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'-dihydrodiol 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) $\psi$ 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 (Guengerich, 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 (Wells 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 (Bajpai 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...

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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 b5 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 cases, insect microsomes expressing only NPR and b5 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.
DECREASE IN PHENYTOIN OXIDATION BY PHENYTOIN-ADMINISTERED RATS

Phenytoin (100 μM) was incubated for 30 min at 37°C with rat liver microsomes (1.0 mg of protein/ml) in the presence of an NADPH-generating system (A), with liver microsomes plus liver cytosol (5.0 mg of protein/ml) and S-adenosyl-l-methionine (100 μM) (B), and 4′-HPPH (100 μM) was incubated as a substrate (C). D, the CYP1A2/NPR membranes (0.20 μM CYP1A2) were used as an enzyme source instead of liver microsomes. E, authentic 4′-HPPH (peak 4), 3′,4′-dihydrodiol (peak 6), 2′′-methyl 3′,4′-diHPPH (peak 7), phenytoin (peak 8), and an unknown peak (peak 3) associated with phenytoin, 4′-HPPH, or 3′-HPPH dissolved in this mobile phase are shown. Peaks 1, 2, and 5 correspond to 3′,4′-dihydrodiol, 3′,4′-diHPPH, and methylated 3′,4′-diHPPH, respectively.

Other Assays. The concentrations of P450 and b5 (Omura and Sato, 1964) and protein (Lowry et al., 1951) were estimated as described above. NADPH-cytochrome c reduction activities were determined using a ΔΔε550 = 21 mM−1 cm−1 and an assumed specific activity of 3.6 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 Oxidation Activities Catalyzed by Recombinant Rat P450 Enzymes Expressed in Baculovirus Systems. Eleven forms of recombinant rat P450 enzymes were used to determine which P450 isoforms are active in catalyzing phenytoin hydroxylation in the presence of NADPH (Fig. 4). CYP2C6 showed the highest activity of 3',4'-diHPPH formation from 100 μM phenytoin as a substrate with rat liver microsomes. The small peak of 3',4'-dihydrodiol (peak 1) was detected when rat liver microsomes were used (Fig. 2A) and CYP1A2 and CYP2A2 weakly inhibited these hydroxylations.

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'-dihydrodiol 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'-dihydrodiol formation from phenytoin (10–200 μM) catalyzed by rat liver microsomes were determined for a 30-min incubation. Apparent \( K_m \) and \( V_{max} \) 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 \( K_m \) and \( V_{max} \) 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',4'-dihydrodiol formation from phenytoin (10–200 μM) catalyzed by rat liver microsomes (1.0 mg/ml) 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'-dihydrodiol formation from 100 μM 4'-HPPH. Anti-CYP2C antibodies inhibited 4'-HPPH formation from phenytoin and 3',4'-dihydrodiol formation from 4'-HPPH to 44 and 28%, respectively. Anti-CYP3A antibodies weakly inhibited these hydroxylations.

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DECREASE IN PHENYTOIN OXIDATION BY PHENYTOIN-ADMINISTERED RATS

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).

<table>
<thead>
<tr>
<th>Additions</th>
<th>4'-HPPH from Phenytoin</th>
<th>3',4'-dihydrodiol from 4'-HPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Chemical</td>
<td>µM</td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>0.08 µM of total P450</td>
<td>0.08 µM of total P450</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>50</td>
<td>144 (100)</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>50</td>
<td>31 (31)</td>
</tr>
<tr>
<td>II Antibody</td>
<td>mg IgG/nmol P450</td>
<td>pmol/min/nmol P450</td>
</tr>
<tr>
<td>None</td>
<td>239 (100)</td>
<td>287 (100)</td>
</tr>
<tr>
<td>Preimmune</td>
<td>20</td>
<td>233 (98)</td>
</tr>
<tr>
<td>Anti-CYP2C</td>
<td>20</td>
<td>105 (44)</td>
</tr>
<tr>
<td>Anti-CYP3A</td>
<td>20</td>
<td>184 (81)</td>
</tr>
</tbody>
</table>

As expected, phenytoin significantly induced by 1.9-fold the total P450 as determined by CO-different spectra (Table 3). The induction of CYP2B and CYP3A enzymes by phenytoin were confirmed both by the immunochemical 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.

An analysis for measuring the mechanism-based inhibition caused by 3',4'-diHPPH (with preincubation of 10 µM 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'-diHPPH.

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 were 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

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).
TABLE 2

Effects of phenytoin administration on the oxidation activities of phenytoin and its primary metabolites in rat liver microsomes

Data are mean ± S.D. for four male rats treated with oral administration of phenytoin (300 mg/kg/day) for 20 days. Values in parentheses indicate percentage of control (untreated).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>μM</th>
<th>Metabolite</th>
<th>Untreated</th>
<th>Phenytoin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenytoin</td>
<td>100</td>
<td>4′-HPPH</td>
<td>95 ± 4</td>
<td>56 ± 6**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′-HPPH</td>
<td>1.0 ± 0.1</td>
<td>3.3 ± 0.6**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′,4′-dHPPH</td>
<td>11 ± 3</td>
<td>6 ± 1**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′,4′-dihydrodiol</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>4′-HPPH</td>
<td>1</td>
<td>3′,4′-dHPPH</td>
<td>10 ± 3</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>3′-HPPH</td>
<td>100</td>
<td>3′,4′-dHPPH</td>
<td>375 ± 68</td>
<td>353 ± 54</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3′,4′-dHPPH</td>
<td>16 ± 3</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>992 ± 158</td>
<td>704 ± 189</td>
</tr>
</tbody>
</table>

** Significantly different from untreated male rats (p < 0.01).

TABLE 3

Effects of phenytoin administration on P450, NPR, and β contents and typical drug oxidation activities in rat liver microsomes

Data are mean ± S.D. for four male rats treated with oral administration of phenytoin (300 mg/kg/day) for 20 days. Values in parentheses indicate percentage of control (untreated).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Phenytoin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(I) Protein Contents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total P450α</td>
<td>0.75 ± 0.08 (100)</td>
<td>1.43 ± 0.24** (191)</td>
</tr>
<tr>
<td>CYP1A1β</td>
<td>0.04 ± 0.01 (100)</td>
<td>0.04 ± 0.02 (100)</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.07 ± 0.02 (100)</td>
<td>1.01 ± 0.16** (1440)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.25 ± 0.05 (100)</td>
<td>0.22 ± 0.05 (89)</td>
</tr>
<tr>
<td>CYP2E1β</td>
<td>0.17 ± 0.02 (100)</td>
<td>0.21 ± 0.05 (123)</td>
</tr>
<tr>
<td>CYP3A6</td>
<td>0.23 ± 0.06 (100)</td>
<td>0.46 ± 0.15* (199)</td>
</tr>
<tr>
<td>NPRα</td>
<td>0.11 ± 0.02 (100)</td>
<td>0.17 ± 0.01** (155)</td>
</tr>
<tr>
<td>β5</td>
<td>0.06 ± 0.02 (100)</td>
<td>0.04 ± 0.02 (67)</td>
</tr>
</tbody>
</table>

** Significantly different from untreated male rats (*p < 0.05; **p < 0.01).
* Determined spectrally.
† Determined immunochemically.
‡ Calculated by NADPH-cytochrome c reduction activities.

** (II) Drug Oxidation Activities

7-Ethoxycoumarin 0-deethylase | 1.05 ± 0.09 (100) | 2.58 ± 0.32** (246) |
Ethoxyresorufin O-deethylase  | 0.26 ± 0.02 (100) | 0.36 ± 0.07* (138) |
Testosterone 16β-hydroxylation | 0.05 ± 0.01 (100) | 1.10 ± 0.24** (2200) |
Testosterone 16a-hydroxylation | 0.42 ± 0.03 (100) | 1.15 ± 0.20** (274) |
Androstenedione 16β-hydroxylation | 0.12 ± 0.01 (100) | 0.12 ± 0.03 (108) |
Testosterone 6β-hydroxylation | 1.10 ± 0.22 (100) | 3.82 ± 0.86** (347) |
Styrene oxide hydration      | 23.4 ± 0.9 (100)  | 51.4 ± 5.7** (220) |

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

TABLE 4

Cumene hydroperoxide-supported rates of phenytoin oxidations in liver microsomes from untreated and phenytoin-administered rats

Phenytoin (100 μM) was incubated with rat liver microsomes at 37°C for 5 min in the presence of 100 μM cumene hydroperoxide. Data are mean ± S.D. for four rats.

<table>
<thead>
<tr>
<th>Rat Liver Microsomes</th>
<th>Product Formation</th>
<th>Untreated</th>
<th>Phenytoin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4′-HPPH</td>
<td>3′,4′-dihydrodiol</td>
<td></td>
</tr>
<tr>
<td>Product formation</td>
<td>pmol/min/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>29 ± 4</td>
<td>310 ± 95</td>
<td></td>
</tr>
<tr>
<td>Phenytoin-treated</td>
<td>44 ± 5**</td>
<td>890 ± 71**</td>
<td></td>
</tr>
</tbody>
</table>

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

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′-dihydrodiol, and 3′,4′-dihydrodiol 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′-dihydrodiol, from phenytoin has been studied with respect to covalent adduction 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 oxides, 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-trichloropropane 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′-dihydrophthalam formation, and the effects of 1,1-trichloropropane 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′-dihydrophthalam 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′-hydroxylation (Fig. 4A). 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 (Table 1). 3′,4′-dihydrophthalam formation from 4′-HPPH was mainly catalyzed by male-specific CYP2C11 (Fig. 4C), which corresponded to sex differences in the rates of 3′,4′-dihydrophthalam formation by liver microsomes (Fig. 5C).

4′-HPPH formation activities catalyzed by rabbit liver microsomes have been reported not to be induced by phenytoin nor by 2,3,7,8-
tetrachloro-p-dibenzodioxin, phenobarbital, acetone, and rifampicin, despite the total P450 induction (Doeecke 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 phenytoin (Fig. 2), which have been reported (Ghosal et al., 1996; Yamazaki et al., 1996) and were confirmed in the present study to be P450 forms inducible by phenytoin after intraperitoneal treatment in rats.

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, 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 to 4′-HPPH and 3′,4′-diHPPH were decreased to ~50% (Table 2). When hydroxylated metabolites were used as substrates, the 3′,4′-diHPPH 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 hydroxylations 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 unmetabolized phenytoin concentrations in the body and/or byproducts of activated oxgenes 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


