Regulatory Action of Nitric Oxide Synthase on Ileal P-glycoprotein Expression under Streptozotocin-Induced Diabetic Condition

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P-glycoprotein (P-gp), a drug efflux transporter, affects the pharmacokinetics of a wide range of substrate drugs. Our previous study clearly revealed that intestinal P-gp expression levels were decreased via an inducible nitric oxide synthase (iNOS)-mediated mechanism in the early phases of diabetes. Here, we focused on changes in ileal P-gp expression and the influences of NOS on the P-gp expression levels in the later phase of diabetic condition using streptozotocin (STZ)-induced diabetic mice. The ileal P-gp expression and activity was analyzed by Western blot analysis and in situ closed loop method, respectively. In STZ-treated mice, ileal P-gp expression levels and activity significantly decreased on the 9th day after STZ administration. Interestingly, the decrease of P-gp function was recovered to the control level on 15th day in same conditioned mice. In addition, the recovery of P-gp expression levels was completely suppressed by a non-selective NOS inhibitor. These results indicate that the diabetic condition-induced decline of P-gp expression levels was temporary, and both decline- and recovery-process of intestinal P-gp expression levels are mediated by NOS. Furthermore, this study shows the bidirectional effect of NOS on regulation of intestinal P-gp expression.

Key words P-glycoprotein; diabetes; nitric oxide synthase

P-glycoprotein (P-gp) is a drug efflux transporter which belongs to the ATP-binding cassette (ABC) transporter family. Because P-gp has a wide range of substrate specificity, P-gp affects the pharmacokinetics of a number of substrate drugs. Although P-gp is located on many tissues such as the blood brain barrier (BBB), intestine, liver and kidney, intestinal P-gp is the first barrier of orally-administered substrate drugs.1) It is known that P-gp expression levels and its activity could be affected by pathophysiological conditions such as cancer or ischemic injury.2,3) We previously reported that ileal P-gp expression levels significantly decreased under streptozotocin (STZ)-induced diabetic condition.4) In addition, we elucidated the involvement of inducible nitric oxide synthase (iNOS) in the mechanism. However, there are bidirectional reports in relation to NOS-mediated change of P-gp expression levels or activity (i.e. one is P-gp up-regulation, and the other is P-gp down-regulation).5,6) Since the difference in NOS-mediated P-gp change seems to be dependent on the periods of exposure to NO in target tissues,5) it is necessary to determine various time points of a diabetic condition. In our previous study, P-gp expression was examined during the early stage of the development of diabetes. Thus, this study was aimed at determining how NOS influences P-gp expression during the later time points of diabetes using STZ-induced diabetic model mice.

MATERIALS AND METHODS

Animals Male ddY mice (Japan SLC Inc., Shizuoka, Japan) (5 weeks old) had free access to food and water 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). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. In addition, all experiments were approved by the ethical committee for animals of Kobe Gakuin University (approval #: A 060601-11).

Mice were injected with streptozotocin (STZ; 230 mg/kg, intraperitoneal (i.p.)) dissolved in citrate buffer (pH 4.28). Some parameters such as body weight, blood glucose levels, and serum insulin levels were measured on the 9th and the 15th day after STZ administration.

Treatments of NOS Inhibitors 1-N®-nitroarginine methyl ester (1-NAME) (1 mg/ml), a non-selective NOS inhibitor, was added to drinking water. Mice had free access to the water from the 9th to the 15th day after STZ administration.

Measurement of Blood Glucose Levels and Serum Insulin Levels The analysis of blood glucose levels and serum insulin levels was carried out as previously described.4) Blood glucose levels were measured using the Glucose Pilot (Aventir Biotech, CA, U.S.A.). Serum insulin levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Kanagawa, Japan).

Preparation of Membrane and Cytoplasmic Fractions of Ileal Mucosa Experiments were performed as described previously.4) Briefly, ileal mucosa obtained from mice was homogenized (400 rpm, 20 strokes) in homogenizing buffer containing 250 mM sucrose, 50 mM Tris–HCl (pH 7.4), 21.73 mM leupeptin, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 U/ml aprotinin. After centrifugation (3000 × g, 10 m, 4 °C), the supernatant was collected and was further centrifuged (15000 × g, 15 m, 4 °C). The cytoplasmic compartment to measure NOS activity. Residual membrane fractions were resuspended with lysis buffer containing 50 mM mannitol, 50 mM Tris–HCl, 1 mM PMSF, 21.73 mM leupeptin, 1 µM pepstatin A, and 0.1 U/ml aprotinin, and was used for P-gp expression analysis.

Western Blot Analysis for P-gp Expression The Western blot analyses were carried out as previously described.4)
Briefly, the proteins of mouse ileal mucosa membrane fraction (50 μg/lane) were separated by electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and the proteins were then electrophoretically transferred to a nitrocellulose membrane. After blocking in blocking buffer containing Tris-buffered saline (TBS, pH 7.6), 0.1% Tween20 and 5% blocking agent (GE Healthcare U.K. Ltd., Bucks, England), the membrane was incubated with primary antibodies for P-gp (nAb C219, 1 : 200 dilution; Calbiochem, CA, U.S.A.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5, 1 : 40000 dilution; Chemicon, CA, U.S.A.).

The membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1 : 2000 dilution; Kirkegaad & Perry Laboratories, MD, U.S.A.). All visualization of immunoreactive bands was performed using Light Capture (ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, ECL Western Blotting system (GE Healthcare U.K. Ltd., Bucks, England). Signal intensity of immunoreactive bands was analyzed with the CS-Analyzer ver. 3.0 (ATTO, Tokyo, Japan).

In Situ Closed Loop Methods
Ileal P-gp activity was evaluated as described previously.4) Briefly, mice were fasted for at least 16 h and then anesthetized with isoflurane (2%). The upper and lower ends of ileum (14 cm) were ligated. Then, 1.2 ml of Krebs Henseleit bicarbonate buffer (KHBB) solution containing Rhodamine123 (52 μm) (Rho123, Sigma, MO, U.S.A.) was administrated into the loop. The fluorescence intensity of Rho123 remaining in the loop was measured with a fluorescence microplate reader (excitation wavelength; 485 nm, absorption wavelength; 535 nm, Perkin Elmer, Kanagawa, Japan). A time–concentration (% of Control) curve was constructed, and the area under the curve (AUC) was calculated from the time versus concentration of Rho123 in the loop.

Measurement of NOS Activity
The NOS activity was measured in the cytoplasmic compartment using the Ultra-sensitive Colorimetric Nitric Oxide Synthase Assay Kit (Oxford Biomedical Research, MI, U.S.A.) according to the manufacturer’s instructions as previously described.3)

Statistical Analysis
Data are expressed as means with S.E.M. 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 the Physiological Parameters and Ileal P-gp and NOS Activity after STZ Administration

<table>
<thead>
<tr>
<th></th>
<th>9 d</th>
<th>STZ</th>
<th>15 d</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>35.3 ±0.38</td>
<td>28.0 ±0.75*</td>
<td>37.5 ±0.53</td>
<td>28.0 ±1.21***</td>
</tr>
<tr>
<td>Blood glucose levels (mg/dl)</td>
<td>160.5 ±5.93</td>
<td>503.8 ±15.3*</td>
<td>153.1 ±8.13</td>
<td>544.8 ±20.8***</td>
</tr>
<tr>
<td>Serum insulin levels (ng/ml)</td>
<td>0.98 ±0.20</td>
<td>0.21 ±0.10*</td>
<td>1.30 ±0.35</td>
<td>0.047 ±0.03****</td>
</tr>
<tr>
<td>P-gp expression (% of Control)</td>
<td>100 ±5.46</td>
<td>56.4 ±12.6*</td>
<td>100 ±1.00</td>
<td>75.5 ±11.8</td>
</tr>
<tr>
<td>P-gp activity (% of Control)</td>
<td>100 ±5.00</td>
<td>69.2 ±4.92*</td>
<td>100 ±9.52</td>
<td>111.4 ±16.5</td>
</tr>
<tr>
<td>NOS activity (% of Control)</td>
<td>100 ±7.37</td>
<td>134.7 ±16.1**</td>
<td>100 ±17.5</td>
<td>179.9 ±24.5****</td>
</tr>
</tbody>
</table>

On the 9th and the 15th day after STZ administration, body weight, blood glucose levels, serum insulin levels, ileal P-gp expression, P-gp activity and NOS activity were measured. n = 4—9, \( p < 0.01, \quad * p < 0.05 \) vs. control (9 d), \(* * p < 0.01, \quad **** p < 0.05 \) vs. control (15 d), unpaired Student’s t-test.

Table 1. Changes in the Physiological Parameters and Ileal P-gp and NOS Activity after STZ Administration

Fig. 1. Effect of Non-selective NOS Inhibitor on the Increase of Ileal NOS Activity after STZ Administration

On the 15th day after STZ administration, NOS activity in the cytosolic fraction of ileal mucosal epithelial cells was measured. L-NAME (1 mg/ml) was added to the drinking water from the 9th to the 15th day. \( n = 4—9, \quad * p < 0.05 \) vs. control (water), \# \( p < 0.05 \) vs. STZ (water), one-way ANOVA and Scheffe’s test.

Effect of a Non-selective NOS Inhibitor on the Increase of Ileal NOS Activity after STZ Administration

In addition, serum insulin levels in the STZ-treated mice were significantly reduced on the 9th and the 15th day after STZ administration. On the contrary, blood glucose levels in the STZ-treated mice were significantly increased compared with the control mice (Table 1).

Ileal P-gp expression levels and activity in the STZ-treated mice were significantly lower level compared with the control group (Table 1). In addition, serum insulin levels in the STZ-treated mice were significantly reduced on the 9th and 15th day after STZ administration. The increase in NOS activity observed on the 15th day after STZ administration was completely suppressed by L-NAME (Fig. 1).
non-selective NOS inhibitor, from the 9th to the 15th day after STZ administration. The recovery of P-gp expression levels and of P-gp Rho123 efflux activity observed on the 15th day after STZ administration was completely suppressed by L-NAME (Fig. 2). In addition, we confirmed that L-NAME (1 mg/ml) was added to the drinking water from the 9th to 15th day.

**DISCUSSION**

This study clearly indicates that the diabetic condition has continued to be aggravated from the 9th to the 15th day after STZ administration in association with a decrease of serum insulin levels and an increase of blood glucose levels. Interestingly, as we reported previously, the ileal P-gp expression levels and activity significantly decreased on the 9th day after STZ administration, but they had recovered on the 15th day after STZ administration. These results suggest that the decrease of P-gp expression and activity was temporary under the diabetic condition. In contrast, NOS activity continued to increase between the 9th and the 15th day after STZ administration. Although the change in NOS activity under diabetic condition is reported to be controversial, the finding that iNOS promoter activity could be up-regulated by high glucose stress in the in vitro intestinal epithelium cell may support our results. Some inflammatory cytokines that are reported to be increased under diabetic conditions may affect the NOS activity in this study.

The recovery of P-gp expression levels was completely suppressed by L-NAME, a non-selective NOS inhibitor, suggesting that NOS is involved in the recovery of P-gp expression levels in the later phase (15 d after STZ administration) of diabetic condition. It is needed to identify the specific NOS isoform involved in the mechanism of recovery of P-gp expression levels. Furthermore, the recovery of P-gp activity was also completely suppressed by L-NAME, suggesting that the recovery of P-gp activity is due to the recovery of P-gp expression levels via NOS. As another possibility, the post-translational modifications such as phosphorylation may be involved in the recovery of P-gp activity.

In consideration of our previous findings, NOS seems to be involved in both the decline- and recovery-process of P-gp expression under diabetic conditions in vivo. This is the first report showing the bidirectional effect of NOS on the P-gp expression levels in vivo, while it has already been reported in in vitro experiments. That is, short term nitrosative stresses after NOS activation (1 h) decrease P-gp activity by protein kinase C (PKC)-mediated P-gp modulation, while long term nitrosative stresses (6 h) up-regulate P-gp expression levels by activation of nuclear factor-kappa B via PKC.

Moreover, other secondary mediators such as interferon-γ or tumor necrosis factor-α may cause an increase and recovery of P-gp expression in the later phase of the diabetic condition.

In conclusion, our study showed that diabetic condition-induced up-regulation of NOS activity bidirectionally modulated intestinal P-gp expression levels in a time-dependent manner. This may influence the pharmacodynamics of orally administered drugs, particularly at the step of intestinal absorption.

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**REFERENCES**