Contribution of Down-Regulation of Intestinal and Hepatic Cytochrome P450 3A to Increased Absorption of Cyclosporine A in a Rat Nephrosis Model

Tomoe Fujita, Shuichi Yasuda, Yuji Kamata, Kazumi Fujita, Yoshio Ohtani, Yuji Kumagai, and Masataka Majima

Departments of Pharmacology (T.F., Y.Ku., M.M.) and Nephrology (K.F.) and Center for Genetic Studies of Integrated Biological Functions (S.Y.), Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan; Department of Endocrinology, Diabetes and Metabolism, Graduate School of Medical Sciences, Kitasato University, Sagamihara, Kanagawa, Japan (Y.Ka.); Syounan Sakura Clinic, Chigasaki, Kanagawa, Japan (Y.O.); and Clinical Investigation Center, Kitasato University East Hospital, Sagamihara, Kanagawa, Japan (T.F., Y.Ku.)

Received June 12, 2008; accepted August 22, 2008

ABSTRACT

This study examined the contribution of changes in regulation of intestinal and hepatic cytochrome P450 3A (CYP3A) and multidrug resistance transporter 1 (Mdr1) to absorption of cyclosporine A (CsA) in a rat nephrosis model. Interleukin (IL)-6 was also measured. Puromycin aminonucleoside at a dose of 20 mg/100 g was administered intravenously. Tissue samples were dissected out from the upper and middle intestines and liver after development of nephrosis to measure the expression levels of mRNA and protein. CsA at a dose of 0.5 mg/100 g was administered into a closed loop of the upper and middle intestines. Blood from the inferior vena cava (IVC) and portal vein was taken until 30 min after administration. The expression levels of CYP3A decreased markedly, whereas those of Mdr1 showed large interindividual variations for all of the tissues in the nephrotic rats. Plasma concentrations of CsA reached higher levels in the nephrotic than in the control rats and were higher when administered from the upper than the middle intestine in both the portal vein and IVC. IL-6 increased in urine in the nephrotic rats. In summary, intestinal and hepatic CYP3A were down-regulated in the nephrosis model accompanying the increased levels of IL-6. Consistent results were not obtained for the regulation of Mdr1. In conclusion, these findings suggest that the down-regulation of CYP3A in the upper intestine and liver predominantly contributes to the increase in CsA absorption, and Mdr1 showed less contribution in this rat nephrosis model.

Metabolism through cytochrome P450 3A (CYP3A) and multidrug resistance transporter (Mdr1) distributed in the intestine and liver is considered to be one of the factors contributing to the variability of cyclosporine bioavailability in patients (Lown et al., 1997; Renton, 2005). Cyclosporine A (CsA) is metabolized by the CYP3A family and transported by the Mdr1 family, a drug efflux pump, in the intestine and liver, which acts as a barrier against drug exposure to the body in the liver as well as the intestine. CsA has been used to treat nephrosis, and interindividual variation in the oral bioavailability of CsA has been reported in patients with nephrosis (Goumenos et al., 2006).

Changes in regulation of P450 enzyme as well as Mdr1 under acute or chronic inflammatory conditions have been extensively studied in both animals and humans (Renton, 2001; Aitken et al., 2006; Fradette et al., 2007). Several studies have reported changes in the pharmacokinetics of CsA in relation to changes in the regulation of intestinal or hepatic CYP3A and Mdr1. The metabolic rate of CsA has been shown to decrease in conjunction with a fall in intestinal and hepatic CYP3A contents in a murine infection model (Berg-Candolfi et al., 1996). In addition, elevated cyclosporine concentrations in blood observed in liver transplant recipients with diarrhea were explained as being due to a possible involvement of the suppression of intestinal CYP3A and Mdr1 with an intestinal inflammation (Maezono et al., 2005).

The aim of the present study was to determine changes in the mRNA and protein expression levels of CYP3A and Mdr1.
in the upper and middle intestines, where drugs are mainly absorbed, and liver in a rat nephrosis model. We then examined whether these changes affect the absorption of CsA in vivo when administered from the upper and middle intestines. Furthermore, to examine the first-pass effects on CsA absorption by the intestine and liver separately, CsA concentrations were measured in blood obtained from the portal vein and inferior vena cava (IVC) across the diaphragm. The changes in interleukin (IL)-6 levels in urine were measured to determine the involvement of IL-6 in this model. Purinomycin aminonucleoside (PAN)-induced nephrosis was adopted as the nephrosis model because it is a representative nephrosis model and is a simple model to make (Koltun et al., 2005).

Materials and Methods

Animals. Seven-week-old male Sprague-Dawley rats (Charles River Laboratories Japan Inc., Kanagawa, Japan) were housed in our animal care facility under constant humidity and temperature and a 12-h light/dark cycle. The animals were maintained on a certified diet of MF pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The experiments were approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine.

Experimental Design. The quantification of expression levels of genes and proteins of intestinal and hepatic CYP3A and Mdr1 was performed using nephrotic (n = 4) and control (n = 4) rats. To examine the first-pass effects on CsA absorption by the intestines and those by the intestine and liver, blood was taken from the portal vein on day 1. The second dose of PAN (10 mg/100 g) was administered from the upper intestine and blood was taken from the IVC (n = 15 for both groups) and IVC (n = 14 and n = 12, respectively). The two groups were further divided according to the administration site in the intestine. Cytokine levels and protein in urine were measured in the nephrosis and control groups, where CsA was administered from the upper intestine and blood was taken from the IVC (n = 7 and n = 6, respectively). Blood biochemistry was examined in two other subgroups (nephrosis, n = 8 and control, n = 9).

Induction of Nephrosis. Nephrosis was induced by administration of PAN (Sigma-Aldrich, St. Louis, MO) in two divided doses. In brief, the rats were placed in a restrainer, and 10 mg/100 g body weight of PAN (3.5% solution in saline) was administered via the tail vein on day 1. The second dose of PAN (10 mg/100 g) was administered on day 14. Control rats received saline. On day 23, the rats were used in the following study. Doses of PAN were determined based on the results of a preliminary experiment in which small decreases in serum protein and albumin levels were observed in nephrotic rats treated with PAN at a dose of 12 mg/100 g (nephrosis, 5.6 ± 0.080 and 3.4 ± 0.048 g/dl; control, 5.8 ± 0.18 and 4.2 ± 0.046; mean ± S.E., n = 6) 15 days after a single intravenous administration. Thus, rats were made nephrotic by sequential doses of PAN similar to a previous study (Kim et al., 2005).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction. After exsanguination under etherization, the intestine and liver tissue specimens were excised. The intestinal samples were cut 20 and 50 cm distal to the pylorus for the upper and middle intestines, respectively. Tissue samples were immediately immersed in ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan), homogenized using a pestle, and then stored at −80°C until RNA extraction. Total RNA was extracted using a phenol-chloroform extraction method. RNA coding for CYP3A23/3A1, CYP3A2, CYP3A9, CYP3A18, Mdr1a, and Mdr1b was used to evaluate two-step quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used as a reference to normalize the quantity of RNA. Specific primer sets were designed and synthesized for each isoform by NIHON Gene Research Laboratories Inc. (Sendai, Japan) (Table 1). It is reported that CYP3A1 may be an allelic variant of CYP3A23, and the sequences of the primers used in the present study were common in both genes (Mahnke et al., 1997). Thus, these primers could amplify both genes. The RT-PCR was performed with a LightCycler DX400 (Roche Diagnostics, Mannheim, Germany). Annealing temperatures were 58°C for CYP3A23/3A1 and CYP3A2 and 60°C for CYP3A9, CYP3A18, and Mdr1a and Mdr1b. Isoforms of CYP3A and Mdr1 in the intestine and liver were determined based on the expression profiles of those reported previously (Takara et al., 2003). Accordingly, CYP3A9 and CYP3A18 mRNA and CYP3A23/3A1, CYP3A2, and CYP3A18 mRNA were determined in the intestines and liver, respectively. Mdr1a and Mdr1b were both examined in the intestine and liver. mRNA levels were quantified by extrapolating the crossing points for the samples to the standard curves. The mRNA levels of CYP3A3 and Mdr1 isoforms were normalized by that of GAPDH. Results are expressed as the relative values to a major isoform of CYP3A in the control for the same region.

Western Blot Analysis. Intestine and liver tissue specimens were excised. Intestinal samples were obtained 10 to 20 and 40 to 50 cm distal to the pylorus for the upper and middle intestines, respectively. Tissue samples were rapidly frozen in liquid N2 and stored at −80°C until protein extraction. Samples were homogenized using a BioMasher (Wako Pure Chemicals, Osaka, Japan). The homogenates were diluted in 0.01 M Tris-HCL, pH 7.4, with leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride and centrifuged at 4000 g for 10 min. The supernatant was further centrifuged at 100,000 g for 30 min, and the supernatant and precipitant were used to measure the protein contents of CYP3A and Mdr1, respectively. Protein content in the supernatant and precipitant was measured using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL), and aliquots of 8 μg of proteins were electrophoresed on SDS-polyacrylamide gels. Separated proteins were transferred to a polyvinylidene difluoride membrane. The blots were incubated with the first antibody of CYP3A (H-300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Mdr1 (H-241; Santa Cruz Biotechnology, Inc.), or actin (H-300; Santa Cruz Biotechnology, Inc.) diluted in 50 mM Tris-HCL, pH 7.5, and 150 mM NaCl containing 0.1% Tween 20 and 1.5% bovine serum albumin (dilution 1:200). After washing, the blots were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Cosmo Bio Co., Ltd., Tokyo, Japan) and diaminobenzidine. The blots were visualized with horseradish peroxidase-labeled goat anti-rabbit IgG (Cosmo Bio Co., Ltd., Tokyo, Japan), and 3',5'-diaminobenzidine. The blots were visualized with horseradish peroxidase-labeled goat anti-rabbit IgG (Cosmo Bio Co., Ltd., Tokyo, Japan), and 3',5'-diaminobenzidine.
the upper end of each loop at a dose of 0.5 mg/100 g. CsA concentrations in the portal vein and IVC were measured after CsA administration from the intestines. For blood sampling from these vessels, two separate experiments were conducted. Due to difficulty in placing a tube exactly at the junction site of the IVC and hepatic vein, blood was collected in the IVC across the diaphragm where the blood from the hepatic vein flows. The drug that was measured was considered to represent the drug that had passed through two sequential barriers, the intestine and liver. Cannulation was conducted as described in a previous study (Nishigaki et al., 1998). In brief, an intramedic polyethylene tube (PE50; Nippon Becton Dickinson Co., Tokyo, Japan) was inserted into the femoral vein toward an IVC. A tube inserted into the blood vessels was filled with saline solution containing heparin. Blood was taken via a tube at 7, 15, and 30 min after administration of CsA. Blood sampling from the portal vein was conducted by obtaining blood directly from the vein 30 min after ingestion of CsA.

**Measurement of Cyclosporine A Concentration.** Plasma concentrations of CsA were determined by radioimmunoassay using CYCLO-Trac (DiaSorin, Inc., Stillwater, MN). This method shows minimal cross-reactivity with CsA metabolites and is more specific to parent CsA (Murthy JN et al., 1998).

**Blood and Urine Chemistry and Cytokine Assay.** Blood was withdrawn from the IVC under ether anesthesia on day 23, and the serum was isolated by centrifugation at 1500 g for 10 min at room temperature. Urine was collected by housing the rats in metabolic cages for 24 h on days 0 to 1, 7 to 8, 13 to 14, and 21 to 22. Assays were carried out by SRL, Inc. (Tachikawa, Tokyo, Japan). IL-6 in the 24-h urine samples was detected by using the Immunoassay Kit (BioSource International, Camarillo, CA).

**Statistical Analysis.** All results are expressed as the mean ± S.E. Differences in physiological parameters, mRNA levels for CYP3A and Mdr1 isoforms, CsA concentrations, and cytokine levels in 24-h urine samples were detected by using the Immunoassay Kit containing heparin. Blood was taken via a tube at 7, 15, and 30 min (data not shown). As shown in Fig. 5A, plasma concentrations of CsA were measured at two sites, the portal vein and IVC from the hepatic vein flows. The drug that was measured was considered to represent the drug that had passed through two sequential barriers, the intestine and liver. Cannulation was conducted as described in a previous study (Nishigaki et al., 1998). In brief, an intramedic polyethylene tube (PE50; Nippon Becton Dickinson Co., Tokyo, Japan) was inserted into the femoral vein toward an IVC. A tube inserted into the blood vessels was filled with saline solution containing heparin. Blood was taken via a tube at 7, 15, and 30 min after administration of CsA. Blood sampling from the portal vein was conducted by obtaining blood directly from the vein 30 min after ingestion of CsA.

**Results**

**Physiological Parameters.** Tables 2 and 3 present the biochemical data, body weights, and urinary protein excretion values in the control and nephrosis group. Nephrotic rats exhibited significant decreases in serum protein and albumin and increases in serum cholesterol and urine protein excretion. Urine protein excretion started to increase 7 days after administration of PAN and further increased 7 days after the second administration. Body weights were lower in the nephrosis group than in the control group.

**mRNA and Protein Levels of CYP3A and Mdr1 Isoforms in the Intestine and Liver.** To examine whether the regulations of CYP3A and Mdr1 isoforms were changed in the nephrosis models in the intestines and liver, mRNA levels were evaluated by quantitative RT-PCR. As shown in Fig. 4A, Expression of Mdr1 protein showed large interindividual differences for all tissues in both groups (Fig. 4B).

**Absorption of Cyclosporine A.** Plasma concentrations of CsA were measured at two sites, the portal vein and IVC after administration of CsA from the upper and middle intestines, to evaluate the changes in absorption of CsA, which first undergoes metabolism in the intestine followed by metabolism in the liver by CYP3A. In a preliminary experiment, blood was taken from the portal vein at 0, 3, 5, 7, 10, 15, 30, 45, and 60 min after administration of CsA, and the drug appeared in plasma at 3 min and reached the maximum at 30 min (data not shown). As shown in Fig. 5A, plasma concentrations of CsA in the portal vein increased significantly in the nephrosis group at 30 min after administration of CsA for both intestinal administration sites. The concentrations were also higher in the group administered via the upper intestine than that via the middle intestine. Similar results were observed for plasma CsA concentrations in the IVC. They were also higher in the nephrosis group than in the control group at all time points tested for both intestinal administration sites (Fig. 5, B and C). The concentrations were higher in the group administered CsA via the upper intestine than that via the middle intestine at ASPET Journals on July 13, 2017 jpet.aspetjournals.org Downloaded from

**Amounts of Cytokine Excreted in Twenty-Four-Hour Urine.** Urinary excretion of IL-6 was measured to determine whether IL-6 was involved in the regulation of CYP3A expression. IL-6 was not detected in the control group throughout the study, whereas it increased gradually with time in the nephrosis group (Fig. 6).

**TABLE 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 9)</th>
<th>Nephrosis (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum total protein (g/dl)</td>
<td>5.8 ± 0.049</td>
<td>5.2 ± 0.28**</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>4.0 ± 0.029</td>
<td>2.2 ± 0.12**</td>
</tr>
<tr>
<td>Serum total cholesterol (mg/dl)</td>
<td>53.3 ± 2.82</td>
<td>44.0 ± 36.5**</td>
</tr>
<tr>
<td>Serum urea nitrogen (mg/dl)</td>
<td>17.4 ± 0.837</td>
<td>28.4 ± 2.80**</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.25 ± 0.015</td>
<td>0.46 ± 0.055**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>391 ± 11.6</td>
<td>317 ± 9.37**</td>
</tr>
</tbody>
</table>

*P < 0.05 and **P < 0.01, significantly different from the control.

**TABLE 3**

<table>
<thead>
<tr>
<th>Day</th>
<th>Control (n = 6)</th>
<th>Nephrosis (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>3.9 ± 0.6</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>7–8</td>
<td>8.6 ± 0.9</td>
<td>323.6 ± 42.9**</td>
</tr>
<tr>
<td>13–14</td>
<td>11.8 ± 1.1</td>
<td>345.1 ± 64.2**</td>
</tr>
<tr>
<td>21–22</td>
<td>12.2 ± 1.1</td>
<td>638.5 ± 74.1**</td>
</tr>
</tbody>
</table>

**P < 0.01, significantly different from the control.**
Discussion

The PAN-induced nephrosis model used in the present study exhibited typical phenotypes of nephrosis in humans, including hypoproteinemia, hypoalbuminemia, hyperlipidemia, and proteinuria. We examined changes in the regulation of intestinal and hepatic CYP3A and Mdr1 in the
nephrotic state and then studied whether the changes affect the absorption of CsA in vivo. To assess the effects of the changes in regulation of CYP3A and Mdr1 in the upper and middle intestines, respectively, on CsA absorption and the differences in CsA absorption between the upper and middle intestines, drug concentrations in blood obtained from the portal vein and IVC were measured after CsA administration into the respective site of the intestine.

In this study, we have shown the following: 1) the protein levels of CYP3A decreased markedly in the intestines and liver in the nephrosis group, an observation that is correlated with the mRNA data; 2) the mRNA levels of Mdr1a decreased in the upper and middle intestines, whereas those of Mdr1b showed a large variation in the nephrosis group, and the protein levels of Mdr1 showed large variations in the intestines and liver in both groups; 3) the plasma concentrations of CsA in the groups administered from the upper intestine were higher in those from the middle intestine; 4) the plasma concentrations of CsA in the portal vein and IVC increased in the nephrosis group compared with the control groups; and 5) the urine levels of IL-6 were higher in the nephrosis group than those in the control groups.

There have been several reports demonstrating considerable decreases in the gene expression of hepatic CYP3A isoforms in inflammatory models. In rat lipopolysaccharide- or Freund's complete adjuvant-induced inflammation, mRNA levels of CYP3A2 were reduced by 60% of the control, and those of CYP3A1 were reduced by approximately 85% of the control (Sewer and Morgan, 1998; Projean et al., 2005). It seems that down-regulation of hepatic CYP3A genes is a common feature of inflammatory states, and it can also be applied to our PAN-induced nephrosis model. Mild uremia, that is, increases in serum creatinine and urea nitrogen by 84 and 63%, respectively, of the control, was observed in the nephrotic rats. Because it has been reported that severe uremic rats produced by 5/6 nephrectomy, which accompanied increases in serum creatinine and urea by 171 and 410%, respectively, of the control, exhibited complete suppression of hepatic CYP3A1 and CYP3A2, a uremic state may partly affect mRNA levels of hepatic CYP3A isoforms (Leblond et al., 2001).

Few studies have examined the changes in protein or mRNA levels of intestinal CYP3A in pathological states. In one study using a lipopolysaccharide-induced intestinal damage model, reduced intestinal CYP3A activity was suggested as shown by the decreased rate of nifedipine oxidation (Maezono et al., 2005). In another study on uremic rat CYP3A2, mRNA expression was observed to have decreased in enterocytes derived from the small intestine (Leblond et al., 2002). These results are in agreement with those of the present study, where CYP3A isoforms were down-regulated in the intestine.

There have been several reports regarding the regulation of intestinal and hepatic Mdr1 in different pathological states. In a lipopolysaccharide-induced intestinal damage model, the activity of Mdr1 was reduced as indicated by enhancement of the mucosal to serosal flux of digoxin and reduction of the efflux of digoxin in the proximal segment of the jejunum (Maezono et al., 2005). A case report of a living donor liver transplant recipient after chronic rejection showed that intestinal mRNA levels of Mdr1 had increased to a greater degree in the second living donor liver transplant recipient than in the first (Masuda et al., 2003). Regulation of hepatic Mdr1 during inflammation has been extensively investigated in rodents. Different results were obtained from a study using rats treated with inflammatory stimuli such as

---

**Fig. 3.** mRNA levels of CYP3A23/3A1, 3A2, and 3A18 (A) and Mdr1a and Mdr1b (B) in rat liver determined by RT-PCR. Rats were treated with 20 mg/100 g PAN. Control rats received saline. The rats were sacrificed 9 days after the second administration. Two-step quantitative RT-PCR reactions were performed on CYP3A23/3A1, CYP3A2, CYP3A18, Mdr1a, and Mdr1b mRNA with GAPDH mRNA as an internal standard. There were four rats in the control group and four in the nephrosis group. Data are normalized by GAPDH and expressed as relative to the control of CYP3A23/3A1. The control and nephrosis groups were compared using the unpaired t test. Data are expressed as the mean ± S.E.
lipopolysaccharide or turpentine. Accordingly, down-regulation of both Mdr1a and Mdr1b, down-regulation of Mdr1a and up-regulation of Mdr1b, and unchanged regulation of Mdr1a and up-regulation of Mdr1b have been reported (Piquette-Miller et al., 1998; Vos et al., 1998; Cherrington et al., 2004). These results suggest that regulation of intestinal or hepatic Mdr1 seems to be different depending on the inflammatory model. In the present study, protein levels of Mdr1 showed a large variation in the intestines and liver, which may be reflected by large variations in the expression levels of Mdr1a and Mdr1b mRNA.

In the present study, examination of the absorption of CsA revealed that CsA was absorbed predominantly from the upper intestine, which was consistent with the findings of previous studies (Cakaloglu et al., 1993; Jin et al., 2006). Increased plasma concentrations of CsA in the nephrotic rats compared with the control rats indicates that the first-pass effects on CsA absorption by the intestine and liver decreased in the nephrosis model. These findings may have resulted from the down-regulation of intestinal and hepatic CYP3A. Jin et al. (2006) reported that the upper intestinal Mdr1 contributes to the inhibition of CsA absorption in mice with a strong induction of Mdr1 by dexamethasone treatment. In this study, significant differences in the up-regulation of Mdr1 in the upper and middle intestines were not demonstrated between the nephrosis and control groups. Thus,
intestinal Mdr1 showed less contribution to the inhibition of the absorption of CsA in this nephrosis model. Changes in hepatic blood flow should be considered as other possible factors that influence the first-pass effect on CsA absorption.

IL-6 has been recognized as an important cytokine that decreases hepatic expression of various P450 isoforms during the inflammatory process (Siewert et al., 2000). In patients undergoing bone marrow transplantation, the time of peak IL-6 levels in serum was correlated with the time of peak CsA levels in the blood (Chen et al., 1994). Similar phenomena were observed in the present nephrosis model, that is, increase in CsA absorption accompanied the increase in IL-6 levels in urine.

In summary, strong down-regulation of CYP3A isoforms was demonstrated for the upper and middle intestines and liver in the present nephrosis model. Absorption of CsA was
Increased Absorption of Cyclosporine A in a Nephrosis Model


Mohan Metab 31:765–771.


Address correspondence to: Dr. Tomoe Fujita, Department of Pharmacology, Kitasato University School of Medicine, Kitasato 1-15-1, Sagamihara, Kanagawa 229-8555, Japan. E-mail address: fujita.t@kitasato-u.ac.jp

References


