PEG-catalase observed in this study are consistent with other in vitro studies showing a protective effect of catalase against ROS production and ROS-initiated HR (Shen et al., 1996; Winn, 2003).

In summary, our results show that valproic acid increases HR frequency after dosing for 24 h in the CHO 3-6 cell line. Mechanistically, we have demonstrated that valproic acid-induced HR occurs through a conservative mechanism with-

out the loss of DNA. Although we have shown that valproic acid does induce a state of oxidative stress in the CHO 3-6 cell line, the precise contribution of valproic acid-mediated ROS formation to the induction of HR requires further analysis. The results of this study suggest that valproic acid-induced neural tube defects could possibly result from aberrant HR, leading to an LOH in genes critical to proper neural tube development.

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