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METABOLISM OF PHENYTOIN AND COVALENT BINDING OF REACTIVE INTERMEDIATES IN ACTIVATED HUMAN NEUTROPHILS

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Abstract—Activation of neutrophils by phorbol-12-myristate-13-acetate (PMA) causes rapid production of superoxide radical (O_2^-), leading to the formation of additional reactive oxygen species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and possibly hydroxyl radical (·OH). These reactive oxygen species have been associated with the oxidation of some drugs. We investigated the metabolism of phenytoin (5,5-diphenylhydantoin) and the covalent binding of reactive intermediates to cellular macromolecules in activated neutrophils. In incubations with 100 μM phenytoin, PMA-stimulated neutrophils from six human subjects produced *p*-, *m*-, and *o*-isomers of 5-(hydroxyphenyl)-5-phenylhydantoin (HPPH) in a ratio of 1.0:2.1:2.8, respectively, as well as unidentified polar products. Analysis of cell pellets demonstrated that phenytoin was bioactivated to reactive intermediates that bound irreversibly to macromolecules in neutrophils. Glutathione, catalase, superoxide dismutase, azide, and indomethacin all diminished the metabolism of phenytoin and the covalent binding of its reactive intermediates. The iron-inactivating chelators desferrioxamine and diethylenetriaminepentaacetic acid had little or no effect on the metabolism of phenytoin by neutrophils, demonstrating that adventitious iron was not contributing via Fenton chemistry. In an ·OH-generating system containing H_2O_2 and Fe^{2+} chelated with ADP, phenytoin was oxidized rapidly to unidentified polar products and to *p*-, *m*-, and *o*-HPPH (ratio 1.0:1.7:1.5, respectively). Reagent HOCl and human myeloperoxidase (MPO), in the presence of Cl^- and H_2O_2, both formed the reactive dichlorophenytoin but no HPPH. However, no chlorinated phenytoin was detected in activated neutrophils, possibly because of its high reactivity. These findings, which demonstrated that activated neutrophils biotransform phenytoin *in vitro* to hydroxylated products and reactive intermediates that bind irreversibly to tissue macromolecules, are consistent with phenytoin hydroxylation by ·OH generated by a transition metal-independent process, chlorination by HOCl generated by MPO, and possibly cooxidation by neutrophil hydroperoxidases. Neutrophils activated *in vivo* may similarly convert phenytoin to reactive intermediates, which could contribute to some of the previously unexplained adverse effects of the drug.

Key words: phenytoin; activated neutrophils; dichlorophenytoin; covalent binding; drug oxidation; myeloperoxidase; hypochlorous acid; hydroxyl radical

Phenytoin was first introduced as an antiseizure medication in 1938 by Merritt and Putnam [1]. Since that time, numerous attempts to elucidate mechanisms of the many adverse effects associated with the drug have yielded only partial explanations. Many of the adverse effects of phenytoin—central nervous system toxicity, cardiac arrhythmias, gastrointestinal disturbances, inhibition of secretion of antidiuretic hormone, hyperglycemia secondary to decreased insulin secretion, osteomalacia, gingival hyperplasia, and fetal abnormalities—may be minimized by adjusting the dosage [2, 3]. Other, less predictable, toxic effects include systemic lupus erythematosus, agranulocytosis, aplastic anemia, lymphadenopathy, potentially fatal hepatic necrosis, and peripheral neuropathy associated with demyelination [3]. A possible contributing factor for some of the toxicity may be the formation of yet unidentified metabolites. Cytochrome P450 is reportedly involved in the bioactivation of phenytoin to reactive intermediates that bind to tissue macromolecules [4, 5]. An arene oxide intermediate, or its electrophilic products, formed by cytochrome P450, may react with protein or other macromolecules, causing cellular damage.

Prostaglandin H synthase has been implicated in the bioactivation of phenytoin; inhibitors of this enzyme and free radical spin traps have reduced the incidence of cleft palate in fetal mice exposed to phenytoin [6]. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate caused a 3-fold increase in the incidence of cleft palate in mice exposed to phenytoin *in utero*, perhaps by stimulating the release of arachidonic acid leading to formation of lipid hydroperoxides and subsequent cooxidation of phenytoin by prostaglandin synthase [7]. *In vitro* studies further support the role for the hydroperoxidase activity of prostaglandin synthase in the bioactivation of phenytoin [8].

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Stimulation of neutrophils by phagocytosis of bacteria or by other immune processes causes a dramatic increase in metabolic oxidation. This phenomenon, the respiratory burst, is initiated when NADPH oxidase reduces molecular oxygen to superoxide anion (O_2^-), which can subsequently form reactive oxygen species such as H_2O_2, HOCl, chloramines, and possibly ·OH* [9, 10]. The principal function of neutrophils is to kill invading organisms by this oxygen-dependent system. Work from our and other laboratories has shown that these reactive oxygen species are also linked to the oxidation of several chemicals, including benzoic acid [11], salicylic acid [12–14], 5-aminosalicylic acid [15], and carbamazepine [16]. Phenytoin is biotransformed by activated neutrophils to reactive intermediates that bind to cellular protein, and *N*-chlorinated phenytoin has been proposed as the reactive intermediate [17]. We report further studies of the biotransformation of phenytoin by activated neutrophils and propose alternative pathways for activation of the drug.

MATERIALS AND METHODS

Chemicals

Phenytoin (Lot No. V) was obtained from the Warner-Lambert Co. (Ann Arbor, MI). The purity determined by HPLC was >99%. [4-^{14}C]Phenytoin (49 μCi/μmol, Lot No. 2596–019) and liquid scintillation mixtures, Econofluor and Atomlight, were purchased from New England Nuclear (Boston, MA). The radiochemical purity of [^{14}C]phenytoin by HPLC was 99.3% prior to purification.

BSTFA and BSA were purchased from Pierce (Rockford, IL). HPLC-grade acetonitrile and reagent grade H_2O_2 were purchased from Fisher Scientific (Cincinnati, OH). *m*-HPPH was purchased from the Aldrich Chemical Co. (Milwaukee, WI). *o*-HPPH was a gift from Drs. Kenneth H. Dudley and James H. Maguire at the University of North Carolina; the synthesis of this compound is described by Butler *et al.* [18]. *N*-1′, *N*-3′-dichlorophenytoin (dichlorophenytoin) and *N*-1′-chlorophenytoin were gifts from Drs. Saul B. Kadin and Christopher A. Lipinski at Pfizer Central Research (Groton, CT). Myeloperoxidase (purity >98%; 180–220 U/mg protein) isolated from human neutrophils was purchased from Calbiochem (La Jolla, CA), and desferrioxamine methane sulfonate (Desferal) was obtained from CIBA Pharmaceutical (Summit, NJ). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO): *p*-HPPH, PMA, catalase from bovine liver (14,000 U/mg protein), superoxide dismutase from bovine liver (15,000 U/mg protein), indomethacin, hypoxanthine, xanthine, xanthine oxidase grade III (12.7 mg protein/mL; 1.2 U/mg), GSH, ferrous chloride, ferric chloride, Me_2SO, and DTPA. PMA was dissolved in Me_2SO at 2 mg/mL, and portions (0.2 mL) were frozen at −80°. Immediately before use, this solution was thawed and a portion diluted with DPBS (Gibco Laboratories, Grand Island, NY) to a final concentration of PMA of 2 μg/mL.

Purification of [^{14}C]*phenytoin*

As supplied, the [^{14}C]phenytoin contained impurities that eluted from the HPLC at retention times corresponding to those of the mono-hydroxylated metabolites of phenytoin. It is possible that the impurities were formed by radiolysis during storage. Although the impurities were quantitatively minor (0.1 to 0.3% of the total radioactivity), [^{14}C]phenytoin was purified to obtain acceptable blanks. The impurities were more polar than phenytoin and easily separated according to differences in their partitioning between 1-chlorobutane and 0.1 M phosphate [19]. Specifically, 125 μCi of [^{14}C]phenytoin was dissolved in 200 mL of 1-chlorobutane, which was equilibrated subsequently with 20 mL of 0.1 M potassium phosphate buffer (pH 6.8). The partition coefficients of phenytoin and *p*-HPPH (the most polar of the mono-hydroxylated metabolites) in this system were 1.9 and 0.021, respectively [20]. The aqueous layer containing the polar impurities was discarded, and the organic layer was washed three more times with 20 mL of phosphate buffer. The organic layer was subsequently evaporated to dryness under vacuum, and the residue containing approximately 100 μCi of [^{14}C]phenytoin was redissolved in 100% ethanol and stored at −20°. The final radiochemical purity of the [^{14}C]phenytoin determined by analyzing 2×10^6 dpm by HPLC was ≥99.94%. There were no detectable peaks of radioactivity (<350 dpm) at retention times corresponding to known metabolites of phenytoin. Solutions of 5 mM [^{14}C]phenytoin were prepared immediately before each experiment by evaporating the ethanol to dryness under nitrogen, redissolving the drug directly in 2 N NaOH (2.5% of reconstitution volume), and diluting the solution to the desired concentration of phenytoin with deionized water (0.05 N NaOH final concentration).

Isolation of neutrophils

Neutrophils from heparinized venous blood (40–100 mL) of healthy volunteers (ages 23–35 years; 4 males and 4 females; one smoker of each gender) who had been medication-free for at least 48 hr were separated by a modification of the method described by Boyum [21]. The viability of cells by trypan blue exclusion was typically >98% and purity was ≥98%.

Metabolism of phenytoin by neutrophils

After stirring for 30 min during preincubation at 37°, the neutrophils, (2×10^7), suspended in 800 μL of DPBS with 50 mg/dL of glucose (pH 7.1), were stimulated by the addition of 100 ng of PMA in 50 μL of DPBS containing 0.1% Me_2SO. Following the preincubation, 20 μL of 5 mM phenytoin in 0.05 N NaOH and 130 μL of DPBS with glucose were added to each sample to yield a final volume of 1 mL. The final pH was 7.1. Some incubations were prepared with proportionately smaller volumes of each component. Control samples, all with equal

* Abbreviations: BSA, *N,O*-bis(trimethylsilyl)acetamide; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DPBS, Dulbecco's phosphate-buffered saline; DTPA, diethylenetriaminepentaacetic acid; GSH, reduced glutathione; ·OH, hydroxyl radical; HPPH, 5-(hydroxyphenyl)-5-phenylhydantoin; PMA, phorbol-12-myristate-13-acetate; SIM, selective ion monitoring; and TMS, trimethylsilyl.

volumes of Me_2SO, DPBS, and deionized-distilled water, were incubated in the absence of one of each of the following: (1) PMA, (2) neutrophils, (3) PMA and neutrophils, and (4) phenytoin. Following a 1-hr incubation (stirring with magnetic stir bars), all samples were immediately cooled to 0°, then centrifuged at 10,000 *g* for 5 min at 4°. Pellets of neutrophils were analyzed for covalent binding, and the supernates were maintained on ice or stored at −20° until analysis by HPLC.

Metabolism of phenytoin in the presence of antioxidants and iron chelators

In further studies, sodium azide, GSH, and indomethacin were added to separate incubations 10 min after the start of the preincubation (without PMA) to yield final concentrations of 0.1, 1 and 0.2 mM, respectively. Superoxide dismutase and catalase, 15 and 100 μg/mL, respectively, were also added separately 10 min after the start of the preincubation and in additional studies were boiled for 15 min prior to introduction into the incubation. Control samples, all with equal volumes of Me_2SO, DPBS, and distilled water, were incubated in the absence of (1) PMA, (2) neutrophils, (3) PMA and neutrophils, or (4) phenytoin. In a separate experiment to determine the effects of free iron on the metabolism of phenytoin, the metal chelators desferrioxamine (0.1 mM) and DTPA (0.1 mM) were added 10 min after the start of the preincubation.

Analysis of metabolites of phenytoin by HPLC

Phenytoin and its metabolites were analyzed by direct injection of 200 μL of cell-free supernates on an HPLC (Hewlett–Packard model 1050 or 1090M, Palo Alto, CA) using a 25 × 0.45 cm IBM C-18 column packed with 5-μm spherical particles. Preliminary experiments showed that profiles of the metabolites of phenytoin in cell supernates were not different when analyzed immediately, after storage on ice for 7 hr, or after storage at −20° for 2 weeks. Most samples were analyzed on the day of the experiment. Mobile phase for the biological samples consisted of 5 mM phosphate buffer (pH 3.0)/acetonitrile (73/27). The flow was 1.2 mL/min, and the UV absorbance was monitored at 210 nm during the 30-min analysis. Radioactivity was measured with a Flo-One Beta model IC Radioactivity Flow Detector (Radiomatic, Tampa, FL) downstream of the UV detector. With a flow rate of 5 mL/min of scintillation mixture (Scintiverse LC, Fisher Scientific) and a 2.5-mL flow cell, the counting efficiency for ^{14}C was 95%. Retention times of the reaction products of phenytoin were compared with those of authentic standards of *o*-, *m*-, and *p*-isomers of HPPH. Recovery by HPLC of the radioactivity added to incubations in a typical experiment was 98 ± 3% (mean ± SD, N = 10).

Identification of metabolites by mass spectrometry

GC–MS analysis was performed on TMS derivatives of *m*- and *o*-isomers of authentic HPPH and presumed HPPH collected and purified by HPLC from the incubation of neutrophils. HPLC fractions were collected from 20 injections of 200 μL each of supernate, lyophilized, and further purified by repeat injections. Samples were collected and lyophilized a total of three times. The mobile phase was H_2O/acetonitrile (73/27). The residues were reconstituted in 5 μL of dichloromethane and 3–5 μL of BSA. The samples were concentrated under nitrogen to about 3 μL of which 1 μL (0.5 min splitless injection) was analyzed by GC–MS. With argon carrier gas, analytes were separated on a DB-1701 GC capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsum, CA) and analyzed on a Finnigan model 4021 mass spectrometer with an Incos data system using electron impact ionization (70 eV). The temperature of the injection port was 250°. The column was maintained at 70° for 1 min and then increased at a rate of 15°/min to 280°. Transline temperature was 260°. The electron multiplier voltage was 2100–2200 V.

In subsequent analyses, GC–MS was performed on an HP 5791A mass selective detector (Hewlett Packard) using SIM of *m/z* 484 (M^+), 469, and 369. Samples were processed as before except that BSTFA was substituted for BSA. Splitless injections (0.5 min) of 0.5 μL (0.1 nmol/μL of standard metabolites) were made on a DB-1 column (28 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific). The temperature of the injection port was 280°. The temperature of the column was maintained at 70° for 3 min and then increased at a rate of 15°/min to 280°. Transline temperature was 280°. Electron impact ionization (70 eV) was used, and the electron multiplier voltage was 2200 V.

Covalent binding of reactive metabolites of phenytoin in neutrophils

After the supernate was removed from the centrifuged incubations containing neutrophils, the remaining pellet of neutrophils was analyzed for covalent binding. The cells were lysed by resuspending in 0.5 mL of methanol, and the mixture was centrifuged at 4000 *g* for 10 min. The pellet was resuspended and washed repeatedly (at least five times to remove unbound radioactivity) with 0.5 mL of methanol until the radioactivity in the wash was less than twice background (background = 45 dpm). Residual radioactivity in the pellet was considered irreversibly bound to macromolecules. The pellet was solubilized in 100 μL of 1 N NaOH at 60° for 1 hr. A 40-μL portion of the sample was counted for radioactivity in 10 mL of Atomlight on a Beckman LS 5000TD liquid scintillation counter, and 50 μL was assayed for total protein by the method of Lowry *et al.* [22] using standards of bovine serum albumin.

Oxidation of phenytoin in chemical and biochemical systems

The potential role of ·OH, O_2^-, HOCl, and H_2O_2 in the oxidation of phenytoin was evaluated in chemical systems (for a discussion of biologically relevant free radical chemistry, see the review of Halliwell and Gutteridge [23]). All samples were incubated at 37° in room air. The products of phenytoin were analyzed by HPLC as described above, except that the mobile phase was water/acetonitrile (73/27).

$ADP/Fe^{2+}/H_2O_2$ system. A well-known source of ·OH is from the univalent reduction of hydrogen

peroxide by metal ions, such as the Fenton reaction between a ferrous chelate and H_2O_2 [24]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH. \quad (1)$$

The chemical system used in our experiments was modified after Floyd *et al.* [25]. The components of the reaction mixture, dissolved in $NaCl/NaHCO_3$ buffer (100 mM/25 mM, pH 7.2) except for phenytoin (5 mM) which was in 0.05 N NaOH, were added in the following sequence at the indicated final concentrations (*the order of addition is critical* [24]): 1.5 mM $FeCl_2$, 2 mM ADP, and 0.1 mM [^{14}C]phenytoin (24.5 μCi/μmol). Enough $NaCl/NaHCO_3$ buffer was added to bring the volume to 0.7 mL. Following preincubation for 30 sec at 37°, 0.3 mL of 10 mM H_2O_2 was added to yield a final volume of 1 mL. Some incubations were prepared with proportionately smaller volumes of each component. The samples were mixed for 1 min, and 100–200 μL was injected directly into the HPLC. In control incubations, H_2O_2, $FeCl_2$, and ADP were each excluded individually.

Hypoxanthine-xanthine oxidase system plus chelated ferric iron. In the presence of molecular oxygen, xanthine oxidase oxidizes hypoxanthine and xanthine to their respective products, xanthine and uric acid. Molecular oxygen is the electron acceptor for these reactions, which generate both O_2^- and H_2O_2; the proportion of each product is dependent on pH and oxygen concentration with higher pH and pO_2 favoring O_2^- [26, 27]. At neutral pH with 21% O_2, approximately one-fifth of the electron flow can be accounted for as O_2^-. The Fe^{3+} complexed with EDTA or ADP is readily reduced to Fe^{2+} by O_2^- [28]:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2. \quad (2)$$

Hydroxyl radical is subsequently generated by a Fenton-type reaction of ADP/Fe^{2+} with H_2O_2, as shown in reaction 1 [27].

The system used in our experiments, modified after Floyd *et al.* [25], contained the following components dissolved in $NaCl/NaHCO_3$ buffer (100 mM/25 mM, pH adjusted to 7.2 with HCl) except for phenytoin which was in 0.05 N NaOH: 25 μL of 2 mM hypoxanthine, 5 μL of 5 mM [^{14}C]-phenytoin (49 μCi/μmol), 25 μL of 20 mM ADP, 25 μL of 1 mM $FeCl_3$, and enough $NaCl/NaHCO_3$ buffer to bring the volume to 215 μL. Samples were preincubated for 15 sec at 37° before addition of 35 μL of xanthine oxidase (15 U/mL) to initiate the reaction. After incubation for 10 min at 37°, 200 μL was injected directly into the HPLC. Control samples contained additional $NaCl/NaHCO_3$ buffer instead of hypoxanthine.

HOCl, H_2O_2, and myeloperoxidase. Previous studies have demonstrated that activated neutrophils produce the equivalent of 0.1 to 1 mM HOCl via the myeloperoxidase system [29, 30]. We studied the reactions of HOCl and its biological precursor, H_2O_2, with phenytoin. Incubations contained final concentrations of 100 μM [^{14}C]phenytoin (49 μCi/μmol), 0.5 mM HOCl or 2.5 mM H_2O_2 in 50 mM sodium phosphate buffer containing 0.15 M NaCl (final pH 7.2 or 6.0). Samples were preincubated for 2 min at 37° prior to the addition of HOCl or H_2O_2, mixed, and incubated for an additional 1.5 to 60 min; 20 to 200-μL samples were analyzed directly by HPLC. In control experiments, HOCl or H_2O_2 was replaced with buffer.

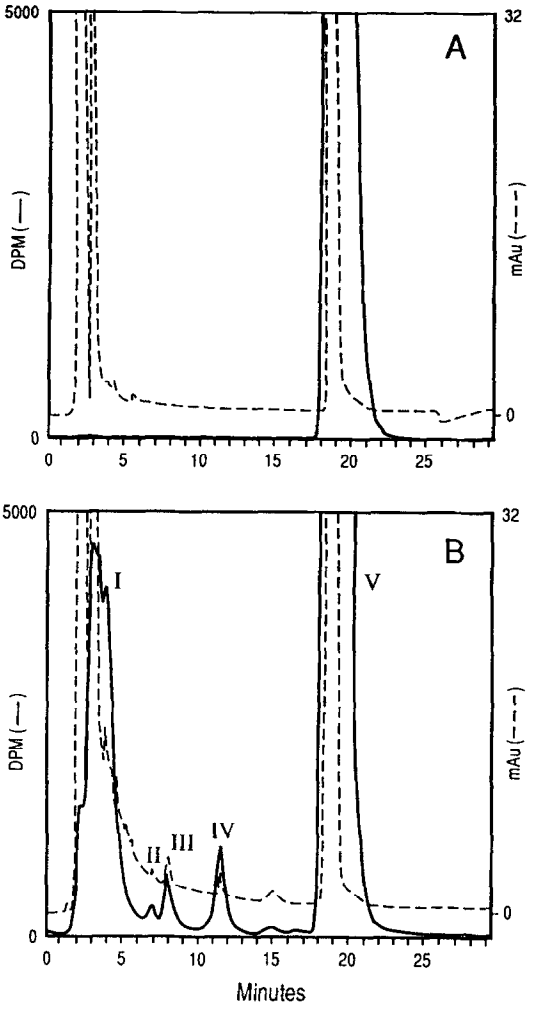

Fig. 1. Metabolism of phenytoin by neutrophils. HPLC-UV/radiochromatograms of cell-free supernates (200 μL) of 60-min incubations of [^{14}C]phenytoin (100 μM, 49 μCi/μmol) with human neutrophils (2×10^7 cells/mL). (A) Unstimulated neutrophils. (B) Neutrophils stimulated with 100 ng/mL of PMA. Key: (I) unidentified polar products; (II) *p*-HPPH; (III) *m*-HPPH; (IV) *o*-HPPH; and (V) phenytoin.

We studied the reaction of myeloperoxidase with phenytoin using the method described by Uetrecht and Zahid [17]. The final concentrations of reactants, dissolved in 0.1 M sodium phosphate buffer (pH 6.0) containing 0.15 M NaCl, except for a 1 mM stock solution of phenytoin which was in 0.05 N NaOH, were: 0.25 U of myeloperoxidase, 0.1 mM [^{14}C]-phenytoin (49 μCi/μmol), and 0.4 mM H_2O_2 in a final incubation volume of 0.20 mL. The myeloperoxidase and phenytoin were preincubated at 37° for 2 min, and the reaction was started by

Table 1. Metabolism of phenytoin by human neutrophils

Subject	*p*-HPPH	*m*-HPPH	*o*-HPPH	HPPH Sum	Polar	Total
	(nmol/2 × 10^7 cells)					
SM	0.070	0.148	0.168	0.386	1.34	1.73
SL	0.053	0.091	0.088	0.232	0.99	1.22
SN	0.115	0.239	0.263	0.617	3.64	4.26
ZS	0.112	0.232	0.263	0.607	2.02	2.63
LP	0.081	0.202	0.380	0.663	3.21	3.88
DS	0.130	0.290	0.483	0.903	4.27	5.17
Mean	0.094	0.200	0.274	0.568	2.58	3.15
SD	0.030	0.071	0.142	0.233	1.32	1.54

Neutrophils (2 × 10^7 cells/mL) from healthy volunteers were stimulated with PMA (100 ng/mL) and incubated with 100 μM [^{14}C]phenytoin for 1 hr at 37°. No metabolites were detected in any control incubations, including those without PMA. Metabolites of phenytoin were quantified by HPLC as described in Materials and Methods. Subjects SM and SL were females; ZS and SL were smokers.

addition of H_2O_2. In separate experiments, a 4 mM phenytoin stock solution in ethanol was added to the incubations to give a final concentration of 0.1 mM phenytoin. Portions (20 μL) of the reaction mixture were analyzed directly by HPLC as described above. To minimize decomposition of labile products during the HPLC run, selected samples were analyzed using a different mobile phase: 44% acetonitrile and 56% water containing 1% glacial acetic acid. In this system the retention times of *p*-HPPH, *m*-HPPH, phenytoin, and dichlorophenytoin were 3.8, 4.0, 5.8 and 14.5 min, respectively.

Statistics

Data are reported as means ± SD. Statistical significance was determined by one-way ANOVA and by Student's unpaired, two-tailed *t*-test.

RESULTS

Metabolism of phenytoin by activated neutrophils

Neutrophils incubated with 100 μM [^{14}C]phenytoin and stimulated *in vitro* with PMA produced metabolites of phenytoin detected by HPLC (Fig. 1). In contrast, no metabolites were observed in any control incubations, including those with [^{14}C]-phenytoin and unstimulated neutrophils (Fig. 1) or with [^{14}C]phenytoin and PMA without neutrophils (not shown). In the cell-free supernates derived from incubations of the complete system, the products of phenytoin eluting from the HPLC at 6.9, 8.1, and 11.7 min corresponded to the respective retention times of *p*-, *m*-, and *o*-isomers of HPPH. Activated neutrophils from six subjects produced 0.57 ± 0.23 μM (mean ± SD) *p*-, *m*-, and *o*-isomers of HPPH in a ratio of 1.0 ± 0.2:2.1 ± 0.2:2.8 ± 0.4 (Table 1). This represents 0.6% conversion of phenytoin to identifiable mono-hydroxylated metabolites. The neutrophils also produced unidentified polar metabolites (2.6 ± 1.3 μM) representing 2.6% conversion.

Identification of HPPH produced from phenytoin by activated neutrophils

The peaks of radioactivity (metabolites or phenytoin) derived from the supernates of activated neutrophils were collected and further purified by HPLC. Analysis by GC–MS established the identity of *m*-HPPH in the biological sample; the mass spectrum of the tri-TMS derivative of the unknown

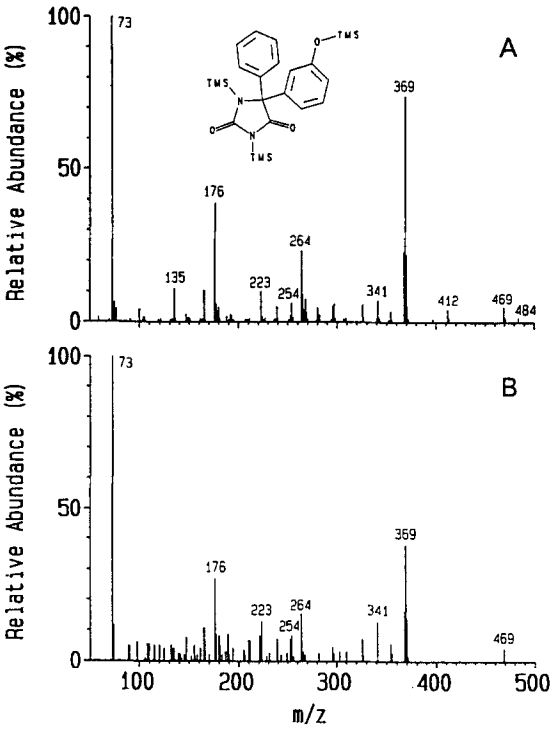

Fig. 2. Identification by mass spectrometry of HPPH produced in activated human neutrophils. Mass spectra (scanning from *m/z* 50 to 500) of the trimethylsilyl (TMS) derivatives of authentic *m*-HPPH (A) and putative *m*-HPPH isolated from an incubation of 100 μM phenytoin with human neutrophils activated with PMA (B). The ions *m/z* 469 [M-15]$^+$, 369, 354, 341, and 264 are characteristic of the tri-TMS derivative of *m*-HPPH.

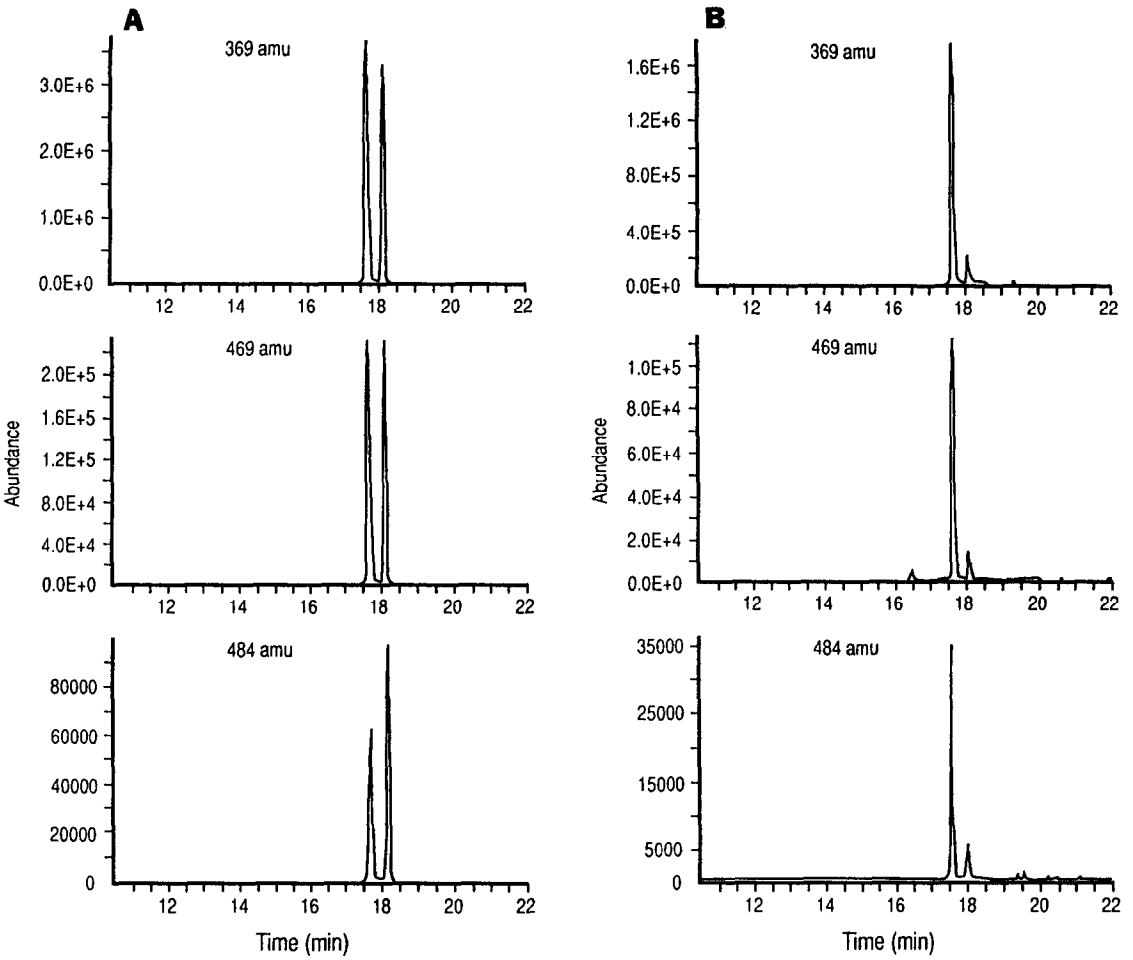

Fig. 3. Selective ion monitoring (SIM) analysis of HPPH isolated from incubations of phenytoin with stimulated neutrophils. SIM analysis (*m/z* 369, 469, and 484) of a mixture of TMS derivatives of HPPH. (A) TMS derivatives of authentic *o*- and *m*-HPPH (coeluting at 17.6 min) and *p*-HPPH (eluting at 18.1 min). (B) TMS derivatives of HPPH fraction isolated and purified by HPLC from an incubation of 100 μM phenytoin with human neutrophils activated with PMA. The peaks at 17.6 and 18.1 min correspond to *o*-/*m*-HPPH and *p*-HPPH, respectively. E + 4, E + 5, etc. = 10^4, 10^5, etc.

was comparable to that of the derivatized standard *m*-HPPH (Fig. 2). The retention time of the unknown by GC (15 m DB-1701 column) was 11.10 min, identical to the derivatized standard *m*-HPPH. The amount of *m*- and *o*-isomers of HPPH in each injection was not sufficient to detect the molecular ion (*m/z* 484) when scanning from *m/z* 45 to *m/z* 500. Analysis of the tri-TMS derivative of the biologic sample believed to be *o*-HPPH, using a Finnigan mass spectrometer with SIM at *m/z* 369, 469, and 484, revealed ions at *m/z* 369 and 469 in a ratio of 12:1, almost identical to the derivatized standard *o*-HPPH (ratio 11:1); the molecular ion was not detected in the biological sample. Retention times of the tri-TMS derivatives of authentic *o*-HPPH and the biological sample were identical, 10.33 min.

Metabolites were also analyzed by GC–MS using a Hewlett–Packard mass selective detector with SIM of *m/z* 484, 469, and 369. The putative HPPH metabolites, eluting from the HPLC between 6 and 15 min, were collected together, further purified by HPLC, and derivatized with BSTFA. The tri-TMS derivatives were analyzed on a 28 m DB-1 column. The tri-TMS derivatives of authentic standards of *o*- and *m*-HPPH coeluted at 17.62 min and *p*-HPPH eluted at 18.13 min (Fig. 3). Two peaks in the biological sample matched the retention times of the *o*-/*m*-HPPH and *p*-HPPH standards. The relative abundance of the selected ions *m/z* 484:469:369 for standards of *o*-HPPH, *m*-HPPH, and *p*-HPPH (analyzed separately) were 3.6:10.3:100, 2.0:6.9:100, and 2.9:6.9:100, respectively, and for the peaks in the biological samples at 17.62 and 18.12 min were 2.1:10.1:100 and 2.6:6.3:100, respectively. Collectively, the GC–MS and HPLC data support the identification of *p*-, *m*-, and *o*-HPPH as oxidation products of phenytoin in neutrophils.

Table 2. Inhibition of metabolism of phenytoin and covalent binding of phenytoin metabolites in neutrophils by antioxidants

Conditions	Metabolite formation (nmol/2 × 10^7 cells)		Covalent binding (nmol/mg protein)
	Unknown polar	Sum of HPPH	
No PMA	<0.01	<0.01	0.002 ± 0.002 (1)
Complete system	3.15 ± 1.13 (100)	0.72 ± 0.16 (100)	0.339 ± 0.297 (100)
Azide (0.1 mM)	0.20 ± 0.06 (6)	0.14 ± 0.02 (19)	0.061 ± 0.024 (18)
Catalase (100 μg/mL)	0.39 ± 0.11 (12)	0.13 ± 0.01 (18)	0.079 ± 0.051 (23)
Catalase/boiled	2.08 ± 0.95 (66)	0.63 ± 0.15 (88)	0.250 ± 0.244 (74)
SOD (15 μg/mL)	0.21 ± 0.02 (7)	0.11 ± 0.05 (15)	0.060 ± 0.037 (18)
GSH (1 mM)	0.51 ± 0.06 (16)	0.20 ± 0.02 (28)	0.051* (15)
Indomethacin (0.2 mM)	0.47 ± 0.04 (15)	0.19 ± 0.02 (26)	0.099 ± 0.027 (29)

Neutrophils (2 × 10^7 cells/mL) from three healthy male volunteers were stimulated with 100 ng/mL of PMA and incubated for 1 hr at 37° with 100 μM [^{14}C]phenytoin (complete system). In the control incubations, PMA was omitted. Azide and other inhibitors were added to incubations containing the complete system before PMA. Metabolism of phenytoin and covalent binding of reactive intermediates of phenytoin in neutrophils were determined as described in Materials and Methods. Abbreviations: SOD, superoxide dismutase; and GSH, reduced glutathione. Each value is the mean ± SD of determinations from three subjects except where noted; percent of complete system is in parentheses.

* One determination.

The more polar products of phenytoin eluting from the HPLC between 1.5 min (void volume) and 6 min (Fig. 1) have not been identified. Dihydroxylated products of phenytoin, such as 5 - (3',4' - dihydroxyphenyl) - 5 - phenylhydantoin (catechol), and hydantoin ring scission products (hydantoic acid analogs) are possible metabolites that, if present, would elute in this early region. Gradient elution of this polar material revealed at least 14 incompletely resolved peaks of radioactivity.

Effects of antioxidants on the formation of HPPH and polar products of phenytoin

Azide inhibits heme-containing enzymes such as catalase and myeloperoxidase [31] but does not block the respiratory burst activity of neutrophils, measured as H_2O_2 production [29]. In our experiments (Table 2), 0.1 mM azide almost completely blocked the formation of unknown polar metabolites of phenytoin and the isomers of HPPH (94 and 81% inhibition, respectively, compared with incubations without inhibitor), indicating that enzymes containing heme iron may contribute to the oxidation of phenytoin. Addition of catalase to incubations with activated neutrophils caused a marked decrease in the oxidation of phenytoin. Superoxide dismutase was an efficient inhibitor of the oxidation of phenytoin to unknown polar metabolites and HPPH (93 and 85% inhibition, respectively), suggesting a role for superoxide or one of its products in the oxidation of phenytoin. GSH (1 mM) blocked >80% of the total oxidation of phenytoin. GSH inactivates many reactive oxidant species, including H_2O_2, HOCl, and ·OH. The anti-inflammatory drug indomethacin, an inhibitor of prostaglandin H synthase, also markedly reduced the metabolism of phenytoin.

Covalent binding of reactive intermediates of phenytoin to neutrophils

Irreversible binding of metabolites of chemicals to tissue macromolecules is evidence of the formation of reactive, potentially harmful, intermediates. Extensive binding of reactive intermediates of [^{14}C]-phenytoin occurred in cells stimulated by PMA, but almost none occurred in cells without PMA (Table 2). Moreover, antioxidants caused a proportionate decrease in metabolism and covalent binding of phenytoin. There was considerable intersubject variation in covalent binding in neutrophils, prompting us to conduct another experiment with replicate determinations of covalent binding in neutrophils from a single individual. The data in Table 3 verify that covalent binding could be measured reproducibly.

Table 3. Covalent binding of reactive intermediates of phenytoin in neutrophils

Conditions	Covalent binding	
	(nmol/mg protein)	% Complete system
No PMA	0.007 ± 0.001*	5 ± 1
Complete system	0.129 ± 0.028	100 ± 22
Azide (0.1 mM)	0.039 ± 0.003*	30 ± 2
Superoxide dismutase (15 μg/mL)	0.049 ± 0.004*	38 ± 3
Catalase (100 μg/mL)	0.070 ± 0.002†	54 ± 2

Experimental details were the same as described in Tables 1 and 2, except that neutrophils were from one subject. Each value is the mean ± SD of three separate incubations.

*,† Significantly different from the complete system: *$P < 0.01$, and †$P < 0.05$.

Oxidation of phenytoin in chemical and biochemical systems

HPPH and polar products of phenytoin were also

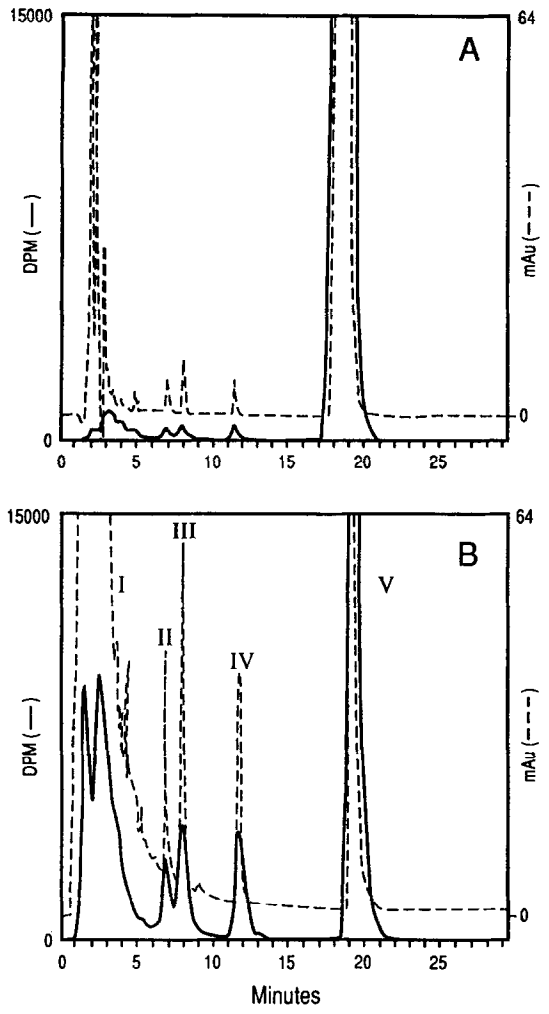

Fig. 4. Formation of HPPH and polar products of phenytoin by an ADP/Fe^{2+}/H_2O_2 hydroxyl radical-generating system. (A) HPLC-UV/radiochromatogram of a 1-min incubation with 1.5 mM $FeCl_2$, 2 mM ADP, bicarbonate buffer, and 100 μM [^{14}C]phenytoin (24 μCi/μmol), omitting H_2O_2. (B) HPLC-UV/radiochromatogram of a 1-min incubation with 1.5 mM $FeCl_2$, 2 mM ADP, bicarbonate buffer, 100 μM [^{14}C]phenytoin (24 μCi/μmol), and 8.8 mM H_2O_2 (complete ·OH-generating system). Peaks are identified in the legend of Fig. 1.

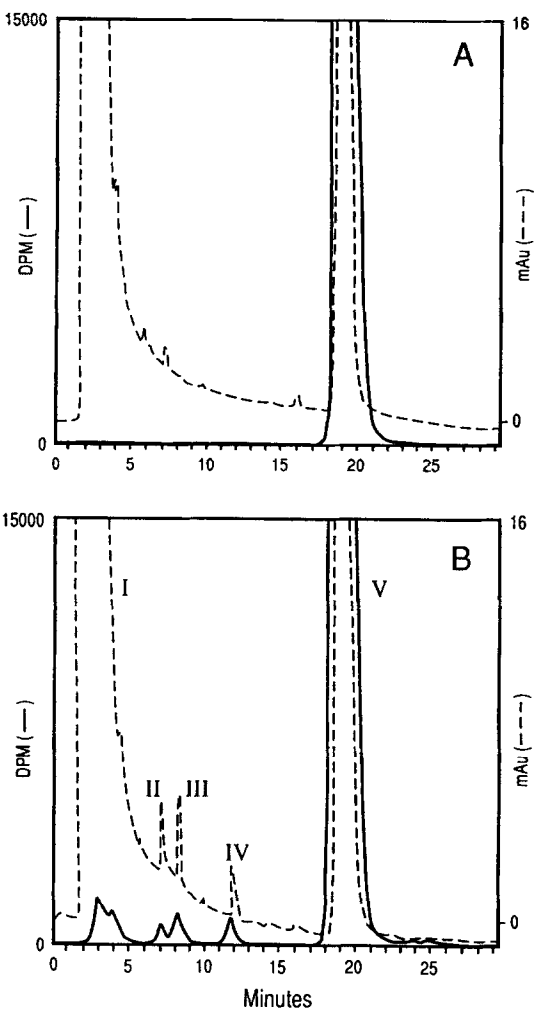

Fig. 5. Formation of HPPH and polar products of phenytoin by a hypoxanthine/xanthine oxidase hydroxyl radical-generating system. (A) HPLC UV/radiochromatogram of a control incubation with 100 μM [^{14}C]phenytoin, 2 mM ADP, 0.1 mM $FeCl_3$, and 19.8 U/mL of xanthine oxidase, omitting hypoxanthine. After 10 min, 200 μL of the incubation was injected directly into the HPLC. (B) HPLC-UV/radiochromatogram of the complete hypoxanthine/xanthine oxidase incubation, including 0.2 mM hypoxanthine. The reaction was stopped after 10 min by direct injection of 200 μL into the HPLC. Peaks are identified in the legend of Fig. 1.

formed in two chemical systems known to generate ·OH. Phenytoin (100 μM) was oxidized rapidly in the incubation with ADP/Fe^{2+}/H_2O_2. In a 1-min incubation, 31 ± 6 μM (N = 4) polar products and 14 ± 1 μM HPPH were formed (Fig. 4). The ratio of the *p*:*m*:*o*-isomers of HPPH was 1.0:1.7:1.5 in this chemical system. Metabolites were also produced, although in much smaller quantities, with ADP and Fe^{2+}, in the absence of H_2O_2 (Fig. 4) and with Fe^{2+} and H_2O_2, in the absence of ADP (data not shown). The former can be explained by auto-oxidation of ferrous iron with subsequent spontaneous dismutation of superoxide anion:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^- \quad (3)$$

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2. \quad (4)$$

The resulting H_2O_2 can react with residual Fe^{2+} as described above for the Fenton reaction (reaction 1). Therefore, ·OH can be generated in this chemical system even without added H_2O_2 [24, 32]. Oxidation of phenytoin in the absence of ADP can be explained by a slower but significant reaction of H_2O_2 with unchelated ferrous iron. No products were detected (<0.02 μM) in incubations when $FeCl_2$ was omitted, demonstrating an absolute requirement for iron in this chemical system.

In 10-min incubations of 100 μM phenytoin with

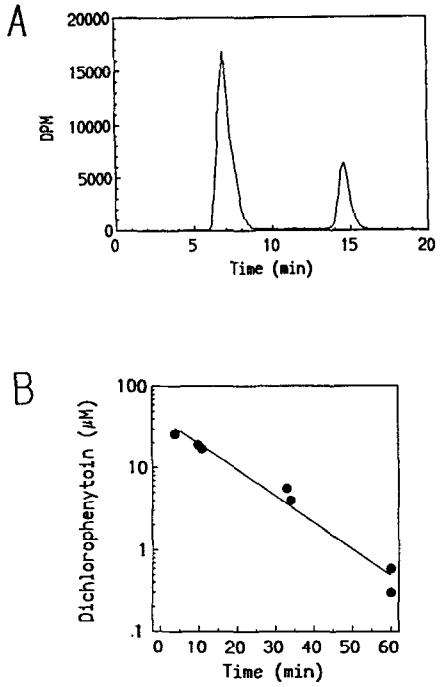

Fig. 6. N-Chlorination of phenytoin by myeloperoxidase *in vitro*. [^{14}C]Phenytoin (100 μM, 49 μCi/μmol), 0.125 U human myeloperoxidase, and 0.4 mM H_2O_2 in 150 mM NaCl/100 mM sodium phosphate buffer (pH 6.0) were incubated at 37° for 60 min. The final incubation volume was 100 μL. At various times, samples (20 μL) were removed and analyzed immediately by HPLC as described in Materials and Methods. (A) HPLC-radiochromatogram of a sample removed from the incubation after 4 min. Phenytoin and *N*-1′,*N*-3′-dichlorophenytoin eluted in this system at 6.9 and 14.5 min, respectively. (B) Dichlorophenytoin concentration in samples removed from the incubation at various times. The data from two separate incubations are combined. Each point is a single determination.

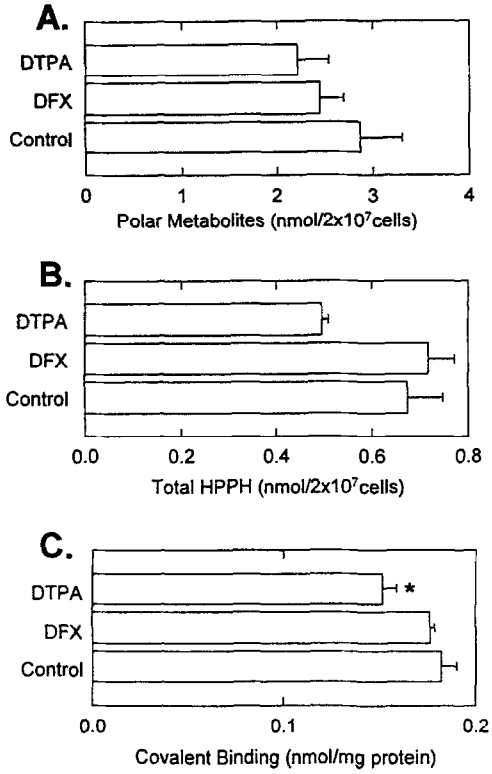

Fig. 7. Effects of iron chelators on the metabolism and covalent binding of phenytoin by activated neutrophils. Neutrophils (2×10^7 cells/mL) isolated from a healthy female volunteer were stimulated with 100 ng/mL of PMA and incubated for 1 hr at 37° with 100 μM [^{14}C]phenytoin (control). In separate incubations 100 μM desferrioxamine (DFX) or 100 μM diethylenetriamine pentaacetic acid (DTPA) was added 20 min before PMA. At the end of the incubation period, 200 μL of the cell-free supernate was analyzed by HPLC for polar metabolites (A) and HPPH (B) as described in Materials and Methods. Covalent binding to protein (C) was measured as radioactivity irreversibly bound to the cell pellet. Values are the means ± SE of three determinations. Key: (*) significantly different from control ($P < 0.05$).

hypoxanthine/xanthine oxidase (mean, N = 2), HPPH (1.0 μM) and polar products of phenytoin (1.3 μM) were generated (Fig. 5). The ratio of *p*:*m*:*o*-HPPH of 1.0:1.8:1.7 was similar to the Fe^{2+}/ADP system. No metabolites were detected (<0.02 μM) in the xanthine oxidase system without hypoxanthine (Fig. 5).

In incubations of 100 μM phenytoin with 0.5 mM HOCl, 65% of phenytoin was converted within 2 min to an apparently less polar product, which coeluted with an authentic standard of dichlorophenytoin. Dichlorophenytoin partially decomposed to phenytoin, or to another compound with the same retention time, during the longer HPLC run using our conventional mobile phase with phosphate buffer (previously optimized for analysis of HPPH isomers). Decomposition of dichlorophenytoin during the HPLC run was minimized by using a different mobile phase [44% acetonitrile and 56% water containing 1% (v/v) glacial acetic acid] as described by Uetrecht and Zahid [17].

Dichlorophenytoin also was formed rapidly in incubations containing 100 μM phenytoin and a human myeloperoxidase HOCl-generating system. The maximum conversion of phenytoin to dichlorophenytoin was 26 μM at 4 min, the earliest sample (Fig. 6). Dichlorophenytoin rapidly decomposed in the incubation mixture to phenytoin (or a closely eluting compound, possibly monochlorophenytoin) with apparent first-order kinetics ($T_{1/2}$ = 10 min; Fig. 6). HPPH was not detected during the 60-min incubation. No products were detected (<0.02 μM) in incubations of 100 μM phenytoin with 2.5 mM H_2O_2 for 30 min in the absence of iron.

Effects of metal chelators on phenytoin metabolism by neutrophils

The potential role of metals, particularly adventitious iron, in the oxidation of phenytoin in preparations of neutrophils was investigated by

adding the strong, inactivating metal chelators desferrioxamine and DTPA to the incubations [23]. Desferrioxamine (100 μM) had no effect on the metabolism or covalent binding of phenytoin in activated neutrophils (Fig. 7), whereas 100 μM DTPA slightly decreased total HPPH production (NS, $P > 0.05$) and covalent binding ($P < 0.05$), suggesting that free iron does not play a major role in the metabolism and covalent binding of phenytoin.

DISCUSSION

Our studies demonstrate that neutrophils activated by PMA metabolize phenytoin to the *p*-, *m*-, and *o*-isomers of HPPH and to other more polar products (Figs. 1–3; Table 1). In humans, metabolism of phenytoin is catalyzed *in vivo* primarily by hepatic cytochrome P450; 60–75% of the dose is excreted in the urine as *p*-HPPH and its conjugates [20, 33, 34]. Aromatic hydroxylation of phenytoin at the *m*-position is a minor pathway in humans (constituting 0–10% of the dose) and in all other species studied except the dog, in which it is the major pathway [18, 19, 33, 35]. To our knowledge, there have been no reports of *o*-HPPH formation in humans. A dihydrodiol metabolite of phenytoin [5-(3,4-dihydroxy - 1,5 - (cyclohexadien-1 - yl)) - 5 - phenylhydantoin], thought to arise from a reactive arene oxide intermediate [36], accounts for about 5–10% of the dose in humans [37].

The ratio of *p*:*m*:*o*-isomers of HPPH generated by hepatic metabolism in humans is approximately 1.0:≤0.1:0.0, consistent with regiospecific hydroxylation catalyzed by cytochrome P450. In the Fe^{2+}/ADP hydroxyl radical-generating system in our studies, the ratio of *p*:*m*:*o*-hydroxylation of phenytoin was 1.0:1.7:1.5 (Fig. 4), similar to the isomer ratio of 1.0:1.9:1.4 for aromatic hydroxylation of benzoic acid exposed to radiation-generated ·OH [38]. In contrast, the ratio of *p*:*m*:*o*-hydroxylated products of phenytoin in activated neutrophils was 1.0:2.1:2.8 (Fig. 1; Table 1), indicating a higher selectivity for *o*-HPPH than observed with ·OH generated in the Fe^{2+}/ADP chemical system and completely different selectivity from that of reactions catalyzed by cytochrome P450 (Table 1, Fig. 1). In neutrophils, *o*-hydroxylation appears to be favored at the expense of *p*- and *m*-hydroxylation. These results support the notion that hydroxylation of phenytoin in neutrophils involves more than a direct chemical reaction with ·OH. Additional, more selective mechanisms, possibly involving enzyme-catalyzed reactions or less reactive oxygen species, may contribute to the higher than expected *o*-hydroxylation.

The metabolism and covalent binding of reactive intermediates of phenytoin in neutrophils occurred only in the presence of PMA (Fig. 1, Tables 2 and 3), which stimulates the cells to produce reactive oxygen species, such as O_2^-, H_2O_2, HOCl, chloramines, and possibly ·OH [10]. These or related oxidants apparently lead to the formation of reactive intermediates of phenytoin that covalently bind to macromolecules in neutrophils (Tables 2 and 3). The significance of these reactive intermediates of phenytoin *in vivo* is not known, although there are several reports in the literature linking metabolic activation of phenytoin by cytochrome P450 to a wide variety of toxic effects [4, 6, 39]. Arene oxides of phenytoin, formed by cytochrome P450, were implicated as the reactive (toxic) intermediates in those studies. A potential role for the activation of phenytoin by the hydroperoxidase activity of prostaglandin H synthase in microsomes from ram seminal vesicles and murine lung and liver was demonstrated in studies by Kubow and Wells [8]. The covalent binding of phenytoin in microsomes stimulated by arachidonic acid could be blocked with indomethacin [8]. There are reports in the literature supporting the importance of peroxidative oxidation of other chemicals during arachidonic acid metabolism by prostaglandin H synthase; this enzyme is thought to play a significant role in the bioactivation of several chemicals, including 2-aminofluorene, diethylstibesterol, and benzo[*a*]pyrene-7,8-dihydrodiol [40]. The extensive work of Uetrecht and colleagues also has demonstrated that myeloperoxidase is involved in the metabolism of phenytoin and several other drugs by activated leukocytes, and that this enzyme may lead to the production of toxic metabolites responsible for some hypersensitivity reactions [17, 41].

In our studies, indomethacin, GSH, azide, catalase, and superoxide dismutase effectively inhibited the metabolism and covalent binding of phenytoin in activated neutrophils (Tables 2 and 3). The effect of indomethacin suggests involvement of prostaglandin synthase in the bioactivation of phenytoin in activated neutrophils. However, indomethacin, like several other inhibitors of prostaglandin synthase, can directly scavenge reactive oxidant species [10, 42, 43] and may also act indirectly by inhibiting O_2^- production in PMA-stimulated neutrophils [44]. The effect of GSH may be explained by reduction of hydroperoxides (e.g. H_2O_2, prostaglandin G_2), or other reactive species generated by activated neutrophils. Azide inhibits the heme-dependent hydroperoxidase activity of prostaglandin synthase and myeloperoxidase but does not affect O_2^- production by NADPH oxidase. Catalase is known to decrease myeloperoxidase activity by depleting H_2O_2, one of its substrates for HOCl production [31]. The mechanism by which superoxide dismutase alters phenytoin metabolism is not known, but superoxide dismutase has been linked to other unexpected findings such as a decrease in myeloperoxidase activity (measured as HOCl production) in incubations of activated neutrophils [45]. Hydroxylation of salicylic acid by a superoxide-dependent reaction catalyzed by myeloperoxidase has been proposed recently [46]. A similar, myeloperoxidase-catalyzed reaction may be contributing to the hydroxylation of phenytoin.

Our results that show chlorination of phenytoin *in vitro* by both reagent HOCl and HOCl generated by myeloperoxidase isolated from human neutrophils (Fig. 6) confirm the findings of Uetrecht and Zahid [17]. Dichlorophenytoin is short-lived in cell-free incubations with myeloperoxidase (Fig. 6) and has a half-life of only 30 sec when added to neutrophil incubations [17], explaining why we and others were unable to detect it by HPLC in the more complex

matrix of incubations with activated neutrophils. Chlorinated products of phenytoin may act as intermediate oxidants, similar to the longer-lived chloramines formed from the reaction of HOCl with amino acids [47]. The chlorohydantoin group of di- and monochlorophenytoin is analogous to a common chlorinating reagent, *N*-chlorosuccinimide [48]. Indeed, intermolecular chlorine transfer to phenytoin forming monochlorophenytoin is a documented reaction of dichlorophenytoin [49]. The relative importance of chlorination of phenytoin in the formation of the ultimate reactants with tissue macromolecules as proposed by Uetrecht and Zahid [17] requires further investigation.

Our chemical studies suggest that reagent-generated ·OH, or another highly reactive oxidant, oxidizes phenytoin to some of the same products observed in neutrophils (compare Fig. 1 with Figs. 4 and 5). While there is much indirect evidence for the production of ·OH by Fenton reactions of O_2^- and H_2O_2 in activated neutrophils [10, 50], there is a growing argument against ·OH production in this biological system [31, 51, 52]. In fact, recent kinetic evidence suggests that the oxidizing intermediate in Fenton oxidations may be a peroxo complex such as FeII(OOH) rather than ·OH [53]. Central to the controversy is that direct study of ·OH has not been possible because of its very short half-life. Therefore, indirect methods are required, such as detection of free radicals by ESR using spin-trapping agents. However, ESR is subject to limitations and artifacts in biological systems [54]. For example, a problem with this method of detection of ·OH in activated neutrophils is that O_2^- rapidly decomposes the ·OH spin-trap adduct [55].

We do not believe that contaminating iron contributes to the hydroxylation of chemicals by activated neutrophils under the conditions used in our laboratory. There are two pieces of evidence from previous studies to support this: (1) the iron-inactivating chelator desferrioxamine does not inhibit hydroxylation of salicylic acid by activated neutrophils, and (2) free iron could not be detected ($<2\ \mu M$) in the neutrophil supernate [13]. In the present investigation, we further examined the role of iron in the oxidation of phenytoin. Addition of $100\ \mu M$ desferrioxamine or $100\ \mu M$ DTPA prior to stimulating neutrophils with PMA had no significant effect on the oxidation of phenytoin and caused only a modest reduction (16% with DTPA) in covalent binding of phenytoin reactive intermediates to macromolecules (Fig. 7). Both metal chelators are known to block the reduction of Fe^{3+} by O_2^- and thereby inhibit the formation of ·OH by the Haber–Weiss reaction [23].

If hydroxylation of phenytoin in activated neutrophils is not caused by Fenton chemistry related to iron contamination, is it possible that ·OH could be formed in these cells by another process? Ramos *et al.* [56] explored this question and proposed that human neutrophils and monocytes generate ·OH through the following transition metal-independent reaction:

$$HOCl + O_2^- \rightarrow \cdot OH + O_2 + Cl^- . \qquad (5)$$

Azide, catalase and superoxide dismutase, but not the iron chelator DTPA, decreased the apparent production of ·OH by neutrophils in their experiments [56]. These agents had similar effects on HPPH production in our studies. Hydroxyl radical from reaction 5 could attack phenytoin, forming a substituted cylcohexadienyl free radical that can be further oxidized to HPPH or participate in free radical reactions, including attacks on macromolecules. Analogous intermediates of phenylalanine hydroxylation by radiation-produced ·OH have been shown to react with molecular oxygen, forming peroxy free radicals [57]. Reaction 5 could also explain the decrease in hydroxylation of phenytoin observed when exogenous superoxide dismutase was added to activated neutrophils. The lack of HPPH formation with either reagent HOCl or the myeloperoxidase HOCl-generating system is consistent with our proposed reactions (see Fig. 8) and can be attributed to the absence of a source of O_2^- in those incubations. Therefore, our results and those of Uetrecht and Zahid [17] support a role for myeloperoxidase-generated HOCl in the formation of potentially reactive species of phenytoin (e.g. dichlorophenytoin) by neutrophils. However, our results also suggest that there are other mechanisms for activation of phenytoin in stimulated neutrophils, including the formation of free radical precursors to hydroxylated phenytoin.

Figure 8 illustrates the possible pathways for the metabolism of phenytoin in activated neutrophils. Hydroxyl radical, formed in neutrophils by the transition metal-independent reaction of HOCl with O_2^-, attacks phenytoin, forming a substituted cyclohexadienyl free radical (**1**) which is oxidized to HPPH (only the *p*-isomer is shown) or undergoes additional reactions, possibly binding to protein or other macromolecules. Phenytoin also reacts directly with HOCl to form dichlorophenytoin. Dichlorophenytoin donates chlorine to a suitable acceptor forming free radical **2**, which may react with protein and possibly other macromolecules. An alternative pathway not shown in Fig. 8 is the oxidation of phenytoin by a superoxide-dependent reaction catalyzed by myeloperoxidase as described by Kettle and Winterbourn [46] for hydroxylation of salicylic acid. This reaction would also be diminished by superoxide dismutase. A further possibility is the oxidation of phenytoin to reactive intermediates by prostaglandin synthase, or one of its lipid hydroperoxide products, similar to the reactions described by Smith *et al.* [40].

The hydroxylation reactions and other undetermined reactions with phenytoin clearly require the presence of PMA. The fact that metabolism of phenytoin by neutrophils occurs only when the neutrophils are activated may explain possible adverse effects following administration of phenytoin in some disease states. It has been proposed that an inflammatory process *in vivo* may stimulate neutrophils and monocytes, resulting in the formation of toxic reactive metabolites of some chemicals, primarily in such areas of inflammation [12, 41, 58].

Oxidation of phenytoin at sites of inflammation may cause some of the adverse effects of phenytoin, for example, gingival hyperplasia. This abnormality occurs only in dentulous gingivae, perhaps because

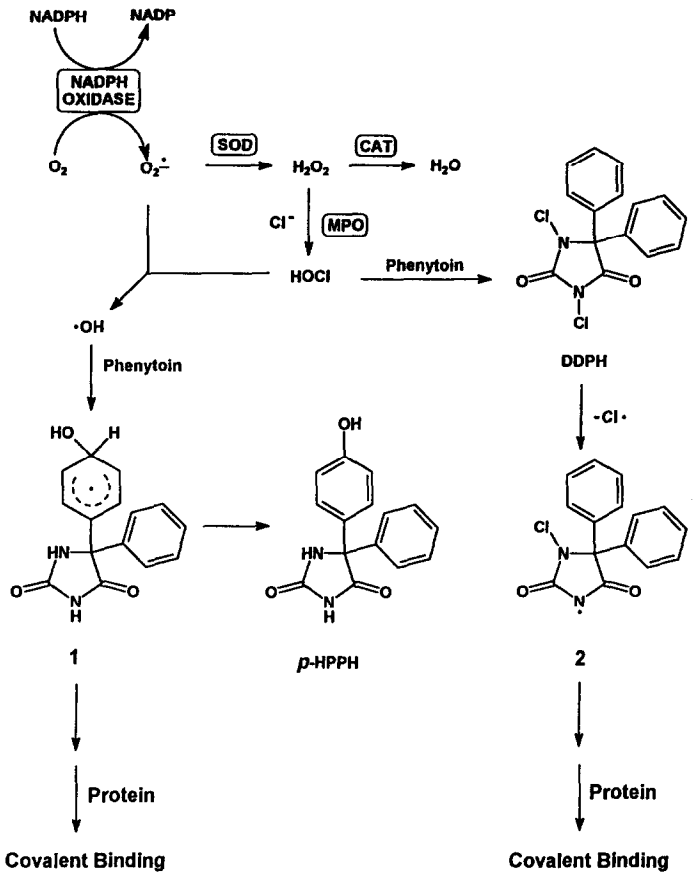

Fig. 8. Proposed pathways of metabolism of phenytoin in activated neutrophils. Abbreviations: SOD, superoxide dismutase; MPO, myeloperoxidase; CAT, catalase; and DDPH, dichlorophenytoin.

edentulous gingivae are not associated with inflammation of chronic bacterial gingivitis. In addition, by forming adducts with proteins, reactive metabolites of drugs may create an antigenic response, contributing to allergic reactions *in vivo*. Further research is needed to determine whether the metabolism of phenytoin observed *in vitro* in neutrophils also occurs *in vivo*, explaining in part the enigma of adverse effects associated with the use of this drug.

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