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Phenytoin-Initiated Hydroxyl Radical Formation: Characterization by Enhanced Salicylate Hydroxylation

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SUMMARY

Bioactivation of phenytoin and related teratogens by peroxidases such as prostaglandin H synthase (PHS) may initiate hydroxyl radical (·OH) formation that is teratogenic. Salicylate is hydroxylated by ·OH at the third and fifth carbon atoms, forming 2,3- and 2,5-dihydroxybenzoic acids (DHBA). *In vivo* salicylate metabolism produces only the 2,5-isomer, so 2,3-DHBA formation may reflect ·OH production. In the present study, we validated the salicylate assay using the known ·OH generator paraquat and evaluated ·OH production by phenytoin. Female CD-1 mice were treated with paraquat (30 mg/kg, intraperitoneally) given 30 min after acetylsalicylic acid (ASA) (200 mg/kg, intraperitoneally). Blood was collected at 5, 15, and 30 min and 1 and 2 hr after paraquat, and plasma was analyzed for DHBA isomers and glucuronide conjugates by high performance liquid chromatography with electrochemical detection. Paraquat increased 2,3-DHBA formation 19.2-fold, with substantial interindividual variability in the time of maximal formation (p = 0.0001). The 2,3-DHBA glucuronide conjugates *in vivo* and in hepatic microsomal studies amounted to ~11% and 0.43%, respectively, of total 2,3-DHBA equivalents. To investigate putative ·OH production initiated via PHS-catalyzed phenytoin bioactivation, ASA was given 30 min before phenytoin (65 or 100 mg/kg, intraperitoneally), resulting in respective 7.6-fold (p = 0.02) and 14.2-fold (p = 0.003) increases in phenytoin-initiated maximal 2,3-DHBA formation. Maximal 2,3-DHBA formation was 2.1-fold higher when ASA was administered after rather than before the same dose (65 mg/kg) of phenytoin (p = 0.03), indicating ASA inhibition of PHS-catalyzed phenytoin bioactivation. Urinary analysis was much less sensitive, and the 2,5-isomer reflected enzymatic rather than ·OH-mediated hydroxylation. The paraquat studies demonstrate the importance of timing in accurately quantifying 2,3-DHBA formation and suggest that glucuronidation does not interfere. The substantial, dose-dependent initiation of 2,3-DHBA formation by phenytoin, and its inhibition by ASA, provide the first *in vivo* evidence that PHS-dependent ·OH formation could contribute to the molecular mechanism of phenytoin teratogenesis.

The treatment of epilepsy generally involves the use of anticonvulsant drugs such as phenytoin (diphenylhydantoin, Dilantin). Although phenytoin is teratogenic in many animal species (1) and humans (2), treatment usually is continued throughout pregnancy to avoid the dangers to both mother and fetus from uncontrolled seizures.

There are several postulated mechanisms by which phenytoin may initiate teratogenicity (3); these include receptor-mediated effects of the parent phenytoin compound, which reversibly binds to the glucocorticoid and/or other receptors, or the bioactivation of phenytoin to an electrophilic arene oxide, catalyzed by the NADPH-dependent P450. Although substantial evidence exists for a P450-catalyzed bioactivating pathway, discrepancies in this hypothesis may be explained by an alternative pathway involving peroxidase-catalyzed bioactivation of phenytoin (1).

Evidence from *in vivo* and *in vitro* studies suggest that peroxidases such as PHS and other enzymes such as LPOs can bioactivate phenytoin and related xenobiotics to a potentially teratogenic, reactive free radical intermediate (1, 4–6) (Fig. 1). The free radical, if not quickly detoxified by GSH, can oxidize and/or covalently bind to essential macromolecules (DNA, proteins, lipids) and/or initiate the formation of ROS, potentially initiating *in utero* death or teratogenesis. This mechanism could contribute to adverse reactions of phenytoin and related drugs in adults, including so-called idiosyncratic reactions (rash, fever, etc.) and reversible lymphoma, although this latter hypothesis has yet to be tested.

A potential molecular mechanism contributing to phenytoin-initiated teratogenesis may involve the production of ROS such as hydroxyl radicals (·OH). Both *in vivo* (7, 8) and in embryo culture (9), phenytoin can initiate oxidation of both

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ABBREVIATIONS: ASA, acetylsalicylic acid; DHBA, dihydroxybenzoic acid; HPLC, high performance liquid chromatography; LPO, lipoxygenase; P450, cytochrome P450; PBS, phosphate-buffered saline; PHS, prostaglandin H synthase; ROS, reactive oxygen species; UDPGA, uridine diphosphate glucuronic acid; APAP, acetaminophen (*N*-acetyl-*p*-aminophenol); TBARS, thiobarbituric acid reactive substances.

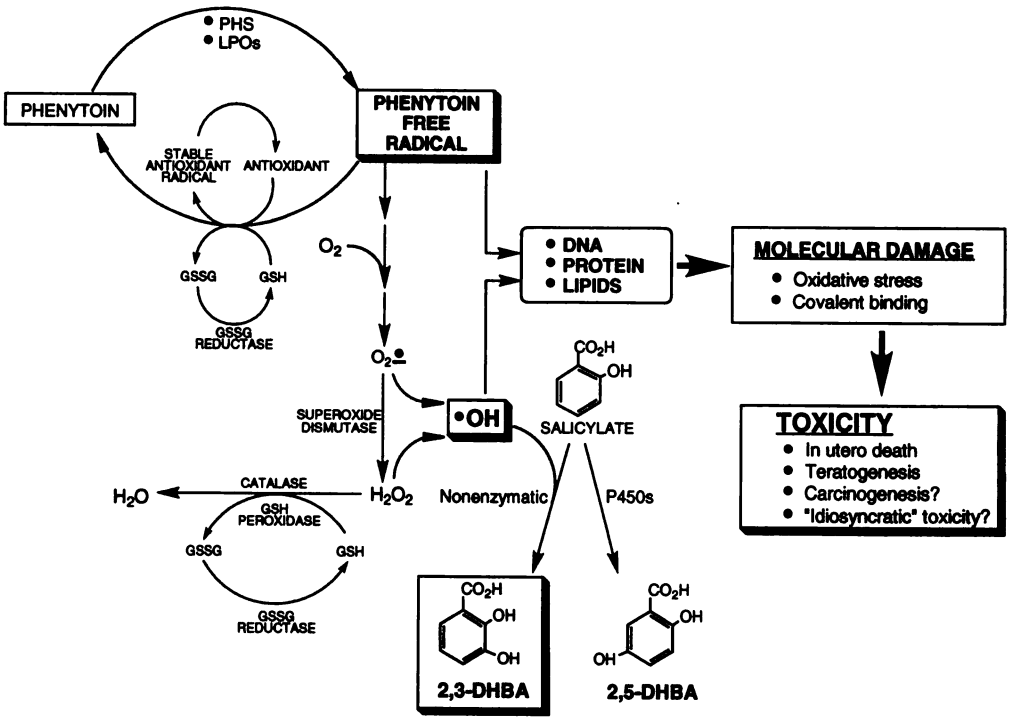

Fig. 1. Phenytoin bioactivation and subsequent salicylate hydroxylation by hydroxyl free radicals (·OH). Phenytoin may be bioactivated by peroxidases such as PHS, as well as by LPOs, to a putative phenytoin free radical. The phenytoin free radical may initiate the formation of reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$), H_2O_2, and ·OH. Both the phenytoin free radical and ROS can initiate molecular damage to DNA, protein, and lipid, potentially leading to teratogenesis and other toxicological sequelae. ·OH also can hydroxylate salicylate forming both the 2,3- and 2,5-DHBA, only the former of which is unique to this reaction. Thus, measurement of 2,3-DHBA may reflect ·OH production. GSH, glutathione; GSSG, oxidized glutathione.

maternal and embryonic DNA, protein, and lipids. The antioxidative enzymes superoxide dismutase and catalase inhibit phenytoin-initiated DNA and protein oxidation, as well as teratogenicity in embryo culture (9), corroborating the teratological relevance of phenytoin-initiated ROS production.

ASA, which has a half-life of ~15 min, is hydrolyzed to salicylate (2-hydroxybenzoic acid) in plasma, liver, erythrocytes, and synovial fluid (10). Salicylate is eliminated in the urine, either unchanged, conjugated with the endogenous amino acid glycine (aminoacetic acid) or with UDPGA, or hydroxylated (oxidized) by P450s, primarily to 2,5-DHBA, which may be conjugated with glycine, forming gentisuric acid (11, 12). Depending on the dose administered, salicylate has a half-life of 2–30 hr (10). Dose-dependent elimination occurs due to saturation of the formation of salicyluric acid (*o*-hydroxyhippuric acid, glycine conjugate at the carboxyl group) and salicyl ether glucuronide (phenolic glucuronide, glucuronidation at hydroxyl group), two of the major salicylate metabolites in humans (11).

Salicylate can be hydroxylated to both 2,5-DHBA and the relatively unique 2,3-isomer (2,3-DHBA) by ·OH, the latter of which appears to be an effective assay for ·OH formation (13). This reaction has been evaluated in many systems that produce ·OH, including hypoxanthine/xanthine oxidase (14) and ischemia/reperfusion (15). The hydroxylated products observed in these systems are 2,3-DHBA and 2,5-DHBA. However, because the latter isomer also is produced *in vivo* by P450s, quantification of 2,5-DHBA likely results in an overestimation of actual ·OH formation (16). Although 2,3-DHBA has been measured in healthy human volunteers, as well as in arthritic patients who had ingested aspirin (12, 17), this isomer was found only in relatively low concentrations and thus was suggested to be due to endogenous *in vivo* ·OH production (18). 2,3-DHBA is not produced by hepatic microsomal fractions obtained from rabbits and rats treated with P450 inducers (19), and no enzyme has been identified that can catalyze its formation (16). Thus, 2,3-DHBA formation appears to be an accurate measure of ·OH production *in vitro*. However, many if not most hydroxylated substrates are substantially conjugated with UDPGA *in vivo* (20), and it is not clear to what extent this glucuronidation may complicate the interpretation of *in vivo* studies of salicylate hydroxylation. Furthermore, in *in vivo* studies, it is unlikely that a single time point will accurately reflect the maximal xenobiotic-initiated formation of ·OH in different subjects.

Another consideration important to this study is that ASA can selectively and irreversibly inhibit the cyclooxygenase component but not the hydroperoxidase component of PHS (21). Previous *in vivo* and *in vitro* studies have demonstrated that pretreatment with ASA and other PHS inhibitors can reduce phenytoin teratogenicity, oxidation of molecular targets, and covalent binding to embryonic protein (1, 4, 5, 7, 8). These results suggest a role for PHS-catalyzed bioactivation of phenytoin to a reactive intermediate that initiates the formation of teratogenic ROS such as ·OH.

This study in CD-1 mice was designed to validate the 3-hydroxylation of salicylate to 2,3-DHBA as an *in vivo* measure of ·OH formation and to use this assay to determine the potential *in vivo* contribution of PHS-dependent ·OH formation to the molecular mechanism of phenytoin teratogenicity. Validation using paraquat, an herbicide known to initiate ·OH formation (22), included an assessment of the importance of interindividual variation in the time of peak DHBA formation in accurately estimating *in vivo* ·OH formation. Potentially confounding effects of glucuronidation were addressed by the direct *in vivo* administration of 2,3-DHBA and by *in vitro* glucuronidation studies using hepatic microsomes. The potential teratogenic contribution of ·OH was characterized according to its dose- and time-dependent initiation by phenytoin. The putative role of PHS-catalyzed phenytoin bioactivation in ·OH formation was determined by comparing 2,3-DHBA formation when ASA was administered after phe-

nytoin with that when ASA was given before, when it can more effectively inhibit phenytoin bioactivation. The results provide insights into both the molecular mechanism of phenytoin teratogenesis and potential approaches in teratological risk assessment.

Materials and Methods

Animals

Virgin female CD-1 mice (Charles River Canada Ltd., St. Constant, Quebec, Canada) were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY) and maintained in a temperature-controlled room with a 12-hr light/dark cycle. Food (Laboratory Rodent Chow 5001, PMI Feeds, St. Louis, MO) and tap water were provided *ad libitum*.

Chemicals

Phenytoin (diphenylhydantoin sodium), paraquat (1,1'-dimethyl-4,4'-bipyridylium), APAP, ASA, 2,3- and 2,5-DHBA, and β-glucuronidase were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium bicarbonate and sodium hydroxide were purchased from BDH (Toronto, Ontario, Canada) and Fisher Scientific Co. (Toronto, Ontario, Canada), respectively. All other reagents used were of analytical or HPLC grade.

2,3-DHBA Glucuronidation Studies

***In vitro* glucuronidation.** Hepatic microsomes (final protein concentration, 1 mg/ml) from TCDD-induced mice were incubated with 2,3-DHBA (50 μM) and UDPGA (10 mM) in 0.1 M PBS, pH 7.4, in glass borosilicate test tubes. Reactions were incubated at 37° for either 30 min, 1 hr, or 3 hr in a shaking water bath (final incubation volume, 300 μl). Reactions were stopped by adding 75 μl of 10% trichloroacetic acid. Samples were then centrifuged at 1000 × *g* for 20 min at 4° (model TJ-6, Beckman,). The pellet was discarded, and supernatants were extracted and analyzed for 2,3-DHBA and its glucuronides as described below.

***In vivo* glucuronidation.** CD-1 mice were injected intraperitoneally with 2,3-DHBA (200 mg/kg) and asphyxiated with CO_2, and blood was collected via cardiac puncture at either 15 or 30 min or 1 hr. Then, ~600–800 μl of whole blood was obtained from each mouse and immediately microcentrifuged (Beckman Microfuge B) at 18,000 × *g* for 2 min. Plasma (100 μl) was removed and divided in half. Both aliquots were incubated for 20 hr at 37° with or without β-glucuronidase (32 μg/ml). Peak disappearance by β-glucuronidase was used to confirm 2,3-DHBA glucuronide peaks. Incubations were similar to that described above except that β-glucuronidase was added instead of microsomes and UDPGA.

In Vivo ·OH-Catalyzed Salicylate Hydroxylation in Murine Plasma

All solutions were prepared immediately before use with an injection volume of 0.01 ml/g body weight, and the route of administration was intraperitoneal. Paraquat was dissolved in 0.9% sodium chloride with final pH 7.5 and administered in a dose of 30 mg/kg, which is the LD_{50} for mice (23). Phenytoin was dissolved in 0.002 N sodium hydroxide with final pH 10.8–10.9 and administered in a teratogenic dose of 65 or 100 mg/kg (1). ASA was dissolved in a vehicle of 0.25 M sodium bicarbonate and injected in an intraperitoneal dose of 200 mg/kg 30 min before paraquat or phenytoin or 30 min after phenytoin. All treatment groups contained a minimum of three mice. Controls received an identical volume of drug vehicle, pH adjusted where appropriate, at times corresponding to ASA, paraquat, or phenytoin administration.

All blood samples were taken via tail tip amputation and analyzed for plasma concentrations of 2,3-DHBA and 2,5-DHBA. In the paraquat study, blood was collected from the same mouse at 5, 15, and 30 min and 1 and 2 hr. In the phenytoin study, blood was collected at 15 and 30 min and 1, 2, 3, and 4 hr after ASA or phenytoin. Blood was collected with heparinized capillary tubes (Drummond Microcaps, Drummond Scientific Co., Broomall, PA) and immediately microcentrifuged (Beckman) at 18,000 × *g* for 2 min to separate plasma from red blood cells. Plasma (40 μl) was then transferred to a new microcentrifuge tube (Sarstedt, Montreal, Quebec, Canada) and kept on ice until the end of the experiment. The plasma was then extracted as described below.

Detection and Quantification

Plasma or supernatants (*in vitro* study) were mixed with 10 μl of 0.1 mM APAP (internal standard), 25 μl of 1 M HCl, (Mallinckrodt, Paris, KY) and 500 μl of diethyl ether (AnalaR, BDH, Toronto, Ontario, Canada). The mixture was vortexed for 10 sec at room temperature and then microcentrifuged for 2 min to allow for separation of diethyl ether and aqueous layers. The top diethyl ether layer (containing 2,3-DHBA, 2,5-DHBA, and APAP) was extracted and placed into a glass borosilicate test tube. A 500-μl aliquot of diethyl ether was added to the remaining bottom layer; the mixture was processed as described above; and the diethyl ether layer was transferred to the same test tube. The addition and removal of diethyl ether were performed at least four times. The diethyl ether was evaporated under a stream of nitrogen. After evaporation, the dried residue was reconstituted with 250 μl of the mobile phase (97% sodium acetate/citric acid 0.03 M, 3% methanol).

HPLC coupled with electrochemical detection (ESA model 5100A) was used to detect the ASA metabolites, including 2,3-DHBA and APAP, according to modified methods of Grootveld and Halliwell (12). Briefly, an isocratic procedure was used, with a mobile phase consisting of 97% 0.03 M sodium acetate/citric acid and 3% methanol. The flow rate was set at 1.0 ml/min. Similar to Floyd *et al.* (14), the electrochemical detector potential (oxidizing potential of +0.8 V) was optimized for ASA metabolites by creating a hydrodynammic voltammogram (data not shown). All samples were measured against authentic standards of 2,3- and 2,5-DHBA.

Statistical Analysis

Statistical significance of differences between treatment groups in each study was determined by Student's *t* test or one-factor analysis of variance as appropriate using a standard, computerized statistical program (Statsview, Abacus Conceptus, Berkeley, CA). DHBA data from paraquat studies were logarithmically normalized before statistical analysis. The level of significance was $p < 0.05$.

Results

2,3-DHBA Glucuronidation Studies

β-Glucuronidase hydrolysis and *in vivo* glucuronidation. HPLC chromatographic peaks for two glucuronide conjugates of 2,3-DHBA in plasma were characterized by HPLC peak disappearance on incubation with β-glucuronidase, which almost completely eliminated both peaks (up to 99%) (Figs. 2 and 3). The peak plasma concentration of both 2,3-DHBA and its two glucuronides occurred at 30 min and was almost completely eliminated within 1 hr. Both glucuronides amounted to a maximum of only 11% of the total 2,3-DHBA plasma concentration (Fig. 3).

***In vitro* glucuronidation.** Further verification of 2,3-DHBA glucuronidation was established in a hepatic microsomal system incubated with the uridine diphosphate-glucuronosyltransferase cofactor UDPGA and 2,3-DHBA for either 30 min or 1 or 3 hr. There was a significant time-dependent increase in 2,3-DHBA glucuronidation that was maximal at the final sampling time (3 hr) ($p = 0.0001$) (Fig. 3). However,

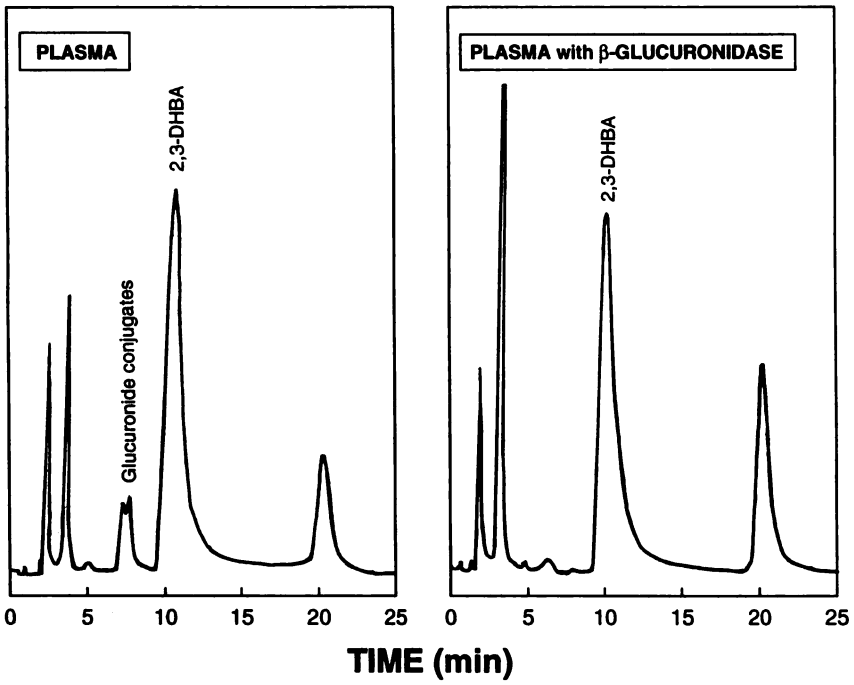

Fig. 2. Analysis of plasma concentrations of 2,3-DHBA and its glucuronide conjugates by HPLC. Incubation of plasma samples with β-glucuronidase was used to cleave and thereby identify glucuronide conjugates. HPLC peaks were identified with the use of authentic standards.

2,3-DHBA glucuronidation amounted to only 0.43% of the 2,3-DHBA concentration.

Paraquat-Initiated ·OH Formation and Salicylate Hydroxylation in Murine Plasma

Paraquat-treated mice had substantially increased plasma 2,3-DHBA formation over the entire 2-hr sampling period ($p \leq 0.04$) (Fig. 4). There was a remarkable interindividual variability in the time of maximal 2,3-DHBA elevation by paraquat, from 0.1 to 1.0 hr, with any single time substantially underestimating formation in some animals. When the maximal value was chosen for each animal, independent of time, there was a 19.2-fold increase in the mean maximal 2,3-DHBA formation in the paraquat-treated group compared with vehicle controls ($p = 0.0001$) (Fig. 4, *inset*).

In contrast, formation of the 2,5-DHBA metabolite, which is catalyzed by both the P450s and ·OH, was decreased in paraquat-treated mice at early sampling times (0.1 and 0.25 hr) ($p < 0.02$) (Fig. 5). However, mean maximal 2,5-DHBA concentration in the paraquat-treated group was not significantly decreased compared with vehicle controls (Fig. 5, *inset*).

Phenytoin-Initiated ·OH Formation and Salicylate Hydroxylation in Murine Plasma

Phenytoin at both low and high doses after ASA pretreatment initiated a dose- and time-dependent increase in 2,3-DHBA formation above that of vehicle controls ($p < 0.05$) (Fig. 6). The low and high phenytoin doses, respectively, increased mean maximal 2,3-DHBA formation by 7.6-fold ($p = 0.02$) and 14.2-fold ($p = 0.003$) above that of vehicle controls (Fig. 6). The time of maximal 2,3-DHBA elevation by phenytoin varied from 0.25 to 1 hr (data not shown). 2,3-DHBA concentrations initiated by the higher dose of phenytoin were significantly higher than with the lower dose at most time points ($p < 0.02$) (Fig. 6), although the mean maximal concentrations were not significantly different (Fig. 6, *inset*).

When the lower phenytoin dose was injected before ASA, there was a higher elevation in 2,3-DHBA formation over the 4-hr sampling period compared with the group given phenytoin after ASA (Fig. 7). The mean maximal 2,3-DHBA formation in the group given phenytoin before ASA was >2-fold higher than the group given ASA before the same dose of phenytoin ($p = 0.03$) (Fig. 7, *inset*).

The 2,5-isomer was differentially affected by pretreatment with the low and high doses of phenytoin (Fig. 5). Although not significant at individual time points, the lower dose of phenytoin appeared to decrease 2,5-DHBA formation over the entire sample period compared with vehicle controls, and there was a significant 49% decrease in mean maximal 2,5-DHBA concentration ($p = 0.04$) (Fig. 5, *inset*). In contrast, the higher phenytoin dose significantly increased 2,5-DHBA formation at 30 min and 1 hr (Fig. 5), although there was no increase in the mean maximal 2,5-DHBA concentration. The mean maximal 2,5-DHBA concentration initiated by the higher dose of phenytoin was 3-fold higher than observed with the lower dose ($p = 0.02$) (Fig. 5, *inset*).

Urinary Dihydroxybenzoic Acid Formation

Mice pretreated with ASA and the lower phenytoin dose showed no increase in urinary 2,3-DHBA concentration over vehicle controls (Fig. 8). However, pretreatment with the same low phenytoin dose given before rather than after ASA showed respective 3-fold ($p = 0.0001$) and 1.8-fold ($p = 0.05$) increases in 2,3- and 2,5-DHBA formation over vehicle controls, and 2,3-DHBA was increased 2.5-fold above the group given the same dose of phenytoin after ASA ($p = 0.002$). Similar increases in both 2,3- and 2,5-DHBA occurred in the group pretreated with ASA followed by the high phenytoin dose ($p < 0.05$) (Fig. 8).

Discussion

Recent attention has focused on free radical-mediated damage as a putative mechanism of various pathological

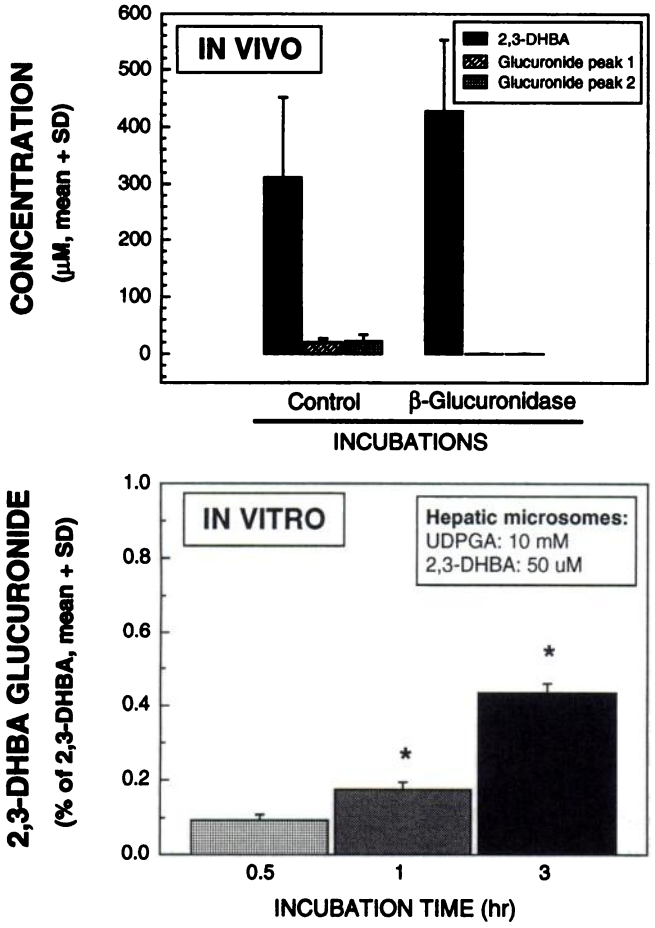

Fig. 3. *In vivo* and *in vitro* characterization of 2,3-DHBA glucuronidation. *Top*, 2,3-DHBA glucuronidation in female CD-1 mice 30 min after injection with 2,3-DHBA (200 mg/kg, intraperitoneally). *Bottom, in vitro* 2,3-DHBA glucuronidation catalyzed by TCDD-induced hepatic microsomal uridine diphosphate-glucuronosyltransferases. 2,3-DHBA (50 μM) and UDPGA (10 mM) were incubated with hepatic microsomal protein (1 mg/ml) for 0.5, 1, and 3 hr at 37° in a shaking water bath. Asterisks indicate a significant difference from other groups ($p < 0.05$).

conditions, including neurodegenerative diseases, carcinogenesis (24), and teratogenesis (1). Salicylate hydroxylation through the formation of 2,3-DHBA, as distinct from 2,5-DHBA, has been postulated to be an effective means of detecting both *in vivo* (12, 17) and *in vitro* (19) ·OH formation. In this study, we characterized salicylate hydroxylation as an *in vivo* probe for xenobiotic-initiated ·OH formation using the known free radical initiator paraquat, and we used this approach in determining the *in vivo* production of ·OH by phenytoin.

Initial *in vivo* and *in vitro* studies were conducted to determine the extent of 2,3-DHBA glucuronidation, which could cause a substantial underestimation of 2,3-DHBA formation. The conjugation of hydroxylated xenobiotics with UDPGA, catalyzed by uridine diphosphate-glucuronosyltransferases, frequently is a major route of elimination (20). Many xenobiotics, such as acetaminophen (25), benzo[*a*]pyrene (26) and, in particular, salicylate (11), are predominantly eliminated via glucuronidation, with glucuronide conjugates excreted in the urine and, to a lesser extent, in the feces. Two glucuronide conjugates of 2,3-DHBA were detected *in vivo* and in *in vitro* hepatic microsomal studies (Figs. 2 and 3). However, even after direct injection of 2,3-DHBA, only 11% was recovered as glucuronide conjugates *in vivo*. *In vitro* studies with UDPGA confirmed the formation of glucuronide conjugates of 2,3-DHBA; the lower percentage of conjugation (0.43%) compared with *in vivo* glucuronidation is expected under *in vitro* conditions, particularly in the absence of detergents. Thus, unlike most xenobiotics, 2,3-DHBA is not substantially glucuronidated in mice, which generally are similar to humans and more active than rats, in their ability to glucuronidate xenobiotics such as the analgesic drug acetaminophen (27, 28). Accordingly, the formation of glucuronide conjugates should not interfere critically with the *in vivo* estimation of ·OH by 2,3-DHBA production.

Xenobiotic-initiated salicylate hydroxylation was investigated with the herbicide paraquat, which can cause lipid peroxidation (23). Paraquat is metabolized by an NADPH-dependent cytochrome reductase that reduces paraquat to a free radical (23). *In vitro* studies with paraquat, using the spin trapping agent 5,5-dimethyl-1-pyrroline-1-oxide, have shown that paraquat initiates superoxide anion and ·OH formation (22). A recent *in vitro* study using either a xanthine oxidase/hypoxanthine or rat hepatic microsomal system investigated paraquat-initiated salicylate hydroxylation (29). This *in vitro* study showed a slight increase in 2,3-DHBA, suggesting increased ·OH formation; however, the results were not significant.

Our study accordingly provides the first evidence for *in vivo* paraquat-initiated ·OH formation measured by 2,3-DHBA formation. Mice treated with paraquat had substantially increased 2,3-DHBA formation over the entire 2-hr sampling period, indicating sustained paraquat-initiated ·OH formation ($p \leq 0.04$) (Fig. 4). Maximal 2,3-DHBA formation occurred at substantially different times and amounts in each individual mouse, which may indicate an important factor for the variable susceptibity seen in paraquat toxicity (30). The mean maximal 2,3-DHBA formation was 19.2-fold higher in paraquat-treated mice over vehicle controls ($p = 0.0001$) (Fig. 4). These results not only corroborate previous studies demonstrating that paraquat initiates ·OH formation but also show that the timing of ·OH production *in vivo* varies considerably in individual mice, indicating the importance of multiple samplings from each mouse for accurate quantification. Multiple sampling in human studies over time may prove to be critical in determining the accurate peak production of ·OH in any single individual. For example, a recent *in vivo* study measuring both plasma salicylate hydroxylation (2,3- and 2,5-DHBA) and oxidative stress (TBARS) in healthy humans failed to show a positive correlation between 2,3-DHBA and TBARS (31). However, this study took measurements at a single point. Given the temporal variability in ·OH formation observed in our study and the expectation that TBARS formation would follow a similarly variable but not necessarily congruent temporal pattern, studies based on single sampling times may prove to be prohibitively inaccurate.

Subsequent *in vivo* studies investigated PHS-dependent phenytoin bioactivation and ·OH formation. PHS contains both a cyclooxygenase and hydroperoxidase component that, respectively, can oxidize arachidonic acid to prostaglandin G_2, which is then reduced to prostaglandin H_2. In the latter reaction, hydroperoxidases can use xenobiotics such as phe-

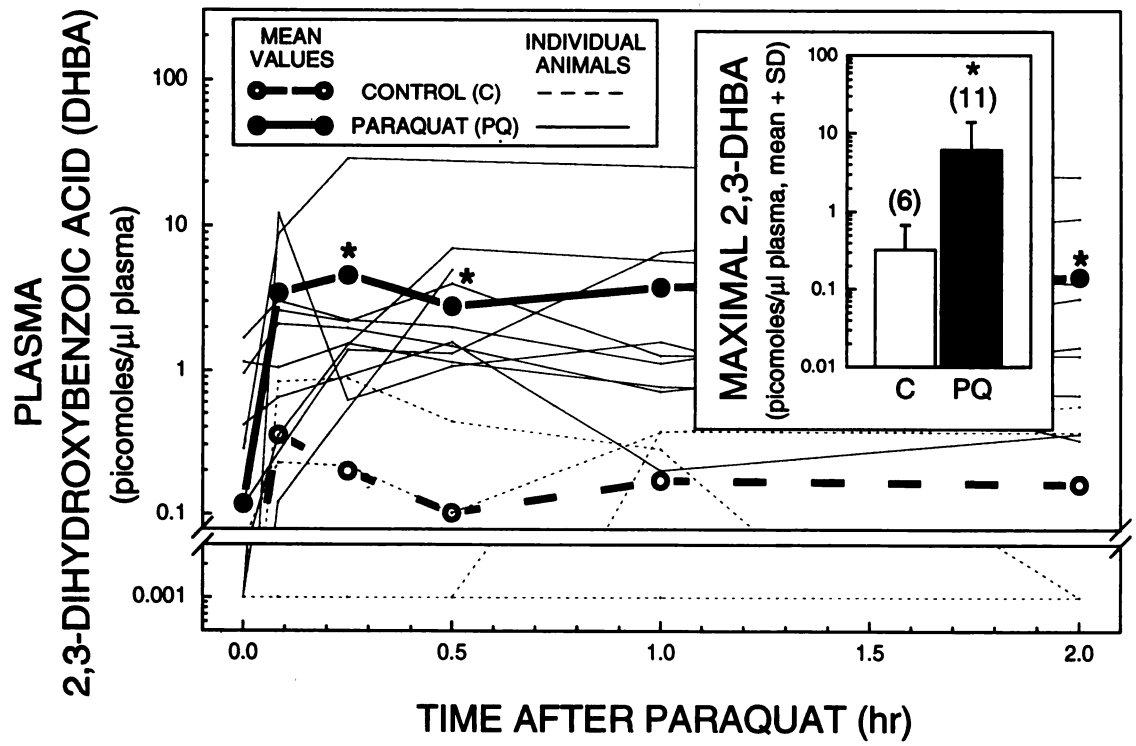

Fig. 4. Paraquat-initiated hydroxyl radical (·OH) formation as measured via 2,3-DHBA production. Mice were injected with ASA (200 mg/kg, intraperitoneally) and 30 min later with paraquat. Blood was collected from each animal at 5, 15, and 30 min and 1 and 2 hr after ASA. *Thin lines*, plasma 2,3-DHBA concentrations in individual mice. *Thick lines*, mean values at each time for the respective paraquat or control groups. In the control group, three of six mice did not have detectable concentrations of 2,3-DHBA at any time. Undetectable concentrations were assigned an arbitrary value of 0.001. *Inset*, mean maximal peak of 2,3-DHBA for individual mice, regardless of the time of occurrence. The number of animals is given in parentheses. *, Difference from respective controls ($p < 0.05$).

nytoin as a source of reducing equivalents, concomitantly cooxidizing the xenobiotic to a free radical (32).

The putative phenytoin free radical (1, 6) potentially may initiate the production of ROS, catalyzed by transition metals such as iron and copper. Free radical intermediates such as the paraquat radical are capable of reducing Fe^{3+} complexes directly, which can further react with H_2O_2 to form ·OH (33). Free radicals in general also may initiate oxidation in the form of lipid peroxidation, which indirectly can lead to the formation of ·OH.

Catalytic iron may be available *in vivo* as iron loosely bound to membrane lipids, DNA, and phosphate complexes such as ADP (18, 34). In addition, ferritin is associated with lipid peroxidation in the presence of ascorbate and H_2O_2, possibly by increasing ·OH formation (35). ·OH production, in the presence of excess H_2O_2, also is increased by methemoglobin (36) and hemosiderin (35); the latter is a weak promoter of ·OH formation. Ascorbate may function in mobilizing iron from ferritin (35), and an excess of H_2O_2 may decompose heme, releasing iron (36), both of which allow iron to participate in the generation of ·OH. On the other hand, transferrin and lactoferrin seem incapable of catalyzing the formation of ·OH (18, 37).

After ASA pretreatment, phenytoin in a dose-dependent fashion increased plasma 2,3-DHBA formation over the 4-hr sampling period (Fig. 6), as well as the mean maximal 2,3-DHBA formation (Fig. 6, *inset*) ($p < 0.05$). Phenytoin-initiated ·OH formation in turn may initiate teratogenic oxidative stress. Previous studies show that phenytoin teratogenicity, oxidation of molecular targets (DNA, protein, and lipids), and covalent binding to protein are reduced by the iron chelator deferoxamine, a variety of antioxidants, and/or free radical spin trapping agents (1, 7, 8). It is remarkable that the mean maximal plasma concentration of 2,3-DHBA initiated by the low dose of phenytoin (Fig. 6, *inset*) was almost half that initiated by an LD_{50} dose of paraquat (Fig. 4, *inset*). This dose of phenytoin is not associated with maternal lethality but is highly teratogenic in mice (1). Thus, although plasma concentrations of 2,3-DHBA may reflect the overall potential for various xenobiotics to initiate ·OH formation in a particular species, the nature and severity of toxicological expression will vary considerably due to other factors, possibly including tissue-specific bioactivation, detoxification, antioxidative cytoprotection, and molecular target repair and redundancy.

The potential toxicological importance of PHS-catalyzed bioactivation was evident in the study by comparison of the same dose of phenytoin given before or after ASA. ASA, in addition to serving as a substrate for 2,3-DHBA formation, is an inhibitor of the cyclooxygenase component of PHS. Maximal 2,3-DHBA formation was 2.1-fold higher when the low dose of phenytoin was given before ASA compared with the same phenytoin dose given after ASA, when PHS was inhibited (Fig. 7). 2,3-DHBA formation initiated by the low dose of phenytoin given before ASA was equivalent to that initiated by the high dose given after ASA (Figs. 6 and 7). Interestingly, putative phenytoin-initiated ·OH formation was not inhibited completely by ASA, suggesting that enzymes other than PHS, such as LPOs, may contribute to phenytoin bioactivation. This hypothesis is consistent with the partial protection by ASA observed in *in vivo* studies of phenytoin

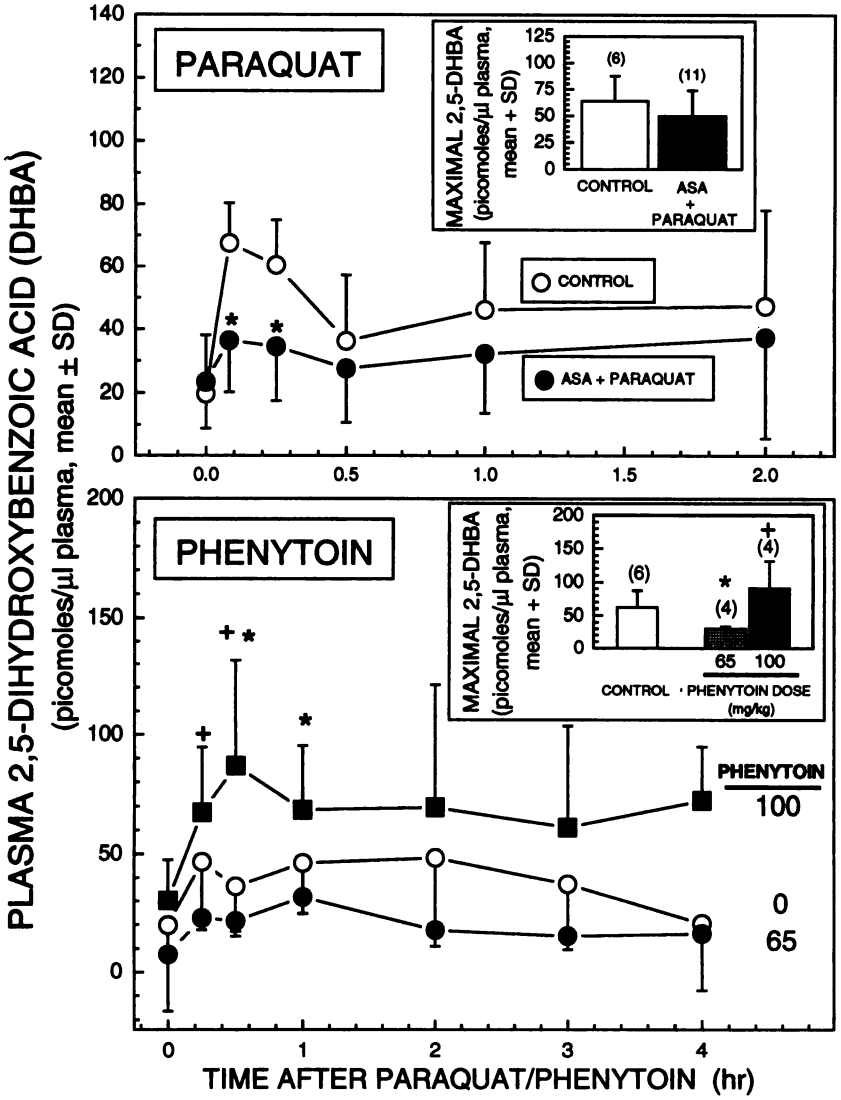

Fig. 5. Formation of the 2,5-isomer of DHBA in mice treated with ASA (200 mg/kg, intraperitoneally) followed by paraquat (30 mg/kg, intraperitoneally) or phenytoin (65 or 100 mg/kg, intraperitoneally). *Top*, plasma 2,5-DHBA formation from paraquat and vehicle control groups. *Bottom*, plasma 2,5-DHBA formation from phenytoin (65 and 100 mg/kg) and vehicle control groups. *Insets*, mean maximal peak of plasma 2,5-DHBA. *, Differences from vehicle control. +, Differences from mice treated with ASA plus the lower dose (65 mg/kg) of phenytoin ($p < 0.05$).

teratogenicity (1) and the apparently more complete protection by eicosatetraynoic acid, a dual inhibitor of PHS and LPOs (5).

Earlier *in vivo* studies using ASA as an inhibitor of PHS and subsequent phenytoin bioactivation and toxicity showed that ASA pretreatment, at a dose of 10 mg/kg (5% of dose in this study), decreased phenytoin-induced cleft palates and phenytoin covalent binding to embryonic protein (1). This suggested PHS-dependent phenytoin bioactivation and teratogenesis, which was corroborated by *in vivo* and embryo culture studies in which phenytoin embryopathy was reduced by the dual PHS/LPO inhibitor eicosatetraynoic acid (4, 5). Similar embryoprotective effects of ASA were seen with the structurally related anticonvulsants trimethadione and dimethadione (1) and the sedative/hypnotic drug thalidomide (38). Recent *in vivo* studies also have demonstrated that phenytoin-initiated lipid and protein oxidation and degradation were decreased by ASA pretreatment at a dose of 10 mg/kg (7).

Subsequent studies have demonstrated that phenytoin can initiate DNA oxidation, as measured by the formation of 8-hydroxy-2′-deoxyguanosine, *in vivo*, *in vitro* (8), and in embryo culture (9). *In vivo* phenytoin-initiated DNA oxidation was inhibited by ASA or the free radical spin trapping agent phenylbutylnitrone, supporting, respectively, the importance of PHS-catalyzed bioactivation and free radical-mediated toxicity. In embryo culture, phenytoin-initiated DNA oxidation and embryopathy were completely inhibited by either superoxide dismutase or catalase, indicating a fundamental role for ROS in mediating phenytoin teratogenesis. DNA is a likely teratologically relevant molecular target because transgenic knock-out mice deficient in the p53 tumor suppressor gene, which facilitates DNA repair, are more susceptible to the teratogenicity of phenytoin (39) and another DNA-damaging teratogen, benzo[*a*]pyrene (40).

Interestingly, unlike 2,3-DHBA, the 2,5-isomer was consistently decreased in paraquat-treated mice over the 2-hr sampling period, suggesting paraquat inhibition of P450-catalyzed salicylate hydroxylation ($p < 0.05$) (Fig. 5). This inhibition may reflect direct paraquat inactivation of the P450 enzyme(s) responsible for salicylate hydroxylation or may be due to intracellular depletion of the P450 cofactor NADPH. Similarly, when the lower dose of phenytoin was administered after ASA pretreatment, there were decreases in 2,5-DHBA both over the 4-hr sampling period and in the mean maximal 2,5-DHBA production ($p < 0.05$) (Fig. 5, *in-*

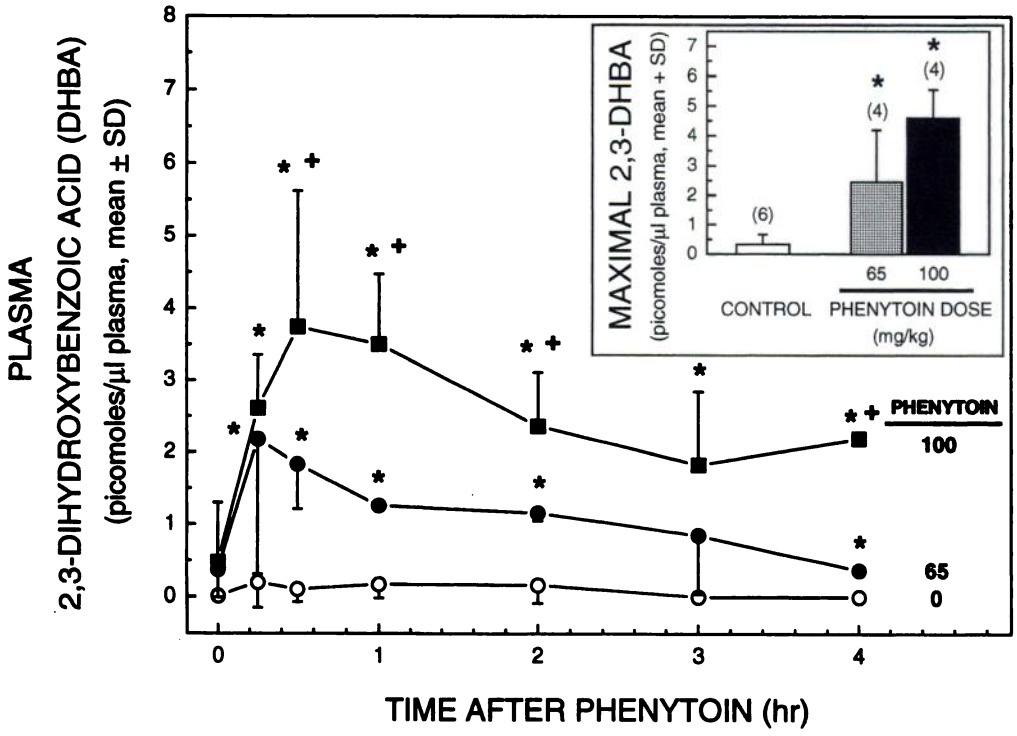

Fig. 6. Phenytoin-initiated, dose-dependent *in vivo* hydroxyl radical (·OH) formation measured via 2,3-DHBA formation. Mice were injected with ASA (200 mg/kg, intraperitoneally) and 30 min later with phenytoin at a dose of either 65 or 100 mg/kg, intraperitoneally. *Inset*, mean maximal peak of 2,3-DHBA from mice treated with either the phenytoin vehicle or the low or high dose of phenytoin. The number of animals is given in parentheses. *, Difference from the time-matched vehicle control. +, Difference from the lower phenytoin dose ($p < 0.05$).

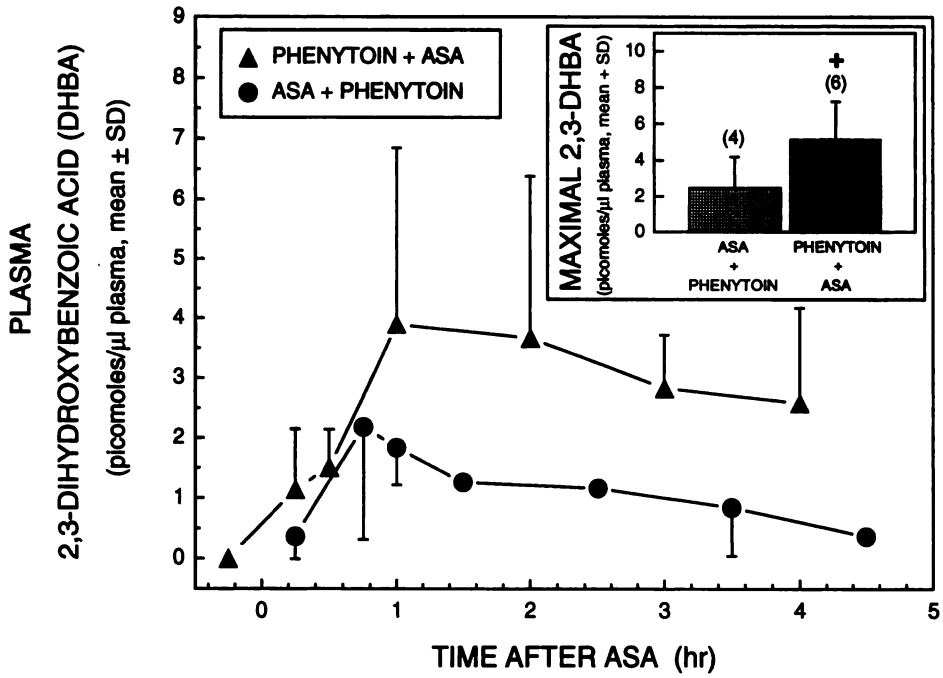

Fig. 7. Comparison of phenytoin-initiated *in vivo* hydroxyl radical (·OH) formation when ASA was administered before or after phenytoin. ASA is an inhibitor of PHS, which catalyzes the bioactivation of phenytoin to a free radical intermediate (see Fig. 1). All animals received the same dose of phenytoin (65 mg/kg, intraperitoneally) and ASA (200 mg/kg, intraperitoneally). *Inset*, mean maximal peak of 2,3-DHBA from mice treated with either ASA followed by phenytoin or phenytoin followed by ASA. +, Difference from mice treated with ASA followed by phenytoin ($p < 0.05$).

set). Although the higher dose of phenytoin did increase 2,5-DHBA formation, likely reflecting ·OH production, the decreased formation of this isomer with paraquat and the lower phenytoin dose show that the 2,5-isomer primarily reflects enzymatic hydroxylation and is not suitable for ·OH estimation. This caution was first raised by Halliwell *et al.* (16), and studies that have used 2,5-DHBA formation to quantify ·OH production (15) are difficult to interpret.

When urine was used to analyze salicylate metabolites, the results were considerably less sensitive than analysis of plasma samples, although they were corroborative. Unlike plasma analysis, the low dose of phenytoin given after ASA did not alter urinary 2,3- or 2,5-DHBA formation over a 4-hr collection period (Fig. 8), demonstrating that urinary analysis is remarkably less sensitive in detecting *in vivo* ·OH production. Otherwise, urinary and plasma analysis were corroborative, with the urinary 2,3- and 2,5-DHBA formation being increased both by the high dose of phenytoin and by the low phenytoin dose when given before ASA. As with the plasma analysis, the 2,3-DHBA formation initiated by the low dose of phenytoin given before ASA, to avoid prior inhibition of PHS by ASA, was equivalent to that initiated by the high dose of phenytoin given after ASA, demonstrating the importance of PHS-catalyzed bioactivation of phenytoin in

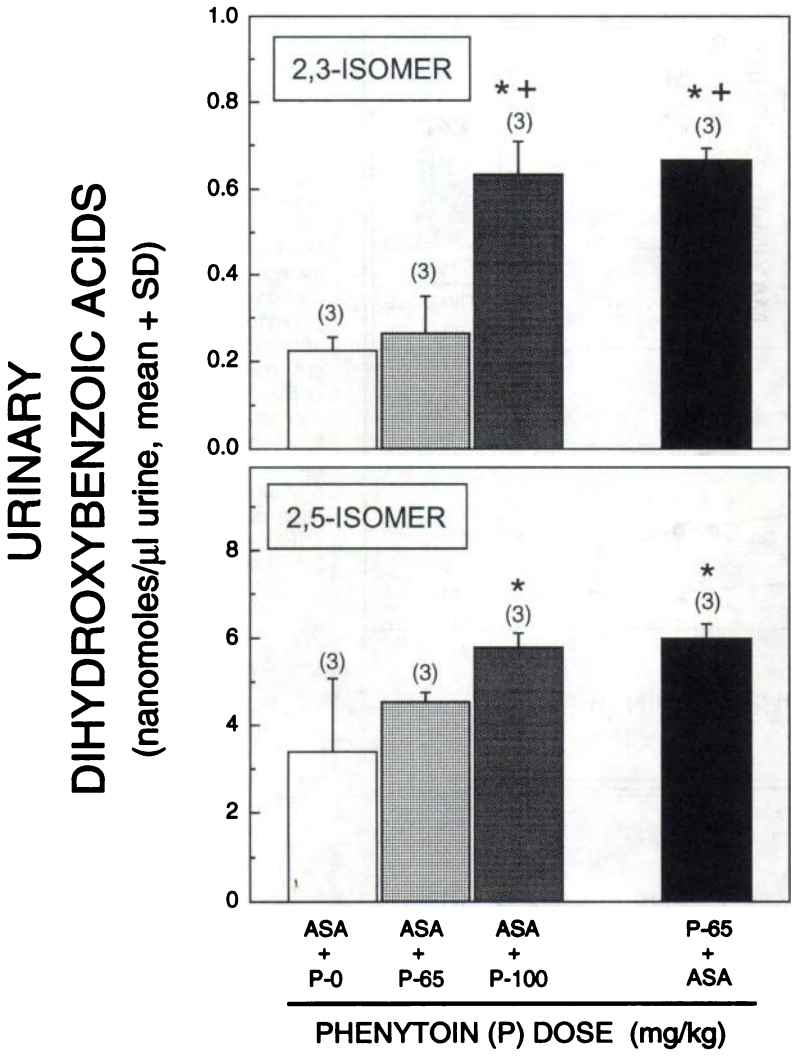

Fig. 8. Urinary concentrations of 2,3- and 2,5-DHBA. Mice were either treated with ASA (200 mg/kg, intraperitoneally) followed by phenytoin vehicle or phenytoin (65 or 100 mg/kg, intraperitoneally) or were first treated with phenytoin (65 mg/kg, intraperitoneally) followed by ASA (200 mg/kg). Urine was collected for 4 hr after the last injection and analyzed. *, Differences from vehicle control. +, Differences from mice treated with ASA followed by the lower dose (65 mg/kg) of phenytoin (*ASA + P-65*) ($p < 0.05$).

·OH formation. In general, however, *in vivo* studies depending on only urinary 2,3-DHBA data may fail to detect ·OH formation at lower levels.

Various techniques (e.g., oxidation of molecular targets, amino acid hydroxylation) have been investigated in an attempt to measure *in vivo* ·OH formation and oxidative stress. This is particularly difficult in humans, where many of the techniques can be invasive or involve toxic probes. We demonstrate that salicylate hydroxylation measured in plasma and urine can be an effective tool not only in determining the molecular mechanism of xenobiotic-initiated toxicity but also in monitoring and assessing individual susceptibility. This technique may prove valuable in cases where drugs such as phenytoin, a known teratogen, must be taken throughout pregnancy to control seizures.

In summary, the paraquat studies demonstrated the importance of multiple sampling in accurately determining 2,3-DHBA formation, the relative insensitivity of urinary compared with plasma analysis, the inappropriateness of the 2,5-isomer of DHBA in estimating ·OH formation, and the low potential for confounding by 2,3-DHBA glucuronidation. In the phenytoin studies, our results provide the first direct *in vivo* evidence for PHS-dependent phenytoin bioactivation and subsequent ·OH formation as a potential molecular mechanism in phenytoin embryotoxicity. This approach may prove useful in human teratological risk assessment.

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