0026-895X/89/040504-08$02.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 35:504-511

MOL PHARM

In Vitro Bioactivation of Phenytoin to a Reactive Free Radical Intermediate by Prostaglandin Synthetase, Horseradish Peroxidase, and Thyroid Peroxidase

STANLEY KUBOW and PETER G. WELLS
Faculty of Pharmacy, University of Toronto, Toronto, Canada M5S 2S2
Received September 26, 1988; Accepted January 10, 1989

SUMMARY

Certain toxic effects of phenytoin are thought to result from its cytochrome P-450-catalyzed bioactivation to a reactive arene oxide intermediate that binds covalently to proteins. Using an *in vitro* system, we examined an alternative hypothesis based upon the cooxidation of phenytoin to a reactive free radical intermediate by prostaglandin synthetase (PGS), horseradish peroxidase, or thyroid peroxidase. Microsomes from hepatic, thyroid, seminal vesicular, or pulmonary tissues, or PGS or horseradish peroxidase, were incubated with the appropriate enzymatic cofactors to study activities of cytochromes P-450 (NADPH), PGS (arachidonic acid), thyroid peroxidase (guiaicol, H_2O_2), and horseradish peroxidase (H_2O_2). The production of potentially teratogenic, reactive phenytoin intermediates during *in vitro* incubations was estimated by the amount of radiolabeled phenytoin bound covalently to microsomal protein or bovine serum albumin and by the detection of a free radical intermediate using ESR spectrometry. Arachidonic acid-dependent bioactivation of phenytoin was demonstrated for purified PGS and ram seminal vesicles (RSV), as well as for liver, lung, and kidney. Optimal arachidonate concentrations varied substantially for different tissues. Arachidonate-dependent binding of phenytoin with PGS and RSV was reduced to baseline levels by coincubation with the cyclooxygenase inhibitor indomethacin. Hydrogen peroxide-dependent covalent binding of phenytoin was observed with thyroid peroxidase and horseradish peroxidase, and binding was significantly reduced in these systems and in PGS and RSV by coincubation with the peroxidase inhibitor methimazole. Glutathione, the antioxidants caffeic acid and butylated hydroxyanisole, and the free radical trapping agent α-phenyl-*N*-*t*-butylnitrone (PBN) all significantly reduced arachidonate-dependent phenytoin binding. Oxygen uptake was increased in a dose-dependent manner by the arachidonate-dependent bioactivation of phenytoin by PGS. ESR spin-trapping techniques using PBN indicated the generation of a free radical intermediate during the metabolism of phenytoin by PGS. These results suggest that the hydroperoxidase component of PGS, as well as thyroid peroxidase and other peroxidases, can bioactivate phenytoin to a reactive free radical intermediate, which may be toxicologically relevant.

Phenytoin (diphenylhydantoin, Dilantin) is the most efficacious and widely used anticonvulsant in North America (1). Phenytoin teratogenicity has been reported and characterized in mice (2), rats (3), and rabbits (4). Similarly, in humans, ingestion of phenytoin during pregnancy has been associated with a variety of fetal defects such as craniofacial malformations and cardiac defects, which have been characterized together as the fetal hydantoin syndrome (1, 5).

There is considerable supporting evidence from several laboratories that phenytoin is bioactivated by microsomal cytochromes P-450 to a reactive arene oxide intermediate, which may be teratogenic (1, 5–7). This reactive intermediate usually is detoxified via a number of mechanisms, including rearrangement of the arene oxide and enzymatic hydration catalyzed by epoxide hydrolase. Under conditions whereby bioactivation exceeds detoxification, this reactive electrophilic intermediate may initiate a teratologic process by binding covalently to nucleophilic sites on fetal macromolecules. However, a number of observations are not readily explained by the cytochromes P-450-arene oxide hypothesis. These observations include 1) the respective inhibition and enhancement of phenytoin teratogenicity by inducers and inhibitors of cytochromes P-450 (1, 8); 2) the embryopathy of mephenytoin and its nirvanol metabolite residing with the *l*-isomers that do not form an arene oxide intermediate (9); 3) the teratogenicity of phenytoin analogs such as trimethadione, which have no phenyl rings and cannot form an arene oxide. These discrepant observations have led to our hypothesis that an alternative bioactivating pathway for phenytoin may involve PGS and other

A preliminary report of this work was presented at the joint meeting of the American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology (*Pharmacologist* **28**:195, 1986), Baltimore, Maryland, August 1986. This work was supported by a grant from the Medical Research Council of Canada.

ABBREVIATIONS: PGS, prostaglandin synthetase; PG, prostaglandin; BHA, butylated hydroxyanisole; PBN, α-phenyl-*N*-*t*-butylnitrone; BSA, bovine serum albumin; RSV, ram seminal vesicles.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

peroxidases, such as thyroid peroxidase. Thyroid peroxidase was evaluated because of both its peroxidase activity and earlier reports that phenytoin-induced decrements in fetal thyroid hormone concentrations could contribute to the teratologic mechanism (1).

An alternative pathway of bioactivation for a number of drugs involves cooxidation of the drug during PGS-catalyzed synthesis of PGs (10, 11). Among these drugs are aminopyrine (12), benzidine (13), acetaminophen (14), and a variety of *N*-alkyl amines (15). PGS is a single protein that contains distinct catalytic sites for cyclooxygenase and hydroperoxidase activities. The cyclooxygenase component catalyzes the conversion of arachidonic acid to the PG hydroperoxy-endoperoxide PGG_2, which is subsequently reduced by the hydroperoxidase component of PGS to the corresponding alcohol PGH_2 (16). In order to catalyze the reduction of PGG_2 to PGH_2, the PG hydroperoxidase requires reducing equivalents, and those drugs undergoing cooxidation have been shown to serve as electron donors to the peroxidase. The latter reaction may lead to the formation of electron-deficient drug metabolites such as free radicals, which may cause oxidant stress, initiate lipid peroxidation, and/or bind covalently to essential cellular macromolecules, thereby causing cytotoxic effects and fetal abnormalities.

The present work describes experiments designed to determine whether phenytoin can be bioactivated by PGS and thyroid peroxidase to a potentially, teratogenic, reactive, free radical intermediate. The potential contribution of phenytoin cooxidation was tested *in vitro* microsomal incubations by measuring the covalent binding of radiolabeled phenytoin, oxygen consumption, and free radical production by ESR spectrometry.

Materials and Methods

Chemicals and tissues. Tritiated phenytoin (5,5-[phenyl-4-^{3}H(*N*)]-diphenylhydantoin; 51.5 Ci/mmol) was purchased from New England Nuclear (Lachine, Quebec). SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was obtained from Smith, Kline and French Canada Ltd. (Mississauga, Ontario). BHA, BSA, NADPH type IV, horseradish peroxidase type VI, caffeic acid, GSH, hydrogen peroxide, indomethacin, methimazole, PBN, and sodium phenytoin (5,5-diphenylhydantoin) were obtained from Sigma Chemical Co. (St. Louis, MO). Arachidonic acid (99% pure) was obtained from Pharmacia (Montreal, Quebec) and Nu-Chek-Prep Inc. (Elysion, MN). Guiaicol and potassium iodine were obtained from BDH Chemicals Canada Ltd. (Toronto, Ontario). PGS, purified from RSV, was purchased from Oxford Biomedical Research (Oxford, MI). Equine thyroid tissue, kindly supplied by Professor M. A. Hayes at the University of Guelph, Ontario, was flash-frozen in liquid nitrogen and stored at −70° until microsomes were prepared as described below. Female CD-1 mice (30–35 g; Charles River Canada Ltd., Lachine, Quebec) were killed by cervical dislocation, the liver and lungs were removed, and microsomes were prepared fresh and stored at −70°. RSV were obtained from a slaughterhouse, flash-frozen in liquid nitrogen, and stored at −70° until used. The arachidonic acid was stored in the dark at −70°.

Microsomal preparation. Tissues were thawed, trimmed of excess fat and connective tissue, and homogenized in ice-cold KCl (1.15%). The homogenates were then centrifuged at 9000 × *g* for 20 min at 4°. The 9000 × *g* supernatant was centrifuged at 100,000 × *g* for 60 min at 4°. The microsomal pellet was resuspended in 2.0 ml of KCl and frozen in liquid nitrogen. The microsomes were stored at −70° until used for the incubation studies described below. Microsomal protein concentrations were measured (17) using a standardised assay kit (Bio-Rad Laboratories, Richmond, CA).

Incubation studies. An initial incubation study was conducted to determine the optimal arachidonic acid concentrations for the PGS-catalyzed covalent binding of phenytoin in various tissues, including murine liver, lung, and kidney, RSV, and PGS. These studies were followed by a comparison of phenytoin binding catalyzed by different peroxidase sources, including RSV, PGS, horseradish peroxidase, and thyroid tissue. A further study compared the covalent binding of phenytoin in murine lung and liver catalyzed by cytochromes P-450 and PGS.

PGS-dependent cooxidation of phenytoin in various tissues was determined as follows. RSV, lung, or liver microsomes, (2.0 mg) were suspended in 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.4. Tritiated phenytoin (5,5[phenyl-4-^{3}H(*N*)]-diphenylhydantoin, (4.24 nM) and 0.1 mM unlabeled phenytoin were then added in 10 μl of 30% ethanol. The reactions were then initiated by adding arachidonic acid (15 μM to 6.0 mM). In appropriate experiments, 0.5 mM NADPH was used to initiate the reaction. When replacing the arachidonic acid, H_2O_2 was present at 160 μM; when horseradish peroxidase was used as the peroxidase, it was present at a concentration of 1.0 mg/ml. PGS also was used to catalyze the covalent binding of phenytoin to protein. The incubation mixture consisted of 100 mM Tris buffer (pH 7.6) containing 5.0 mg of crystallized BSA as the target binding protein, 1000 units of PGS, 0.5 mM phenol, 1 μM hematin, and water to make 1.0 ml. In these experiments, tritiated phenytoin was added as described above but unlabeled phenytoin was omitted. When thyroid tissue, microsomes, (2.0 mg) were suspended in 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.4, containing 33 mM guiaicol or 5 mM KL. The reactions were initiated by the addition of 160 μM H_2O_2. In other experiments, reaction mixtures were preincubated for 5 min with different enzymatic inhibitors or free radical scavengers, including SKF 525A, indomethacin, methimazole, GSH, caffeic acid, BHA, or PBN before the addition of [^{3}H]phenytoin. The reactions were terminated after 60 min at 37° with the addition of 5 volumes of ice-cold acetone. Metabolic blanks were run by omitting arachidonic acid, horseradish peroxidase, guiaicol, or NADPH. Radioactive phenytoin covalently bound to the microsomal protein was assayed as described below.

Measurement of covalent binding. The determination of covalent binding of tritiated phenytoin to protein involved an exhaustive washing procedure (7) modified as follows. After addition of acetone to the microsomal mixture, the suspension was centrifuged at 25,000 × *g* for 20 min. The precipitate was resuspended in 2.0 ml of hot methanol (50°) and the entire content was transferred onto a Nylon$_{66}$ membrane filter held by a vacuum filtration apparatus (Millipore, Mississauga, Ontario). The sample was filtered with hot methanol to give a total of 10.0 ml of filtrate or until the washings contained no radioactivity. The entire filter paper was placed in 0.5 ml of tissue solubilizer (BTS-450; New England Nuclear) and incubated in a shaking water bath for 30 min at 50° or until all the precipitate was dissolved. The samples were counted in 10.0 ml of scintillation cocktail (Ready-Solv HP/b; Beckman) containing 0.7% glacial acetic acid.

Spin trapping study. The incubation medium contained PGS (1000 units/ml), 1.0 μM hematin, 0.1 mM PBN, and 200 mM phenytoin in a 100 mM Tris buffer (pH 7.6) containing 0.5 mM phenol, in a total volume of 1.0 ml. The PGS was first dissolved in the buffer and preincubated for 1 min at 37°. The hematin was added and preincubated for 30 sec. Before the addition of arachidonic acid to initiate the reaction, phenytoin and PBN were added. The reaction mixture was incubated at 37° for 15 min. The incubation reaction was stopped by extraction with ethyl acetate. Spin adducts formed in the incubation mixtures were extracted with an equal volume of ethyl acetate and concentrated under a stream of nitrogen to 200 ml. The ESR spectrum of the extracted spin-trapped radicals was recorded with a Varian E-102 spectrometer. The instrument settings were as follows: microwave power, 10 mW; modulation amplitude, 1 G; time constant, 3 sec; scan range, 100 G; and scan time, 30 min.

Oxygen consumption studies. Oxygen consumption studies were used to monitor the oxidation of phenytoin by PGS. All incubations were performed using an oxygen consumption monitor (Model 5300;

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Yellow Springs Instrument Company, Yellow Springs, OH) with a 2.0-ml stirred cuvette maintained at 37°. Each assay contained 0.05 M sodium phosphate buffer, pH 7.4, hepatic microsomal protein (750 μg of protein/ml), unlabeled phenytoin at varying concentrations as indicated, and water to a final volume of 2.0 ml. Oxygen uptake was recorded on a standard chart recorder (Model 482; Pharmacia, Montreal, Quebec) and slopes were compared to determine stimulation of oxygen uptake. Oxygen uptake was initiated by the addition of 3.2 mM arachidonic acid.

Statistical analysis. Statistical comparisons of differences were determined using a standard computerized statistical program (SPSS Inc., Chicago, Illinois) modified for microcomputers (SPSS-PC). Statistical analysis of multiple comparisons was performed using analysis of variance followed by a range test, whereas single comparisons were analyzed by Student's t test with $p < 0.05$ as the minimal level of significance.

Results

Incubation of radiolabeled phenytoin with microsomal protein or BSA and appropriate cofactors for both peroxidases (Figs. 1, 2, and 3) and cytochromes P-450 (Fig. 3) resulted in a significant increase in irreversible binding of phenytoin to protein after a 1.0-hr incubation period. Peroxidase activities tested included PGS (Figs. 1, 2, and 3), thyroid peroxidase (Fig. 2), and horseradish peroxidase (Fig. 2). Phenytoin was found to be bioactivated by peroxidases from a number of tissues, including RSV, murine lung, liver, and kidney, and equine thyroid (Figs. 1, 2, and 3). Cytochromes P-450-catalyzed bioactivation of phenytoin was observed in murine liver and lung and was equivalent to that catalyzed by PGS.

The requirement for PGS in the bioactivation of phenytoin was indicated by dependence upon arachidonic acid (Figs. 1, and 2) and by the reduction in covalent binding of phenytoin by PGS inhibitors (Fig. 2). Indomethacin, an inhibitor of the cyclooxygenase component of PGS (18), and methimazole, an inhibitor of hydroperoxidases (19), inhibited arachidonate-dependent covalent binding of phenytoin catalyzed by PGS, RSV, liver microsomes, and horseradish peroxidase (Fig. 2; Table 1). The covalent binding of [^{3}H]phenytoin to BSA catalyzed by horseradish peroxidase further supported the hypothesis that phenytoin was cooxidized by the hydroperoxidase component of PGS during arachidonic acid-induced covalent binding of [^{3}H]phenytoin to microsomal protein (Fig. 2). In experiments using liver microsomes, it was evident that two different drug-metabolizing enzymes associated with the liver microsomal fraction, PGS and the cytochromes P-450, played distinct functional roles in catalyzing the irreversible binding of [^{3}H]phenytoin (Fig. 3). Preincubation of microsomal protein with 100 μM indomethacin inhibited PGS-dependent covalent binding by 95% in lung tissue and 90% in liver tissue. Omission of arachidonic acid or NADPH from the microsomal system led to significantly reduced levels of covalently bound phenytoin.

In addition to microsomes from liver (20) and RSV (21), many other mammalian tissue microsomes such as lung (22) and kidney (23) possess significant PG activity. We, therefore, examined PGS-dependent covalent binding of radiolabeled phenytoin at various arachidonic acid concentrations in microsomes from different tissues (Fig. 1). The addition of arachidonic acid was found to stimulate the covalent binding of phenytoin, indicating PGS-catalyzed bioactivation to a reactive intermediate. Maximal covalent binding was achieved with arachidonate concentrations of 6.0 mM for lung and kidney microsomes, 3.0 mM for liver microsomes, 150 μM for RSV microsomes, and 15 μM for PGS. These optimal concentrations of arachidonic acid were employed in subsequent studies.

Table 1 illustrates the dependence of covalent binding of [^{3}H]phenytoin on either PGS or cytochromes P-450 by the use of known inhibitors of these pathways. SKF 525A, a reversible inhibitor of many hepatic monooxygenase reactions, inhibited NADPH-dependent covalent binding of radiolabeled phenytoin but did not inhibit arachidonic acid-induced covalent binding. In fact, SKF 525A slightly stimulated covalent binding when arachidonic acid was used as cofactor. Conversely, indometha-

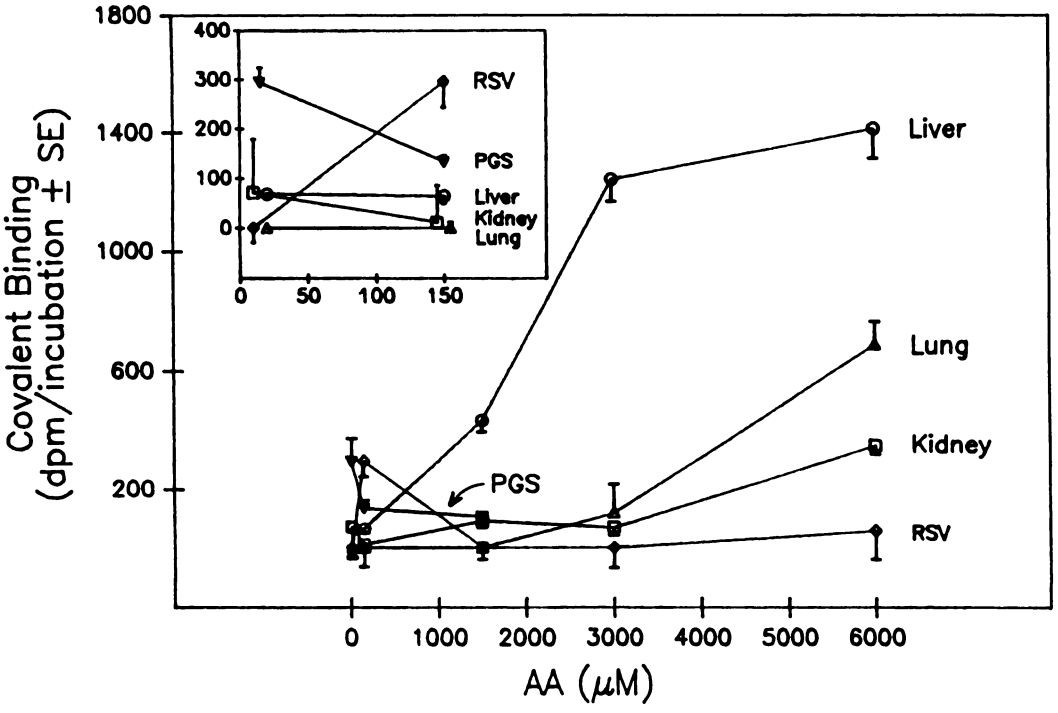

Fig. 1. Effect of arachidonic acid (*AA*) concentration on phenytoin covalent binding catalyzed by PGS and by microsomes from liver, lung, kidney, and RSV. The PGS incubation system consisted of 100 mM Tris buffer (pH 7.6), 5.0 mg of BSA, 1000 units of PGS, 0.5 mM phenol, 4.24 nM [^{3}H]phenytoin, 1 μM hematin, and water to make 1.0 ml. Incubations of liver, lung, kidney, or RSV microsomes (2.0 mg) contained 4.24 nM [^{3}H]phenytoin and 0.1 mM unlabeled phenytoin, 0.05 M sodium phosphate buffer (pH 7.4), and water to make 1.0 ml. Reactions were initiated with 15 μM to 6.0 mM arachidonic acid and were terminated after 60 min at 37°. *Symbols* represent the mean ± standard error of at least triplicate determinations.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

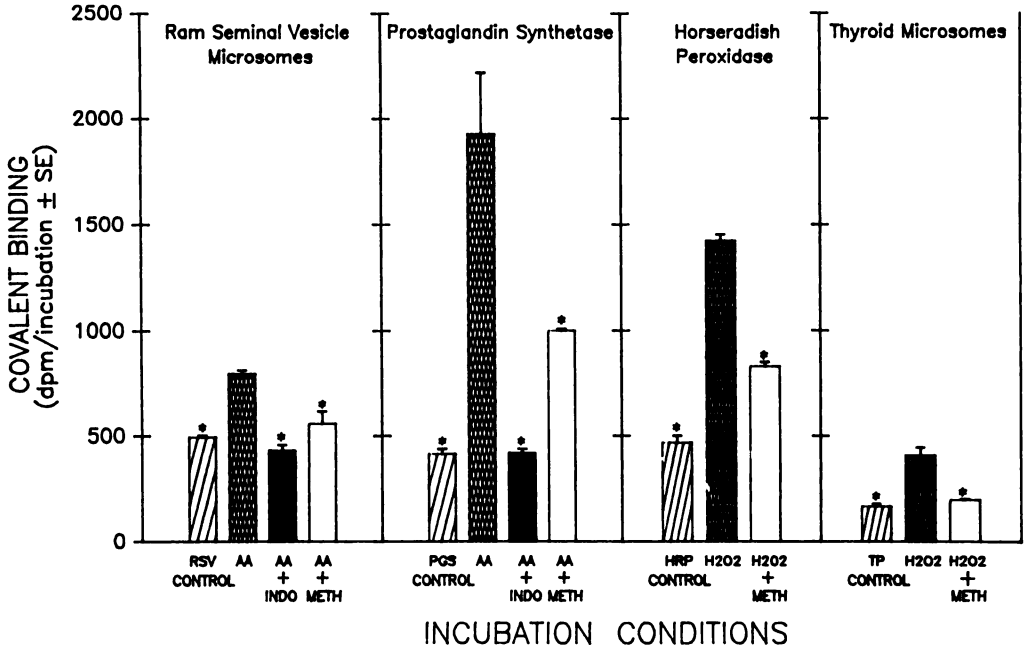

Fig. 2. Peroxidase-dependent covalent binding of phenytoin catalyzed by RSV, PGS, horseradish peroxidase (*HRP*), and thyroid peroxidase (*TP*). Both of the enzymatic inhibitors, indomethacin (*INDO*) and methimazole (*METH*), were employed in a concentration of 0.2 mM. RSV microsomes mixture contained 2.0 mg of RSV microsomal protein, 4.24 nM [^{3}H]phenytoin, and 0.1 mM unlabeled phenytoin suspended in 1.0 ml of 0.05 M sodium phosphate buffer (pH 7.4). Reactions were initiated with 150 μM arachidonic acid (*AA*). The PGS incubation system consisted of 100 mM Tris buffer (pH 7.6), 2 mg of crystallized BSA, 1000 units of PGS, 0.5 μM phenol, 4.24 nM [^{3}H]phenytoin, 1 μM hematin, and water to make 1.0 ml. Reactions were initiated with 15 μM arachidonic acid. The horseradish peroxidase incubation mixture consisted of 1.0 mg of horseradish peroxidase suspended in 1.0 ml of 0.05 M sodium phosphate buffer (pH 7.4) containing 5.0 mg of crystallized BSA. Reactions were initiated with 160 μM H_2O_2. The thyroid microsomal mixture contained 2.0 mg of thyroid microsomal protein, 4.24 nM [^{3}H]phenytoin, 0.1 mM unlabeled phenytoin, and 33 mM guiaicol suspended in 1.0 ml of 0.05 M sodium phosphate buffer (pH 7.4). Reactions were initiated with 160 μM H_2O_2. All reactions were terminated after 60 min at 37°. *Bars* represent the mean + SE of at least triplicate determinations. *Asterisks* indicate groups that are significantly different from the incubation activated with cofactor (arachidonic acid or H_2O_2) ($p < 0.05$).

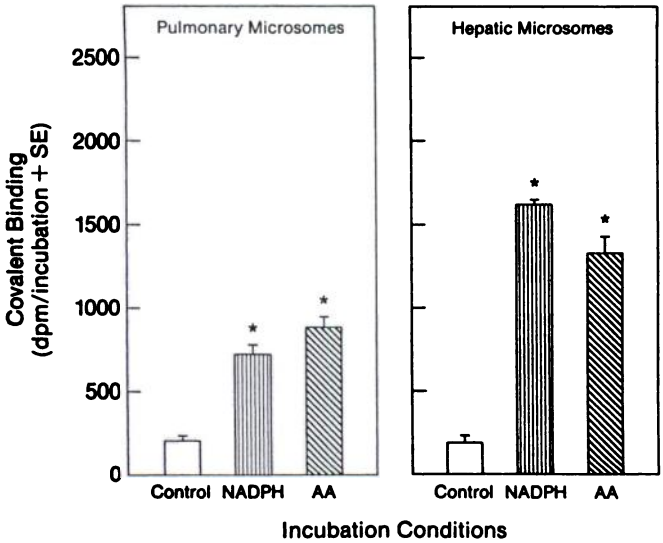

Fig. 3. Comparison of covalent binding of [^{3}H]phenytoin in murine lung and liver catalyzed by cytochromes P-450 and PGS. Reaction mixtures contained 2.0 mg of microsomal protein, 4.24 nM [^{3}H]phenytoin, and 0.1 mM unlabeled phenytoin, suspended in 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.4. Reactions were initiated with 3.2 mM arachidonic acid (*AA*) for PGS, or 0.5 mM NADPH for cytochromes P-450, and were terminated after incubation for 60 min at 37°. *Bars* represent the mean + SE of at least triplicate determinations. *Asterisks* indicate groups that are significantly different from controls ($p < 0.05$).

TABLE 1

Effects of various enzyme inhibitors on the covalent binding of radiolabeled phenytoin to hepatic microsomal protein

Incubations were conducted for 60 min at 37° under aerobic conditions. The values are given as percentages of the control value, 1180 ± 114 [dpm/2 mg of protein (mean ± standard error)], in the absence of cofactors. Results are the averages of at least two experiments done in triplicate. Experimental conditions were as described in the legend to Fig. 3.

Inhibitor	Concentration	Covalent Binding	
		Arachidonic acid	NADPH
	mM	% OF CONTROL	
Indomethacin	0.2	10	91
Methimazole	0.2	2	100
SKF 525A	1.0	134	6

cin and methimazole inhibited covalent binding of phenytoin in the presence of arachidonic acid but had no effect when NADPH was utilized as the cofactor.

To examine bioactivation of phenytoin by tissue peroxidases apart from the hydroperoxidase component of PGS, the ability of thyroid peroxidase to stimulate covalent binding of radiolabeled phenytoin was studied. The addition of H_2O_2 to thyroid microsomes containing guiaicol resulted in a significant increase in irreversible binding of radiolabeled phenytoin to protein (Fig. 2). A similar increase in covalent binding of tritiated phenytoin was observed with KI was used as a cofactor for thyroid peroxidase (data not shown). Omission of the necessary cofactor to activate thyroid peroxidase, either guiaicol or KI, resulted in no increase in the covalent binding of radiolabeled

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

phenytoin. Incubation with the thyroid peroxidase inhibitor methimazole reduced the covalent binding of phenytoin to baseline levels.

Phenytoin stimulated arachidonic acid-induced oxygen consumption by PGS. The stimulation of PGS was concentration dependent (Table 2). At a phenytoin concentration of 200 μM, a 49% increase in oxygen uptake was observed (Fig. 4). Phenylbutazone, a known cofactor of PGS, stimulated oxygen consumption by 58% in hepatic microsomes at a concentration of 200 μM.

To evaluate whether a reactive free radical intermediate may be formed from phenytoin by PGS, we examined the effect of antioxidants on covalent binding of phenytoin in the microsomal system (Table 3). GSH and the free radical scavenging agent PBN substantially inhibited covalent binding of [^{3}H] phenytoin to microsomal protein. This inhibition was greater for PGS-dependent covalent binding than for covalent binding catalyzed by cytochromes P-450. Addition of the phenolic antioxidants BHA and caffeic acid to the incubation mixtures

TABLE 2

Effect of phenytoin and phenylbutazone on arachidonic acid-dependent oxygen consumption in hepatic microsomes

Assays were performed in 0.05 M sodium phosphate buffer, pH 7.4, containing hepatic microsomal protein (750 μg of protein/ml) and phenytoin in a total volume of 2.0 ml. After a 4-min preincubation period at 37°, the reaction was initiated by the addition of 3.2 mM arachidonic acid. Results are the mean ± standard error for at least three experiments done in triplicate.

Phenytoin	Phenylbutazone	Oxygen Consumption	Increase
μM	μM	μmol/min	%
0		103 ± 4	
100		118 ± 12	15
200		153 ± 11	49
400		115 ± 6	12
	200	163 ± 13	58

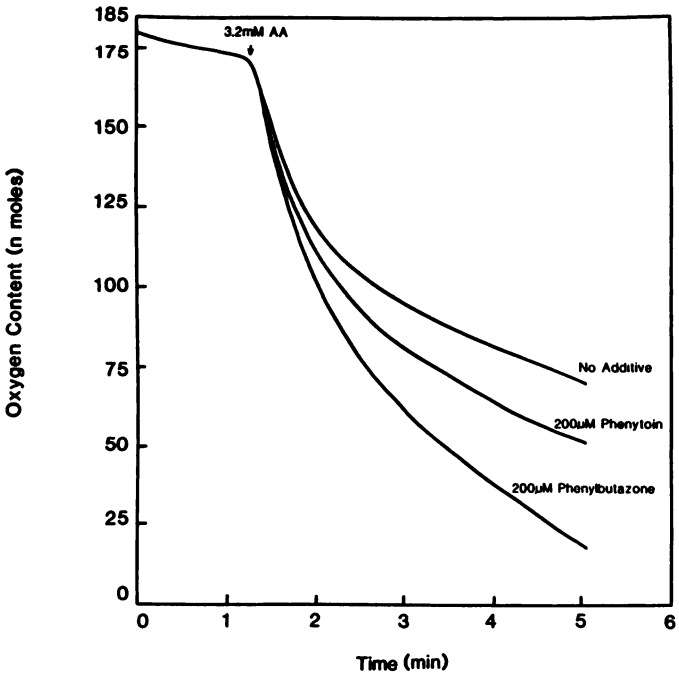

Fig. 4. The stimulation of oxygen consumption during PGS-catalyzed oxidation of phenytoin. Reaction mixtures contained 0.05 M sodium phosphate buffer, pH 7.4, hepatic microsomal protein (750 μg of protein/ml), 200 μM concentrations of phenytoin or phenybutazone, and water to a final volume of 2.0 ml.

TABLE 3

Effects of free radical scavengers on the covalent binding of radiolabeled phenytoin to hepatic microsomal protein

Incubations were conducted for 60 min at 37° under aerobic conditions. All scavengers were used in a concentration of 10 mM. The data are given as percentages of the control value in the absence of scavenger. Results are averages of at least two experiments done in triplicate. Control values (mean ± standard error) for the incubations were for NADPH-dependent covalent binding, 1997 ± 70 dpm/2 mg of protein; for arachidonic acid-dependent covalent binding, 1821 ± 291 dpm/2 mg protein; and for horseradish peroxidase-dependent covalent binding, 1331 ± 313 dpm/2 mg of protein. Experimental conditions were as described in the legend to Fig. 3.

Scavenger	Covalent Binding		
	NADPH	Arachidonic acid	Horseradish Peroxidase
	% of control		
PBN	51	44	27
BHA	47	31	33
GSH	31	20	13
Caffeic acid	5	6	5

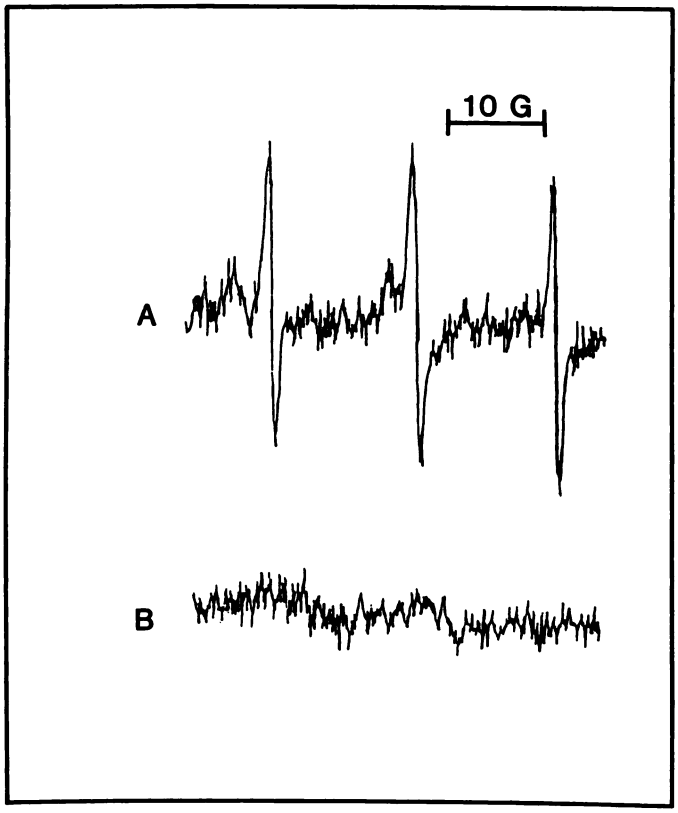

Fig. 5. ESR spectra of PGS incubation systems. The incubation systems contain 1000 units of PGS, 0.24 nM arachidonic acid, 1.0 μM hematin, 200 μM phenytoin, 0.2 M PBS, 0.5 μM phenol, and 100 mM Tris buffer in a total volume of 1.0 ml. The spectra represent the free radical PBN spin adduct obtained after a 15-min incubation at 37° (A) or when phenytoin was omitted from the incubation system (B).

decreased covalent binding to a similar degree in both the arachidonic acid- and the NADPH-dependent systems. Additional and direct support for PGS-catalyzed bioactivation of phenytoin to a free radical intermediate was obtained from the ESR spin-trapping study. The results from this experiment in which PGS was incubated with phenytoin and the spin trap PBN indicated the presence of a free radical adduct of PBN with narrow unresolved hyperfine splittings for the a_βH and with a_N = 14.5 G (Fig. 5A). No ESR signal was observed if phenytoin was omitted from the incubation system (Fig. 5B).

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

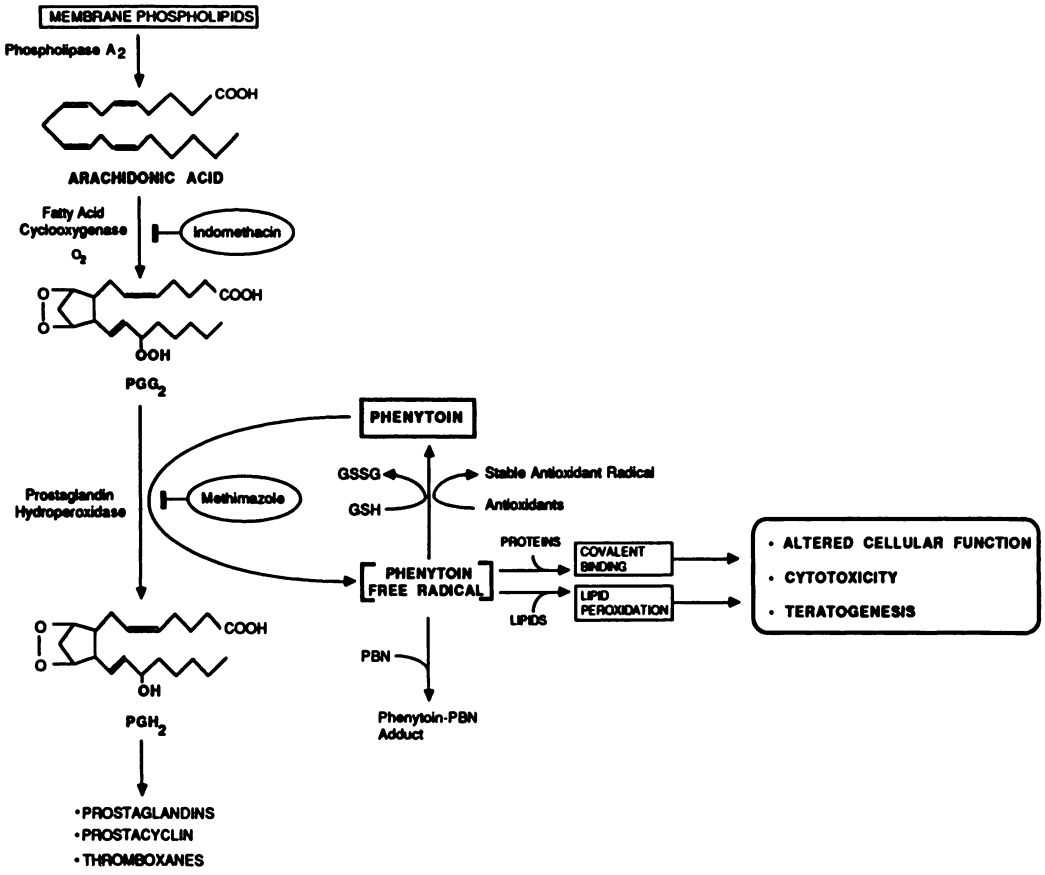

Fig. 6. Postulated relation of teratogenicity to the cooxidation of phenytoin by PGS. Arachidonic acid is oxidized by the cyclooxygenase activity of PGS to PGG_2, a hydroperoxy endoperoxide. This compound then is reduced by the hydroperoxidase activity of PGS to PGH_2, a hydroxy endoperoxide. Phenytoin may serve as a reducing cofactor for this reaction, being cooxidized to an electrophilic intermediate via a free radical pathway. The electrophilic species may bind covalently to tissue macromolecules. The free radical intermediate may initiate lipid peroxidation, react with PBN to form an adduct, or react with GSH or intracellular antioxidants to regenerate the parent compound phenytoin. Indomethacin and methimazole are, respectively, inhibitors of fatty acid cyclooxygenase and hydroperoxidase activities. Covalent binding, oxidant stress, and/or lipid peroxidation could lead to altered cellular function, cytotoxicity, and/or teratogenesis.

Discussion

The aim of this study was to evaluate the potential role of PGS and other enzymes with peroxidase activity in the biotransformation of phenytoin to reactive intermediates capable of interacting irreversibly with cellular macromolecules. Our results demonstrated the formation of reactive phenytoin intermediates produced by RSV, PGS, thyroid peroxidase, and horseradish peroxidase, as indicated by the covalent binding of radiolabeled phenytoin to tissue protein, the stimulation of oxygen consumption, and the detection of a free radical intermediate using ESR spectrometry. The dependence of covalent binding on arachidonic acid and its inhibition by the cyclooxygenase inhibitor indomethacin indicate that PGS is involved in the cooxidation of phenytoin in mouse lung, kidney and liver microsomes. Our data using PGS provide corroborative evidence that phenytoin is metabolized by this enzyme system, which supported a relatively large amount of phenytoin covalent binding to the BSA added to the incubation system. The ability of the hydroperoxide H_2O_2, with either thyroid peroxidase or horseradish peroxidase, to substitute for arachidonic acid in initiating covalent binding and the ability of the hydroperoxidase inhibitor methimazole to inhibit arachidonic acid-dependent covalent binding together indicate that the hydroperoxidase component of PGS is responsible for the cooxidative metabolism of phenytoin. This is consistent with the bioactivation of other substrates by PGS (10, 11).

The arachidonate-dependent stimulation of oxygen uptake observed with phenytoin supports the role of phenytoin as a reducing cofactor for the peroxidase activity of PGS. The mechanism could involve increased incorporation of oxygen into arachidonic acid during PGG_2 biosynthesis, because such stimulation of PGS by its chemical substrates is commonly observed (24, 25). Alternatively, the enhanced oxygen uptake could be the result of autooxidation of phenytoin by PGS, which would result in the incorporation of oxygen into phenytoin, as has been observed for such substrates such as phenylbutazone (26).

In addition to NADPH, various hydroperoxy compounds also can support cytochromes P-450-dependent drug oxidations (27–29). The possibility, therefore, exists that the covalent binding observed during PG biosynthesis was due to the interaction of PGG_2 with cytochrome P-450 in liver and lung microsomes. However, our evidence argues against this possibility. SKF 525A, which is an inhibitor of cytochromes P-450, was not inhibitory to covalent binding of radiolabeled phenytoin in microsomes fortified with arachidonic acid (Table 1). The modest enhancement on arachidonic acid-dependent binding may have been due to inhibition by SKF 525A of alternative cytochromes P-450-catalyzed routes of arachidonic acid oxidation (28, 29) and/or inhibition of the autooxidation of PGS (25).

The nature of the reactive intermediate of phenytoin formed from PG metabolism is a matter of speculation. The inhibition of covalent binding by GSH further indicates that an electrophilic or free radical intermediate is generated during cooxidation of phenytoin (Fig. 6). The inhibition of covalent binding by the free radical scavengers BSH, caffeic acid, and PBN in the cooxidation of phenytoin suggests that the reactive intermediate may be a free radical, as has been observed for many other compounds activated by the PGS system (11). In such a mechanism, the initial step is believed to be the interaction of

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

PGG_2 (formed from arachidonic acid) with the peroxidase, generating the corresponding alcohol and a compound I-type derivative of PGS. This enzymatic derivative would be two oxidizing equivalents above the resting state of the enzyme (30). Sequential one-electron reductions of the enzyme with reducing equivalents provided by the electron abstraction of phenytoin would reduce the protein back to the resting state and yield a free radical from phenytoin. A chemical precedent for a nitrogen-centered hydantoin free radical exists in the formation of the succinimidyl free radical (31), which involves a ring structure similar to that for hydantoin compounds. In those studies, the succinimidyl free radical was a nitrogen-centered free radical generated chemically from *N*-bromosuccinimide. A similar mechanism could hold true for horseradish peroxidase-catalyzed activation of phenytoin to a free radical. The ESR spin-trapping experiment provided direct evidence that the metabolism of phenytoin by PGS generated a free radical intermediate. An alternative possibility is that an epoxide of phenytoin could be formed by a free radical mechanism from PGS metabolism, as has been previously shown to occur with PGS-catalyzed metabolism of benzo(*a*)pyrene (32).

GSH, BHA, and caffeic acid could partially inhibit total phenytoin metabolism by serving as alternative cooxidizable substrates (33, 34). The ability of these free radical scavengers to inhibit NADPH-induced covalent binding of phenytoin metabolites may be due, in part, to the known capability of such compounds to inhibit mixed function oxidase activity at the concentrations used (35).

The extent of PGS-catalyzed covalent binding of radiolabeled phenytoin was observed to vary among different tissues. This result may be due to differences in relative concentrations of PGS. These preparations may also differ in endogenous peroxide content of microsomal lipid, which has been shown to support cooxidation reactions (36, 37). Small increases in the level of cellular peroxides can cause dramatic increases in cyclooxygenase activity (36, 37). Such variables could alter the absolute dose-response relation for arachidonic acid stimulation of phenytoin cooxidation.

PGS-dependent metabolism of drugs and other foreign chemicals has important implications with regard to the disposition of such compounds, because the biosynthesis of PGs occurs in many cell types (38). In the case of phenytoin, cooxidative bioactivation of phenytoin by PGS and other peroxidases may allow such extrahepatic tissues as lung, fetus, and thyroid to metabolize phenytoin to reactive intermediates (Figs. 2, 3, and 6). Such peroxidase-catalyzed bioactivation of phenytoin may play an important role in many of the multitude of toxic effects of phenytoin and other structurally related drugs, which in humans affect virtually every organ system. This pathway minimally may be important for phenytoin teratogenicity, which at least in mice, is reduced by *in vivo* pretreatment with either the irreversible PGS inhibitor acetylsalicylic acid, the antioxidant caffeic acid, or the free radical spin-trapping agent PBN (39). Conversely, murine phenytoin teratogenicity is enhanced by *in vivo* treatment with 12-*O*-tetradecanoylphorbol-13-acetate, a phorbol ester that stimulates phospholipase A_2, thereby releasing arachidonic acid intracellularly (40). Further *in vivo* support for the involvement of PGS-catalyzed bioactivation of phenytoin and structurally similar teratogens is the protection afforded by pretreatment with the PGS inhibitor acetylsalicylic acid against the murine teratogenicity of trimethadione and its *N*-demethylated metabolite, dimethadione (41). Peroxidase-catalyzed bioactivation also could explain differences in the embryopathic activities of the asymmetric hydantoin anticonvulsants mephenytoin and its *N*-demethylated metabolite nirvanol, wherein embryopathic activity resides with the *l*-isomers of each drug, which do not form an arene oxide intermediate (9). Bioactivation of phenytoin by thyroid peroxidase also could play a role in phenytoin teratogenicity, in that a reduction in fetal concentration of thyroid hormones, possibly due to thyroid dysfunction, has been implicated in the teratologic mechanism (1). Site-specific bioactivation by peroxidases in other tissues such as bone marrow could be involved in other toxic effects of phenytoin, including the pseudolymphoma syndrome. Additional studies are warranted to ascertain further the pharmacologic and/or toxicologic importance of cooxidation of phenytoin by PGS and other peroxidases.

In conclusion, our studies show that the cooxidation of phenytoin by PGS, thyroid peroxidase, or horseradish peroxidase results in the formation of a reactive intermediate that binds covalently to proteins, apparently via a free radical pathway. This mechanism of bioactivation may be relevant to the teratogenicity and possibly other toxic effects of phenytoin and structurally related compounds.

References

1. Wells, P. G., and R. D. Harbison. Significance of the phenytoin reactive arene oxide intermediate, its oxepin tautomer, and clinical factors modifying their roles in phenytoin-induced teratology, *Phenytoin-Induced Teratology and Gingival Pathology*, (T. M. Hassell, M. C. Johnston, and K. C. Dudley, eds.) Raven Press, New York, 83–108 (1980).
2. Gibson, J. E., and B. A. Becker. Teratogenic effects of diphenylhydantoin in Swiss-Webster and A/J Mice. *Proc. Soc. Exp. Biol. Med.* **128**:906–909 (1968).
3. Harbison, R. D., and B. A. Becker. Diphenylhydantoin teratogenicity in rats. *Toxicol. Appl. Pharmacol.* **22**:193–200 (1972).
4. McClain, R. M., and L. Langhoff. Teratogenicity in the New Zealand white rabbit. *Teratology* **21**:371–279 (1980).
5. Strickler, S. M., M. A. Miller, E. Andermann, L. V. Dansky, M.-H. Seni, and S. P. Spielberg. Genetic predisposition to phenytoin-induced birth defects. *Lancet* **1**:746–749 (1985).
6. Pantarotto, C., M. Arboix, P. Sezzano, and R. Abbruzzi. Studies on 5,5-diphenylhydantoin irreversible binding to rat liver microsomal proteins. *Biochem. Pharmacol.* **31**:1501–1507 (1982).
7. Lum, J. T., and P. G. Wells. Pharmacological studies in the potentiation of phenytoin teratogenicity by acetaminophen. *Teratology* **33**:53–72 (1986).
8. Wells, P. G., and I. B. Gesicki. Modulation of phenytoin teratogenicity by exogenous and endogenous inhibitors of oxidative drug metabolism. *Proc. Int. Congr. Pharmacol.* **9**:1772P (1984).
9. Wells, P. G., A. Kupfer, J. A. Lawson, and R. D. Harbison. Relation of *in vivo* drug metabolism to stereoselective fetal hydantoin toxicology in mouse: evaluation of mephenytoin and its metabolite, nirvanol. *J. Pharmacol. Exp. Ther.* **221**:228–234 (1982).
10. Marnett, L. J., P. Wlodawer, and B. Samuelsson. Co-oxygenation of organic substrates by the prostaglandin synthetase of sheep vesicular gland. *J. Biol. Chem.* **257**:8510–8517 (1975).
11. Eling, T. E., J. A. Boyd, G. A. Reed, R. P. Mason, and K. Sivarajah. Xenobiotic metabolism by prostaglandin endoperoxide synthetase. *Drug Metab. Rev.* **14**: 1023–1053 (1983).
12. Lasker, J. M., B. Sivarajah, R. P. Mason, B. Kalyanarayan, M. B. Abou-Donia, and T. E. Eling. A free radical mechanism of prostaglandin synthetase-dependent aminopyrine demethylation. *J. Biol. Chem.* **256**:7764–7767 (1981).
13. Rapp, N. S., T. V. Zenser, W. W. Brown, and B. B. Davis. Metabolism of benzidine by a prostaglandin-mediated process in renal inner medullary slices. *J. Pharmacol. Exp. Ther.* **215**:401–406 (1980).
14. Boyd, J. A., and T. E. Eling. Prostaglandin endoperoxide synthetase-dependent co-oxidation of acetaminophen to intermediates which covalently bind *in vitro* to rabbit renal medullary microsomes. *J. Pharmacol. Exp. Ther.* **219**: 659–664 (1981).
15. Savarajah, K., J. M. Lasker, T. E. Eling, and M. B. Abou-Donia. Metabolism of *N*-alkyl compounds during the biosynthesis of prostaglandins. *Mol. Pharmacol.* **21**:133–141 (1982).
16. Samuelsson, B. Biosynthesis of prostaglandins. *Biochem. Pharmacol.* **5**:109–128 (1969).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

18. Vane, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* **231**:232–235 (1971).
19. Balazs, C., E. Kiss, A. Leovey, and N. R. Farid. The immunosuppressive effect of methimazole on cell-mediated immunity is mediated by its capacity to inhibit peroxidase and to scavenge free oxygen radicals. *Clin. Endocrinol.* **25**:7–16 (1986).
20. Murota, S., and I. Morita. Prostaglandin synthesising system in rat liver: changes with aging and various stimuli. *Adv. Prostaglandin Thromboxane Res.* **8**:1495–1506 (1980).
21. Cottee, F., R. J. Flower, S. Moncada, J. A. Salmon, and J. R. Vane. Synthesis of 6-keto-$PGF_{1\alpha}$ by ram seminal vesicle microsomes. *Prostaglandins* **14**:413 (1977).
22. Korbut, R., J. Boyd, and T. Eling. Respiratory movements altered the generation of prostacyclin and thromboxane A_2 in isolated rat lungs: the influence of arachidonic acid pathway inhibitors on the ratio between pulmonary prostacyclin and thromboxane A_2. *Prostaglandins* **21**:491–503 (1981).
23. Sraer, J., W. Siess, L. Moulonguet-Doleris, J. Oudinet, F. Dray, and R. Ardaillou. *In vitro* prostaglandin synthesis by various rat renal preparations. *Biochem. Biophys. Acta* **710**:45–52 (1982).
24. Egan, R. W., P. H. Gale, and F. A. Kuehl. Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase. *J. Biol. Chem.* **254**:3295–3302 (1979).
25. Egan, R. W., J. Paxton, and F. A. Kuehl, Jr. Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J. Biol. Chem.* **251**:7329–7335 (1976).
26. Reed, G. A., I. O. Griffin, and T. E. Eling. Evaluation of prostaglandin H synthetase and prostacyclin synthetase by phenylbutazone. *Mol. Pharmacol.* **27**:109–114 (1985).
27. Kadlubar, F. F., K. C. Morton, and D. M. Zeigler. Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines. *Biochem. Biophys. Res. Commun.* **54**:1255–1261 (1973).
28. Oliw, E. H., and J. A. Oates. Oxygenation of arachidonic acid by hepatic monooxygenases. *Biochem. Biophys. Acta* **666**:327–340 (1981).
29. Oliw, E. H., P. Guengerich, and J. A. Oates. Oxygenation of arachidonic acid by hepatic monooxygenases. *J. Biol. Chem.* **257**:3771–3781 (1982).
30. Dolphin, D., A. Forman, D. C. Bang, J. Fajer, and R. H. Felton. Compounds I of catalase and horseradish peroxidase: π-cation radicals. *Proc. Natl. Acad. Sci. USA* **68**:614–618 (1971).
31. Chalfont, G. R., and M. J. Perkins. A probe for homolytic reactions in solution. IV. The succinimidyl radical. J. Chem. Soc. Sect. (B) 401–404 (1970).
32. Reed, G. A., E. A. Brooks, and T. E. Eling. Phenylbutazone-dependent epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene. *J. Biol. Chem.* **259**:5591–5595 (1984).
33. Duniec, Z., J. Robak, and R. Gryglewski. Antioxidant properties of some chemicals vs their influence on cyclooxygenase and lipooxidase activities. *Biochem. Pharmacol.* **32**:2283–2286 (1983).
34. Eling, T. E., J. R. Curtis, L. S. Harman, and R. P. Mason. Oxidation of glutathione to the thiyl free radical metabolite by prostaglandin H synthase: a potential endogenous substrate for the hydroperoxidase. *J. Biol. Chem.* **261**:5023–5028 (1986).
35. Albano, E., K. A. K. Lott, T. F. Slater, A. Stier, M. C. R. Stymons, and A. Tomasi. Spin-trapping studies on the free-radical products formed by metabolic activation of carbon tetrachloride in rat liver microsomal fractions, isolated hepatocytes and *in vivo* in the rat. *Biochem. J.* **204**:593–603 (1982).
36. Hemler, M. E., H. W. Cook, and W. E. M. Lands. Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch. Biochem. Biophys.* **193**:340–345 (1979).
37. Lands, W. E. M., and M. E. Hemler. *Biochemical and Clinical Aspects of Oxygen*, (W. Caughey, ed.). Academic Press, New York, 213 (1979).
38. Mitchell, M. D., S. P. Brennecke, S. Saeed, and D. M. Strickland. Arachidonic acid metabolism in the fetus and neonate, in *Biological Protection with Prostaglandins*, (M. Cohen, ed.). CRC Press, Boca Raton, FL 27–44 (1985).
39. Wells, P. G., J. T. Zubovits, S. Z. Wong, L. M. Molinari, and S. Ali. Modulation of phenytoin teratogenicity and embryonic covalent binding by acetylsalicylic acid, caffeic acid and α-phenyl-*N*-*t*-butylnitrone: implications for bioactivation by prostaglandin synthetase. *Toxicol. Appl. Pharmacol.* **97**:192–202 (1988).
40. Wells, P. G., and H. P. N. Vo. Effects of the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on phenytoin-induced embryopathy in mice. *Toxicol. Appl. Pharmacol.* **97**:398–405 (1988).
41. Wells, P. G., M. K. Nagai, and G. Spano Greco. Inhibition of trimethadione and dimethadione teratogenicity by the cyclooxygenase inhibitor acetylsalicylic acid: a unifying hypothesis for hydantoin anticonvulsants and structurally related compounds. *Toxicol. Appl. Pharmacol*, **97**:406–414 (1988).

Send reprint requests to: Peter G. Wells, Faculty of Pharmacy, University of Toronto, 19 Russell St., Toronto, Canada M5S 2S2.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012