Original Research Paper

Effects of duration of phenytoin administration on mRNA expression of cytochrome P450 and P-glycoprotein in the liver and small intestine of rats

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ABSTRACT
Phenytoin (5,5-diphenylhydantoin; DPH) induces expression of cytochromes P450 (CYPs). Interactions between DPH and tacrolimus suggested that the persistence of CYP induction after discontinuation of DPH is dependent on the history of administration and dosing period of DPH. However, the relationship between the duration of DPH administration and expression of CYPs in the liver and small intestine of rats is not known. Alterations in levels of P-glycoprotein (P-gp; MDR1; ABCB1) as well as CYPs cause drug interactions in the small intestine. We examined the effects of the duration of DPH administration on expression of CYPs and P-gp in the liver and small intestine of rats. Rats were treated with DPH (100 mg/kg, peroral (p.o.) twice a day (b.d.)) for 2, 4, 8, and 16 d. mRNA levels of CYPs and P-gp were examined using the total RNA extracted from the liver and duodenum 2 h and 24 h after the final administration of DPH. CYP3A activities were determined using microsomes. DPH administration for 2 d and 4 d markedly increased mRNA levels of CYPs such as CYP3A1, CYP3A2, CYP2B1, and CYP2B2 in the liver. A relatively long duration of DPH administration (8 d and 16 d) resulted in abolition of the induction of hepatic CYP but increased CYP3A activities were maintained. These results suggest that the duration of DPH administration could be an important determinant of hepatic CYP induction.

1. Introduction
Cytochromes P450 (CYPs) belong to a superfamily of proteins containing a heme cofactor. CYPs are expressed abundantly in the liver and small intestine. CYPs are involved in the metabolism of various endogenous and xenobiotic substrates. CYP inhibition and induction by xenobiotics are the causes of drug–drug interactions and can result in adverse effects. It is known that carbamazepine [1,2], phenobarbital [3–5], and phenytoin (5,5-diphenylhydantoin; DPH) [6,7] are potent inducers of CYPs. DPH also shows competitive inhibition for the hydroxylation of...
tolbutamide [8]. CYP3A4 in humans as well as CYP3A1 and CYP3A2 in rats accounts for ≤30% of hepatic CYPs [9]. CYP3A is very important in hepatic drug metabolism because CYP3A4 participates in the metabolism of ~50% of marketed drugs [10,11]. Intestinal CYPs are involved in first-pass metabolism in addition to hepatic CYPs. The major intestinal isoforms of CYP3A in rats are CYP3A9, CYP3A18, and CYP3A62 [12].

DPH is an anticonvulsant agent. Interactions between DPH and tacrolimus have been reported [13]. Wada et al. demonstrated that the persistence of CYP induction after discontinuation of DPH was dependent on the history of administration and dosing period of DPH [14]. Patients treated consecutively with DPH display sustained interactions between DPH and tacrolimus. However, the relationship between the duration of DPH administration and CYP expression in the liver and small intestine of rats is not known.

In addition to the metabolism of drugs by CYP3A in the small intestine, drugs are subject to active efflux from cells by adenosine triphosphate-binding cassette transporters such as P-glycoprotein (P-gp; MDR1; ABCB1). Substrates for CYP3A and P-gp overlap [15], showing a concerted barrier function for drug absorption. Identification of changes in the expression and function of P-gp during drug therapy is important because P-gp is the cause of drug interactions in the small intestine [16,17]. Therefore, we examined the effects of the duration of DPH administration on expression of P-gp in the small intestine.

Expression of CYPs and P-gp are regulated on the transcription level by nuclear receptors such as the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [18–20]. We also determined the effects of the duration of DPH administration on expression of PXR and CAR.

2. Material and methods

2.1. Ethical approval of the study protocol

The study protocol was approved by the Committee for the Care and Use of Laboratory Animals of the Faculty of Pharmacy of Kinki University (Osaka, Japan).

2.2. Compounds and reagents

DPH was purchased from Sigma-Aldrich (Saint Louis, MO, USA). TRizol and a Bicinchoninic Acid (BCA) Protein Assay kit were obtained from Life Technologies (Carlsbad, CA, USA) and Pierce Biotechnology (Rockford, IL, USA), respectively. A P450-Glo CYP3A4 Assay kit and a regeneration system for the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Promega (Madison, WI, USA). All other chemicals and solvents were of the best purity available.

2.3. Animals and treatments

Six-week-old female Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan). Animals were housed in a temperature-controlled room with free access to standard laboratory chow (MF diet; Oriental Yeast, Tokyo, Japan) and water. Rats were treated with DPH (100 mg/kg, peroral (p.o.) twice a day (b.d.)) for 2, 4, 8, and 16 d [21]. At 2 h and 24 h after the final administration of DPH, animals were anesthetized with diethyl ether. Then, the liver and the proximal 2–3 cm of the duodenum were perfused with ice-cold physiologic (0.9%) saline and removed. After flash freezing in liquid nitrogen, each sample was preserved at ~80 °C until used for RNA extraction and microsome preparation.

2.4. Determination of mRNA levels by real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from samples of liver tissue and small intestine tissue using TRizol. mRNA expression was measured using RT-PCR, as described previously [22,23]. Oligonucleotide sequences for each mRNA target are shown in Table 1. Data were analyzed using ABI Prism 7000 SDS (Life Technologies) using the multiplex comparative method.

2.5. Preparation of hepatic microsomes

Liver samples were perfused with ice-cold 0.9% saline and chopped into small pieces. A 25% (w/v) homogenate was made in ice-cold 1.15% KCl solution using a Physcotron Homog- enizer (Microtec, Chiba, Japan). The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C. The supernatant was centrifuged further at 105,000 × g for 60 min at 4 °C to obtain a microsomal pellet. The latter was washed by resuspension in 3 ml of 1.15% KCl. The suspension was centrifuged at 105,000 × g for 30 min at 4 °C to obtain the final microsomal pellet, which was resuspended in 1.5 ml of 1.15% KCl and stored at ~80 °C until use. Protein concentrations were determined using a BCA Protein Assay kit.

### Table 1 – Primer sequences used in PCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B1</td>
<td>Forward: CAAGGAGAATGCGATTTGGAAAAA</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>Forward: CACAGGAAAAGGGCTTTTTC</td>
</tr>
<tr>
<td>CYP2C2</td>
<td>Forward: GTCCTTTCCTTGCGATTGCT</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>Forward: GCTTTTTTTTGGCAGCTTGGT</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>Forward: TTTGCCATCATGGACACAGAGA</td>
</tr>
<tr>
<td>CYP3A9</td>
<td>Forward: CCATACATCATCTTTATAGT</td>
</tr>
<tr>
<td>CYP3A18</td>
<td>Forward: CCAATCATATCTTATAGT</td>
</tr>
<tr>
<td>CYP3A62</td>
<td>Forward: CCCGGGAATTTCACTGCAG</td>
</tr>
<tr>
<td>Mdr1a</td>
<td>Forward: TGGAGACGTCATCTGTGAGC</td>
</tr>
<tr>
<td>Mdr1b</td>
<td>Forward: TGGAGACGTCATCTGTGAGC</td>
</tr>
<tr>
<td>PXR</td>
<td>Forward: GACGGGAGCATCTGGAATAC</td>
</tr>
<tr>
<td>CAR</td>
<td>Forward: CCACGCGTACATTCTCATAC</td>
</tr>
<tr>
<td>18S ribosomal RNA</td>
<td>Forward: CGGCCGCTAGGGTAAATTC</td>
</tr>
</tbody>
</table>
2.6. Measurement of CYP3A activity

CYP3A activity was determined using a P450-Glo CYP3A4 Assay kit. CYP3A1 and CYP3A2 activities in rats and CYP3A4 activity in humans were measured according to manufacturer instructions. Briefly, CYP3A reactions were carried out in a 96-well plate (OptiPlate-96; PerkinElmer, Waltham, MA, USA). An incubation mixture (total volume, 50 μl) was prepared that contained 200 mM potassium phosphate buffer (pH 7.4), a NADPH regeneration system, 20 μg rat liver microsomes, and 50 μM luciferin 6′ benzyl ether (luciferin-BE) as a substrate for CYP3A1 and CYP3A2. After preincubation for 10 min at 37 °C, the reaction was initiated by addition of the NADPH regeneration system and then incubated for 30 min at 37 °C with constant shaking. The reconstituted luciferin detection reagent (50 μl) was added to terminate the reaction and generate chemiluminescence. Luminescence was measured using FLUOstar Optima (Moritex, Tokyo, Japan).

2.7. Statistical analyses

Statistical analyses were carried out using the Dunnett’s test after analysis of variance.

3. Results and discussion

mRNA levels of CYP3A1 and CYP3A2 (Fig. 1a) and CYP3A activities (Fig. 1b) in the liver after DPH administration were determined. mRNA levels of CYP3A1 and CYP3A2 were increased significantly 2 h and 24 h after the final administration of DPH for 4 d. Longer duration of DPH administration (8 d and 16 d) resulted in recovery to the mRNA levels observed in controls. CYP3A activities were increased significantly upon DPH administration for 4, 8, and 16 d. At 24 h (but not 2 h) after the final DPH administration for 2 d, significant increases in CYP3A activities were observed. In preliminary study, the larger variations between individuals were observed in the longer term after last administration of DPH (data not shown). These findings suggested that 2 h after DPH administration for 2 d was not a sufficient time for increases in levels of protein and activity of CYP3A to be observed. Increased activities of CYP3A showed persistence compared with those of CYP3A1 and CYP3A2 mRNA after DPH administration. We primarily examined the mRNA levels and activities but not protein levels of CYP3A. It has been reported that the expression of CYP1A, CYP2B, CYP2C, and CYP3A mRNA were correlated with those protein levels and metabolic activities in human liver [24,25] and rat and human hepatocytes [26].

We examined the effects of duration of DPH administration on the mRNA levels of CYP2B1, CYP2B2, and CYP2C12 (Fig. 2). mRNA levels of CYP2B1 and CYP2B2 were increased significantly at relatively short durations of DPH administration (2 d and 4 d) and tended to return to control levels after DPH administration for 8 d and 16 d. These results for CYP2B1 and CYP2B2 were similar to those for CYP3A1 and CYP3A2. In CYP2C12, slight changes in mRNA levels were observed upon DPH administration. In control rats, CYP2C is highly expressed in the liver compared with CYP3A, which is different.
to the situation in humans [27]. Reports of a marked induction of expression of CYP2C12 mRNA upon DPH administration are lacking.

Hepatic mRNA levels of PXR and CAR were examined (Fig. 3). Induction of expression of CYP mRNA by DPH is mediated primarily through activation of CAR (but not PXR) in humans [28–30]. At 2 h and 24 h after DPH administration for 2 d and 4 d, mRNA levels of PXR and CAR did not change, but mRNA levels of CYP3A1, CYP3A2, CYP2B1, and CYP2B2 were increased. The ligand-activated transcriptional activities of PXR and CAR could have increased without affecting alterations in the mRNA levels of PXR and CAR. Further studies are needed to clarify the precise effects of duration of DPH administration on the activities of nuclear receptors. Interestingly, longer duration of DPH administration (8 d and 16 d) resulted in transcriptional negative feedback for CYP3A1, CYP3A2, CYP2B1, and CYP2B2. CYP3A activities in the small intestine did not change upon DPH administration (data not shown). Some drug interactions are mediated via P-gp in the small intestine. mRNA levels of Mdr1a and Mdr1b in the small intestine did not change upon DPH administration. In preliminary experiments, mRNA levels of Mdr1a and Mdr1b in the liver did not change (data not shown). The protein levels of P-gp showed little correlation with the mRNA levels of Mdr1a and Mdr1b [25]. Further studies are needed to clarify the effects of DPH administration on P-gp activities.

### 4. Conclusion

DPH administration for 2 d and 4 d increased the mRNA levels of hepatic CYPs such as CYP3A1, CYP3A2, CYP2B1, and CYP2B2 markedly. Relatively long duration of DPH administration (8 d and 16 d) resulted in transcriptional negative feedback for CYP3A1, CYP3A2, CYP2B1, and CYP2B2. However, the increased activities of CYP3A were maintained. These results suggest that the duration of DPH administration could be an important determinant of CYP induction in the liver.

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**Fig. 2** – Levels of CYP2B1, CYP2B2, and CYP2C12 mRNA in the liver of rats treated with DPH (p.o., b.d.) for 2, 4, 8, and 16 d. At 2 h and 24 h after the final administration of DPH, the liver was excised. Data are the mean ± SEM (n = 3–4). *p < 0.05, **p < 0.01, and ***p < 0.001 vs control.

**Fig. 3** – Levels of PXR and CAR mRNA in the liver of rats treated with DPH (p.o., b.d.) for 2, 4, 8, and 16 d. At 2 h and 24 h after the final administration of DPH, the liver was excised. Data are the mean ± SEM (n = 3–4). *p < 0.05, **p < 0.01, and ***p < 0.001 vs control.
Acknowledgments

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REFERENCES


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