The Disposition of Pravastatin in a Rat Model of Streptozotocin-Induced Diabetes and Organic Anion Transporting Polypeptide 2 and Multidrug Resistance-Associated Protein 2 Expression in the Liver

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The combination of diabetes and hyperlipidemia promotes the development of atherosclerosis. Therefore, it is important for diabetic patients to control blood fat. 3-Hydroxy-3-methylglutaryl enzyme A (HMG-CoA) reductase inhibitors (statins), like pravastatin, are frequently administered to diabetic patients for this purpose. Although the alterations of metabolic enzymes and transporters in the diabetic liver may change the disposition of pravastatin, the effect has not been fully investigated. In the present study, we investigated the disposition of pravastatin and the mRNA expression of transporters in the liver. Pravastatin (5 mg·kg⁻¹ body weight) was administered intravenously to diabetic rats, and the pravastatin concentrations in the plasma, urine, and bile were measured by high-performance liquid chromatography. Changes in the mRNA expressions of multidrug resistance-associated protein 2 (MRP2) and organic anion transporting polypeptide 2 (OATP2) in the liver were also estimated using reverse transcriptase-polymerase chain reaction (RT-PCR). We found that the plasma pravastatin concentration was lower in the diabetic rat because the transportation of pravastatin into hepatocytes was promoted along with increased expression of OATP2. The biliary excretion ratio of pravastatin was significantly lower in the diabetic rat because the pravastatin transportation into bile was reduced along with the decreased expression of MRP2. To clarify these phenomena, the analysis of mRNA expression using real-time PCR and the measurement of the amount and the activity of proteins are necessary in future study.

Key words diabetes; disposition; pravastatin; organic anion transporting polypeptide 2; multidrug resistance-associated protein 2

In recent years, morbidity of diabetes, which is a metabolic disorder caused by the absence of insulin and/or organ response to this lack of insulin, has been increasing. Diabetes, hyperlipidemia, and hypertension accelerate atherosclerosis and increase risk of stroke and heart disease. The major cause of diabetes, hyperlipidemia, and hypertension is obesity. The combination of those 4 factors (the obesity syndrome, diabetes, hypertension, and hyperlipidemia) is a risky condition called “the deadly quartet.” The combination of diabetes and hyperlipidemia is especially dangerous because it promotes the development of atherosclerosis. Therefore, it is important for diabetic patients to control blood fat. Drugs such as 3-hydroxy-3-methylglutaryl enzyme A (HMG-CoA) reductase inhibitors (statins) are frequently administered to diabetic patients for this reason.

Pravastatin, a hydrophilic statin, has a high safety profile and is often used for treating hyperlipidemia in diabetic patients. However, serious symptoms like a rhabdomyolysis may be caused by pravastatin administration as the side effect.

In diabetes, the expression of metabolic enzymes and transporters in the liver is altered. Shimojo demonstrated that the expressions of cytochrome P450 (CYP) 1A2, 2B1, and 4A were altered in rat models of type 1 diabetes induced by streptozotocin (STZ) treatment.1) Furthermore, van Waarde et al. reported that the expression of multidrug resistance-associated protein 2 (MRP2) was decreased, whereas the expression of multidrug resistance-2 (MDR2) was increased, and that of the bile salt export pump (BSEP) was unchanged.2) It is possible that alterations of metabolic enzymes and transporters in the diabetic liver may change the disposition of pravastatin. The alteration of pravastatin disposition may lead to the decline or increase of the effect and the risk of the side effect. However, the disposition of pravastatin in diabetes has not been fully investigated. We consider that it is important to examine the change of pravastatin disposition in the diabetes for appropriate treatment of diabetic patients with pravastatin.

Therefore, in the present study, we investigated the disposition of pravastatin in a rat model of streptozotocin-induced diabetes. Moreover, we also evaluated the changes in the mRNA expression of organic anion transporting polypeptide 2 (OATP2) and MRP2 in the liver and discussed these changes with regard to pravastatin disposition changes.

MATERIALS AND METHODS

Materials Pravastatin and triamcinolone acetonide were purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. STZ and Sepasol RNA I Super were obtained from Nacalai Tesque Inc., Kyoto, Japan. Glycogen solution and the SuperScript III first-strand synthesis system for reverse transcriptase-polymerase chain reaction (RT-PCR) were obtained from Life Technologies Inc., Tokyo, Japan. The KOD Dash kit was obtained from Toyobo Inc., Osaka, Japan. Sense and antisense primers for OATP2, MRP2, and β-actin were purchased from Bex Inc., Tokyo, Japan. All the other reagents used were molecular biology grade and the highest quality available.

Animals Fourteen male 6-week-old Donryu rats were purchased from Japan SLC Inc., Shizuoka, Japan, and were housed for 1 week to exclude any abnormal animals. Seven
rats (STZ-4w group) were injected intraperitoneally (i.p.) with STZ at a dose of 50 mg·kg⁻¹ body weight after an overnight fast and bred for 4 weeks after STZ administration. Other 7 rats (control group) were injected i.p. with saline and were bred for 4 weeks after saline administration. Three rats from each group were killed under pentobarbital anesthesia 4 weeks after STZ or saline administration; liver tissue and blood samples were collected and stored at −80 °C until analysis. The liver tissue was stored in RNA stabilization solution to prevent the decomposition of total RNA by RNase. The blood samples were centrifuged at 12000 rpm for 10 min to obtain plasma samples. Samples were dispatched to FALCO Biosystems Ltd., Kyoto, Japan, to determine the concentrations of total bile acids and serum albumin. The blood glucose level was measured using Precision Q.I.D™ (Abbott Japan Co., Ltd., Tokyo, Japan). All rats were bred under the free feeding and taking water condition. The operations were done at the same time (13:00) for all groups. All rats were handled in accordance with the Guidelines for the Care of Laboratory Animals established by Kobe Gakuin University. The protocol for this animal study was approved by the Animal Experimentation Ethics Committee of Kobe Gakuin University.

Disposition of Pravastatin Four male rats from each group were continuously injected intravenously (i.v.) with pravastatin at a dose of 5 mg·kg⁻¹ body weight via the lower limb vein, and under isoflurane anesthesia, 0.5 ml blood samples were collected from the arteries of the contralateral limb. Samples were obtained at 0, 5, 10, 20, 30, 45, and 60 min after pravastatin administration; these samples were centrifuged at 12000 rpm for 10 min to obtain the plasma. Excreted urine was collected during the experiments, and bladder urine was directly collected at the 180 min from pravastatin administration. Bile excreted during the 180 min from pravastatin administration was collected via the bile duct. The plasma, urine, and bile samples were stored at −80 °C until analysis.

Solid-phase extraction was performed for these samples using Inertsep C18-C FF cartridges (GL Sciences Inc., Tokyo, Japan). The cartridges were conditioned with 6 ml of methanol, 6 ml of acetonitrile, and 6 ml of water. After this conditioning, 200 μl of the sample was added to 20 μl of 10 μg·ml⁻¹ triamincolone acetonide as an internal standard and loaded onto the cartridges. The plasma samples were loaded undiluted, while the urine and bile samples were loaded after dilution in saline. The cartridges were then washed twice with 3 ml of water. Samples were eluted with 300 μl of acetonitrile, and the eluent was evaporated at 50 °C under reduced pressure. The residue was dissolved in 100 μl of the mobile phase, and aliquot of the sample was injected into a high-performance liquid chromatography. Pravastatin was detected using an ultra violet (UV)–visible spectrophotometer (254 nm; SPD-10A, Shimadzu Corp., Kyoto, Japan). The reverse-phase column (Inertisil ODS-3, GL Sciences, Inc., Tokyo, Japan) was maintained at 40 °C. The mobile phase used was a mixed solution of 20 mM phosphate buffer (pH 2.0) and methanol (55:45 by volume). The flow rate of the mobile phase was kept constant at 1 ml·min⁻¹ for a total run time of 40 min.

The pharmacokinetic parameters of pravastatin in plasma were calculated using 1-compartment model fitting with MULTI.

Analysis of mRNA Expression by RT-PCR Total RNA was extracted from the rat liver tissue (100 mg) using a Sepasol RNA I Super, chloroform, and isopropanol. cDNA was synthesized by the SuperScript III first-strand synthesis system using KOD Dash DNA polymerase. To a 24.5 μl reaction mixture containing KOD Dash DNA polymerase, 0.5 μl of synthetic cDNA and the sense and antisense primers were added. Denaturation, annealing, and extension were performed for 25—40 cycles using the iCycler system.

Degenerate PCR primers were designed and chemically synthesized on the basis of the amino acid sequences of rat OATP2 (496 bp; sense primer, 5'–TGC ACA CTG AGC AGC ATT CTG GC–3'; antisense primer, 5'–TGC ATG TAA CCC AAC TCC AA–3'), MRP2 (421 bp; sense primer, 5'–ATC CTC AGC TGC TGA AGT TG–3'; antisense primer, 5'–CTG ATC TTG GAT GCC AGA AC–3') and β-actin (646 bp; sense primer, 5'–ATG TAC TGA GCC ATC CAG GC–3'; antisense primer, 5'–TCC ACA CAG AGT ACT TGC GC–3').

The PCR products were resolved by subjecting them to electrophoresis on 2% agarose gels for 50 min. Subsequently, the agarose gels were stained with ethidium bromide. The bands were visualized using a UV transilluminator (Atto Corp., Tokyo, Japan), and specific bands were quantified using densitometric analysis.

Statistical Analyses The data are presented as the mean±standard deviation (S.D.). Significant differences were evaluated using Dunnett’s test. Values of p<0.05 were considered statistically significant.

RESULTS

A Rat Model of Diabetes The blood glucose level in the STZ-4w group (401.3±43.3 mg·dl⁻¹) was significantly higher than in the control group (134.5±27.4 mg·dl⁻¹). Although the volume of the urine in the STZ-4w group (8.0±1.8 ml) was approximately 2.7-fold higher than in the control group (2.8±0.57 ml), the volume of the bile in the STZ-4w group (2.2±0.89 ml) was similar to that in the control group (2.2±0.48 ml). The volume of drinking water and food intake was approximately 7-fold and 2.5-fold higher in the STZ-4w group, respectively (data not shown). In contrast, the body weight was significantly lower in the STZ-4w group (360.0±31.4 g) than in the control group (426.6±27.0 g). These results indicated that a STZ-administered rat could be used as a rat model of diabetes type 1. The concentration of total bile acids was higher in the STZ-4w group (131.9±119.4 μM) than in the control group (36.2±22.5 μM). The concentration of serum albumin in the STZ-4w group (2.1±0.1 g·dl⁻¹) was similar to that in the control group (2.3±0.1 g·dl⁻¹).

Disposition of Pravastatin The plasma concentration–time profile of pravastatin in plasma was shown in Fig. 1, and the pharmacokinetic parameters calculated as 1-compartment model are shown in Table 1. The extrapolated initial concentration (C₀) and half-life (T₁/₂) were lower in the STZ-4w group than in the control group. The distribution volume (Vd) and total body clearance (CL) were significantly higher in the STZ-4w group than in the control group. Area under the blood concentration–time curve (AUC) was significantly lower in the STZ-4w group than in the control group.
The excreted amount of pravastatin in bile over 180 min which showed as a percentage of administered dose was significantly lower in the STZ-4w group (7.2 ± 2.5%) than in the control group (23.2 ± 5.6%). On the other hand, the excreted amount of pravastatin in urine over 180 min which showed as a percentage of administered dose were very small in both groups (2.1 ± 1.9% in the STZ-4w group, 0.53 ± 0.68% in the control group).

Analysis of mRNA Expression by RT-PCR  In rat models of STZ-induced diabetes, we investigated the mRNA expression of OATP2, which transports pravastatin from blood to hepatocytes, and MRP2, which transports pravastatin from hepatocytes into bile. The corresponding electrophoretic profiles and band strengths are shown in Fig. 2. The mRNA expression of OATP2 was approximately 1.6-fold higher in the STZ-4w group than in the control group (Fig. 2A). In contrast, the mRNA expression of MRP2 in the STZ-4w group was approximately half of the control group (Fig. 2B).

DISCUSSION

Although urinary and stercoraceous excretion contribute to pravastatin elimination at the same level in humans, pravastatin is primarily eliminated by stercoraceous excretion in rats.3,4) The excretion route via bile is defined as a main stercoraceous excretion route.5) It is thought that pravastatin is mainly eliminated as an unmetabolized form because pravastatin is hardly metabolized by CYP in human and rat liver.6,7)

The pravastatin disposition markedly changed in the STZ-4w group: the plasma concentration and biliary excretion ratio in the STZ-4w group was significantly lower than in the control group (Fig. 1). We analyzed the plasma concentration-time profiles of pravastatin using 1-compartment model fitting and found that $V_d$ and $CL_{tot}$ were higher in the STZ group than in the control group. We also found that $C_0$, $AUC_0→\infty$ and $T_{1/2}$ were lower in the STZ-4w group than in the control group (Table 1). As the serum albumin levels in the both groups were comparable, these results indicated that the plasma concentration of pravastatin reduced in the STZ-4w group because the distribution volume and the elimination process of pravastatin were changed in the STZ-4w group. In addition, pravastatin excretion to outside of the body might be delayed in diabetes because biliary excretion ratio was lower in the STZ-4w group.

To explain these disposition changes in the STZ-4w group, we investigated the alteration of mRNA expression of OATP2 and MRP2 in liver. In the liver of the rat, pravastatin is transported from blood into hepatocytes by OATP2 and is transported from blood into bile by MRP2.8—10) Although the mRNA expression level of OATP2 was notably higher in the STZ-4w group than in the control group, the MRP2 mRNA

Table 1. Pharmacokinetic Parameters of Pravastatin Calculated as 1-Compartment Model

<table>
<thead>
<tr>
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<th>Control</th>
<th>STZ-4w</th>
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<tr>
<td>$C_0$ (µg·ml$^{-1}$)</td>
<td>5.0±2.3</td>
<td>2.3±0.80</td>
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<tr>
<td>$T_{1/2}$ (min)</td>
<td>14.2±4.7</td>
<td>9.8±3.4</td>
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<tr>
<td>$V_d$ (l·kg$^{-1}$)</td>
<td>1.2±0.50</td>
<td>2.4±0.83*</td>
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<tr>
<td>$CL_{tot}$ (ml·min$^{-1}$·kg$^{-1}$)</td>
<td>0.056±0.013</td>
<td>0.19±0.10*</td>
</tr>
<tr>
<td>$AUC_{0→\infty}$ (mg·min$^{-1}$·L$^{-1}$)</td>
<td>92.8±21.5</td>
<td>34.4±21.0*</td>
</tr>
</tbody>
</table>

$C_0$, extrapolated initial concentration; $T_{1/2}$, half-time; $V_d$, distribution volume; $CL_{tot}$, total body clearance; $AUC$, area under the blood concentration-time curve. Data are expressed as mean±S.D. $n$=4. *p<0.05 compared with the control rats.

Fig. 1. The Plasma Concentration–Time Profile of Pravastatin in the Control and STZ-4w Groups

The rats received pravastatin i.v. at a dose of 5 mg·kg$^{-1}$ body weight. The plasma concentration of pravastatin was determined at the indicated time points. $n$=4. Data are expressed as mean±S.D. *p<0.05 as compared to the control group rats.

Fig. 2. Electrophoretic Profiles of the mRNA Expression of OATP2 (a) and MRP2 (b) in the Liver after STZ Administration, Expressed as a Percentage of the Control Value

Data are expressed as mean±S.D. $n$=3. *p<0.05, compared with the control rats.
expression level was significantly lower in the STZ-4w group (Fig. 2). These results indicated that the plasma pravastatin concentration was lower in diabetes because the transport of pravastatin from blood into hepatocytes was promoted along with the increased expression of OATP2. In addition, the biliary excretion ratio of pravastatin from hepatocytes was significantly lower in the STZ-4w group than in the control group because the transport of pravastatin into bile was reduced along with the decreased expression of MRP2. It is known that the bile acids were associated with the expression of OATP2 and MRP2.11) We consider that the increase of serum bile acid enhanced the mRNA expression of OATP2 because the concentration of total bile acids in plasma was higher in the STZ group than in the control group in this study (Fig. 2A). On the other hand, the mRNA expression of MRP2 was decreased (Fig. 2B). The glutathione in the cell also plays a role in the transport by MRP2. The glutathione conjugate in the cell is excreted via MRP2.12) A decline in glutathione levels in both blood and liver and an increase of the glutathione-S-transferase activity were reported in the STZ-4w rats.2,13) Therefore, we think that the reduced concentration of glutathione in the diabetes might have decreased the mRNA expressions of MRP2 in this study.

In human, pravastatin was transported from blood into the liver via OATP1B1.8) It is known that the bile acids are associated with the expression of human OATP1B1 as well as rat OATP2.11) Therefore, we think that the expression OATP1B1 may be increased in the liver of human diabetic patient. This alteration may change the pravastatin disposition in human diabetic patient. The alteration of pravastatin disposition in diabetes has the possibility to increase the pravastatin accumulation in the liver, although the alteration of pravastatin disposition in human may be not remarkable because the urinary excretion of pravastatin in human is about 40% while that in rats is low.8) The hepatic accumulation might enhance the effect of pravastatin because pravastain mainly inhibit the synthesis of cholesterol in the liver. Therefore, we assume that the dosage of pravastatin in diabetic patient can be reduced compared to the non-diabetic patient to obtain the same effectiveness, resulting to decrease the risk of the side effect.

In present study, we analyzed mRNA expression using RT-PCR and observed that alterations in the expressions of OATP2 and MRP2 might be largely responsible for the alteration of pravastatin disposition in diabetes. To clarify these phenomena, the analysis of mRNA expression using real-time PCR and the measurement of the amount and the activity of proteins are necessary in future study.

REFERENCES