Effects of Repeated Morphine Treatment on the Antinociceptive Effects, Intestinal Absorption, and Efflux from Intestinal Epithelial Cells of Morphine

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The present study was conducted to investigate the effects of repeated treatment with morphine on the drug’s antinociceptive effects, intestinal absorption, and transepithelial transport. The antinociceptive effects of morphine in rats were markedly decreased after repeated oral administration of the drug for 5 d, indicating the development of tolerance. In the morphine-tolerant rats, intestinal absorption of morphine was determined using the in situ loop method. Absorption of morphine from the jejunum was significantly decreased after repeated administration. The permeability of human intestinal epithelial Caco-2 cells was increased in the efflux direction after repeated treatment. The repeated administration of morphine also reduced the cellular accumulation and efflux of P-glycoprotein substrates ([3H]vincristine and rhodamine123) from Caco-2 cells, suggesting that it enhances P-glycoprotein-mediated efflux in Caco-2 cells. These results suggest that repeated use enhances the efflux of morphine in the epithelial cells of the small intestine, subsequently decreasing its intestinal absorption.

Key words morphine; tolerance; absorption; P-glycoprotein

Chronic treatment with morphine is associated with the development of tolerance. The mechanism underlying this phenomenon has been investigated at the cellular and molecular level, but is poorly understood at the in vivo level. In vivo pharmacokinetic actions of drugs are generally dependent on both pharmacokinetic and pharmacodynamic factors.3) Pharmacokinetic processes should be considered to elucidate the mechanism of morphine tolerance in vivo. In particular, P-glycoprotein (ABC1B, an MDR1 gene product), a drug resistance-related transporter, affects the intestinal absorption of morphine in humans and the distribution of morphine in the brain in mice and rats.4–8)

Aquilante et al. reported that repeated morphine administration caused a two-fold increase in the level of P-glycoprotein in the rat brain associated with a decrease in the antinociceptive effects.9) King et al. also reported that an antisense-oligo against mdr1a suppressed the development of antinociceptive tolerance to intracerebroventricularly administered morphine.10) Those results suggest that P-glycoprotein at the brain barrier is involved in the development of morphine tolerance. On the other hand, Yousif et al. reported that chronic morphine treatment did not alter P-glycoprotein activity in the blood–brain barrier in rats.11) In patients treated with a sustained-release dosage form of morphine, P-glycoprotein in the intestine is more significantly affected than that at the blood–brain barrier, since the intestinal epithelium might be exposed to a relatively high concentration of morphine in a sustained manner. It was reported that intestinal P-glycoprotein affects morphine absorption in humans4) and rats.12,13) The previous studies led to the assumption that repeated treatment with morphine causes an increase in intestinal P-glycoprotein-mediated transport, subsequently leading to a decrease in the oral bioavailability of this drug. Such a pharmacokinetic mechanism may be partly associated with the development of morphine tolerance.

The present study was undertaken to examine the effects of repeated treatment with morphine on its antinociception and on the intestinal absorption of this drug in rats. Further, we examined the effects of repeated treatment with morphine on transepithelial transport and on P-glycoprotein-mediated transport activity in Caco-2 cells, an in vitro model often used to evaluate intestinal drug permeability.

MATERIALS AND METHODS

Materials Male Sprague-Dawley rats weighing about 250 g were housed 3 to 4 per cage with free access to food and water and maintained on a 12 h-light/dark cycle in a room with controlled temperature (24±1 °C) and humidity (55±5%). Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), maintained at subconfluence in cell culture flasks, and subcultured twice a week. Caco-2 cells were used between passages 45 and 55. This study was conducted in accordance with the guidelines for the care and use of laboratory animals adopted by the U.S. National Institutes of Health. Morphine hydrochloride was purchased from Takeda Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were purchased from commercial sources.

Antinociceptive Test The development of morphine tolerance was measured according to a method described previously14) with a slight modification. Briefly, rats received morphine (100 mg/kg) orally once a day for 5 d, and the antinociceptive effects were determined in a tail-flick test with a thermal stimulus applied to the tail15) once a day (120 min after morphine administration) to avoid tissue damage from repeated measurements. Before drug administration, baseline antinociceptive testing was performed. A maximum tail-flick latency of 10 s was used to minimize tissue damage to the tail. The tail-flick latency values were converted to a percent-
age of the maximum possible effect (%MPE) as follows: 

\[
\text{%MPE} = \left( \frac{\text{postdrug latency} - \text{predrug latency}}{\text{maximum latency} - \text{predrug latency}} \right) \times 100
\]

**Intestinal Absorption of Morphine Evaluated Using the in Situ Loop Method**

Rats received oral morphine (100 mg/kg) or saline once a day for 5 d. On the day (day 6) after the last administration, the intestinal absorption of morphine was determined using the in situ loop method. Under sodium pentobarbital anaesthesia, three loops (each 5 cm) were prepared in rats. The proximal ligature of the first loop (duodenal loop) was placed about 1 cm from the pylorus, that of the second loop (jejunal loop) was about 20 cm from the pylorus, and that of the third loop (ileal loop) was about 7 cm above the ileoceleal junction. The bile duct was ligated in all experiments. After the contents of the loops were gently washed out with Tyrode’s buffer (NaCl 137 mM, KCl 3 mM, CaCl₂ 2 mM, MgCl₂ 2 mM, NaH₂PO₄ 0.4 mM, and D-glucose 6 mM, pH 7.4), 0.5 ml of Tyrode’s buffer containing morphine 10 μM was administered into each loop using a syringe. After 30 min, the solution in the loop was collected. Then, the mucosal side of the loop was rinsed with buffer to give a total volume of 2 ml. The fraction of absorbed morphine was estimated from the difference between the dose administered and the amount remaining in the loops. The absorption of phenol red, an unabsorbable marker, did not change after the repeated oral administration of morphine.

**Transport Experiments**

Caco-2 cells were seeded at 1 × 10⁵ cells/cm² onto polycarbonate membrane Transwell inserts (Corning Co., Acton, MA, U.S.A.) in 12-well plates. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, penicillin 50 units/ml, and streptomycin 0.05 mg/ml. DMEM with or without morphine (10 μM) was changed every 2 d. After 21 d of culture, the cells were washed and incubated for 4 h with morphine-free DMEM for the washout of morphine before transport experiments. Hanks’ balanced salt solution (HBSS) containing morphine 10 μM was added to the apical or basolateral chamber. The cells were incubated for 60, 120, and 180 min, and the morphine concentration in the other (receiver side) chamber was determined using the Millicell-ERS system (Millipore Co., Bedford, MA, U.S.A.). Statistical analysis of the data was performed employing Student’s t-test and one-way analysis of variance, followed by Dunnett’s test for single and multiple comparisons. The differences were considered statistically significant at p<0.05.

**RESULTS**

**Antinociceptive Effects after Repeated Oral Administration of Morphine in Rats**

Rats were orally administered morphine (100 mg/kg) once a day for 5 d. The antinociceptive effects were measured using the tail-flick test 2 h after administration. The antinociceptive effects were 100% MPE on days 1 and 2, and 65%, 20%, and 5% MPE, respectively, on days 3, 4, and 5 (Fig. 1). Thus, pharmacologic tol-
erance developed after the repeated oral administration of morphine in rats. 

**Rat Intestinal Absorption of Morphine** The intestinal absorption of morphine in morphine-tolerant rats (100 mg/kg per os (p.o.), once a day for 5 d) was measured using the in situ loop method. Morphine absorption from the jejunum was significantly (53%) decreased in the morphine-treated rats (Fig. 2). The absorption from the duodenum and ileum was decreased by 38% and 17%, respectively, but these changes were not significant.

**Effects of Repeated Treatment with Morphine on the Transepithelial Transport of Morphine through Caco-2 Cell Monolayers** Morphine (10 μM) showed little differential transport between the apical to basolateral and the basolateral to apical direction through untreated Caco-2 cell monolayers (Fig. 3). After repeated treatment with morphine (10 μM for 21 d), permeability to the drug (10 μM) was significantly (two-fold) greater in the basolateral to apical direction than in the apical to basolateral direction.

**Effects of Repeated Treatment with Morphine on the Transport of [3H]Vincristine and Rhodamine123 in Caco-2 Cells** The accumulation of [3H]vincristine in Caco-2 cells increased with incubation time, and the cell-to-medium ratio reached a steady state at a value of 15 μl/mg protein after 60 min. The accumulation at 60 min increased significantly in the presence of verapamil (500 μM) and cyclosporin A (17 μM) by 18% and 64%, respectively (data not shown). The repeated addition of morphine (0.1—500 μM) to Caco-2 cells significantly reduced the amount of [3H]vincristine accumulated by 12—20% in a concentration-dependent manner (Fig. 4A). A significant decrease (approximately 10%) was seen after 12-h culture with morphine (10 μM), and a similar decrease (10—14%) was observed after culture for 1 to 14 d (Fig. 4B). Moreover, cyclosporin A (17 μM) increased the accumulation. The decrease in the accumulation of [3H]vincristine in Caco-2 cells after culture with morphine did not occur in the presence of cyclosporin A (Fig. 5). The accumulation of rhodamine123 was also significantly inhibited in the presence of cyclosporin A, and was decreased by 10—15% after culture with morphine for 1—7 d (data not shown). The efflux of rhodamine123 from Caco-2 cells was significantly (1.4-fold and 2.5-fold, respectively) increased by preculture with morphine (10 μM) and digoxin (1 μM) (Fig. 6).
We also investigated the effects of morphine on transepithelial transport in Caco-2 cells. The Caco-2 line is frequently used as an intestinal model for the prediction of drug absorption.\(^{20}\) Repeated morphine administration caused an increase in the permeability of Caco-2 monolayers in the basolateral to apical direction and a decrease in the apical to basolateral direction. Thus the basolateral to apical transport was 2-fold greater than the apical to basolateral transport after repeated administration of morphine, suggesting an increase in the efflux of morphine after repeated administration. To our knowledge, this is the first report to demonstrate significant effects of repeated morphine administration on transepithelial permeability.

P-glycoprotein mediates the efflux of morphine and affects the intestinal absorption of morphine in humans.\(^{4}\) Thus the effects of morphine administration on P-glycoprotein-mediated transport was characterized in Caco-2 cells using \([^{3}H]\)vincristine and rhodamine123 as substrates of P-glycoprotein. The level of P-glycoprotein varies with culture conditions and time.\(^{23}\) The cellular accumulation of both \([^{3}H]\)vincristine and rhodamine123 was significantly increased in the presence of cyclosporin A, a P-glycoprotein inhibitor. These results suggest that P-glycoprotein operates as the efflux transporter in Caco-2 cells.

After Caco-2 cells were cultured with morphine (0.1—500 \(\mu M\)) for 14 d, the accumulation of \([^{3}H]\)vincristine was significantly decreased. A reduction in the accumulation was also observed after culture with morphine for 12 h and 14 d. Since the accumulation was restored to the control level by the addition of cyclosporin A, it is suggested that the decrease in \([^{3}H]\)vincristine accumulation is due to the stimulatory effects on P-glycoprotein-mediated efflux. The accumulation of rhodamine123 was also decreased after culture with morphine, and the efflux of rhodamine123 from Caco-2 cells was significantly increased after culture with morphine. These results suggest that the repeated culture with morphine enhances P-glycoprotein-mediated efflux in Caco-2 cells. It was reported that the addition of digoxin increases the efflux of rhodamine123 and reduces the cytotoxicity of paclitaxel, a P-glycoprotein substrate, in Caco-2 cells.\(^{17}\) Although the increase (1.4-fold) in the efflux of rhodamine123 with the addition of digoxin, the enhancement of P-glycoprotein-mediated efflux is considered to affect significantly the transepithelial transport of morphine through Caco-2 cell monolayers after repeated culture with morphine.

In relation to the mechanism underlying the stimulation of P-glycoprotein-mediated efflux, we measured \(MDR1\) mRNA expression in Caco-2 cells after culture with morphine (10 \(\mu M\)) for 1—3 d) or digoxin (1 \(\mu M\) for 24 h) (our unpublished data). \(MDR1\) mRNA expression in Caco-2 cells was increased after the addition of digoxin but not after that of morphine, suggesting that digoxin upregulates \(MDR1\) mRNA while morphine does not regulate transcriptional expression of P-glycoprotein in Caco-2 cells.

The morphine concentration (10 \(\mu M\)) used in the current transport experiment was about 50-fold greater than the mean plasma concentration (223 nm) in patients with chronic cancer pain receiving oral morphine.\(^{24}\) However, the concentration in the intestinal lumen after oral administration was
much higher than the plasma concentration. Repeated administration caused significant decreases in the antinociceptive effects and intestinal absorption of morphine in rats, suggesting that a reduction in the absorption of morphine occurred at pharmacologically relevant doses. Therefore repeated oral administration may stimulate the intestinal efflux of morphine, leading to a decrease in the oral bioavailability and antinociceptive effects of the drug.

In conclusion, the results of the current study suggest that repeated morphine administration in rats reduces the intestinal absorption of this drug, subsequently decreasing its antinociceptive effects. The decrease in absorption may be related, at least in part, to the stimulation of P-glycoprotein-mediated efflux. These findings may be relevant to the development of morphine tolerance after oral administration. Further study will be required to clarify the mechanisms underlying morphine tolerance in relation to intestinal absorption.

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REFERENCES AND NOTES

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