Effect of Pregnenolone 16α-Carbonitrile on the Expression of P-Glycoprotein in the Intestine, Brain and Liver of Mice

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P-Glycoprotein (P-gp), encoded by the MDR1 (ABCB1) gene in humans and by Mdr1a and Mdr1b genes in rodents, is a member of the superfamily of ATP-binding cassette transporters. Since P-gp is constitutively expressed in numerous tissues and exhibits a broad specificity in substrate recognition, it can play a crucial role in limiting the absorption and distribution of xenobiotics by decreasing their intracellular accumulation. The expression of P-gp is regulated by various nuclear receptors such as pregnane X receptor (PXR). Although the characterization of P-gp induction by PXR ligands is a crucial goal for predicting pharmacokinetics of drugs, findings regarding the induction of P-gp by PXR ligands in vivo are still controversial. In this study, we examined the effect of pregnenolone 16α-carbonitrile (PCN), a murine PXR ligand, on the expression of Mdr1a/1b mRNA and P-gp protein in the intestine, brain and liver of mice. The results showed that PCN increased the expression of both Mdr1a/1b mRNA and P-gp protein in the intestine and the brain. The present study provided the first evidence that P-gp is inducible by PCN in the large intestine. The results also showed that P-gp protein was induced by PCN in the cortex but not in the whole brain. On the other hand, PCN increased the expression of Mdr1a/1b mRNA in the liver, although no increase was observed in the expression of P-gp protein. These results suggested different effect of PCN on the expression of P-gp protein in the intestine, brain and liver of mice.

Key words P-glycoprotein; induction; pregnane X receptor; intestine; brain; liver

Note

P-Glycoprotein (P-gp), encoded by the MDR1 (ABCB1) gene in humans and by Mdr1a and Mdr1b genes in rodents, is a member of the superfamily of ATP-binding cassette transporters. Since P-gp is constitutively expressed in numerous tissues and exhibits a broad specificity in substrate recognition, it can play a crucial role in limiting the absorption and distribution of xenobiotics by decreasing their intracellular accumulation. The expression of P-gp is regulated by various nuclear receptors such as pregnane X receptor (PXR). It is known that the expression of P-gp is regulated by various nuclear receptors such as pregnane X receptor (PXR). Activation of PXR by ligands causes transcriptional activation of the MDR1 gene and results in induction of P-gp. Since P-gp plays important roles in regulation of the pharmacokinetics of many drugs, the induction of P-gp by PXR ligands can lead to drug–drug interactions. The characterization of P-gp induction by PXR ligands is a crucial goal for predicting pharmacokinetics of drugs.

Several in vivo studies have shown that the expression of P-gp was induced by PXR ligands in the small intestine, brain and liver of humans and rodents. However, controversial results have been reported for the induction of P-gp in the brain and the liver, and there have been no reports on the induction of P-gp by PXR ligands in the large intestine. We therefore examined the effect of pregnenolone 16α-carbonitrile (PCN) on the expression levels of Mdr1a/1b mRNA and P-gp protein in the intestine, brain and liver of mice. PCN is a typical ligand of rodent PXR and is usually used in numerous in vivo studies. In previous studies, the dosage of PCN used ranged 10 to 200mg/kg, which may result in the controversial results for the induction of P-gp in the brain and liver. To clarify whether P-gp in the intestine, brain and liver of mice can be induced by sufficient dose of PCN, PCN was administered at a dose of 100 or 200mg/kg.

MATERIALS AND METHODS

Chemicals Pregnenolone-16α-carbonitrile (PCN) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Corn oil was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Treatments Male C57BL/6 mice (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained under a standard 12-h light/12-h dark cycle with water and chow provided ad libitum. All animal studies were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals adopted by the Committee on Animal Research of Chiba University. Groups of mice (4 or 5 mice per group) were administered corn oil as vehicle control (10mL/kg body weight) or PCN (100 or 200mg/kg/d, intraperitoneally (i.p.)) once daily for 4d. After the final administration of corn oil or PCN, the mice were fasted for 24h, and intestine, brain and liver tissues were then collected. The intestine was divided into five segments of equal lengths (Segments 1–5, S1–5). S1 and S4 encompassed the duodenum and cecum, respectively. S5 and the part behind the cecum of S4 corresponded to the large intestine. The right-side end of each segment was excised to a smaller segment of 3–4mm and used for extraction of total RNA for real-time PCR analysis. The residues of S1, S3 and S5 were immediately used for preparation of enterocytes for Western blot analysis. The brain was divided into the cortex and a half of the whole brain. All tissues were pooled from each group of mice, snap-frozen in liquid nitrogen, and then stored at −80°C until use. It was confirmed that treatment with 100 or
200 mg/kg PCN increased the expression levels of CYP3A11 mRNA, a positive control of the effect of PCN, by 4.2–15.9 fold in the intestine and the liver of mice.

**mRNA Analysis**

Total RNAs were extracted from tissues using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Isolated total RNA was then
treated with ribonuclease (RNase)-free deoxyribonuclease (DNase) I (Roche, Basal, Switzerland) to remove contaminating genomic DNA. The cDNA was generated with a random hexamer by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, U.S.A.). To measure the levels of Mdr1a/1b mRNA, real-time PCR analysis was performed as described in Supplementary Materials and Methods.

**Protein Analysis** Crude membrane fractions of the tissues were prepared as described in Supplementary Materials and Methods. Protein concentrations of the samples were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer’s instructions. To measure the levels of P-gp protein, Western blot analysis was performed as described in Supplementary Materials and Methods.

**Statistical Analysis** Statistical analyses were performed by using Statcel3 (OMS, Tokyo, Japan). Comparison of multiple groups was made with one-way ANOVA followed by Scheffé’s F test. p<0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

In this study, we examined the effects of PCN on the expression levels of Mdr1a/1b mRNA and P-gp protein in the intestine, brain and liver of mice. PCN (100 or 200 mg/kg) increased the expression levels of Mdr1a/1b mRNA in all of the tissues examined in this study, suggesting that 200 mg/kg PCN is sufficient dose to activate the transcription of Mdr1a/1b genes. On the other hand, 200 mg/kg PCN increased the expression levels of P-gp protein in the intestine and brain but not in the liver (Figs. 1–3). These findings suggested that the effect of PCN on the expression of P-gp protein was different among these tissues.
PCN increased the expression levels of both Mdr1a/1b mRNA and P-gp protein in all segments of the mouse intestine examined in this study (Fig. 1). These findings are consistent with previous results for the small intestine of mice, rats and humans.3–6,8,10,11,13,14 Thus, induction of P-gp in the small intestine by a PXR ligand appears to be a common phenomenon that was found in both rodents and humans. One point worthy of special mention in this study is that the inductive effects of PXR ligands on the intestinal expression of Mdr1a/1b mRNA and P-gp protein were found for the large intestine, while the effects only for the small intestine were examined in previous studies. Therefore, as far as we know, these are the first results showing that PCN can increase the expression of P-gp in the large intestine (Fig. 1). Since the expression of Mdr1a mRNA was detected in all segments of intestine (data not shown), PXR is probably involved in the induction of P-gp by PCN both in the small and large intestine. On the other hand, the present study also showed that the extent of induction of Mdr1a mRNA and P-gp protein by PCN tends to be greater in the lower intestine than the upper intestine (Figs. 1A, C). Since the expression of Mdr1a/1b gene is regulated by numerous factors other than PXR, the factors except for PXR may be responsible for the difference in extent of P-gp induction between lower and upper intestine. Although the physiological significance of the induction of P-gp in the large intestine remains unclear, the induction may be pharmacologically significant. Rectal administration of drugs has been used for patients with a gastrointestinal movement problem since rectal administration causes less nausea than oral administration and bypasses the hepatic first pass metabolism through the portal vein. Thus, the induction of P-gp by PXR ligands in the large intestine may accelerate the excretion of absorbed drugs to feces and reduce bioavailability of P-gp substrate drugs when they are administered rectally, although further studies are clearly needed to clarify this possibility. The relative expression of Mdr1a mRNA was almost equally between S1 and S5, and that of Mdr1b mRNA in S5 was lower than that in S1 (Figs. 1A, B). However, the relative expression of P-gp protein in S5 was much higher that in S1 (Fig. 1C). This contradiction is assumed to be caused by the difference in the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which was used as an internal control, between intestinal segment S1 and S5. As for the brain, the present study showed that 200 mg/kg
PCN increased the expression levels of both Mdr1a/1b mRNA and P-gp protein in the cortex, whereas 200 mg/kg PCN only slightly increased Mdr1a/1b mRNA expression and did not increase P-gp protein expression in the whole brain (Fig. 2). The findings suggest that P-gp protein is inducible by 200 mg/kg PCN through transcriptional activation of Mdr1a/1b genes in the cortex, whereas such induction could hardly be found in the whole brain. Since the extent of increase in Mdr1a/1b mRNA in the whole brain was lower than that in the cortex, it may be insufficient to detect the significant increase of P-gp protein in the whole brain. In contrast to the intestine and liver, information on the induction of P-gp in the brain has been limited. Only two reports indicated that PCN increased expression levels of Mdr1a/1b mRNA but not P-gp protein in the whole brain,\(^{13,15}\) being consistent with the present findings. On the other hand, studies using brain capillaries indicated that PCN and rifampicin increased the expression levels of P-gp protein in rats and human PXR-transgenic mice, respectively.\(^7,9\) Interestingly, a similar phenomenon was observed in P-gp protein in rats and human PXR-transgenic mice, respectively.\(^7,9\) The results for Mdr1a/1b mRNA and P-gp protein were induced by PCN in the cortex, in which the density of brain capillaries is higher than that in white matter,\(^20\) but were hardly induced in the whole brain. It is known that P-gp is broadly expressed in various regions of the brain.\(^21,22\) Thus, the possible induction of P-gp by PCN in a brain capillaries-specific manner may be masked by the expression of P-gp in other regions of the brain.

As for the liver, previous studies showed that PXR ligands induced the expression of Mdr1a/1b mRNA or P-gp protein in the liver of mice and rats.\(^6,7,9,12\) However, there are also controversial results showing no increase in Mdr1a/1b mRNA or P-gp protein in the liver.\(^13,15,16\) In addition, there has been no study to examine whether PXR ligands affect the expression of both Mdr1a/1b mRNA and P-gp protein in the liver. The results of present study showed that PCN significantly increased the expression levels of Mdr1a/1b mRNA (Fig. 3), suggesting that PCN can clearly up-regulate the transcription of Mdr1a/1b genes in the liver of mice. On the other hand, the induction of Mdr1a/1b mRNA by PCN did not result in the induction of P-gp protein in the liver of mice (Fig. 3). These findings suggest that PCN can repress the expression of P-gp protein through post-transcriptional/translational processes of P-gp in the liver. PCN may affect the membrane sorting of P-gp protein. Further studies are needed to clarify the effects of PXR ligands on the regulation of P-gp protein expression in the liver of mice.

In conclusion, present study showed that PCN increased the expression of Mdr1a/1b mRNA in the intestine, brain and liver of mice, while PCN increased the expression of P-gp protein in the intestine and brain but not in the liver. These findings suggested that the effect of PCN on the expression of P-gp protein was different among these tissues. There are several notable points in this study: 1) this study provided the first evidence that P-gp is inducible by PCN in the large intestine, 2) this study also showed that P-gp protein was induced by PCN in the cortex but not in the whole brain, and 3) P-gp protein was not inducible by PCN in the liver of mice, being inconsistent with the results for Mdr1a/1b mRNA. These findings can help to clarify P-gp-mediated drug–drug interactions (e.g., changes in intestinal absorption, brain distribution and biliary excretion of P-gp substrates) caused by PXR ligands.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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