Note

Down-Regulation of Intestinal Multidrug Resistance-Associated Protein 2 in Long-Evans Cinnamon Rats

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Summary: Wilson’s disease is an inherited, autosomal recessive disorder of copper accumulation and toxicity. Lifelong chelation therapy is essential in all Wilson’s disease patients. Intestinal absorption of some compounds is limited partly because they are preferentially transported in the secretory direction. Several ATP-binding cassette (ABC) transporters are expressed in the apical membrane of the small intestine and secrete various drugs into the lumen. In this study, we investigated the characteristics of the intestinal efflux ABC transporters in LEC rats. We found that the expression of multidrug resistance-associated protein 2 (Mrp2) in the jejunum of Long-Evans Cinnamon (LEC) rats, an animal model for Wilson’s disease, is decreased.

Key words: LEC rats; Mrp2; ABC transporter; Wilson’s disease

Introduction

The Long-Evans Cinnamon (LEC) strain, has been established at the Center for Experimental Plants and Animals of Hokkaido University.1) The liver plays important roles in the detoxification of xenobiotics. Hepatobiliary transporters contribute to hepatic uptake and efflux processes of xenobiotics. Expressions of these transporters may be modulated under the condition of hepatic failure. We have found that hepatic expressions of three sinusoidal organic anion transporters, Na+/taurocholate cotransporting polypeptide (Ntcp/Slc10a1), organic anion transporting polypeptide 1a1 (Oatp1a1/Slc01a1) and Oatp1a4 (Slco1a4), were decreased in LEC rats.2) On the other hand, the expression levels of hepatic canalicular transporters, multidrug resistance-associated protein 2 (Mrp2/Abcc2), P-glycoprotein (P-gp/Abcb1) and bile salt export pump (Bsep/Abcb11), in LEC rats were similar to those in Wistar rats. In this study, we focused on the expression patterns of transporters in other tissues.

Intestinal absorption of ionic drugs is also controlled by a number of drug transporters in the intestine. It is possible that the function(s) of these transporters in LEC rats is changed. LEC rats show biochemical features that are very similar to those found in patients with Wilson’s disease, an autosomal recessive disorder of copper accumulation and toxicity.3) It has been reported that LEC rats are deficient in the expression of Wilson’s disease gene (ATP7B).4,5) LEC rats are therefore a good animal model for the study of Wilson’s disease in humans. Oral administration of copper chelating agents, including D-penicillamine and trientine, results in complete reversal or alleviation of hepatic, neurologic and psychiatric abnormalities in most patients with Wilson’s disease.6,7) However, lifelong chelation therapy is essential in all Wilson’s disease patients.

Although oral drug delivery is generally the most desirable means of administration, mainly because of patient acceptance, convenience in administration, and cost-effective manufacturing, various mechanisms can influence the intestinal absorption and oral bioavailability of drugs. Permeation by diffusion is often predictable from a drug’s physicochemical properties. However, numerous drugs exhibit lower absorption rates after oral administration than expected from their physicochemical properties. It has been reported that intestinal absorption of some compounds is limited partly because they are preferentially transported in the secretory direction.8) Studies on the mechanisms of intestinal ab-
Absorption of various ionic drugs have revealed that drug transporters that are expressed at the apical membrane play an important role in the secretion of drugs. Thus, it is important to elucidate the expressions of these transporters in the intestine of the patients with Wilson’s disease. Among the transporters involved in secretion, P-gp has been the most extensively investigated. In addition to P-gp, Mrp2 and breast cancer resistance protein (Bcrp/Abcg2) are expressed in the apical membrane of the epithelium of the small intestine and secrete various drugs into the lumen. Generally, a substrate of P-gp is thought to be a lipophilic and neutral or cationic drug. On the other hand, Mrp2 and Bcrp recognize organic anions. It has been reported that Mrp2 and Bcrp mediate the ATP-dependent unidirectional transport of substrates. These transporters belong to the superfamily of ATP-binding cassette (ABC) transporters. Absorption of drugs from the intestine is one of the important determinants of oral bioavailability. However, only a few reports describing the properties of intestinal efflux ABC transporters in Wilson’s disease patients have appeared in the literature. The aim of this study was to clarify the characteristics of the intestinal efflux ABC transporters in LEC rats. In this study, we used Wistar rats as a control.

**Materials and Methods**

**Chemicals:** Pravastatin, sulfochromthalein, sodium azide and sodium fluoride were purchased from Wako Pure Chemical (Osaka, Japan). Probenecid was purchased from Sigma Chemical Co. (St Louis, MO). D-[3H]-Mannitol was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). All other reagents were of the highest grade available and used without further purification.

**Animals:** Male LEC rats, aged 6 weeks (150–200 g in weight), were obtained from the Center for Experimental Plants and Animals of Hokkaido University (Sapporo, Japan). Male Wistar rats, aged 6 weeks (200–250 g in weight), were obtained from Jla (Tokyo, Japan). The housing conditions were described previously. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals” as adopted by the National Institutes of Health.

**RT-PCR analysis:** Total RNA was prepared from the rat small intestine using Isogen (Nippon Gene, Tokyo, Japan) including DNase digestion according to the manufacturer’s instructions. Single-strand cDNA was made from 2 µg total RNA by reverse transcription using Rever Tra Ace (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using an ABI PRISM® 7700 sequence detector (Applied Biosystems, Foster City, CA) with 2 x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) as per manufacturer’s protocol. PCR was performed using P-gp, Mrp2, Bcrp or GAPDH-specific primers through 40 cycles of 95°C for 15 s, 52°C for 30 s and 72°C for 30 s after pre-incubation at 50°C for 2 min and 95°C for 2 min. The sequences of the specific primers were as follows: sense sequence was 5’-GCAGGTTGGCTGGACAAGT-3’ and antisense sequence was 5’-GGAGCGCAATTCCATGGATA-3’ for P-gp (accession no.: NM_133401); sense sequence was 5’-TGATCGGTTTGCGTGAAGAGCT-3’ and antisense sequence was 5’-ACGCACATTCCACACAAACAAAC-3’ for Bcrp (accession no.: NM_012833); sense sequence was 5’-GTGACTCAAGCACAAGCA-3’ and antisense sequence was 5’-TGAGTTTCCCCAGAAGCCAGT-3’ for Bcrp (accession no.: AB094089); sense sequence was 5’-ATGGGAAGCTGGTCATCAAC-3’ and antisense sequence was 5’-GTGGTTCCACACCACATCAAC-3’ for GAPDH (accession no.: AF106860). The PCR products were normalized to amplified GAPDH, the internal reference gene.

**Western blot analysis:** Brush-border membrane vesicles were prepared from the rat intestine by the calcium precipitation method with some modification as described previously. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Western blotting was performed as described in a previous report using a monoclonal anti-P-gp antibody (JSB-1) (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution of 1:200), anti-Mrp2 antibody (M2III-5) (Abcom, Cambridge, UK) (dilution of 1:500), anti-Bcrp antibody (BXP-21) (Sigma, St Louis, MO) (dilution of 1:200) or an anti-actin monoclonal antibody (MAB1501) (Chemicon, Temecula, CA) (dilution of 1:500).

**Everted sac studies:** Transport studies were carried out as described in a previous report. The amount of the substrate (100 µM pravastatin or D-mannitol) transported from the serosal to mucosal surfaces across the intestine was measured by sampling the mucosal buffer periodically for 60 min. In the energy-dependency studies, the buffer was preincubated at 37°C for 30 min in the presence of 10 mM sodium fluoride and 10 mM sodium azide.

**Analysis:** Pravastatin concentration was determined using an HPLC system. D-[3H]-Mannitol was determined using a liquid scintillation counter. Statistical significance was evaluated using ANOVA followed by a post hoc test or Student’s t-test. A value of p < 0.05 was considered significant.

**Results**

It is known that duodenum mainly plays a role in the digestion of nutrients and that jejunum and ileum play major roles in the absorption of digested products.
Moreover, colon mainly plays a role in the absorption of water. In this study, we therefore focused on jejunum and ileum. In the first part of this study, we investigated whether expression of apical-localized ABC transporters is changed in LEC rats. The expression levels of ABC transporters were determined by real-time RT-PCR. We found that the expression level of Mrp2 in the jejunum of LEC rats was lower than that in the jejunum of Wistar rats (Fig. 1). However, no quantitative difference in P-gp and Bcrp levels between Wistar rats and LEC rats was found in the jejunum or ileum (Fig. 1). We examined the expression levels of Mrp2 protein in jejunums from Wistar rats and LEC rats by Western blot analysis. The expression level of Mrp2 protein was decreased in the jejunum from LEC rats (Fig. 2). On the other hand, the expression levels of P-gp and Bcrp protein in the ileum from LEC rats were similar to those from Wistar rats (Fig. 2).

We then investigated the effect of down-regulation of intestinal Mrp2 on the Mrp2-mediated efflux transport in LEC rats by everted sac experiments. The serosal-to-mucosal permeation of pravastatin, a substrate for Mrp2, across the jejunum from LEC rats (2.48 ± 0.77 nmol/5-cm sac) was similar to that from Wistar rats (2.93 ± 0.59 nmol/5-cm sac). On the other hand, serosal-to-mucosal permeation of mannitol, a marker of the paracellular route, across the jejunum from LEC rats (7.03 ± 1.54 nmol/5-cm sac) was significantly higher than that from Wistar rats (4.02 ± 0.52 nmol/5-cm sac). We then compared the inhibitory effect of several compounds on the serosal-to-mucosal permeation of pravastatin across the jejunum from Wistar rats and that from LEC rats. In the jejunum from Wistar rats, the serosal-to-mucosal permeation of pravastatin was significantly decreased in the presence of metabolic inhibitors or two known Mrp2 inhibitors, probenecid and sulfobromophthalein, (Fig. 3). In the jejunum from LEC rats, a significant difference was not observed in the presence of probenecid (Fig. 3). In the presence of sulfobromophthalein, serosal-to-mucosal permeation of pravastatin across the jejunum from LEC rats was significantly decreased, but by 15% at most (Fig. 3). Pravastatin permeation in the jejunum from LEC rats was reduced in an ATP-depleted condition (Fig. 3). These compounds did not affect the serosal-to-mucosal permeation of mannitol across the jejunum from either Wistar rats or LEC rats (data not shown).

**Discussion**

Wilson’s disease, an inherited, autosomal recessive disorder of copper accumulation and toxicity, occurs in about one of every 40,000 people. In this study, we investigated the expressions of ABC transporters in the intestine of LEC rats, an animal model of Wilson’s disease.

In the first part of this study, we investigated the expression of intestinal ABC transporters in LEC rats. mRNA and protein levels of Mrp2 were decreased in the jejunum of LEC rats. In contrast, mRNA and protein levels of P-gp and Bcrp were not significantly different between Wistar rats and LEC rats. We then character-
Expression of Intestinal Transporters in LEC Rats

Fig. 2. Western blot analysis of P-gp, Mrp2 and Bcrp expression in the brush-border membrane. Ten μg protein was applied per lane.

Fig. 3. Effects of various compounds on the permeation of pravastatin from serosal to mucosal surfaces across the everted jejunum from Wistar rats and LEC rats. The concentration of pravastatin was 100 μM. Results were obtained at the end of a 60-min experiment. Each column represents the mean with S.D. of three to five determinations. The control value for the permeation of pravastatin on Wistar rats and LEC rats were 2.48 ± 0.77 nmol/5-cm sac and 2.93 ± 0.59 nmol/5-cm sac, respectively. *p < 0.05, significantly different from the control.

The Mrp2-mediated efflux transport in LEC rats was significantly higher than that from Wistar rats. Since the thickness of the muscle layers of Wistar rat jejunum was greater than that of LEC rats, this difference would affect the permeation of compounds including mannitol. However, the serosal-to-mucosal permeation of pravastatin across the jejunum from LEC rats was not significant higher than that from Wistar rats. Moreover, we found the decreased or missing inhibitory effects of Mrp2 inhibitors on the serosal-to-mucosal permeation of pravastatin across the jejunum from LEC rats. These findings suggest that the Mrp2-mediated efflux transport in LEC rats is impaired due to the decreased expression of Mrp2. Thus, it is possible that the expression of intestinal Mrp2 in Wilson's disease patients is decreased. In clinical, patients usually take many kinds of drugs at the same time. It is important to be aware of the contribution of Mrp2 to the drug secretion in case of medication in Wilson's disease patients. In the presence of metabolic inhibitors, the serosal-to-mucosal permeation of pravastatin across the jejunum from LEC rats was similar to that from Wistar rats. It is
possible that an ATP-dependent transport system, which is distinct from Mrp2, plays a role in the serosal-to-mucosal permeation of pravastatin across the jejunum from LEC rats.

In this study, we focused on the intestinal efflux ABC transporters in LEC rats at the stage of pre-hepatitis. It has been reported that increased conjugated bilirubin in plasma may suppress Mrp2 function in rats with hepatic failure. Moreover, LEC rats and Wilson’s disease patients also exhibit hyperbilirubinemia. It is possible that expressions of Mrp2 in LEC rats and Wilson’s disease patients are further decreased at the stage of hepatitis. Further studies are needed to elucidate the expression of Mrp2 in the intestine of the patients with Wilson’s disease.

In addition to hepatic failure, expression of intestinal Mrp2 has been reported to be down-regulated by bile duct obstruction. In bile duct-ligated rats, down-regulation of intestinal Mrp2 is due to reduced binding of the heterodimer retinoid X receptor to the DR5 element, and IL-1β plays a role as a central mediator for transcriptional and posttranscriptional intestinal Mrp2 down-regulation. It is possible that these factors are responsible for the down-regulation of intestinal Mrp2 in LEC rats. Further studies are needed to elucidate the mechanisms of down-regulation of intestinal Mrp2 in LEC rats and to evaluate the expression of intestinal MR2 in Wilson’s disease patients.

In summary, we found that the expression of Mrp2 in the jejunum of LEC rats is decreased. The clinical consequences of our observations may comprise increased oral bioavailability of MR2 substrates, including numerous drugs as well as carcinogens with genotoxic effects, in Wilson’s disease patients.

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References
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