Decreased Expression of Intestinal P-glycoprotein Increases the Analgesic Effects of Oral Morphine in a Streptozotocin-induced Diabetic Mouse Model

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Summary: Morphine is one of the strongest analgesics and is commonly used for the treatment of chronic pain. The pharmacokinetic properties of morphine are, in part, modulated by P-glycoprotein (P-gp). We previously reported that intestinal P-gp expression levels are influenced via the activation of inducible nitric oxide synthase (iNOS) in streptozotocin (STZ)-induced diabetic mice. Herein, we examine the analgesic effects of orally administered morphine and its pharmacokinetic properties under diabetic conditions, specifically we focusing on the involvement of intestinal P-gp in a type 1 diabetic mouse model. We assessed the analgesic effect of morphine using the tail-flick test. Serum and brain morphine levels were determined using a HPLC-ECD system. Oral morphine analgesic effects and serum and brain morphine content were significantly increased 9 days after STZ administration. The increase in the analgesic effects of morphine, as well as serum and brain morphine content, was suppressed by aminoguanidine, a specific iNOS inhibitor. Conversely, there were no changes in the analgesic effects obtained with subcutaneous morphine in STZ-treated mice. Our findings suggest that the analgesic effects of oral morphine are dependent on intestinal P-gp expression, and this may be one of the reasons that it is difficult to obtain stable pharmacological effects of morphine in diabetic patients.

Keywords: P-glycoprotein; diabetes; intestine; inducible nitric oxide synthase; morphine

Introduction

Opioids are the strongest analgesics available and are commonly used for the treatment of chronic pain in the palliative care of cancer patients. However, the severe side effects associated with opioid use, such as vomiting, respiratory depression, constipation and drowsiness, may outweigh their therapeutic benefits. Thus, it is critical that the pharmacokinetics and/or pharmacodynamics of opioids are appropriately regulated. It was previously reported that the pharmacokinetics and pharmacodynamics of opioids may be regulated not only by opioid receptor sensitivity, but also by other factors, such as metabolizing enzymes (e.g. UDP-glucuronyltransferase) and drug efflux transporters.

P-glycoprotein (P-gp), a drug efflux pump that transports a large number of drugs, including opioids, is expressed in numerous tissues, such as the blood-brain barrier (BBB), intestines and liver. It has been reported that opioid disposition and analgesic effects were significantly enhanced in a P-gp knockout mouse. Furthermore, many reports have demonstrated that functional differences in P-gp may result in differences in the analgesic effects produced by opioids. These findings indicate that the analgesic effects of opioids are highly dependent on the functional differences in P-gp. Interestingly, the majority of research in the area has focused on P-gp function within the BBB, and only a few reports have focused on intestinal P-gp. Given that the current guidelines of the World Health Organization (WHO) recommend the oral administration of opioids in the palliative care setting, and that the absorptive processes of the small intestine influence the pharmacological effects of orally administered drugs, the present study focused on the influence of intestinal P-gp on the analgesic effects of opioids.

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P-gp expression and activity levels are affected by certain pathophysiological conditions, such as cancer, diabetes or ischemic injury.6,8 We previously reported that ileal P-gp expression levels are transiently decreased via the activation of inducible nitric oxide synthase (iNOS) in a streptozotocin (STZ)-induced type 1 diabetic mouse model. These changes may affect the pharmacokinetics and pharmacodynamics of orally administered opioids. Additionally, diabetic patients are susceptible to developing cancer,9 and thus it is important to investigate alterations in the pharmacokinetic and pharmacodynamic properties of opioids in the diabetic condition.

Herein, we examine the analgesic effects of orally administered morphine and its altered pharmacokinetics in the diabetic condition. Specifically, we focus on the involvement of intestinal P-gp in a type 1 diabetic mouse model.

Methods

Animals: Male ddY mice (4–5 weeks old, Japan SLC Inc., Shizuoka, Japan) 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 Gakuen University (approval number: A 060601-11).

Mice were injected with streptozotocin (STZ; Sigma, MO., USA) (230 mg/kg, i. p.) dissolved in citrate buffer (pH 4.28). On the 9th and 15th day after STZ administration, mice with non-fasting blood glucose levels above 400 mg/dL were used in the study. Control mice were administrated morphine and sampled for 9 days immediately after STZ administration.

Drug administration: Morphine (Takeda, Co., Ltd., Osaka, Japan; 30 mg/kg) or vehicle (water) was provided orally to mice. Additionally, morphine was administered subcutaneously (1–10 mg/kg) or intravenously (0.5–5 mg/kg). Aminoguanidine 1 mg/mL (AG; Sigma, MO., USA), a specific iNOS inhibitor, was added to drinking water and provided to mice ad libitum for 9 days immediately after STZ administration.

Tail-flick test: Morphine analgesia against noxious thermal stimuli was assessed with the tail-flick test. Mice were gently held with the tail positioned with the tail-flick apparatus (MK-330B; Muromachi Kikai Co., Ltd., Tokyo, Japan) for radiant thermal stimulation of the dorsal surface of the tail. The intensity of the thermal stimulus was adjusted to cause the animal to flick its tail within 3–4 s as the base line of the tail-flick latency. The tail-flick latency was measured before and 30, 60 and 90 min after morphine administration. The cutoff time was set at 10 s to minimize tissue damage. The area under the curve (AUC) value for the morphine analgesia on each mouse was calculated for the relevant experiments.

Morphine content: Experiments were performed as previously described by Shimizu et al.10 Briefly, blood and brain samples were collected 15 and 30 min after morphine administration. Serum was separated by centrifugation (3000 rpm for 10 min at 4°C). The brain was homogenized in 1 ml of pure water by sonication (20s). The mixture of sample (100 µl of serum or brain homogenate) and 40% K2HPO4 solution (1 ml) was shaken with 5 ml of ethyl acetate for 20 min and then centrifuged at 2000g for 5 min at 4°C. The organic layer was collected, and the aqueous layer was re-extracted with 5 ml of ethyl acetate. Then, the morphine in the organic layer was extracted with 1 ml of 1 M acetic acid, and a 0.9-ml portion of the aqueous layer was lyophilized. The samples were dissolved in 200 µl of 0.01 M HCl, and 20 µl was analyzed by the HPLC-ECD system. The HPLC-ECD system conditions were as follows: column, Eicompak MA-ODS (Eicom, Kyoto, Japan); mobile phase, 0.1 M citric acid buffer (pH 3.9)/methanol (82/18) containing 3 mg/l of EDTA and 150 mg/l of sodium octane sulfonate; flow rate, 1 ml/min; detector, ECD-100 (Eicom) 750 mV Ag/AgCl; temperature, 25°C.

Preparation of membrane fractions from intestinal mucosa: Experiments were performed as previously described by Kageyama et al.11 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 3,000g for 10 min at 4°C. The supernatant was then further centrifuged at 15,000g 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.

Western blot analysis for intestinal P-gp expression: Western blot analysis was carried out as previously described.12 Briefly, proteins extracted from the ileal mucosal membrane fraction (50 µg/lane) were separated by electrophoresis on a 7.5% sodium dodecyl sulfate-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% Tween20 and 5% blocking agent (GE Healthcare UK Ltd, Little Chalfont, 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-conjugated anti-mouse secondary antibody (1:2,000 dilution; Kierkegaard & Perry Laboratories, MD, USA). Immunoreactive bands were visualized using Light Capture (ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for horseradish peroxidase detection (ECL Western Blotting system, GE Healthcare UK Ltd). Signal intensity of the immunoreactive bands
Intestinal P-gp expression levels and the analgesic effect of oral morphine in STZ-treated mice

A typical Western blot image (A) and the effects of oral morphine analgesia (B and C) are shown. Mice were treated with morphine (30 mg/kg, p.o.). The cutoff time for the tail-flick experiments was set as 10 s to prevent injury to the tail. Data are presented as mean ± SEM. Control (water) n = 10, control (morphine) n = 10, STZ (water) n = 9, and STZ (morphine) n = 10. *p < 0.05, **p < 0.01 vs. STZ (water). †p < 0.05 vs. control (morphine), according to Scheffe’s test.

Results

Intestinal P-gp expression levels and analgesic effects of oral morphine in STZ-treated mice: Ileal P-gp expression levels were significantly lower in STZ-treated mice compared to control mice (Fig. 1A), and tail-flick latency following oral morphine administration was significantly greater in STZ-treated versus control mice (Fig. 1B). Additionally, the analgesic effects of morphine, as determined by the area under the tail-flick latency curve (AUC0–90), were significantly greater in STZ-treated mice than in control mice (Fig. 1C).

Serum morphine concentrations and brain morphine content in STZ-treated mice following oral morphine administration: Serum morphine concentrations 30 min after oral morphine administration were significantly greater in STZ-treated mice than in control mice (Fig. 2A). Similarly, brain morphine content was also significantly higher in STZ-treated mice (Fig. 2B). However, the brain/plasma ratio was not different between control and STZ-treated mice (Fig. 2C).

Analgesic effects of subcutaneous morphine and the serum morphine concentrations and brain morphine content in STZ-treated mice: The analgesic effects of subcutaneous morphine (1–10 mg/kg) in STZ-treated mice were not significantly different from control mice (Fig. 3A). Additionally, serum morphine concentrations and brain morphine content were not significantly different between control and STZ-treated mice 30 min after subcutaneous administration of morphine (5 mg/kg) (Figs. 3B and 3C).

Dose-dependent changes in serum and brain morphine levels in STZ-treated mice following intravenous morphine administration: There were dose-dependent changes in both serum and brain morphine levels following intravenous administration of morphine in both control and STZ-treated mice (Fig. 4). Furthermore, there were no differences in the slopes of the dose-response curves between control and STZ-treated mice.

Effects of an iNOS-specific inhibitor on ileal P-gp expression levels and oral morphine analgesia in STZ-treated mice: The significant decrease in P-gp expression levels, which is apparent 9 days after STZ administration, was suppressed by AG, a selective iNOS inhibitor. However, AG had no effects on P-gp expression levels in control mice (Fig. 5A).

Furthermore, the significant increase in the analgesic effects of oral morphine were suppressed by AG in STZ-treated mice. However, AG had no effects on analgesia induced by oral morphine in control mice (Fig. 5B). Interestingly, AG treatment did not alter the analgesic effects of subcutaneous morphine in STZ-treated mice (Fig. 5C).

Effects of an iNOS-specific inhibitor on serum and brain morphine levels in STZ-treated mice after oral morphine administration: The increase in serum morphine concentrations and brain morphine content 30 min after oral morphine administration was suppressed by AG in STZ-treated mice (Fig. 6).

Analgesic effects of oral morphine and serum and brain morphine levels 15 days after STZ treatment: On the 15th day after STZ treatment, ileal P-gp expression levels were not significantly different from control mice (Fig. 7A), and the analgesic effects of oral morphine were similar in both control and STZ-treated mice (Fig. 7B).
Additionally, serum morphine concentrations and brain morphine content 30 min after oral morphine administration were not significantly different between control and STZ-treated mice (Figs. 7C and 7D).

**Discussion**

Although numerous drugs, including morphine, are available in an oral dosage form, the bioavailability of orally administered drugs is lower than that of subcutaneous or intravenous forms due to the first-pass effect within the intestines and liver.13 In the present study, it was found that the analgesic effects of oral morphine were significantly altered in STZ-treated mice. Given that the analgesic effects of oral morphine appear to be coupled to intestinal P-gp expression levels, then alterations in the analgesic effects of oral morphine may be due to changes in intestinal morphine-absorptive processes. Indeed, serum morphine concentrations were significantly increased 9 days after STZ treatment, at which time intestinal P-gp levels had decreased. These findings suggest that intestinal absorption of morphine is enhanced in the diabetic condition because the drug efflux activity of P-gp is down-regulated. Although there are other types of drug-sensitive transporters,2,14 only a few reports indicate morphine as their substrate. Thus, P-gp appears to be a key regulator of the pharmacological effects of morphine.

Nine days after STZ treatment, the brain content and analgesic effects of morphine were significantly increased following oral morphine administration. Since the analgesic effects of morphine are dependent on the binding of morphine to its receptor within the central nervous system, it may be that the enhancement of morphine analgesia in STZ-treated mice was a result of the breakdown of the BBB in the diabetic condition.15 Indeed, it has been reported that P-gp expression and activity levels within the BBB were decreased in the diabetic condition.16 However, our results clearly demonstrate that the analgesic effect, serum concentration and brain content of morphine following its
subcutaneous administration i.e., bypassing the intestinal absorption process were not different in STZ-treated versus control mice. Moreover, the dose-dependent increase in serum concentrations and brain content of morphine following intravenous administration of morphine in STZ-treated mice did not differ from those of control mice. Thus, it appears that the BBB had not broken down 9 days after STZ administration. In fact, after oral morphine administration, the brain/plasma ratio of morphine contents was not different between control and STZ-treated mice. This finding strongly support the hypothesis that the altered intestinal absorption of morphine may increase morphine brain content and serum morphine concentrations, and thereby enhance its analgesic effects in STZ-treated mice.

Contrary to our present findings, it was previously reported that the analgesic effects of subcutaneous and oral morphine were reduced in animals with diabetes-induced hyperalgesia.17,18 As observed in these previous studies,17,18 diabetes-induced hyperalgesia may also be present in our diabetic mouse model. In the present study, however, the enhancement of oral morphine analgesia associated with the decrease in intestinal P-gp expression levels 9 days after STZ treatment may mask the diabetes-induced hyperalgesia. On the other hand, since morphine levels following subcutaneous administration did not differ between the STZ-treated and control group, then the diabetes-induced decrease in subcutaneous morphine analgesia could be observed when estimated using more appropriate experimental methods for estimating hyperalgesia, such as tactile mechanical or weak thermal stimuli.

With respect to the mechanism of the altered intestinal P-gp levels in the diabetic condition, we previously reported the involvement of NOS.12,19 In particular, intestinal NOS activity gradually and continuously increased after STZ administration accompanied with an increase of blood glucose levels.12 Additionally, when mice were administered NOR5, a known NO donor, to reproduce the increment of NO levels in intestine, intestinal P-gp expression levels were significantly decreased, clearly suggesting that NOS activation is essential for the significant decrease of intestinal P-gp expression levels. Importantly, it has been reported that “high glucose stress” per se can increase NOS activity in vitro.20

As is well known, NOS is classified into three isoform types as neuronal NOS (nNOS), endothelial NOS (eNOS) and iNOS.21 In our previous study, we reported that

![Image](image-url)

Fig. 5. Effects of an iNOS-specific inhibitor on ileal P-gp expression levels and analgesic effects of oral morphine analgesia in STZ-treated mice
A typical Western blot image (A), effects of oral morphine analgesia (B) and subcutaneous morphine analgesia (C) are shown. Mice were treated with morphine (30 mg/kg, p.o.). The cutoff time for the tail-flick experiments was set as 10 s to prevent injury to the tail. Aminoguanidine (AG; 1 mg/mL) was provided in the drinking water for 9 days. Data are presented as the mean ± SEM. (B): Control (water/water) n = 8; control (morphine/water) n = 10; control (morphine/AG) n = 12; STZ (water/water) n = 11; STZ (morphine/water) n = 12. **p < 0.01 vs. Control (water/water), ***p < 0.01 vs. STZ (water/water), ††p < 0.01 vs. STZ (morphine/water), Scheffe’s test. (C): saline (SAL) (water) n = 5; SAL (AG) n = 4; morphine (water) n = 9; and morphine (AG) n = 4. **p < 0.01 vs. SAL, Scheffe’s test.
The activation of iNOS plays an important role in decreasing intestinal P-gp levels in STZ-treated mice. Thus, in the present study, we used AG to inhibit decreases in intestinal P-gp expression levels. Since AG has anti-glycation effects, it may also affect the diabetic state; however, in our previous study, AG was found not to affect blood glucose levels. Despite the fact that AG was previously reported to attenuate the development of morphine tolerance and dependence, it did not affect the analgesic effects of morphine, as was shown in the present study. Thus, AG treatment appears to completely suppress the enhancement of morphine analgesia in the diabetic state. It is hypothesized that the analgesic effects of oral morphine are dependent on alterations in intestinal P-gp expression. Although the precise mechanism for alteration of intestinal P-gp expression via iNOS activation under diabetic conditions was not fully elucidated in this study, it is expected that both transcriptional and post-translational regulation may be involved in the mechanisms. For example, several transcription factors, such as NF-κB, YB-1 and PXR that could bind elements of mdr1 promoter, might be repressed under NOS activation. With respect to post-translational regulation, we now hypothesize the involvement of the ubiquitin-proteasome system that could be activated by the NOS-mediated pathway in the degradation of P-gp.

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**Fig. 6. Effects of an iNOS-specific inhibitor on serum and brain morphine levels in STZ-treated mice following oral morphine administration**

Serum morphine concentration (A), brain morphine contents (B). Blood and brain samples were harvested 30 min after morphine administration (30 mg/kg, p.o.). Aminoguanidine (AG; 1 mg/mL) was provided in the drinking water for 9 days. Data are presented as mean ± SEM. Control n = 14; STZ (Water) n = 14; and STZ (AG) n = 19, *p < 0.05, **p < 0.01 vs. Control, *p < 0.05, ##p < 0.01 vs. STZ (Water), Scheffe’s test.

**Fig. 7. The analgesic effects of morphine, serum morphine concentrations and brain morphine content after oral morphine administration 15 days after STZ administration**

A typical Western blot image (A), effects of oral morphine analgesia (B) serum morphine concentration (C), and brain morphine contents (D) are shown. Mice were treated with morphine (30 mg/kg, p.o.). The cutoff time for the tail-flick experiments was set at 10 s in the test to avoid injury to the tail. Blood and brain were harvested 30 min after morphine administration (30 mg/kg, p.o.). Data are presented as mean ± SEM. (B): Control (water) n = 5; control (morphine) n = 7; STZ (water) n = 7; and STZ (morphine) n = 14. (C and D): control mice, n = 11; and STZ-treated mice, n = 14.
Furthermore, 15 days after STZ treatment, ileal P-gp expression levels, oral morphine analgesic effects and serum and brain morphine levels had recovered back to control levels. Interestingly, our previous study clearly demonstrated that this recovery of ileal P-gp expression levels was also mediated by NOS activation. In particular, when mice were administered L-NAME, a non-selective NOS inhibitor, the expression levels of P-gp were significantly enhanced.

These findings suggest that NOS is involved not only in the decrease but also in the recovery of intestinal P-gp expression levels observed on the 9th and 15 days after STZ administration, respectively. Interestingly, it has been reported that the effect of NO on P-gp function and expression levels was different between short-term and long-term exposure to NO in vitro. Specifically, short-term exposure to NO in rat brain capillary endothelial cells decreased the P-gp function through PKC activation, while long-term exposure to NO increased the function and expression levels of P-gp.

In conclusion, our results clearly indicate that the analgesic effects of oral morphine are significantly enhanced by the iNOS-mediated transient decrease in intestinal P-gp expression. Furthermore, expression levels of P-gp appear to fluctuate throughout the developmental course of diabetes. Unfortunately, there are few descriptions of clinical evidence for the changes of pharmacokinetics of P-gp-substrate drugs in diabetic patients. As we have shown here, the significant changes in pharmacokinetics and pharmacodynamics of oral morphine in the diabetic condition appear to be period- and severity-dependent. This may make it hard to obtain clear and critical evidence in the clinical setting. This study underlines the fact that it is important for physicians to be aware of the hidden pharmacological changes of P-gp-substrate drugs in diabetic patients during advancement of the clinical stage. In summary, alteration of intestinal P-gp expression levels in the diabetic condition may be one of the reasons that it is difficult to obtain stable pharmacological effects of morphine in diabetic patients.

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References


