Free Radical Intermediates of Phenytoin and Related Teratogens

PROSTAGLANDIN H SYNTHASE-CATALYZED BIOACTIVATION, ELECTRON PARAMAGNETIC RESONANCE SPECTROMETRY, AND PHOTOCHEMICAL PRODUCT ANALYSIS*

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Phenytoin and related xenobiotics can be bioactivated by embryonic prostaglandin H synthase (PHS) to a teratogenic free radical intermediate. The mechanism of free radical formation was evaluated using photolytic oxidation with sodium persulfate and by EPR spectrometry. Characterization of the products by mass spectrometry suggested that phenytoin photolyzes to a nitrogen-centered radical that rapidly undergoes ring opening to form a carbon-centered radical. PHS-1 was incubated with teratogen (phenytoin, mephénytoin, trimethadione, phenobarbital, and major metabolites) or its vehicle and the free radical spin trap α-phenyl-N-β-butyl nitronate, and incubations were analyzed by EPR spectrometry. There was no α-phenyl-N-β-butyl nitronate radical adduct in control incubations. For phenytoin, a putative unstable nitrogen-centered radical adduct and a stable carbon-centered radical adduct were detected. Free radical spin adducts were also detected for all other teratogens and metabolites except carbamazepine. The PHS inhibitor eicosatetraynoic acid abolished the free radical EPR signal. Incubation of 2'-deoxyguanosine with phenytoin and PHS-1 resulted in a 5-fold increase in its oxidation to 8-hydroxy-2'-deoxyguanosine. This is the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related teratogens to a free radical intermediate that initiates DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation.

Phenytoin (diphenylhydantoin; Dilantin) is a widely used anticonvulsant drug that is teratogenic in animals and humans (1–5). Several teratologic mechanisms have been proposed, including the bioactivation of phenytoin by embryonic cytochrome P450 to an electrophilic arene oxide reactive intermediate that covalently binds to embryonic protein, thereby altering cellular function (1–6). However, these hypotheses are not consistent with a number of published observations, including 1) the association of embryopathic activities of the structurally similar, asymmetric hydantoin anticonvulsants mephénytoin (Mesantoin) and its N-demethylated active metabolite nirvanol with the L-isomers that primarily do not form the arene oxide (Fig. 1) (7); 2) the teratogenicity of structurally similar anticonvulsants, such as trimethadione (Tridione) and its N-demethylated pharmacologically active metabolite, dimethadione, that lack the phenyl substituent necessary for the formation of an arene oxide; and 3) the relatively low embryonic activity of most cytochrome P450s during organogenesis (4, 5, 9), including CYP2C9, which is known to bioactivate phenytoin (10).

We have investigated an alternative hypothesis involving the bioactivation of prototergenides by peroxidases such as prostaglandin H synthase (PHS)1 to teratogenic free radical intermediates (Fig. 2) (3–6, 9). PHS and related potential bioactivating enzymes such as lipoxygenases are present with high activity in the embryo during organogenesis, the period of major teratologic susceptibility. Xenobiotic free radicals can bind covalently to cellular macromolecules (DNA, protein) and can initiate the formation of reactive oxygen species (ROS) that cause oxidative stress and oxidative damage to DNA, protein, and lipid. As detailed in the above reviews (3–6, 9), there is evidence in vivo, in embryo culture, and in vitro for embryonic PHS-catalyzed bioactivation of phenytoin to a free radical intermediate that initiates embryotoxic ROS formation. Phenyltoin initiates hydroxyl radical formation and the oxidation of embryonic DNA, protein, thios, and lipid. Conversely, phenytoin-initiated oxidation of embryonic cellular macromolecules and teratogenicity or embryotoxicity are reduced by PHS inhibitors, free radical spin trapping agents, iron chelators, antioxidants, and antioxidative enzymes, including GSH reductase, GSH peroxidase, superoxide dismutase, and catalase (3–6, 9, 11–13).

On the other hand, little is known about the chemical nature of the putative free radical intermediate of phenytoin and related xenobiotics in biological systems. Phenytoin and other hydantoins, as well as the structurally related succinimides, contain an imidyl group as shown in boxes in Fig. 1. The generation of imidyl radicals (a nitrogen-centered radical flanked by two acyl groups) from N-halohydantoines and N-bromosuccinimide has been studied and used in synthetic chemistry for a bromination process since 1942 (14). N-Bromosuccinimide undergoes photodecomposition to generate an imidyl radical that opens to form a carbon-centered radical with an isocyanate moiety (14–20). The characteristic reactions for both of these radicals are hydrogen abstraction and addition to double bonds (14, 16, 19, 20).

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1 The abbreviations used are: PHS, prostaglandin H synthase; AA, arachidonic acid; 2'-dG, 2'-deoxyguanosine; ETYA, 5,8,11,14-eicosatetraynoic acid; HFSC, hyperfine splitting constant; HPLC, high performance liquid chromatography; 8-OH-2'-dG, 8-hydroxy-2'-deoxyguanosine; PBN, α-phenyl-N-β-butyl nitronate; ROS, reactive oxygen species; HPPH, 5-(p-hydroxyphenyl)-5-diphenylhydantoin; MS, mass spectrometry.
Given that chemical studies indicate that \(N\)-halohydantoins can form a nitrogen-centered radical on their imidyl moiety and biochemical studies have shown that hydantoins and related compounds can initiate hydroxyl radical formation and oxidation of macromolecular targets, we hypothesized that phenytoin and its analogs can be bioactivated by PHS to an imidyl free radical that can undergo ring opening to generate a carbon-centered free radical with an isocyanate group. These radicals may covalently bind to embryonic macromolecules with carbon-carbon double bonds, such as DNA and protein, and/or initiate embryonic ROS formation and oxidative macromolecular damage, thereby initiating teratogenesis. This hypothesis was investigated using two approaches. The first involved the chemical characterization of products following the photolytic oxidation of phenytoin using sodium persulfate. The second approach involved direct characterization of teratogen free radical that can undergo ring opening to generate a carbon-centered free radical with an isocyanate group. These radicals may covalently bind to embryonic macromolecules with carbon-carbon double bonds, such as DNA and protein, and/or initiate embryonic ROS formation and oxidative macromolecular damage, thereby initiating teratogenesis. This hypothesis was investigated using two approaches. The first involved the chemical characterization of products following the photolytic oxidation of phenytoin using sodium persulfate. The second approach involved direct characterization of teratogen free radical intermediates by EPR spectrometry and phenytoin-initiated DNA oxidation, following in vitro incubation with PHS. The results provide the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related proteratogens to a potentially embryotoxic free radical intermediate.

EXPERIMENTAL PROCEDURES

Materials

Purified PHS-1 and 8-hydroxy-2'-deoxyguanosine were obtained from Cayman Chemicals Co. (Ann Arbor, MI); phenytoin (diphenylhydantoin acid), 5-(\(p\)-hydroxyphenyl)-5-diphenylhydantoin (HPPH), dimethadione, \(\alpha\)-phenyl-N-\(\tau\)-butylnitro (PBN), hematin, hydroxylmine hydrochloride, sodium persulfate, benzophenone, and \(2'\)-deoxyguanosine were obtained from Sigma-Aldrich (Oakville, Canada); re-distilled phenol was from Aldrich. Mephenytoin and nirvanol isomers were gifts from Dr. A. Küpfner (Switzerland); trimethadione was a gift from Abbott. 5,8,11,14-Eicosatetraynoic acid (ETYA) was a gift from Hoffmann-La Roche. All other reagents used were of analytical or HPLC grade.

Methods

Photochemical Generation of Phenytoin Free Radical—A 3-ml solution containing 100 \(\mu\)m phenytoin and 100 \(\mu\)m sodium persulfate in 6.0 mM NaOH (pH 11.8) was photolyzed at 300 nm for 0, 10, 20, 30, and 40 min in a Rayonet chamber. The reaction mixture of each time interval was analyzed for product formation by HPLC equipped with a model 222 solvent delivery system (Scientific Systems, Inc.), a 5-\(\mu\)m Spheri-sorb ODS II C-18 column (15 cm \(\times\) 4.6 mm, Jones Chromatography, Lakewood, CO), a model SPD-6AV UV/Vis detector (Shimadzu, Kyoto, Japan), and an integrator (Chromapac model CR501; Shimadzu). The mobile phase consisted of 59% water, 1% glacial acetic acid, and 40% acetonitrile, at a flow rate of 1 ml/min. The product separation was performed at 240 nm.

Identification of Photolysis Products by Thin Layer Chromatography—The photolysis products were separated by preparative TLC using 30:70 ethyl acetate/hexane as the eluting solvent. Authentic samples of some products were synthesized or purchased and co-eluted to confirm the identity of products. The separated products were then scraped off the TLC plate and analyzed by HPLC in line with a tandem mass spectrometer (HPLC-MS/MS).

Identification of Photolysis Products by HPLC-MS/MS—The reaction mixture of each time interval as well as the separated products obtained from TLC studies were analyzed by HPLC-MS/MS (API II, FIG. 1. Structures of phenytoin and related drugs and metabolites. Structural similarities are indicated by the boxes. In vivo murine studies have shown that the \(N\)-demethylated drugs and metabolites are substantially more teratogenic than their respective methylated parent molecules, suggesting that in vivo \(N\)-demethylation is a prerequisite for peroxidase-catalyzed bioactivation to a teratogenic reactive intermediate (3). P450, cytochrome P450.
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Perkin-Elmer Sciex, Concord, Canada). The instrument was set in ion spray mode, and the collision activation spectra of the products were obtained using argon as the target gas at an energy of 80 eV. The mean mass ± S.E. was calculated from the multiply charged ions by the software Mass spec (version 3.3). The HPLC conditions were the same as above.

Synthesis of Benzophenone Oxime (21)—A mixture of 1 g of benzophenone, 1 g of hydroxylamine hydrochloride, 5 ml of pyridine, and 5 ml of absolute ethanol was heated under reflux for 2 h in a water bath. The solvents were removed by roteovaporation. The residue was precipitated with 5 ml of ice cold water, and the mixture was vacuum-filtered. The oxime was recrystallized from ethanol. Melting point was 142–144 °C; HPLC-MS: m/z (MH+) 198, 180, 77.

Bioactivation of Phenytoin and Its Analogs to a Free Radical Reactive Intermediate by PHS—PHS-1 (1000 units/ml) was incubated with 5 ml of ice cold water, and the mixture was vacuum-filtered. The oxime was recrystallized from ethanol. Melting point was 142–144 °C; HPLC-MS: m/z (MH+) 198, 180, 77.

Bioactivation of Phenytoin and Its Analogs to a Free Radical Reactive Intermediate by PHS—PHS-1 (1000 units/ml) was incubated with hematin (10 μM) and phenol (0.5 mM) for 1 min at 37 °C in 80 mM potassium phosphate buffer, pH 7.9. After addition of the teratogen (500 μM) and the free radical spin trap PBN (1 mM), arachidonic acid (AA) (67 μM) was added to start the reaction. After 30 min at 37 °C, reactions were terminated and extracted twice with 2 ml of ethyl acetate. The combined ethyl acetate layers were reduced under nitrogen to 500 μl and analyzed by EPR spectrometry. To obtain information on a less stable, putative nitrogen-centered radical, phenytoin also was incubated with PHS-1 for shorter intervals of 2 and 15 min. To block PHS-1-catalyzed bioactivation of phenytoin, the PHS inhibitor ETV (40 μM) was incubated with the enzyme at 37 °C for 1 min prior to the addition of phenytoin and AA.

The controls for all incubations lacked the respective teratogen but contained all other components of the incubation including the vehicle for the teratogen. For phenytoin and its major in vivo metabolite, HPPH, saline/NaOH was the vehicle. Trimethadione, dimethadione, and phenobarbital were dissolved in saline. The vehicle for nirvanol, mephenytoin, and carbamazepine was Me2SO. The concentration of Me2SO in these incubations did not exceed 0.5% (v/v).

The free radical adducts of PBN were detected at room temperature in a ST-EPR cavity with a Bruker ER-200 DX band spectrometer. The instrument settings were as follows: microwave power, 20.5 milliwatts; modulation amplitude, 1 G; time constant, 50 ms; sweep range, 100 G; sweep time, 50 s; accumulation, 5 scans; receiver gain, 5.00 × 10 (5); field center, 3475 G; frequency, 9.81 GHz.

The EPR spectrum for the mixture of the carbon-centered and putative nitrogen-centered free radicals was simulated using a standard software (ESR 42) developed by Dr. Uwe Oehler (Department of Chemistry, University of Guelph, Canada).

Oxidation of 2′-Deoxyguanosine (2′-dG) was incubated with or without phenytoin in the presence of PHS-1 using the conditions given above with the following modifications: 250 μM phenytoin was used, PBN was replaced with 2′-deoxyguanosine, and 140 μM arachidonic acid was added to start the reaction. The resulting mixture was analyzed by HPLC.

Detection of 8-Hydroxy-2′-deoxyguanosine (8-OH-2′-dG)—Oxidation of 2′-dG to 8-OH-2′-dG was quantified using an isocratic HPLC system (Scientific Systems, Inc.) equipped with a 5-μm Spherisorb ODS II C-18 column (15 cm × 4.6 mm, Jones Chromatography), an electrochemical detector (model 5100A), a guard cell (model 5020), an analytical cell (model 5010) (Coulotech, ESA, Chelmsford, MA) and an integrator (Chrompack model CR501, Shimadzu). Samples were eluted using a mobile phase consisting of 50 mM KH2PO4 buffer (pH 5.5) and 5% methanol at a flow rate of 0.8 ml/min with a detector oxidation potential of +0.4 V.

Statistical Analysis—Statistical significance of differences between treatment groups was determined by Student’s t test using a standard computerized statistical program (Statsview, Abacus Concepts, Inc.). The level of significance was p < 0.05.

RESULTS

Products of Photochemical Reactions—Over the period of 40 min, more than 50% of phenytoin was photolyzed to one major and three minor products, which were identified by HPLC-
MS/MS (Fig. 3). The major product of this reaction was 1,2,3,4-tetrahydro-2-oxo-4-phenylquinazoline (1), and the minor products were benzophenone (2), benzophenone oxime (3), and 1-phenyl-1-[2-hydroxyphenyl]methyl imine (4). The HPLC retention times of these compounds are summarized in Table 1.

The fragmentation pattern for compound 1 (Table I) was consistent with concomitant loss of a phenyl ring, carbon monoxide, and hydrogen cyanide from this compound. The fragmentation pattern for compound 2 was consistent with the loss of a phenyl ring. Compounds 3 and 4 had the same molecular weight but showed different fragmentation patterns and were detected at different retention times (Table I). The fragmentation pattern for compound 3 was consistent with a loss of water and phenyl ring, while that of compound 4 was consistent with a loss of a phenol group and a phenyl ring.

The products of the photolysis reactions were separated on the TLC plate and characterized by HPLC-MS/MS. The fragmentation pattern observed for each product was the same as that observed for the reaction mixture. The authentic samples containing all components except the enzyme also were analyzed. The products of the photolysis reaction were identified by tandem mass spectrometry.

**Bioactivation of Phenytoin and Its Analogs to a Free Radical Intermediate by PHS—1—PHS-catalyzed formation of free radical spin adducts were obtained for phenytoin and its in vivo hydroxylated metabolite, HPPH (Fig. 4). The EPR signal for phenytoin after a 30-min incubation (Fig. 4B) revealed the presence of a carbon-centered free radical. The triplet of doublets observed for this radical adduct of phenytoin had hyperfine splitting constants (HFSCs) of $a_N = 13.75 \text{ G}$ and $a_H = 2.13 \text{ G}$. HPPH also gave rise to a carbon-centered free radical (Fig. 4C) with similar HFSCs, $a_N = 13.79 \text{ G}$ and $a_H = 2.38 \text{ G}$. Preincubation of PHS-1 with the PHS/lipoxygenase inhibitor ETYA (40 $\mu M$) abolished the free radical EPR signal for phenytoin (Fig. 4D). The 40 $\mu M$ concentration of ETYA is well above the $K_i$ value for PHS inhibition in isolated cells and purified enzyme preparations (22, 23), is not embroytotoxic, and inhibits phenytoin embryotoxicity in embryo culture (24). The control incubation, which contained saline/sodium hydroxide (Fig. 4A), the vehicle of phenytoin and HPPH, did not show the presence of any radical other than the two peaks that are always observed, probably from PHS.

To explore this possibility and that of the generation of teratogen free radicals by other components, incubations containing all components except the enzyme also were analyzed. Free radicals were not detected in these incubations, indicating that the two signals at either end of the spectra were due to PHS and that free radicals of teratogens were not formed in the absence of PHS-1 (Fig. 5A).

Time-dependent incubation of phenytoin revealed the early simultaneous existence of carbon- and putative nitrogen-centered free radicals at 15 min (Fig. 5C) and maximally at 2 min (Fig. 5D), with HFSCs of $a_N = 13.75 \text{ G}$ and $a_H = 2.13 \text{ G}$ for the carbon-centered radical adduct and $a_N = 14.2 \text{ G}$, $a_H = 0.79 \text{ G}$, and $a_N = 1.90 \text{ G}$ for the nitrogen-centered radical adduct. The arrows in Fig. 5D identify the presence of additional lines that were formed due to the overlapping signals of the nitrogen- and carbon-centered radical adducts. These additional lines were not present in the signal observed for the carbon-centered radical at 30 min of incubation (Fig. 5B). This characterization was confirmed by computer simulation of the signal (Fig. 5E).

The EPR spectra of isomers of mephenytoin and nirvanol indicated carbon-centered free radicals in varying amounts (Fig. 6) with HFSCs similar to those observed for phenytoin (Table II). There was no radical adduct of PBN in the control incubations of these compounds (Fig. 6A). We found in our system that concentrations higher than 0.5% of Me$_3$SO, a radical scavenger, resulted in elimination of the signal, possibly by inhibiting PHS. We also found that the addition of more than 1 mM PBN to incubations resulted in a decrease in intensity of

**Table I: Retention times and fragmentation patterns of phenytoin photolysis products**

| Compound                        | Retention time | Molecular ion (MH$^+$) | Fragmentation pattern |
|---------------------------------|----------------|------------------------|-----------------------|
| Phenytoin                       | 4.25           | 253                    | 225, 182              |
| 1,2,3,4-Tetrahydro-2-oxo-4-phenylquinazoline (1) | 3.37           | 223                    | 145, 117, 90, 77      |
| Benzophenone (2)                | 19.34          | 183                    | 78                    |
| Benzophenone oxime (3)          | 12.15          | 198                    | 180, 77               |
| 1-phenyl-1-[2-hydroxyphenyl]methyl imine (4) | 8.25           | 198                    | 105, 77               |

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the signal, suggesting that higher concentrations of PBN could inhibit PHS. Both L-mephenytoin and D-mephenytoin were bioactivated by PHS-1 to carbon-centered free radicals, with a stronger signal observed for D-mephenytoin compared with its L-isomer (Fig. 6, B and C). The embryotoxic L-isomer of nirvanol produced slightly more free radical in the presence of PHS-1 than the nonteratogenic D-isomer (Fig. 6, B and C).

Trimethadione and dimethadione were both bioactivated by PHS-1 to carbon-centered free radicals (Fig. 7), with HFSCs similar to phenytoin (Table II). Dimethadione is the pharmacologically active metabolite of trimethadione and, due to its longer half-life, accumulates in humans and animals. Separate administration of these two compounds in pregnant mice suggests that the teratogenicity of trimethadione results from in vivo N-demethylation to dimethadione, which is the penultimate teratogenic species (8). This hypothesis is consistent with the observation in the present study that dimethadione produced a strong EPR signal (Fig. 7B), while the less teratogenic parent compound trimethadione produced a very weak signal (Fig. 7C). A free radical signal was not detected in control incubations for these two drugs (Fig. 7A).

Phenobarbital was bioactivated by PHS-1 to a carbon-centered free radical intermediate, which was detected as a weak EPR signal (Table II). Free radical intermediates were not detected in PHS-1 incubations containing the anticonvulsant drug carbamazepine (data not shown).

In Vitro Phenytoin-initiated Oxidation of 2'-dG—Using the reaction conditions above employed for free radical characterization, phenytoin was bioactivated by PHS-1 in vitro to a free radical reactive intermediate that oxidized 2'-dG to 8-OH-2'-dG. Incubations containing phenytoin had a 5.2-fold increase in 2'-dG oxidation compared with incubations with the saline vehicle control (Fig. 8).

**DISCUSSION**

Results obtained from the photolysis of phenytoin in the presence of a strong oxidizing agent, sodium persulfate, suggest that phenytoin is first oxidized to a nitrogen-centered radical that can rapidly undergo ring opening to form a carbon-centered free radical with an isocyanate moiety (Fig. 9). Radical b and its resonance contributor radical e both react with an oxygen molecule to generate compounds 2 and 4. Analysis of an authentic sample of compound 4 was not attempted, since imines in which the nitrogen is attached to a hydrogen are generally unstable and rapidly decompose or polymerize (25). Cyclization of radical e produces compound 1. Compound 3 may be formed after oxidation of the decomposition product of the radical b. The mechanism postulated in Fig. 9 provides a potential chemical basis for the generation and characteristics of the putative phenytoin free radicals.

Bioactivation of phenytoin by the purified PHS-1/AA system generated a putative nitrogen-centered free radical and a definitive carbon-centered free radical. Inhibition of the phenytoin EPR signal by the PHS inhibitor ETYA indicated that the bioactivation of phenytoin to a free radical intermediate was catalyzed by PHS. ETYA is a dual inhibitor of PHS and lipoxygenases (26–30) and has been shown in vitro at the same concentration to inhibit both the bioactivation of phenytoin by PHS and lipoxygenase and the embryotoxicity of phenytoin in embryo culture, as well as inhibiting phenytoin teratogenicity in vivo (24, 31). The absence of an EPR signal when PHS-1 was omitted from the medium indicated that bioactivation was due
Components of this in vitro system are detailed in the legend to Fig. 5, with a 30-min incubation. The control incubation contained all components, except the drug was excluded from the vehicle (0.5% Me2SO).

The control incubation contained all components, except the drug was excluded from the vehicle (saline).

The in vitro system contained prostaglandin H synthase, hematin, and phenol. After preincubation for 1 min at 37 °C, arachidonic acid, α-phenyl-N-t-butylnitroine, and xenobiotic or vehicle was added, and the system was incubated for 30 min.

The components of this in vitro system are detailed in the legend to Fig. 5, with a 30-min incubation. The control incubation contained all components, except the drug was excluded from the vehicle (saline).

to PHS rather than other components of the system. The absence of a signal in the control incubations also confirms that signals observed in each spectrum were the result of bioactivation by PHS.

Carbon-centered free radicals also were observed in varying amounts for the analogs of phenytoin (Table II). The observed similarity between the HFSCs observed for the carbon-centered free radical of phenytoin and its structurally related analogs is due to the similarities in the environment of the radical center. All observed carbon-centered radicals in this study have either two phenyl, one phenyl, or two methyl groups around the radical center, as well as a nitrogen or an oxygen attached to them. For some but not all of these compounds, there was an interesting correlation between their reported teratogenicity and the amount of free radical intermediate formed via the PHS bioactivating system. For instance, the highly teratogenic dimethadione (8) produced substantially more free radicals than its minimally teratogenic parent compound trimethadione. This substantial free radical formation from dimethadione, compared with none by trimethadione, probably account for the teratogenicity observed with trimethadione therapy during pregnancy. This is in agreement with the substantial in vitro oxidation of DNA initiated by dimethadione, compared with none by trimethadione, using a horseradish peroxidase bioactivating system (32). Similarly, phenytoin and its major parahydroxylated metabolite HPPH produced similar amounts of free radicals. While HPPH has been reported to be nonteratogenic following maternal administration, this probably is due to maternal glucoronidation preventing HPPH from reaching the embryo (3–6, 9), since in embryo culture, phenytoin and HPPH demonstrate similar embryotoxic potencies (34). Phenobarbital produced only minimal free radical formation, while no free radicals were detected with carbamazepine, and these drugs are believed to be less teratogenic in humans (34–37) and animals (38, 39) than phenytoin and trimethadione.

![Fig. 6. EPR spectra for vehicle control (A), l-mephenytoin (B), d-mephenytoin (C), l-nirvanol (D), and d-nirvanol (E) observed after their bioactivation by PHS. Components of this in vitro system are detailed in the legend to Fig. 5, with a 30-min incubation. The control incubation contained all components, except the drug was excluded from the vehicle (0.5% Me2SO).](image)

![Fig. 7. EPR spectra for vehicle control (A), trimethadione (B), and dimethadione (C) after their bioactivation by PHS. Components of this in vitro system are detailed in the legend to Fig. 5, with a 30-min incubation. The control incubation contained all components, except the drug was excluded from the vehicle (saline).](image)

![Fig. 8. Formation of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG) during the bioactivation of phenytoin by PHS-1. Incubations consisted of 2'-dG, phenytoin or its vehicle, PHS-1, hematin, and AA as described in the legend to Fig. 5. The mixtures were incubated for 30 min and analyzed by high performance liquid chromatography with electrochemical detection. The asterisk indicates a difference from control (p < 0.05).](image)
On the other hand, both D- and L-isomers of mephenytoin were bioactivated to carbon-centered free radicals, and the amount of free radicals formed was higher for the D-isomer, which is less embryotoxic in mice (7). Furthermore, the amount of free radicals formed by D-mephenytoin and both the D- and L-isomers of its N-demethylated metabolite nirvanol were similar, although L-nirvanol is substantially more embryotoxic than either mephenytoin or D-nirvanol. These results suggest that factors in addition to PHS-catalyzed bioactivation to a free radical may contribute to the relative teratologic potencies of mephenytoin and nirvanol isomers. Mephenytoin is administered as a racemic mixture, and in humans, the D-isomer of mephenytoin is preferentially and rapidly hydroxylated and excreted, with virtually no D-nirvanol being produced via N-demethylation, while the L-isomer of mephenytoin is stereospecifically N-demethylated to L-nirvanol (40, 41). A similar stereoselective elimination is observed in mice (7). This rapid elimination of D-mephenytoin via maternal hydroxylation may prevent its transport to the embryo, where it can be bioactivated by PHS. In turn, while D-nirvanol produced slightly more free radicals than the highly embryotoxic L-isomer, most mephenytoin administered as an anticonvulsant is preferentially and rapidly hydroxylated and excreted, with very little being N-demethylated to D-nirvanol (7). Furthermore, D-nirvanol itself is hydroxylated and excreted more rapidly than its L-isomer, leaving less to reach the embryo (7). Thus, for several reasons, little of the D-isomers of either mephenytoin or nirvanol should reach the embryo. Nevertheless, in an in vitro horseradish peroxidase bioactivation system, the highly embryotoxic L-nirvanol produced substantial DNA oxidation, compared with minimal oxidation by L-mephenytoin or D-nirvanol (32), so both drug isomer disposition and embryonic bioactivation may play important roles in teratologic potency.

In the cells of normal untreated animals, there is considerable oxidative damage (42). However, excessive oxidative DNA damage caused by xenobiotic-initiated ROS can cause irreversible modifications to DNA (43). These modifications to DNA have been shown to disrupt transcription, translation, and DNA replication, which can ultimately lead to mutation and cell death (44–46). Oxidative damage to embryonic cellular macromolecules (DNA, protein, lipid) also may play an important role in the mechanism of embryotoxicity for a number of proteratogens (3–6, 9, 47, 48). The potential teratologic role of damage to DNA in particular is suggested by a number of lines of evidence (3–6, 9), including particularly 1) the oxidation of embryonic DNA in embryo culture by proteratogens like phenytoin and benzo[a]pyrene (3, 5); 2) the abolition of embryonic DNA oxidation and embryotoxicity for both of these proteratogens by the addition of the antioxidative enzymes superoxide dismutase or catalase to the culture medium; and 3) the enhanced in vivo teratogenicity of both these proteratogens in knockout mice deficient in the p53 tumor suppressor gene, which facilitates DNA repair (49–51). Generally, hydroxyl radical (‘OH) generated chemically or by ionizing radiation can add across the double bonds of a DNA base, forming a hydroxylated product. The oxidized guanine analog 8-OH-2'-dG is thought to be formed in DNA via hydroxylation of deoxyguanosine residues by 'OH at the C-8 position (52), and phenytoin has been shown to initiate the in vivo formation of 'OH, measured by salicylate hydroxylation (12). Accordingly, 8-OH-2'-dG formation can be used as a biological marker of oxidative DNA damage, as well as providing an insight into potential molecul-
FIG. 10. Postulated role of peroxidases and NADH in the formation of ROS during the bioactivation of phenytoin by PHS-1 to a free radical reactive intermediate.

FIG. 11. Postulated role of a Fenton-like mechanism for the generation of ROS resulting from the bioactivation of phenytoin by PHS-1 to a free radical. The phenytoin hydroperoxide is postulated to form via a carbon-centered free radical intermediate as shown in Fig. 10. 1. Fenton-like pathway, wherein hydroxyl radical is generated during the reduction of phenytoin hydroperoxide by Fe$^{2+}$ to an alcohol. 2, pathway for generation of superoxide anion, whereby hydroxyl radical reduces phenytoin hydroperoxide to an alcohol and produces superoxide anion, which subsequently regenerates hydroxyl radical.
lar mechanisms of toxicological initiation. In the current study, under in vitro conditions similar to those used for the formation and characterization of teratogen free radical intermediates, arachidonate-dependent, PHS-catalyzed bioactivation of phenytoin resulted in over a 5-fold increase in the oxidation of 2'-dG to 8-OH-2'-dG. These results suggest that the free radical intermediates characterized herein for phenytoin and related proteratogens are relevant to their molecular mechanism of teratologic initiation, which may involve oxidative damage to embryonic DNA.

Based on the results of these studies, at least two biochemical pathways, summarized in Figs. 10 and 11, could account for the generation of $O_2^\bullet$ and other ROS during the bioactivation of phenytoin by PHS-1. In both pathways, first a nitrogen-centered free radical of phenytoin (a) is generated by PHS-1. This nitrogen-centered radical is most likely unstable, since a shorter incubation time (2 min) was required for its detection by EPR, and undergoes ring opening to generate the isoxygenate-containing carbon-centered radical (b). The equilibrium between radicals a and b is probably shifted toward radical b, since the radical a (nitrogen-centered) was observed in a very low concentration, as was evidenced by the low intensity of the EPR signal. The nitrogen- and carbon-centered radicals of phenytoin can be reduced to metabolite e by hydrogen abstraction from DNA, lipids, and GSH. This process generates DNA (DNA') and lipid (L') radicals as well as oxidized GSH (GSSG). Phenytoin-initiated lipid peroxidation and GSSG formation have been demonstrated in vitro, in embryo culture, and in vivo (24, 32, 53). Generation of L' can lead to lipid peroxidation and ultimately damage cellular membranes, while generation of DNA' causes DNA strand breaks and DNA-DNA and DNA-protein cross-links (54). Metabolite e is an isocyanate, which as a class are highly electrophilic and biologically active (55, 56). The carbon-centered free radical of phenytoin can also interact with one molecule of oxygen to generate the hydroperoxide d.

In the first postulated pathway for generation of $O_2^\bullet$, as summarized in Fig. 10, hydroperoxide d can be reduced by peroxidases to the alcohol e, during which compound I and compound II intermediates of peroxidases are formed and two molecules of NAD' are generated. Further oxidation of NAD' by a molecule of oxygen generates NAD$^+$ and $O_2$. This pathway is based upon the mechanism previously proposed for generation of $O_2^\bullet$ from lipid hydroperoxides (57, 58). Generation of superoxide anion in this pathway is consistent with our previous observation that the addition of antioxidative enzymes such as superoxide dismutase, which removes superoxide anion, abolishes embryonic DNA oxidation and embryotoxicity initiated by phenytoin and benzo[a]pyrene in embryo culture (3, 5).

A second pathway by which $O_2^\bullet$ can be generated involves a Fenton-like reaction (58). In this process, as proposed in Fig. 11, hydroperoxide d can oxidize Fe$^{3+}$ to Fe$^{2+}$, producing ‘OH and alcohol e. Subsequently, the ‘OH can react with hydroperoxide d to generate alcohol e, $O_2^\bullet$, and H$^+$. This is consistent with our observation that the iron chelator desferoxamine inhibited phenytoin teratogenicity in mice (59), possibly by blocking step 1 of this pathway and preventing hydroxyl radical formation. The alcohol e, which is produced by both proposed pathways, can undergo spontaneous and water-assisted decomposition to generate hydrogen isocyanate, benzophenone, carbon dioxide, and urea. Besides generation of superoxide, this mechanism also provides an explanation for the distal effects of unstable hydroxyl radical across a cell. Once formed, hydroxyl radical can interact with cellular components nearest to its site of formation and generate superoxide anion, which is more stable and can travel to distal parts of a cell, where it can regenerate hydroxyl radical.

In summary, the radical spin trapped adducts of hydantoins and related proteratogens are formed via PHS-catalyzed bioactivation in varying amounts and with similar HFSCs, consistent with a common mechanism of teratogenesis. In some cases (phenytoin, HPPH, trimethadione, dimethadione, phenobarbital, carbamazepine), the amount of free radicals formed correlated well with the teratologic potency of the drugs, while for other drugs (mephenytoin and nirvanol isomers), additional factors appeared to be involved. The free radical detected for all hydantoins and related compounds was carbon-centered, and for phenytoin, a putative, unstable nitrogen-centered radical was also detected. This study provides the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related proteratogens to free radical intermediates that can initiate DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation.

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