# Alteration of Pharmacokinetics of Grepafloxacin in Type 2 Diabetic Rats

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**ABSTRACT - Purpose.** Patients with type 2 diabetes are generally treated with various pharmacological compounds and are exposed to a high risk of drug-drug interactions. However, alterations of pharmacokinetics in a type 2 diabetes model have been obscure. The present study was undertaken to investigate the effects of type 2 diabetes on the pharmacokinetics of the fluoroquinolone grepafloxacin (GPFX) and the expression level of P-glycoprotein (P-gp), one of the drug efflux transporters. **Methods.** We used Goto-Kakizaki (GK) rats, a lean model of type 2 diabetes. Plasma concentration and intestinal, renal, and biliary clearance of GPFX were measured after intravenous and intraintestinal administration in Wistar and GK rats. Real-time PCR and Western blotting were used to assess mRNA and protein expression levels. **Results.** We found a significant increase in the plasma concentrations of GPFX at 90, 120 and 240 minutes after intraintestinal administration in GK rats compared with the concentrations in Wistar rats but not after intravenous administration. The increase in plasma GPFX concentration was associated with reduction in jejunal clearance of GPFX caused by a decrease in secretory transport of GPFX. However, there was no correlation between the decrease in secretory transport of GPFX and P-gp expression level. **Conclusion.** Type 2 diabetic conditions alter P-gp function as well as expression level and correlate poorly with each other.

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## INTRODUCTION

Diabetes mellitus (DM), which is mainly a glucose metabolism disorder, is associated with the development and progression of pathological changes in various organ systems. There are several distinct types of diabetes, but the two most common types are type I and type 2 DM, of which type 2 DM constitutes more than 90% of diabetes cases. Patients with type 2 DM are generally treated with various pharmacological compounds and are exposed to a high risk of drug-drug interactions (1).

Quinolones are antibacterial drugs with potent activities against a broad spectrum of bacteria and are well absorbed from the intestine and distributed to many tissues (2). However, the bioavailability of grepafloxacin (GPFX), one of the quinolones, in humans is 72% (3). It was reported that secretion of GPFX across human intestinal epithelial Caco-2 cells was mediated by P-glycoprotein (P-gp) (4) and that P-gp mediated the intestinal secretion of GPFX and limited the bioavailability of this drug in vivo (5).

Efflux transporters mainly belong to the ATP binding cassette (ABC) superfamilies, which comprise P-gp, multidrug resistance protein 2 (Mrp2) and breast cancer resistance protein (Bcrp). P-gp, a product of the mdr1 gene, is expressed in various tissues, including the brain, lung, liver, kidney, gastrointestinal tract, skin and muscle tissue (6, 7) and plays a critical role in drug absorption, biliary excretion, renal secretion, and central nervous system (CNS) entry of a broad range of hydrophobic substrates (8).

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Therefore, concomitant administration of drugs usually has been shown to cause drug-drug interactions via the inhibition of P-gp-mediated transport (9). Although some studies using streptozocin (STZ)-induced diabetic rats model of type 1 DM have shown that the pharmacokinetic behavior of many drugs may also be affected (10-12), alterations of the pharmacokinetics or expression level of efflux transporters in a type 2 DM model have been obscure. Goto-Kakizaki (GK) rats, a lean model of type 2 diabetes, exhibit a spontaneous polygenic disease (13). They were produced by repeated inbreeding of Wistar rats using glucose intolerance as the selection index. The GK rats show hyperglycemia, mild insulin resistance, impaired glucose-induced insulin secretion, and a decrease of β-cell mass. We therefore selected GPFX as a marker for evaluating P-gp function.

In the present study, we investigated pharmacokinetic alterations of GPFX and P-gp expression level in Wistar and GK rats.

## MATERIALS AND METHODS

### Chemicals
All other reagents were of the highest grade available and used without further purification.

### Animals
Male Wistar and Goto-Kakizaki (GK) rats, aged 6 weeks, were obtained from SLC Inc. (Hamamatsu, Japan). 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". GK rats, which exhibited hyperglycemia (>180 mg/dl) for 14 days in the non-fasting state, were used in the study. Blood samples of the rats in a non-fasting state were collected from the tail vein. Plasma glucose concentrations were measured and the values are shown in Table 1.

### Pharmacokinetic studies in rats
Pharmacokinetic studies were performed as described previously (5). The femoral artery and vein were cannulated with polyethylene tubing filled with heparin solution (100 U/ml) for blood sampling and drug administration, respectively. GPFX was injected intravenously at a dose of 10 mg/kg. In a separate experiment for intraintestinal administration of GPFX, the abdominal cavity of rats was opened via a midline incision, and the upper site of the duodenum was exposed to administer the drug. GPFX was injected into the lumen of the duodenum at a dose of 10 mg/kg. Blood samples were collected from the left femoral artery at 5, 15, 30, 45, 60, 90, 120 and 240 min after the end of the injection.

### Intestinal, renal, and biliary clearance in rats
The femoral artery and vein were cannulated as described above for the pharmacokinetic studies. The abdominal cavity of rats was opened via a midline incision to gain access to the small intestine. The common bile duct was cannulated for bile collection. The bladder was also cannulated for urine collection.

**Table 1.** Body weight, blood glucose and pharmacokinetic parameters of grepafloxacin (GPFX) after intravenous or intraintestinal administration in Wistar and Goto-Kakizaki (GK) rats.

|  | Wistar | GK |
|---|---|---|
| Body weight (g) | 288 ± 36 | 319 ± 37* |
| Blood glucose (mg/dL) | 114 ± 13 | 283 ± 80* |
| $CL_T$ (mL/min/kg) | 39.34 ± 3.51 | 35.11 ± 2.15 |
| $V_1$ (L/kg) | 1.96 ± 0.13 | 1.79 ± 0.07 |
| Q (mL/min/kg) | 99.72 ± 9.30 | 92.04 ± 4.63 |
| $V_2$ (L/kg) | 3.89 ± 0.60 | 2.99 ± 0.22 |
| $V_{ss}$ (L/kg) | 5.84 ± 0.69 | 4.78 ± 0.16 |
| $CL_T/F$ (mL/min/kg) | 83.30 ± 7.17 | 54.48 ± 5.44* |
| Tmax (min) | 21.00 ± 3.67 | 24.00 ± 3.67 |
| Cmax (μg/mL) | 1.19 ± 0.09 | 1.32 ± 0.17 |
| F (%) | 47.22 | 64.44 |

Body weight or blood glucose value represents the mean ± S.D. of 24-25 rats.
GPFX was injected at a dose of 10 mg/kg. Each parameter value represents the mean ± S.E. of 4-5 rats.
*, significantly different fromWistar rats at $p < 0.05$.

The whole small intestine starting from the Treitz ligament was used to make an intestinal loop. After washing the loop with saline until the efflux was clear, 2.5 ml of saline was injected into the loop. GPFX was injected at a dose of 10 mg/kg intravenously *via* the catheterized right femoral vein. Blood samples were collected at 2, 5, 15, 30, 45 and 60 min after the injection from the left femoral artery. After 60 min, the contents of the loop were withdrawn as much as possible, and the lumen was washed with saline to give a volume of 15 ml.

### Analytical methods

The concentrations of GPFX in plasma, intestinal fluid, urine, bile, and tissue homogenate were measured by high-performance liquid chromatography (HPLC). The system used was an L-7110 pump and an L-7485 detector (Hitachi, Tokyo, Japan). The flow rate was 0.8 mL/min and injection volume was 50 µL. Fluorescence detection at 448 nm with excitation at 285 nm was used. Separation of GPFX was performed at 40 °C on a TOSOH TSK-gel ODS-80$T_M$. Mobile phase was 20 mM $Na_2SO_4$ (0.1% $H_3PO_4$) - acetonitrile (74:26, v/v). For sample preparation, 100 µL of plasma was placed into a 1.5-mL Eppendorf tube and methanol (200 µL) was added for protein precipitation, and then the mixture was vortexed and centrifuged for 10 min at 3,000 g. After the supernatant (150 µL) had been filtered through a membrane filter with a pore size of 0.45 µm (Cosmonice filter W; Nacalai, Kyoto, Japan), the sample was dried and the residue was reconstituted in 150 µL of mobile phase. The sample (50 µL) was injected into the HPLC system.

The minimum quantifiable concentrate was 0.1 µg/mL (% CV < 10%).

### Transport experiments

Transport experiments were performed as described previously (14). The intestine was quickly removed and the longitudinal muscle layer was carefully stripped off with scissors. Prepared intestinal sheets were filled with Hanks' balanced salt sodium (HBSS) buffer (137 mM NaCl, 5.4 mM KCl, 1.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.4 mM $KH_2PO_4$, 0.3 mM $NaH_2PO_4$ and 25 mM D-glucose). The pH of the buffer was adjusted to 6.0 or 7.4 with 1N HCl or NaOH. The prepared intestinal sheets were mounted between two Ussing-type diffusion chambers that provided an exposed area of 0.64 $cm^2$. HBSS buffer was added to the chambers of the mucosal and serosal sides. The volume of bathing solution on each side was 1.5 ml, and the solution temperature was maintained at 37 °C in a water-jacketed reservoir. The solution was bubbled with a 95 : 5 mixture of $O_2/CO_2$ before and during the transport experiment. The buffer solution in one of the chambers contained GPFX. Samples of 0.5 ml were taken from the receptor side at 15, 30, 45 and 60 min after incubation. The permeation rate of GPFX was expressed as an apparent permeability coefficient (Papp) according to the following equation: Papp=dQ/dt/SC0, where dQ/dt is the linear appearance rate of mass in the receiver solution, S is the exposed area (0.64 $cm^2$), and $C_0$ is the initial concentration of GPFX (10 µM). Papp is expressed in centimeters per minute.

### Quantitative real-time PCR

Quantitative real-time PCR was performed using an Mx3000TM Real-time PCR System with Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. PCR was performed using rat mdr1a-specific primers through 40 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 30 s or using rat 18S rRNA-specific primers after pre-incubation at 95°C for 15 min. The primers specific to mdr1a were designed on the basis of sequences in the GenBankTM database (accession no.: NM_133401). The sequences of the specific primers were as follows: the sense sequence was 5'- GCA GGT TGG CTG GAC AGA TT -3' and the antisense sequence was 5'- GGA GCG CAA TTC CAT GGA TA -3' for mdr1a, and the sense sequence was 5'- GTT GGT TTT CGG AAC TGA GGC -3' and the antisense sequence was 5'- GTC GGC ATC GTT TAT GGT CG -3' for 18S rRNA. The PCR products were normalized to amplified 18S rRNA, which was the internal reference gene (housekeeping gene).

### Western blot analysis

The samples used for Western blot analysis were intestinal brush-border-membrane vesicles (BBMVs). These samples were prepared as described previously (15). The protein concentration in the clear supernatant was determined by the method of Lowry et al. (16).

Each sample was denatured at 100°C for 3 min in a loading buffer containing 0.1 M Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% BPB and 9 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with Mdr1 (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:500) or mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA) (diluted 1:500) for 24 h at room temperature and washed three times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at room temperature with goat anti-mouse $IgG_1$-HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences Corp., Piscataway, NJ).

**STATISTICAL ANALYSES**

Student's t-test was used to determine the significance of differences between two group means at $p < 0.05$.

**RESULTS**

**Pharmacokinetics of grepafloxacin (GPFX) in Wistar and GK rats**

Time courses of plasma GPFX concentration after intravenous administration and intraintestinal administration in Wistar and GK rats are shown in Fig. 1. Plasma GPFX concentrations after intravenous administration in Wistar and GK rats were almost unchanged (Fig. 1A). On the other hand, plasma GPFX concentrations at 90, 120 and 240 minutes after intraintestinal administration in GK rats were significantly higher than that in control rats (Fig. 1B). Pharmacokinetic parameters of GPFX after intravenous administration and intraintestinal administration are summarized in Table 1. CL/F of GPFX in GK rats was significantly decreased to 65% of that in Wistar rats. *F* of GPFX was increased to 64% from 47% in the GK rats.
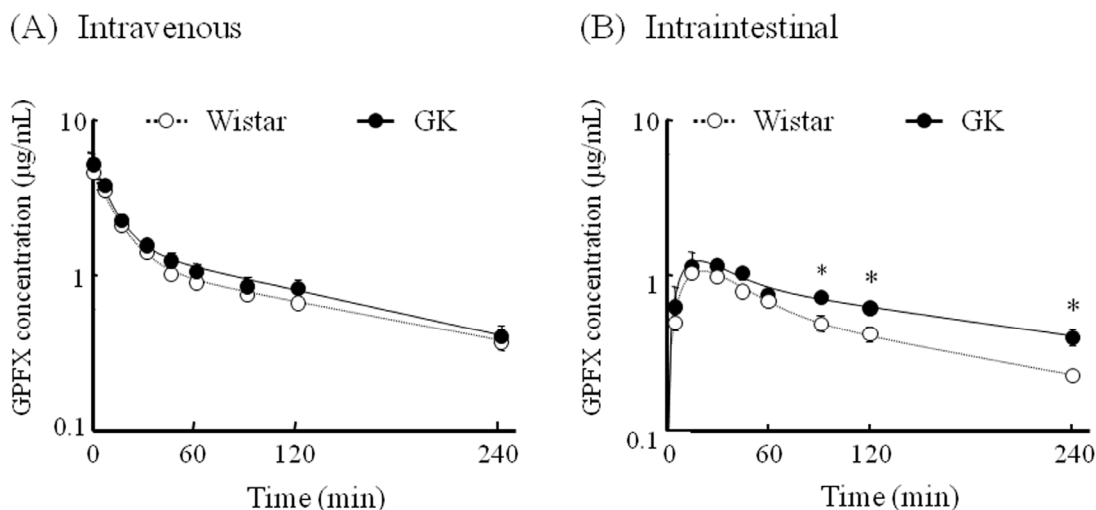


**Figure. 1.** Plasma concentrations of grepafloxacin (GPFX) after intravenous administration (A) and intraintestinal administration (B) in Wistar and Goto-Kakizaki (GK) rats.
GPFX was injected at a dose of 10 mg/kg. Blood samples were collected at specified times after injection. Each point represents the mean with S.E. of 4-5 rats. *, significantly different from Wistar rats at p < 0.05.

**Intestinal, renal, and biliary clearance of GPFX in Wistar and GK rats**

To clarify the elimination mechanisms in a diabetic state, excretion of GPFX into gastrointestinal fluid, urine, and bile of Wistar and GK rats was examined. In GK rats, jejunal clearance of GPFX was significantly lower than that in Wistar rats, and renal clearance of GPFX was significantly higher than that in Wistar rats (Fig. 2).

**Papp of GPFX across the intestinal membrane in Wistar and GK rats**

Since jejunal clearance of GPFX in GK rats was significantly lower than that in Wistar rats, the transport of GPFX in GK and Wistar rat intestinal tissues was further examined by using the Ussing-type chamber method. Permeation of GPFX across a rat jejunal sheet was measured in the mucosal-to-serosal and serosal-to-mucosal directions and the permeation coefficient (Papp) was obtained as the slope of the time course of appearance of GPFX. As is clearly shown in Fig.

3B, serosal-to-mucosal Papp in GK rats was significantly lower than that in control rats, whereas mucosal-to-serosal Papp in Wistar rats and that in GK rats were similar (Fig. 3A). These results suggest that the decrease of GPFX clearance in the jejunum was associated with the decrease of secretory transport of GPFX in GK rats.

**Expression of MDR1/P-gp in the jejunum and ileum of Wistar and GK rats**

To clarify the cause of the decrease in secretory transport in GK rats, we focused on efflux transporter expression. We therefore examined mdr1/P-gp expression in GK and Wistar rat intestinal tissues. As shown in Fig. 4, Mdr1 mRNA level in the jejunum of GK rats was significantly higher than that in Wistar rats. P-gp expression level was also increased in the jejunum of GK rats compared with that in Wistar rats. In the ileum, mdr1/P-gp levels in Wistar and GK rats were not altered.
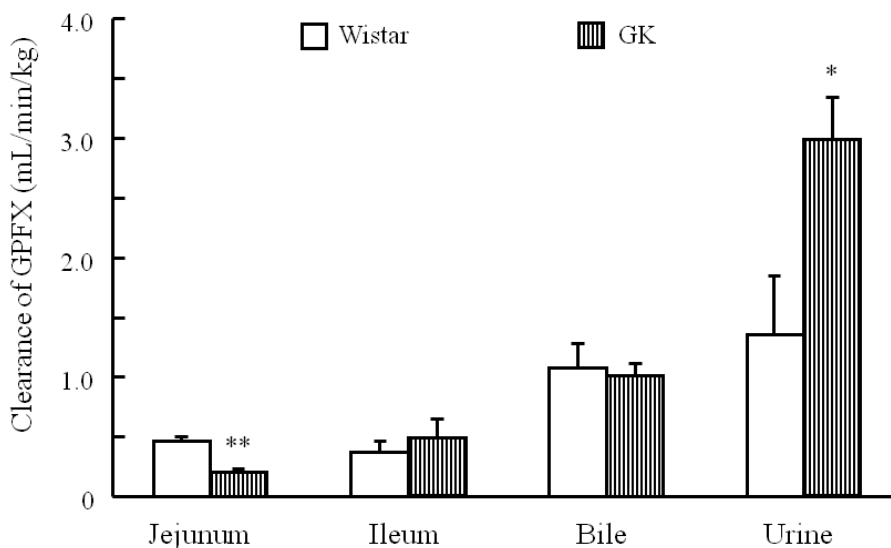


**Figure. 2.** Jejunal, ileal, biliary and renal clearance of grepafloxacin (GPFX) over a period of 60 min after intravenous administration in Wistar and Goto-Kakizaki (GK) rats.
GPFX was injected at a dose of 10 mg/kg. Each column represents the mean with S.E. of 6 rats. *, **, significantly different from Wistar rats at $p < 0.05$, 0.01, respectively.
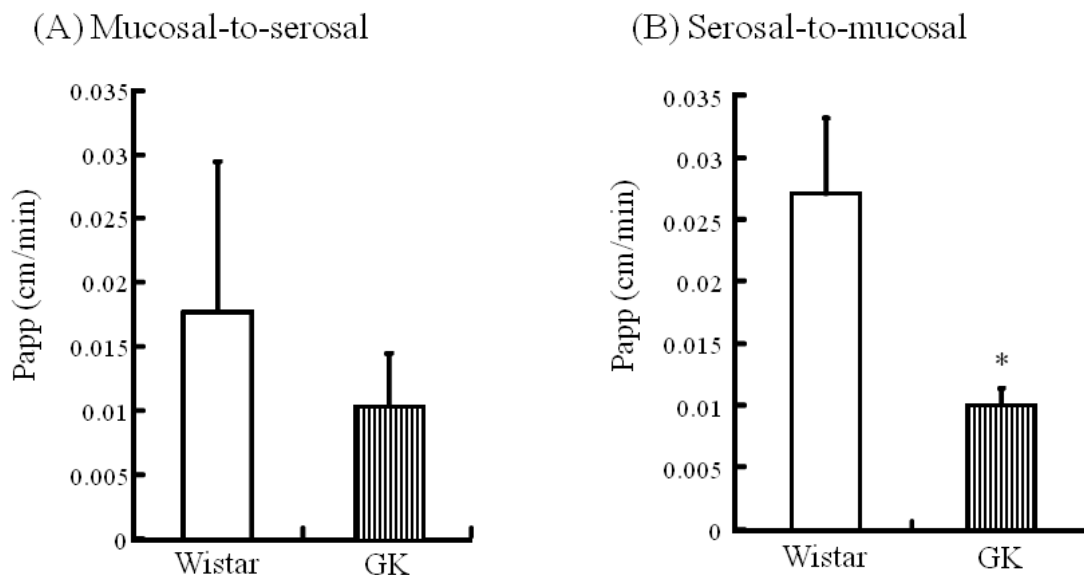
**Figure. 3.** Mucosal-to-serosal (A) and serosal-to-mucosal (B) transport of grepafloxacin (GPFX) in intestine tissues isolated from Wistar and Goto-Kakizaki (GK) rats and mounted in diffusion chambers. The permeation rate of GPFX (10 μM) was expressed as an apparent permeability coefficient (Papp). Each column represents the mean with S.E. of 3-4 experiments. *, significantly different from Wistar rats at $p < 0.01$.
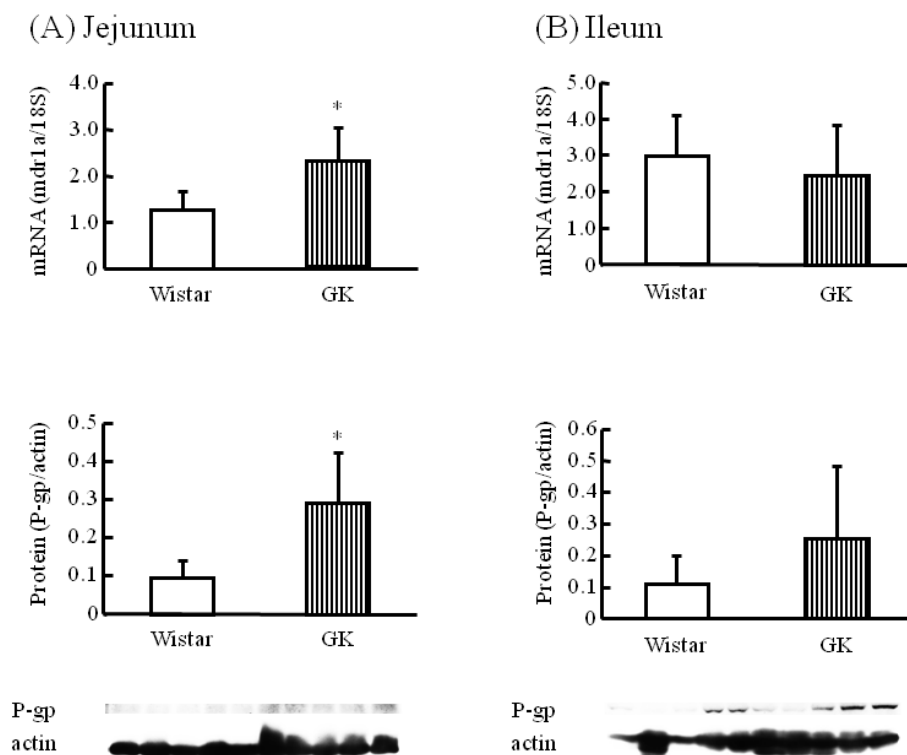


**Figure. 4.** Expression of mdr1a/ P-glycoprotein (P-gp) in the jejunum (A) and ileum (B) of Wistar and Goto-Kakizaki (GK) rats. The column shows the mRNA level (up) and protein expression (down). Each column represents the mean with S.D. of 5 experiments. *, significantly different from Wistar rats at $p < 0.05$.

## DISCUSSION

Several studies have shown that the function and/or expression of various membrane transporters are either up-regulated or down-regulated in diabetes (17-19). Diabetes has been shown to be associated with changes in both barrier function and transport function of cerebral microvessels. The present study was undertaken to investigate the effects of type 2 diabetes on the pharmacokinetics of grepafloxacin (GPFX), a P-glycoprotein (P-gp) substrate. The results clearly demonstrated that GPFX permeation through the intestinal membrane and P-gp expression were altered under diabetic conditions. Plasma GPFX concentrations at 90, 120 and 240 minutes after intraintestinal administration in GK rats were significantly higher than that in Wistar rats. The increase in renal clearance of GPFX in GK rats did not reflect the plasma concentration of GPFX because GPFX is metabolized largely in the liver. Clear increases in the plasma concentration of GPFX caused by decreases in secretory transport were found in the jejunum of GK rats. Serosal to mucosal transport of GPFX through intestinal membranes in GK rats was significantly lower than that in Wistar rats. On the other hand, mucosal to serosal transport of GPFX in Wistar rats and that in GK rats were almost unchanged. Hence, we focused on efflux transporters including P-gp contributed the decreases in secretory transport in jejunum of GK rats, but not uptake transporter. However, P-gp expression level was increased in the jejunum of GK rats compared with that in Wistar rats. This result suggests the P-gp expression level does not correspond with that of function. Since GPFX has been reported to be transported via MRP2 as well as P-gp (20), we checked whether MK571, MRPs inhibitor blocked the secretory transport of GPFX in Wistar rats. MK571 did not inhibit the serosal-to-mucosal transport of GPFX (data not shown). We examined the expression levels of other efflux transporters, Mrp2 and Bcrp, in the jejunum and ileum of Wistar and GK rats (Supplemental Fig. 1). Mrp2 and Bcrp expression levels were also increased in the jejunum but not in the ileum. Expression levels of efflux transporters in the liver and kidney of Wistar and GK rats are also shown in Supplemental Fig. 2. P-gp expression in the liver and Mrp2 expression in the kidney of GK rats were increased compared

with those in Wistar rats. These results also did not reflect the pharmacokinetics of GPFX. Moriguchi et al reported that the P-gp protein expression was decreased in inflammatory conditions (21) and it was reported the endogenous P-gp substrate compounds are produced under inflammatory conditions. The reason why the protein expression did not correlate with the activity might be the existence of endogenous P-gp substrate compounds. And it has been reported that P-gp expression in the jejunum was markedly decreased in rats with streptozocin-induced diabetes (22) and was associated with P-gp function (23). On the other hand, Nawa et al later reported that P-gp expression levels in the upper part of the intestine were increased in obese mice treated with hyperglycemia/hyperinsulinemia (i.e, type 2 diabetes) -associated monosodium glutamate (MSG) and that these results are completely different from those found in the STZ-induced type 1 diabetic mouse model (24). Nawa et al suggested that some differences between the MSG-treated obese mouse model and STZ-induced type 1 diabetic mouse model including duration of hyperglycemic condition or their associated complications with hyperglycemia may be multiply involved in the differences of changes in P-gp expression levels. Our results corresponded to their results. It has recently been shown that radixin, one of the membrane-cytoskeleton cross-linkers (25), is involved in posttranslational regulation of P-gp (26) and that radixin might play an important role in regulating P-gp functional activity at the intestinal membrane (27). Moreover, Lopez et al previously reported that diabetic mouse islets contained less active radixin (28). Collectively, the results suggest that type 2 diabetes conditions may alter P-gp function via radixin inactivation. Further studies are needed to elucidate the mechanism of alteration of P-gp function in diabetes.

In summary, we have reported for the first time that P-gp substrate permeation through the intestinal membrane and expression of efflux transporters including P-gp are altered under type 2 diabetic conditions.

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