DECREASES IN PHENYTOIN HYDROXYLATION ACTIVITIES CATALYZED BY LIVER MICROSONAL CYTOCHROME P450 ENZYMES IN PHENYTOIN-TREATED RATS

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ABSTRACT:
Phenytoin, 5,5-diphenylhydantoin, is a widely used anticonvulsant agent with a variety of toxicities, including drug interactions. The formation of four oxidative metabolites, 4'-hydroxylated (4'-HPPH), 3'-hydroxylated (3'-HPPH), a catechol (3',4'-diHPPH), and the 3',4'-dihydrodiol form of phenytoin was examined in rat liver microsomes. In 11 cDNA-expressed rat P450 enzymes tested, CYP2C6 had the highest activities in 4'- and 3'-HPPH formation from phenytoin, followed only by CYP2C11. In contrast, CYP2C11 had high activity for 3',4'-diHPPH formation from 4'-HPPH, followed by CYP2C6. The rates of 4'-HPPH and 3',4'-diHPPH formation from phenytoin in liver microsomes in the presence of NADPH were significantly decreased by oral administration of phenytoin (300 mg/kg for 20 days) to rats, despite the increase in P450 contents.

However, the cumene hydroperoxide-supported formation of 3',4'-dihydriodiol and 4'-HPPH from phenytoin was induced by phenytoin administration. Hydrogen peroxide formation in reaction mixtures with NADPH was induced by the administration of phenytoin; however, the coupling ratio of phenytoin oxidation was decreased in phenytoin-induced liver microsomal P450 systems. These results suggested that phenytoin could not stimulate its own apparent oxidative metabolism by liver P450s induced with phenytoin administration. The increase of unmetabolized phenytoin and by-products of oxygen generated in the phenytoin-induced liver microsomal P450 system may be involved in phenytoin-related drug toxicity.

Cytochrome P450 (P450) comprises a super gene family of enzymes that catalyze the oxidation of a great number of xenobiotic chemicals, such as drugs, toxic chemicals, and carcinogens, as well as endobiotic chemicals (Wrighton and Stevens, 1992). The contributions of P450s to metabolism in rats and humans have been well demonstrated, particularly regarding the issue of drug clearance (Gueneger, 1997). P450s are not self-sufficient enzymes, and the microsomal enzymes require NPR as an electron carrier from NADPH to function as monooxygenases. In the catalytic cycle of P450s, some of the consumed NADPH is converted to hydrogen peroxide or water instead of oxidized substrate (Gorsky et al., 1984).

Phenytoin, 5,5-diphenylhydantoin, is widely used as an anticonvulsant agent. It has received much attention concerning its variety of toxicities, e.g., teratogenicity (Wellis et al., 1989), carcinogenicity in animals (Diwan et al., 1993), drug-induced hepatitis (Haruda, 1997), the nonlinearity in its blood concentrations in humans (Odani et al., 1997), autoantibody formation in phenytoin-treated patients (Leeder et al., 1992, 1998), and drug-drug interactions with different kinds of medicines (Nation et al., 1990). It has been pointed out that the phenytoin epoxide shown in Fig. 1 is an active intermediate. A secondary hydroxylated metabolite, 3',4'-diHPPH, from phenytoin can bind to microsomal proteins to cause autoimmunity (Munns et al., 1997). There are many drugs that may increase and/or decrease phenytoin serum levels or that may be affected by phenytoin (Nation et al., 1990); for example, rifampicin (Kay et al., 1985), ticlopidine (Klaassen, 1998), theophylline (Adebayo, 1988), valproic acid, ibuprofen, and naproxen (Dasgupta and Timmerman, 1996). Although most of these adverse interactions are thought to be attributable to the induction and inhibition of P450-mediated drug metabolism, some of the causative mechanisms are still unknown (Klaassen, 1998).

Phenytoin metabolism in humans has been extensively reported in vitro (Yasumori et al., 1999; Komatsu et al., 2000) and in vivo (Maguire, 1988; Szabo et al., 1990). Four oxidative metabolites are reported, i.e., 4'-HPPH, 3'-HPPH, 3',4'-diHPPH, and 3',4'-dihydrodiol (Maguire, 1988; Szabo et al., 1990). It has been suggested that phenytoin is mainly oxidized to 4'-HPPH by CYP2C9 and to a minor extent by CYP2C19 in humans (Baijai et al., 1996). However, the roles of P450s responsible for the formation of other metabolites are still unclear. Recently, we reported that CYP2C9, CYP2C19, and CYP3A4 have different contributions to liver microsomal 3',4'-diHPPH formation from primary hydroxylated metabolites in individual
humans (Komatsu et al., 2000). Studies of phenytoin metabolism in animals have been also performed (Chow et al., 1980; Billings, 1983; Doecke et al., 1990). Although it was shown that the rates of 4′-HPPH formation from phenytoin by rat liver microsomes were 10 times higher than those by human liver microsomes (Munns et al., 1997), species difference between rats and humans with regard to the roles of P450 in phenytoin oxidation are unclear. Phenytoin is known to induce some P450 forms (Fleishaker et al., 1995; Ghosal et al., 1996; Yamazaki et al., 1996), but there is little information regarding whether phenytoin administration might stimulate its own (phenytoin) metabolism in rats and humans (autoinduction) (Edeki and Brase, 1995; Chetty et al., 1998).

To understand the mechanisms of phenytoin toxicity such as P450-mediated drug interactions, we investigated phenytoin metabolism in rats using rat liver microsomes and rat P450 enzymes. In the present study, oxidative metabolites from phenytoin formed by liver microsomes and the effects of P450 induction by phenytoin on phenytoin hydroxylation activities in rat liver microsomes were investigated. Total phenytoin oxidation activities in the presence of NADPH were decreased in liver microsomes that had P450s induced by phenytoin.

**Materials and Methods**

**Chemicals.** Phenytoin and styrene 7,8-oxide were obtained from Wako Pure Chemicals (Osaka, Japan). 4′-HPPH, 3′-HPPH, and styrene glycol were from Aldrich (Milwaukee, WI). Racemic mephenytoin, testosterone, its metabolites, and other reagents used in this study were obtained from sources described previously or were of the highest qualities commercially available (Komatsu et al., 2000).

**Enzyme Preparations.** Male Wistar rats (6 weeks old, Japan SLC, Hamamatsu, Japan) were intraperitoneally treated daily for 5 days with phenytoin (100 mg/kg) as well as typical P450 inducers, including β-naphthoflavone (50 mg/kg), phenobarbital (80 mg/kg), isoniazid (100 mg/kg), and dexamethasone (50 mg/kg). Liver microsomes and cytosol from untreated male and female rats and those treated with the chemicals mentioned above (7 weeks old) were prepared as described previously (Guengerich, 1994). Liver microsomes were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol. In separate experiments, male rats (7 weeks old) were treated with phenytoin (300 mg/kg) suspended in 0.5% carboxymethyl cellulose sodium salt solution via oral administration for 20 days. Liver microsomes from these rats (10 weeks old) were prepared as mentioned above. CYP1A2/NPR membranes were prepared as reported by Parikh et al. (1997); this cDNA plasmid was kindly provided by Dr. F. Peter Guengerich (Vanderbilt University School of Medicine, Nashville, TN). Recombinant P450 enzymes expressed in microsomes of insect cells infected with baculovirus containing rat P450 and NPR cDNA inserts (Supersomes) were obtained from GENTEST (Woburn, MA). We used them without further modifications in this study because appropriate catalytic activities by those P450 enzymes are provided in the manufacturer’s data sheets. Insect microsomes expressing only NPR and β were also used as a control. Anti-rat CYP2C13 and anti-CYP3A2 antibodies for inhibition studies were from Daiichi Pure Chemicals (Tokyo, Japan). The cross-reactivities of these anti CYP2C- and CYP3A-antibodies to the same CYP2C subfamily members (CYP2C6, -2C11, -2C12, and -2C13) and CYP3A subfamily members (CYP3A1 and -3A2) of rat P450 enzymes are provided in the data sheets by the manufacturer.

**Enzyme Assays.** Phenytoin, 4′-HPPH, and 3′-HPPH hydroxylation activities were determined as described previously (Komatsu et al., 2000). Substrates (1–100 μM) were incubated at 37°C for 30 min with rat liver microsomes (1.0 mg/ml) and an NADPH-generating system in 50 mM potassium phosphate buffer (pH 7.4). In some experiments, insect microsomes containing recombinant rat P450 enzymes (0.20 μM) coexpressing NPR and β were also used as a control. Anti-rat CYP2C13 and anti-CYP3A2 antibodies for inhibition studies were from Daiichi Pure Chemicals (Tokyo, Japan). The cross-reactivities of these anti CYP2C- and CYP3A-antibodies to the same CYP2C subfamily members (CYP2C6, -2C11, -2C12, and -2C13) and CYP3A subfamily members (CYP3A1 and -3A2) of rat P450 enzymes are provided in the data sheets by the manufacturer.
mained by HPLC with a C18 (5-μm) analytical column (150- × 4.6-mm i.d.; Mightysil RP-18, Kanto Chemical, Tokyo, Japan). We present the activities for the formation of 3',4'-diHPPH and 3',4'-dihydrodiol on the basis of the chromatographic peak height, using 4'-HPPH as a standard. The basic substrate concentration of 100 μM phenytoin was chosen because steady-state therapeutic concentrations of phenytoin are ~10 to 20 μg/ml (~40–80 μM) in humans (Bajpai et al., 1996).

Styrene oxide hydration activities were determined as described previously (Kerr et al., 1989) with minor modifications. Briefly, rat liver microsomes (0.20 mg/ml) were incubated at 37°C for 10 min with 0.5 mM styrene oxide in 0.1 M Tris-HCl buffer (pH 9.0). The reaction (total volume, 0.5 ml) was terminated by adding n-hexane (0.5 ml). After centrifugation at 900g for 10 min, 1 N HCl (25 μl) was added to a portion of the aqueous phase (0.25 ml). The formed styrene glycol was measured by HPLC with a C18 (5-μm) analytical column. The elution was conducted with a mixture of 25% methanol at a flow rate of 1.5 ml/min, and the eluate was monitored at 208 nm. The activities of epoxide hydrolase were corrected by subtracting their nonenzymatic components.

NADPH oxidation (in the absence of a regenerating system) was measured by the rate of the decrease of absorbance at 340 nm at 25°C with rat liver microsomes (0.2 mg/ml) in the presence of 0.4 mM NADPH (Cummings and Prough, 1983). H2O2 formation was determined by the methods described by Hildebrandt et al. (1978). Liver microsomes (1.0 mg/ml) were incubated at 37°C for 10 min with or without substrates in the presence of 0.5 mM NaN3 and the NADPH-generating system. Reactions (total volume, 0.5 ml) were terminated by adding 0.75 ml of trichloroacetic acid (3% w/v). The precipitated samples were allowed to stand at 4°C for 20 min before centrifugation at 9,000g. The supernatant samples were incubated at 25°C for 10 min and were measured for absorbance at 480 nm.

LC/MS/MS Analysis. LC/MS analysis was performed using a Hewlett Packard 1100 series HPLC system equipped with a Finnigan MAT TSQ7000. The capillary potential was 4.5 kV, and the source temperature was 200°C. The metabolites were separated on an Inertsil ODS-2 column (150- × 2.1-mm i.d.) using a mobile phase of 30% CH3CN:0.05% HCO2H (v/v) at a flow rate of 0.2 ml/min. Identification of 3',4'-dihydrodiol and 3',4'-diHPPH was based on the MS/MS characteristics.

Other Assays. The concentrations of P450 and b5 (Omura and Sato, 1964) and protein (Lowry et al., 1951) were estimated as described previously. NADPH-cytochrome c reduction activities were determined using Δε550 = 21 mM−1 cm−1 and an assumed specific activity of 3.0 μmol of cytochrome c reduced/min/mmol of NPR based on purified human and rabbit NPR preparations (Parikh et al., 1997). The contents of rat P450 enzymes in liver microsomes were estimated by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development (Western blotting) (Guengerich et al., 1982).

Statistical Analysis. Kinetic analysis for substrate oxidations by P450 enzymes was performed using a computer program KaleidaGraph (Synergy Software, Reading, PA) designed for nonlinear regression analysis. Statistical analysis was carried out using the computer program Instat (GraphPad Software, San Diego, CA) designed for Student’s t test after the assumption of equal variance with an F test.

Results

Identification of Oxidative Metabolites from Phenytoin by Rat Liver Microsomes. Phenytoin metabolites formed by liver microsomes were determined by HPLC analysis as shown in Fig. 2. Phenytoin (100 μM) was incubated at 37°C for 30 min with rat liver microsomes (1.0 mg of protein/ml) in the presence of an NADPH-
Phenytoin (100 μM) was incubated at 37°C for 30 min with recombinant rat P450 enzymes (0.20 μM P450) in the presence of an NADPH-generating system, and the formation of 4′-HPPH (A) and 3′-HPPH (B) are shown. 4′-HPPH (100 μM) was incubated as mentioned above, and 3′,4′-diHPPH formation is shown (C). Insect microsomes expressing only NPR and βs were used as control. Results are presented as means of duplicate determinations.

Phenytoin Oxidation Activities Catalyzed by Liver Microsomes from Rats Treated with Typical P450 Inducers. The effects of P450 inhibitors and anti-P450 antibodies on phenytoin oxidation by rat liver microsomes were determined (Table 1). 4′-HPPH formation from 100 μM phenytoin was markedly inhibited by the addition of SKF-525A and weakly by metyrapone. These chemicals similarly inhibited the 3′,4′-diHPPH formation from 100 μM 4′-HPPH. Anti-CYP2C antibodies inhibited 4′-HPPH formation from phenytoin and 3′,4′-diHPPH formation from 4′-HPPH to 44 and 28%, respectively. Anti-CYP3A antibodies weakly inhibited these hydroxylations.

4′-HPPH formation was increased linearly up to 60 min under the conditions used. The kinetic parameters for 4′-HPPH and 3′,4′-diHPPH formation from phenytoin (10–200 μM) catalyzed by rat liver microsomes were determined for a 30-min incubation. Apparent Kₘ and Vₘₐₓ values (±S.E.) of 4′-HPPH formation were 60 ± 8 μM and 202 ± 12 pmol/min/mg of microsomal protein, respectively. For 3′,4′-HPPH formation from phenytoin, the apparent Kₘ and Vₘₐₓ values were 13 ± 1 μM and 21 ± 1 pmol/min/mg of microsomal protein, respectively.

The formation of four metabolites from phenytoin by rat liver microsomes was observed. The effects of typical P450 inducers on phenytoin oxidation were also examined (Fig. 5). The formation of 4′-HPPH, a main metabolite, was not induced by treatment with any of the drugs tested and decreased by phenobarbital treatment (Fig. 5A). On the other hand, 3′-HPPH formation was induced by phenobarbital (Fig. 5B). The rates of 3′,4′-diHPPH formation, a second major metabolite, catalyzed by female rat liver microsomes were lower than those by male rats, and β-naphthoflavone treatment decreased 3′,4′-diHPPH formation (Fig. 5C). 3′,4′-Dihydrodiol formation was increased by treatment with dexamethasone, phenobarbital, and β-naphthoflavone (Fig. 5D).

Phenytoin (100 mg/kg for 5 days) was treated intraperitoneally to male rats as typical P450 inducers as described above, and the effects on phenytoin oxidation were also examined (Fig. 5). Phenytoin treatment significantly decreased 4′-HPPH and 3′,4′-diHPPH formation to ~40 and 30%, respectively (Fig. 5, A and C). Although the formation of 3′-HPPH and 3′,4′-dihydrodiol was significantly induced by phe...
and 100 μM of 4'-HPPH (100 μM) was incubated with rat liver microsomes in the presence of chemicals or antibodies. Results are presented as means of duplicate determinations. Values in parentheses indicate percentage of control activities (none).

### Table 1

**Effects of P450 inhibitors and anti-P450 antibodies on oxidation of phenytoin and 4'-HPPH by rat liver microsomes**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Product Formation</th>
<th>4'-HPPH from Phenytoin</th>
<th>3',4'-dihydrodiol from 4'-HPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>pmol/min/mg protein</td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>(I) Chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKF-525A</td>
<td>50</td>
<td>233 (98)</td>
<td>105 (44)</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>50</td>
<td>233 (98)</td>
<td>105 (44)</td>
</tr>
<tr>
<td>None*</td>
<td>50</td>
<td>144 (100)</td>
<td>92 (31)</td>
</tr>
<tr>
<td>Anti-CYP2C</td>
<td>20</td>
<td>105 (44)</td>
<td>102 (28)</td>
</tr>
<tr>
<td>Anti-CYP3A</td>
<td>20</td>
<td>239 (100)</td>
<td>287 (100)</td>
</tr>
<tr>
<td>Preimmune</td>
<td>233 (98)</td>
<td>287 (100)</td>
<td></td>
</tr>
<tr>
<td>None†</td>
<td>20</td>
<td>102 (28)</td>
<td>105 (44)</td>
</tr>
<tr>
<td>None‡</td>
<td>20</td>
<td>102 (28)</td>
<td>105 (44)</td>
</tr>
<tr>
<td>None</td>
<td>233 (98)</td>
<td>287 (100)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>144 (100)</td>
<td>92 (31)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>112 (78)</td>
<td>207 (71)</td>
<td></td>
</tr>
</tbody>
</table>

| (II) Antibody    |                   |                         |                             |
| None*            | 20                | 233 (98)                | 102 (28)                    |
| Preimmune        | 20                | 105 (44)                | 102 (28)                    |
| Anti-CYP2C       | 239 (100)         | 287 (100)               |                             |
| Anti-CYP3A       | 239 (100)         | 287 (100)               |                             |
| Preimmune        | 233 (98)          | 105 (44)                | 102 (28)                    |

*0.8 μM of total P450.
†0.08 μM of total P450.

Phenytoin (100 μM) or 4'-HPPH (100 μM) was incubated with rat liver microsomes in the presence of chemicals or antibodies. Results are presented as means of duplicate determinations. Values in parentheses indicate percentage of control activities (none).

Activities of Phenytoin Oxidation and Catechol Formation from Hydroxylated Phenytoin in Liver Microsomes from Phenytoin-Administered Rats. To investigate the effects of chronic administration of phenytoin on its metabolism, male rats were orally administered phenytoin (300 mg/kg for 20 days). The rates of 4'-HPPH and 3',4'-dihydrodiol formation from phenytoin catalyzed by liver microsomes were significantly decreased to one half by rats treated with phenytoin (Table 2). Although the rates of 3',4'-dihydrodiol formation from phenytoin catalyzed by liver microsomes were not affected, testosterone 16β-dehydroxylation activities were increased by the administration of phenytoin. NPR content, O-deethylation activities of 7-ethoxycoumarin and ethoxyresorufin, and microsomal epoxide hydrolase activities toward styrene oxide were also induced by phenytoin.

An analysis for measuring the mechanism-based inhibition caused by 3',4'-dihydrodiol formation (10 μM of 3'-HPPH) revealed that the inhibition was less than 20% in 4'-HPPH formation from phenytoin in either untreated or phenytoin-administered rats. These results suggested that the low catalytic activities of phenytoin oxidation in liver microsomes induced by phenytoin would not be involved in the mechanism-based inhibition caused by reactive 3',4'-dihydrodiol.

**Comparison of Phenytoin Oxidation Activities by Liver Microsomes from Phenytoin-Treated Rats Supported by CuOOH and NADPH.** The CuOOH-supported phenytoin oxidation activities catalyzed by rat liver microsomes were significantly induced by phenytoin (Table 4). The activities of 3',4'-dihydrodiol formation were 10-fold higher than those of 4'-HPPH formation. These results suggested that the decrease of phenytoin oxidation activities by NADPH-dependent phenytoin-induced liver microsomes was due to the low coupling ratio. To determine the activated oxygen that was not used for substrate oxidations, the formation of hydrogen peroxide in liver microsomes was measured with an NADPH-generating system (Table 5). Oxidized NADPH was measured in the absence of a regenerating system. In the absence of exogenous substrates, NADPH oxidation and hydrogen peroxide formation were induced by phenytoin, indicated by the P450 contents. In the presence of testosterone, both product formation activities (2.1-fold) and the coupling ratio (testosterone oxidations per NADPH oxidation, 1.7-fold) were increased in the phenytoin-treated rats. In contrast, when phenytoin was used as a substrate, NADPH oxidation and hydrogen peroxide formation were increased to 155 and 262%, respectively, but phenytoin oxidations were decreased to 27% by the treatment with phenytoin. In the

As expected, phenytoin significantly induced by 1.9-fold the total P450 as determined by CO-sensitive spectra (Table 3). The induction of CYP2B and CYP3A enzymes by phenytoin were confirmed both by the immunocytchemical determination and by the testosterone 16β- and 6β-hydroxylation activities. Although the levels of CYP2C enzymes were not affected, testosterone 16α-hydroxylation activities were increased by the administration of phenytoin. NPR content, O-deethylation activities of 7-ethoxycoumarin and ethoxyresorufin, and microsomal epoxide hydrolase activities toward styrene oxide were also induced by phenytoin.

**Fig. 5.** Effects of phenytoin and typical P450 inducers on phenytoin oxidation in rat liver microsomes.

Phenytoin (100 μM) was incubated at 37°C for 30 min with rat liver microsomes (1.0 mg/ml) in the presence of an NADPH-generating system. Data are mean ± S.D. for five rats untreated or treated intraperitoneally with indicated drugs as described under Materials and Methods. Significantly different from untreated male rats (*p < 0.05; **p < 0.01).
presence of 100 m of 4-HPPH, 3'-diHPPH formation activities as indicated, 4, 3'-diHPPH, and 3', 4'-di-HPPH formation catalyzed by rat liver microsomes. These peaks were confirmed by comigration with authentic standards, LC/MS analysis, and/or the effects of typical cofactors for catechol O-methyltransferase (Figs. 2 and 3). The secondary hydroxylated metabolite, 3', 4'-diHPPH, from phenytoin has been studied with respect to covalent adduct binding to microsomal proteins to cause an autoimmune reaction (Munns et al., 1997). Similarly, the importance of catechols (primary hydroxylated metabolites) of estrogens with regard to their toxicity has also been reported (Zhu and Conney, 1998). Hydroxysterogens formed by P450 enzymes could be converted to semiquinone and activated oxygen, which would cause free radical-mediated damage to lipids, proteins, and DNA. A catechol metabolite of phenytoin may also generate activated oxygen and work as a toxic intermediate.

In previously reported covalent binding studies of phenytoin (Roy and Snodgrass, 1988, 1990), glutathione (0.5–5 mM), an inhibitor that can trap epoxide intermediate(s), and 1,1,1-trichloropropene 2,3-epoxide (1–2 mM), an inhibitor for epoxide hydrolase, had been used. In our preliminary study, glutathione (3–5 mM) weakly decreased 4'-HPPH, 3'-HPPH, and 3', 4'-diHPPH formation, and the effects of 1,1,1-trichloropropene 2,3-epoxide were small in rat liver microsomes (results not shown). These results suggested that the role of epoxide hydrolase in phenytoin oxidation would be minor and that a phenytoin epoxide intermediate would be generated by liver microsomal P450 systems (Fig. 1), but it would have a small contribution to phenytoin oxidation or, if formed, convert rapidly to the phenolic form.

It has been reported that 4'-HPPH formation from phenytoin was mainly catalyzed by CYP2C9 and partly by CYP2C19 in humans (Bajpai et al., 1996). Recently, we showed the involvement of CYP2C9, CYP2C19, and CYP3A4 in 3', 4'-diHPPH formation from primary hydroxylated phenytoin by human liver microsomes (Komatsu et al., 2000). In this study, the important roles of the CYP2C subfamily in phenytoin oxidation by rat liver microsomal P450 enzymes were shown. When recombinant rat P450 enzymes were used, CYP2C6 had the highest catalytic activities for 4'-HPPH, 3'-HPPH, and 3', 4'-diHPPH formation (Table 1). The level of CYP2C6 expression has been reported not to be affected by age and sex (Waxman et al., 1985). Phenytoin 4'-hydroxylation was inhibited by anti-CYP2C antibodies in rat liver microsomes (Komatsu et al., 2000). This fact suggested that the role of epoxide hydrolase in phenytoin oxidation would be minor and that a phenytoin epoxide intermediate would be generated by liver microsomal P450 systems (Fig. 1), but it would have a small contribution to phenytoin oxidation or, if formed, convert rapidly to the phenolic form.

**Discussion**

Phenytoin is commonly used as an anticonvulsant agent and has been studied with regard to its toxicity from wide research viewpoints. However, in vitro studies of its metabolism have been mainly concerned with the rates of primary 4'-hydroxylation of phenytoin, except for a few reports on the formation of 3'-HPPH (Yasumori et al., 1999) and the catechol metabolite (Munns et al., 1997). In the present experiments, we determined 4'-HPPH, 3'-HPPH, 3', 4'-diHPPH, and 3', 4'-di-HPPH formation catalyzed by rat liver microsomes. These peaks were confirmed by comigration with authentic standards, LC/MS analysis, and/or the effects of typical cofactors for catechol O-methyltransferase (Figs. 2 and 3). The secondary hydroxylated metabolite, 3', 4'-diHPPH, from phenytoin has been studied with respect to covalent adduct binding to microsomal proteins to cause an autoimmune reaction (Munns et al., 1997). Similarly, the importance of catechols (primary hydroxylated metabolites) of estrogens with regard to their toxicity has also been reported (Zhu and Conney, 1998). Hydroxysterogens formed by P450 enzymes could be converted to semiquinone and activated oxygen, which would cause free radical-mediated damage to lipids, proteins, and DNA. A catechol metabolite of phenytoin may also generate activated oxygen and work as a toxic intermediate.
tetrachloro-p-dibenzodioxin, phenobarbital, acetone, and rifampin, despite the total P450 induction (Doecke et al., 1990). In the present study, no increase in phenytoin 4'-hydroxylation activities was observed by intraperitoneal treatment of rats with typical P450 inducers (Fig. 5). Phenytoin and phenobarbital treatment resulted in a decrease in phenytoin 4'-hydroxylation activities. It is interesting that recombinant rat CYP2B1 or CYP3A1 showed slow oxidation rates of oxidized phenytoin. As well as total P450 and NPR in rats (Table 3). Although the total P450 induction (Doecke et al., 1990; Yamazaki et al., 1996) and were confirmed in the present study to be related to male rats to investigate the effects of phenytoin administration on phenytoin oxidation. In the present study, it was confirmed that phenytoin induced the contents of CYP2B and CYP3A enzymes as well as total P450 and NPR in rats (Table 3). Although the total P450 contents in rat liver microsomes were increased ~2-fold by phenytoin administered orally, the rates of oxidation of phenytoin 4'-HPHP and 3',4'-dihydroxylation were decreased to ~50% (Table 2). This extends the extent of phenytoin into P450 enzymes induced by phenytoin.

We administered an increased amount of phenytoin (300 mg/kg for 20 days) to male rats to investigate the effects of phenytoin administration on phenytoin oxidation. In the present study, we confirmed that phenytoin induced the contents of CYP2B2 and CYP3A enzymes as well as total P450 and NPR in rats (Table 3). Although the total P450 contents in rat liver microsomes were increased ~2-fold by phenytoin administered orally, the rates of oxidation of phenytoin 4'-HPHP and 3',4'-dihydroxylation were decreased to ~50% (Table 2). When hydroxylated metabolites were used as substrates, the 3',4'-dihydroxylation formation by phenytoin-induced rats was not suppressed (Table 2). The extent of the decrease in phenytoin oxidation seemed not to differ between short and long term phenytoin treatment. This is the first report, to our knowledge, on the suppression of hydroxylation of phenytoin itself by P450 enzymes induced with phenytoin.

To investigate the decreased activities of total phenytoin hydroxylation in the phenytoin-induced rat liver microsomal P450s, we focused on the total substrate oxidations and byproducts in the P450 cycle with regard to the coupling ratios. There was a considerable difference in the catalytic activities of phenytoin when using rat liver microsomes supported by CuOOH or NADPH (Tables 4 and 5). The activities of CuOOH-supported phenytoin oxidation and NADPH-supported testosterone hydroxylation catalyzed by liver microsomes were significantly induced by phenytoin. A low coupling ratio of phenytoin oxidation would result in the formation of hydrogen peroxide in liver microsomes from rats untreated and treated with phenytoin in the presence of NADPH. The mechanism for the small efficiency in the transfer of activated oxygen to phenytoin via P450 enzymes in the primary hydroxylation is unknown. This area is under investigation in our laboratory to clarify phenytoin metabolism in animals and humans.

In conclusion, phenytoin can induce liver microsomal P450 enzymes, but the induced P450 could not increase the total oxidation activities of phenytoin itself supported by the NADPH and NPR systems. These results suggest that chronic administration of phenytoin might extensively induce P450 enzymes in the liver, resulting in a decrease of phenytoin oxidative metabolism. The increase of unconjugated phenytoin concentrations in the body and/or byproducts of activated oxides generated in phenytoin-induced liver microsomal P450 system may be pertinent to an understanding of the phenytoin and its related drug toxicity.

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References


TABLE 5
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Untreated</th>
<th>Phenytoin-Treated</th>
<th>Fold Increase in Coupling Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Oxidized NADPH</td>
<td>13 ± 3</td>
<td>9 ± 3*</td>
<td>1.00</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Oxidized NADPH</td>
<td>2.0 ± 0.7</td>
<td>4.2 ± 0.9**</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Oxidized NADPH</td>
<td>15 ± 2</td>
<td>19 ± 3</td>
<td>1.00</td>
</tr>
<tr>
<td>4',6'-HPHP</td>
<td>Oxidized NADPH</td>
<td>0.15 ± 0.04 (1.4)</td>
<td>0.04 ± 0.01** (0.2)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Significantly different from untreated group (* p < 0.05; ** p < 0.01).

^ Sum of rates of 6',16a-, 16b-, 2a-, and 2b-hydroxylated testosterone formation at 100 μM testosterone.

^ Sum of rates of 4',6'-HPHP, 3',4'-dihydroxylation, and 3',4'-dihydroxylation formation at 100 μM phenytoin.

Data are mean ± S.D. for four rats. The number in parentheses indicate percentage of product formation to oxidized NADPH (coupling ratio).


