Involvement of Ubiquitination in the Decrease of Intestinal P-Glycoprotein in a Streptozotocin-induced Diabetic Mouse Model

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Summary: P-Glycoprotein (P-gp), one of the drug efflux pumps, is expressed in some tissues and may affect the pharmacokinetics of its substrates. We have previously reported that a decrease of intestinal P-gp expression affects the pharmacokinetics of orally-administered P-gp substrate drugs in a streptozotocin (STZ)-induced type 1 diabetic mice model. Although we have found the participation of nitric oxide synthase (NOS) activation as a mechanism of the decrease in intestinal P-gp expression under diabetic conditions, more detailed mechanisms other than NOS remain unknown. Here, we studied the involvement of the ubiquitin-proteasome system in the mechanism of the decrease in intestinal P-gp expression under diabetic conditions. Nine days after STZ administration (diabetic condition), ubiquitination levels of ileal P-gp were significantly increased, accompanied by a decrease of intestinal P-gp protein expression levels. Furthermore, treatment with an NO donor could increase the intestinal ubiquitinated P-gp levels. On the other hand, activity of 26S proteasome, an important enzyme in ubiquitin-proteasome system, did not change, suggesting the first step of the system (i.e., ubiquitination) but not the second step (i.e., degradation)-specific up-regulation under diabetic conditions. Our results reveal the participation of the acceleration of the ubiquitin-proteasome system by NO in the decrease of intestinal P-gp expression levels under diabetic conditions.

Keywords: P-glycoprotein; diabetes; intestine; ubiquitination; nitric oxide

Introduction

P-Glycoprotein (P-gp), belonging to the ATP-binding cassette (ABC) transporter family, is a drug efflux pump and is expressed in some tissues such as brain capillary endothelial cells [known as the blood brain barrier (BBB)], liver and intestine.1-3 Additionally, P-gp may affect the pharmacokinetics of its substrates.4,5 In particular, intestinal P-gp primarily affects the pharmacokinetics of orally-administered substrate drugs.6 We have already reported that intestinal P-gp expression levels are transiently decreased in a streptozotocin (STZ)-induced type 1 diabetic mouse model only on the 9th day after STZ treatment.7,8 As we have already found, the intestinal absorption of orally-administered morphine, a P-gp substrate drug, was significantly increased in accordance with a decrease in intestinal P-gp protein expression under diabetic conditions.7,9 Furthermore, the analgesic effect of morphine was also significantly increased in the STZ-induced type 1 diabetic mice model,7,9 indicating that the alteration of P-gp expression levels under pathophysiological conditions may affect the pharmacokinetics and pharmacodynamics of substrate drugs. Since it is important to clarify the precise mechanism for the changes in P-gp expression levels under pathophysiological conditions, we have focused on it under diabetic conditions. Although we have clarified the participation of nitric oxide synthase (NOS) activation as a mechanism of the decrease in intestinal P-gp expression under diabetic conditions,9 more detailed mechanisms other than NOS remain unknown.

The regulatory system of protein expression includes a transcriptional system and a proteolytic system. Although there are many reports about the transcriptional regulatory system for P-gp,10,11 there are few about its proteolytic system. The proteolytic system, that is, protein degradation, is classified roughly into lysosomal proteolysis and an
ubiquitin-proteasome system. Ubiquitin-proteasome system-mediated degradation of damaged, oxidized and/or misfolded proteins plays a key role in maintaining protein quality and regulating various cellular processes such as cell cycle and protein turnover. Under diabetic conditions, the ubiquitin-proteasome system is reported to be up-regulated and to contribute to insulin resistance and impaired insulin secretion, while there are few reports about its contribution to the regulation of P-gp expression levels. Here, in this study, we studied the involvement of the ubiquitin-proteasome system in the mechanism of the decrease in intestinal P-gp expression under diabetic conditions.

**Methods**

**Animals:** Male ddY mice (Japan SLC Inc., Shizuoka, Japan) (4–5 weeks old) were provided with food and water ad libitum, and housed in an animal room that was maintained at 24°C and 55 ± 5% humidity with a 12 h light/dark cycle (light phase 8:00–20:00). All procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. Additionally, all experiments were approved by the ethical committee for animals of Kobe Gakuin University (approval number: A 060601-11).

Mice were injected with STZ (Sigma, MO, USA) (230 mg/kg, i.p.) dissolved in citrate buffer (pH 4.28). On the 9th day after STZ administration, mice with non-fasting blood glucose levels above 400 mg/dL were used in the study. Control mice were injected with citrate buffer (0.1 mL/10 g).

**Preparation of membrane fractions from intestinal mucosa:** Experiments were performed as previously described by Kageyama et al. with some modifications. Briefly, the ileal mucosa membrane was obtained from STZ or citrate buffer-treated (Control) mice. After homogenization (400 rpm, 20 strokes) in homogenizing buffer, the homogenate was centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was then further centrifuged at 15,000 × g for 15 min at 4°C. Residual membrane fractions were resuspended with lysis buffer. Protein concentrations were measured using the Lowry method (DC Protein Assay kit II, Bio-Rad, CA, USA). The membrane fraction was used for P-gp expression quantification.

**Immunoprecipitation:** Membrane fraction samples were lysed in TNE buffer including 10 mM Tris-HCl, 1 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), 1% NP-40, PMSF, 21.73 mM leupeptin, 1 µM pepstatin A and 0.1 U/mL aprotinin. Lysate (1,500 µg) was incubated with 50 µL of Protein G Sepharose 4 Fast Flow (GE Healthcare UK Ltd, Buckinghamshire, England) for 10 min at 4°C on a rotating wheel. The beads were then pelleted by centrifugation at 14,000 rpm for 20 min at 4°C, and the supernatant transferred to a fresh tube, to which was added a 1:10 dilution of P-gp antibody (mAb C219; Calbiochem, CA, USA). After overnight incubation at 4°C on a rotating wheel, 20 µL of protein G sepharose was added before a further 2 h incubation at room temperature on the rotating wheel. The beads were pelleted at 5,000 rpm for 1 min at 4°C and the supernatants carefully removed. The beads were resuspended in 20 µL sodium dodecyl sulfate (SDS) sample buffer and boiled at 97°C. The beads were then pelleted by centrifugation for 15,000 rpm for 1 min at 4°C. The supernatant fraction was stored at −80°C until use.

**Western blot analysis for intestinal P-gp expression:** Western blot analysis was carried out as previously described. Briefly, proteins extracted from the ileal mucosal membrane fraction (50 µg/lane) were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, and then electrophoretically transferred onto a nitrocellulose membrane. After blocking in blocking buffer containing Tris-buffered saline (TBS, pH 7.6), 0.1% Tween 20 and 5% blocking agent (GE Healthcare UK Ltd., Buckinghamshire, England), the membrane was incubated with primary antibodies for P-gp (mAb C219, 1:200 dilution; Calbiochem, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5, 1:20,000 dilution; Chemicon, CA, USA). The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:2,000 dilution; Kirkegaard & Perry Laboratories, MD, USA). Immunoreactive bands were visualized using Light Capture (ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for HRP detection (ECL Western Blotting system, GE Healthcare UK Ltd., Buckinghamshire, England). Signal intensity of the immunoreactive bands was determined using specialized software (CS-Analyzer version 3.0, ATTO, Tokyo, Japan).

**Preparation of tissue homogenates for proteasome activity measurement:** Experiments were performed as previously described by Kwak et al. with some modifications. Briefly, the ileal mucosa membrane was obtained from STZ or citrate buffer-treated (control) mice. After homogenization (400 rpm, 20 strokes) in homogenizing buffer, containing 50 mM Tris-HCl (pH 7.8), 200 mM KCl, 5 mM, MgCl2, and 1 mM DTT, the homogenate was centrifuged at 9,000 × g for 15 min at 4°C. Protein concentrations were measured using the Lowry method (DC Protein Assay kit II, Bio-Rad, CA, USA).

**26S proteasome activity measurement:** Experiments were performed as previously described by Kwak et al. with some modifications. Peptidase activity of the proteasome was measured by mixing tissue homogenates with 50 µM of fluorogenic peptide succinyl-Leu-Leu-Val-Thy-7-amino-4-methylcoumarin (Suc-LLVY-MAC) in a final volume of 100 µM with a reaction buffer consisting of 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM MgCl2, and 1 mM DTT. The mixture was incubated at 37°C for 20 min and then the reaction was stopped by addition of an equal volume of 125 mM sodium borate buffer (pH 9.0) contain-
ing 7.5% ethanol. Released fluorogenic Suc-LLVY-MAC was measured at 360 nm excitation and 460 nm emission (Gemini EM, Molecular Devices Japan, Tokyo, Japan).

**Treatment with NO donor:** Mice were injected with (±)-N-{[E]-4-ethyl-3-[(Z)-hydroxyimino]-6-methyl-5-nitro-3-heptenyl]-3-pyridine-carboxamide (NOR5, Dojindo, Kumamoto, Japan) (t½ = 20 h) (10 mg/kg, p.o.) dissolved in 0.5% sodium carboxymethyl cellulose twice a day for 5 days.

**Treatment with iNOS specific inhibitor:** Aminoguanidine (AG; Sigma, MO, USA) (1 mg/mL), a specific iNOS inhibitor, was added to drinking water, and provided to mice ad libitum for nine days immediately after STZ administration.

**Statistical analysis:** Data are expressed as mean ± SEM. Statistical significance was assessed with an unpaired Student’s *t*-test or one way analysis of variance (ANOVA) followed by Scheffe’s test. Differences were regarded as statistically significant when the p value was less than 0.05.

**Results**

**Changes in ileal P-gp expression level at 9th day after STZ administration:** On the 9th day after STZ administration, the P-gp expression level in the membrane fraction of ileal mucosa was significantly decreased compared with that of control mice (Fig. 1).

**Changes in ubiquitination level of ileal P-gp and in ileal 26S proteasome activity at 9th day after STZ administration:** The ileal ubiquitinated P-gp level in the STZ-treated mice was significantly increased compared with that of control mice (Fig. 2A). However, 26S proteasome activity was not significantly changed compared with that of control mice (Fig. 2B).

**Effects of NO donor on ubiquitination level of ileal P-gp level:** The ileal ubiquitinated P-gp level in the NOR5, a NO donor, -treated mice was significantly increased compared with that of control mice (Fig. 3).

**Effects of iNOS specific inhibitor on the increase in ubiquitination level of ileal P-gp on 9th day after STZ administration:** The significant increase in ubiquitination levels of ileal P-gp was significantly suppressed by aminoguanidine (Fig. 4). Aminoguanidine did not affect the blood glucose levels (data not shown).

**Discussion**

In this study, we found the participation of the ubiquitin-proteasome system in the decrease of intestinal P-gp...

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**Fig. 1. Changes in ileal P-gp expression level at 9th day after STZ administration**

On the 9th day after STZ administration, the P-gp expression level was analyzed by Western blotting. Control *n* = 8; and STZ *n* = 6. **p < 0.01 vs. Control, unpaired Student’s *t*-test.

**Fig. 2. Changes in ubiquitination level of ileal P-gp and in ileal 26S proteasome activity at 9th day after STZ administration**

On the 9th day after STZ administration, ubiquitinated P-gp levels (A) and 26S proteasome activity (B) were analyzed by immunoprecipitation and Western blot or 26S proteasome activity assay, respectively. Relative levels of ubiquitinated P-gp were analyzed by the ratio of ubiquitinated P-gp/total P-gp. A: Control *n* = 8; and STZ *n* = 6. B: Control *n* = 22; and STZ *n* = 23, *p < 0.05 vs. Control, unpaired Student’s *t*-test.
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On the 9th day after NOR5 administration, ubiquitinated P-gp levels were analyzed by immunoprecipitation and Western blot. Relative levels of ubiquitinated P-gp were analyzed by the ratio of ubiquitinated P-gp/total P-gp. Control n = 6; and NOR5 n = 4, *p < 0.05 vs. Control, unpaired Student’s t-test.

On the 6th day after NOR5 (10 mg/kg, p.o.) administration, ubiquitinated P-gp levels were analyzed by immunoprecipitation and Western blot. Relative levels of ubiquitinated P-gp were analyzed by the ratio of ubiquitinated P-gp/total P-gp. Control n = 6; and NOR5 n = 4, *p < 0.05 vs. Control, unpaired Student’s t-test.

An NO donor could increase the ubiquitination levels of intestinal P-gp, corresponding to a previous report showing the involvement of NO in protein ubiquitination. Furthermore, as we have previously demonstrated, the inducible NOS (iNOS) participates in the decrease of intestinal P-gp expression under diabetic conditions. Our results also showed an iNOS specific inhibitor suppressed ubiquitination levels of intestinal P-gp increase, suggesting that the increment of intestinal iNOS activity participated in the ubiquitination of intestinal P-gp, leading to degradation of and decrease of intestinal P-gp under diabetic conditions. Among the known ubiquitin targeting lysine residues, lysine 48 (K48)-linked polyubiquitination is associated with 26S proteasome-mediated protein degradation. On the other hand, K63-linked ubiquitination is associated with intracellular signaling but not with protein degradation. As Oustwani et al. reported in in vivo and in vitro experiments, NO increases K48-linked ubiquitination but not K63-linked ubiquitination, it is possible that an increase in NO-mediated ubiquitination of intestinal P-gp under diabetic conditions may lead to the degradation and decrease of P-gp expression.

A recent report suggests that the Abcb1 mRNA level was significantly decreased in intestinal mucosa in STZ-induced diabetic rats, indicating that not only the degradation system but also the transcription system may be changed and involved in the decrease in intestinal P-gp under diabetic conditions. However, as we showed here for the first time, ubiquitination should be one of the important mechanisms for the decrease in P-gp expression under diabetic conditions.

Additionally, it has been reported that the basal P-gp expression level was different in every part of the intestine, i.e., duodenum < jejunum < ileum. However, as we have already reported, the alteration patterns of STZ-induced duodenal, jejunal and ileal P-gp expression levels were similar. That is, all of them are significantly decreased on the 9th day after STZ administration. Since the ileum has the highest level of P-gp expression among the three sites, it is thought that the capacity for substrate efflux is also high in the ileum, being the strong barrier for intestinal absorption of P-gp substrate drugs. Considering that the following degradation, intestinal P-gp might be specifically modified, making it easier to be ubiquitinated under diabetic conditions. Although it comprises two steps including the first phase, a protein ubiquitinating step, and the second phase, a degrading step with 26S proteasome complex, our results indicate that in this diabetic condition, only the first step but not second step was accelerated for degradation of ileal P-gp because there were no significant changes in ileal 26S proteasome activity. Since it is reported that protein ubiquitination involves three classes of enzymes, namely ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligases (E3) enzymes, it is possible that these enzymes may be activated under diabetic conditions, but this remains under determination.

Interestingly, a NO donor could increase the ubiquitination levels of intestinal P-gp, corresponding to a previous report showing the involvement of NO in protein ubiquitination. Furthermore, as we have previously demonstrated, the inducible NOS (iNOS) participates in the decrease of intestinal P-gp expression under diabetic conditions. Our results also showed an iNOS specific inhibitor suppressed ubiquitination levels of intestinal P-gp increase, suggesting that the increment of intestinal iNOS activity participated in the ubiquitination of intestinal P-gp, leading to degradation of and decrease of intestinal P-gp under diabetic conditions.

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alterations of expression levels of ileal P-gp may have a great deal of influence on the transport of P-gp substrate drugs, we focused on their changes in the ileum under type 1 diabetic conditions in this study.

In conclusion, we clarified the participation of acceleration of the ubiquitin-proteasome system by NO in the decrease of intestinal P-gp expression under the diabetic conditions. As we have already demonstrated, this change in intestinal P-gp may affect the pharmacokinetics and pharmacodynamics of orally-administered P-gp substrate drugs. Furthermore, since the ubiquitin-proteasome system is also considered to be important for the regulation of the transport of P-gp substrate drugs, including cytochrome P450, the regulation of the ubiquitin-proteasome system is considered to be important for the achievement of appropriate drug disposition and pharmacological effect.

References