Full Paper

Negative Relationship Between Morphine Analgesia and P-Glycoprotein Expression Levels in the Brain

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Abstract. It is known that opioid analgesics given systemically have limited distribution into the brain because of their interaction with P-glycoprotein (P-gp), an ATP-dependent efflux pump acting at the blood-brain barrier (BBB). We previously found that morphine and fentanyl showed higher analgesic potencies in P-gp–deficient mice compared with those in wild-type mice, suggesting that their analgesic effects are considerably dependent on P-gp expression. In this study, we focused on individual differences in the analgesic effectiveness of morphine, in cortical P-gp expression, and in basal P-gp ATPase activity in male ICR mice. We found that there were 3- to 10-fold differences between the magnitude of morphine analgesia (3 mg/kg, s.c.; tail-pinch method) in mice. Furthermore, there was a significant negative correlation between morphine's analgesic effects and individual P-gp expression in the cortex as estimated by western blot analysis. In addition, basal P-gp ATPase activities in isolated membrane preparations of brain capillary endothelial cells (BCECs) were negatively correlated with the magnitude of the analgesic effect of morphine. These results indicate that the individual differences in morphine analgesia may be due to some functional or quantitative differences in individual P-gp in BCECs, acting at the BBB.

Keywords: morphine, analgesia, individual difference, P-glycoprotein, blood-brain barrier

Introduction

Opioid analgesics, generally used for the treatment of acute and chronic pain, have widely variable clinical responses in humans (1). Recent studies revealed that inherited differences in drug-metabolizing enzymes (e.g., cytochrome P-450), drug-transporters [e.g., P-glycoprotein (P-gp)], and/or opioid receptors (\(\mu\), \(\delta\), and \(\kappa\)-opioid), may affect the effectiveness of opioid drugs in individual patients (2). Furthermore, some variability in nociceptive sensitivity or in the activity of descending inhibitory systems may also cause interindividual variability in response to these drugs (3, 4), indicating that further research is required to clarify the interindividual variability in drug effectiveness. In this study, we focused on interindividual variability of morphine analgesia in mice.

Although there are many factors that can influence the pharmacokinetics/pharmacodynamics of a drug, transport of morphine across the blood-brain barrier (BBB) seems to be the most important step in permitting morphine to exert a centrally-mediated analgesic effect (5, 6). The limitation of drug transport into the brain is determined by characteristics of the BBB, which in turn is affected by the physicochemical properties of drugs such as size, charge, and lipid solubility. Brain endothelial cells express numerous influx and efflux transporters. P-gp is one of the drug efflux transporters systemically expressed, not only in the BBB, but also in several tissues such as liver, kidney, and intestine (7–9). P-gp acts as an energy-dependent efflux pump, which transports a wide array of structurally divergent...
Effect (%MPE) was calculated using the following formula: %MPE = 100 × (each latency – baseline latency) / (15 – baseline latency).

The clip was applied prior to and every 15 min following morphine administration over a 120-min observation period. A time-antinociception (%MPE) curve was constructed and the area under the curve (AUC) was calculated from time versus %MPE. The morphine antinociceptive analysis was performed close to the same time of day for all respective groupings.

**Western blot analysis for P-gp expression**

One week after analgesic analysis, mice were decapitated and their brains were removed. A part of the forebrain was dissected and immediately lysed in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol), boiled, and reduced with β-mercaptoethanol. Samples (10 µg) and molecular weight standards (BioRad, Hercules, CA, USA) were electrophoresed in 7.5% SDS-PAGE acrylamide gels and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked for 2 h at 25°C with 5% skim milk in TBS containing 0.1% Tween 20, incubated with a rabbit anti-P-gp antibody (1:100) (H-241; Santa Cruz Biotechnology, CA, USA) and a rabbit anti-β-actin antibody (1:1000) (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C and then reacted with horseradish peroxidase (HRP)–conjugated antirabbit IgG (1:2000) for 2 h at 25°C. All visualization of immunoreactive bands was performed by using LuminoCapture (ATTO, Tokyo) with an enhanced chemiluminescent substrate for the detection of horse-radish peroxidase, LumiGLO (Pierce Chemical, Rockford, IL, USA). The signal intensity of immunoreactive bands was analyzed by use of NIH-image analysis software (Image J; National Institutes of Health, USA). We confirmed that the signal intensity was linear function of the amount of transporter protein (data not shown).

**Isolation of mouse brain capillaries**

Mouse brain capillaries were isolated from mouse cerebrum, as described previously with some modifications (16, 17). Briefly, individual mouse brain cortices (for one experiment, mixed brains of 2 to 4 mice were used) were stripped of the pial membrane, meninges, superficial large blood vessels, and choroid plexus and then gently homogenized in 3 volumes (v/w) of ice-cold physiological buffer (PB), consisting of 147 mM NaCl, 4 mM KCl, 3 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, and 15 mM HEPES, pH 7.4, with a tissue homogenizer run at 400 rpm for 20 strokes. Then, the homogenate was centrifuged (5800 × g for 10 min, 4°C) after adding dextran (15%). The resulting pellet was resuspended in PB and filtered through a 200-µm nylon mesh. The filtrate was passed over a 1.5 × 3 cm column of 170–250-µm glass beads and washed twice with 20 ml of PB. The capillaries adhering to the beads were collected by agitation for 15 min followed by centrifugation at...
500 × g for 10 min. All steps in the isolation procedure were carried out at 4°C. In regard to this method, the prepared brain capillary fraction has been morphologically characterized by means of phase-contrast microscopy (17). The capillary preparation was resuspended in 1 ml of 0.1% (w/v) collagenase II in phosphate-buffered saline (PBS) and incubated at 37°C for 30 min. The suspension was centrifuged at 1000 × g for 10 min at 4°C and the pellet was washed twice with PBS, and then BCECs were obtained (18).

Preparation of plasma membranes
Plasma membranes were prepared from BCECs as previously described (19). Isolated cells were suspended in hypotonic lysis buffer [10 mM Tris-HCl (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), and 1 mM EGTA] and allowed to swell for 20 min at 4°C. Swollen cells were disrupted by sonication for 10 s at 20% of maximum power (Branson Sonifier 250; Ultrasonic Corporation, Danbury, CA, USA) and the resulting homogenate was centrifuged (1400 × g for 10 min, 4°C). The supernatant was then laid on a 46% sucrose cushion in lysis buffer and centrifuged (7000 × g for 20 min, 4°C). The layer at the sucrose interface was collected, diluted twice with lysis buffer, and then sedimented (13,500 × g for 15 min, 4°C). The pellet of total membranes was resuspended in lysis buffer supplemented with 100 mM NaCl at a total membrane protein concentration of about 0.5 and 1 mg/ml. The protein concentration was determined with the Coomassie (Bradford) Protein Assay Kit (Pierce Chemical), with bovine serum albumin as the standard. The membrane fraction obtained by this method is known to be highly enriched for the marker enzymes gamma-glutamyl transpeptidase and alkaline phosphatases (17). We confirmed the expression of P-gp in our membrane preparation of BCECs using western blot analysis (data not shown).

Measurement of basal P-gp ATPase activity
The basal P-gp ATPase activity of the isolated BCEC membranes was estimated by measuring inorganic phosphate (Pi) liberation (18). Membrane suspensions (0.3 µg of membrane protein) were incubated at 37°C for 60 min in 0.05 ml of a reaction buffer containing 50 mM Tris-HCl (pH 6.8), 2 mM DTT, 5 mM MgCl₂, 2 mM ouabain (to eliminate Na⁺/K⁺-ATPase activity), 2 mM EGTA (to eliminate Ca²⁺-ATPase activity), and 5 mM sodium azide (to eliminate F₁-F₀-ATPase activity). The ATPase reaction was initiated by the addition of 0.5 mM MgATP. Pi was measured by a sensitive colorimetric reaction using Biomol Green reagent according to the manufacturer’s instructions (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA). Activities were calculated from the absorbance of the initial linear rate of Pi production read at 630 nm. All reactions were performed in the presence of ouabain, sodium azide, and EGTA to inhibit ATPases other than P-gp as described above (11, 18). Furthermore, we confirmed that vanadate, an inhibitor of many ATPases including P-gp, significantly inhibited this ATPase activity (11).

Drugs
The drugs used and their suppliers were as follows: morphine hydrochloride (Takeda Chemical Industries, Ltd., Osaka); ouabain (Sigma-Aldrich, St. Louis, MO, USA); and sodium azide (Nacalai Tesque, Tokyo). For analgesic analysis, morphine was dissolved in saline and administered subcutaneously. For measuring basal P-gp ATPase activity, all drugs except ouabain were dissolved in water. Ouabain was dissolved in dimethylsulfoxide.

Statistical analysis
Data were analyzed by Pearson’s correlation coefficient (r). The differences were regarded as statistically significant when the P value was less than 0.05.

Results
Individual differences in morphine analgesia in mice
The analgesic effect of morphine (3 mg/kg, s.c.) was measured by the tail-pinching method using 26 individual male ICR mice (Fig. 1). The time-antinociception (%MPE) course analysis revealed that the analgesic effect of morphine in the 26 mice peaked at 15 min in 2 mice (Fig. 1A), 30 min in 17 mice (Fig. 1: B and C), 45 min in 5 mice (Fig. 1D), and 60 min in 2 mice (Fig. 1E). When we determined the total amount of morphine analgesia by the AUC assay in each mouse, there was as much as a 10.7-fold difference in analgesic responses between the mouse “a” with the smallest response and the mouse “z” with the largest response to morphine (Fig. 2).

Negative correlation between cortical P-gp expression levels and morphine analgesia
The individual cortical P-gp expression levels relative to β-actin estimated by western blot analysis showed a 10.6-fold maximal difference between low- and high-P-gp expressions (Fig. 3). We observed a significant negative correlation between P-gp expression levels and morphine analgesia (Fig. 4A), while there was no relationship between β-actin expression levels and the analgesic effects of morphine (data not shown).
other hand, there were no positive/negative relationships between baseline pain threshold and the analgesic effect of morphine or P-gp expression levels (Fig. 4: B and C).

Correlation between basal P-gp ATPase activity and morphine analgesia
To determine the basal P-gp ATPase activity in the BCEC membrane fraction, we divided 13 mice into four groups according to the amount of morphine analgesia in each mouse (Table 1). Interestingly, the basal P-gp ATPase activity in the BCEC membrane fraction of each

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**Fig. 1.** Time course of morphine analgesia in 26 individual mice. Mice were labeled alphabetically. The effect of morphine (3 mg/kg, s.c.) was measured by the tail-pinch method every 15 min for 120 min after morphine administration. The vertical bar indicates the peak time of morphine analgesia (15 min, A; 30 min, B and C; 45 min, D; 60 min, E).

**Fig. 2.** Comparison of analgesic effect of morphine in 26 individual mice. The analgesic effect of morphine is presented as the area under the curve (AUC; %MPE × h). Each letter on the X-axis indicates an individual mouse.

**Fig. 3.** The levels of P-glycoprotein expression in cortex of 26 individual mice estimated by western blot analysis. The relative levels of P-gp expression were analyzed by determining the ratio of P-gp/β-actin (the endogenous internal standard protein).
Morphine Analgesia and P-Glycoprotein Expression

Discussion

In this study, we found individual differences in morphine (3 mg/kg, s.c.)-induced analgesic effects in male ICR mice, although our experiments were performed close to the same time of day for all respec-
tive groupings to preclude the possibility of “time-
dependent differences in morphine analgesia”, which had been reported previously (20, 21). Moreover, the individual differences in morphine analgesia were negatively correlated with relative cortical P-gp expression levels and basal P-gp ATPase activity in isolated membrane preparations of BCECs. These results clearly demonstrated that individual differences in morphine analgesia were P-gp–dependent.

We evaluated the P-gp expression level and the basal P-gp ATPase activity at 1 week after analgesic analysis, so that morphine could be completely eliminated from the mouse body. Although we confirmed that the single

Table 1. Morphine analgesia (AUC) and basal P-gp ATPase activity in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse i.d.</th>
<th>AUC (%MPE x h)</th>
<th>AUC average (%MPE x h)</th>
<th>Basal P-gp ATPase activity (Pi [nmol])</th>
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<tr>
<td>1</td>
<td>a</td>
<td>5.97</td>
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<td></td>
<td>b</td>
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<td>c</td>
<td>13.17</td>
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<td>d</td>
<td>16.30</td>
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<td>i</td>
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The mice were divided into four groups according to the morphine analgesia (3 mg/kg, s.c.). AUC: the area under the curve (%MPE x h).

Fig. 4. Overall correlation between the relative P-gp expression levels and AUC of morphine analgesia (A), between AUC of morphine analgesia and the baseline tail-pinching latency (B), and between the relative P-gp expression levels and the baseline tail-pinching latency (C). Pearson correlation coefficients were used for data analysis.

Fig. 5. Overall correlation between the basal P-gp ATPase activity and AUC of morphine analgesia. The AUC data of each point represents the average of each group as shown in Table 1. For the estimation of basal P-gp ATPase activity, mixed brains of 2 – 4 mice were used as shown in Table 1. Pearson correlation coefficients were used for data analysis.
injection of morphine does not stimulate the expression levels of P-gp in the BCECs when compared to the saline treatment (data not shown), we previously reported that the in vivo basal P-gp ATPase activity might be increased by the single morphine treatment (11). However, as others had reported, the activated P-gp ATPase activity might return to the basal level after elimination of substrate drug (22), suggesting that the P-gp ATPase activity could not be affected by morphine (3 mg/kg) administered for analgesic analysis 1 week before. In other words, it is considered that the significant antinociceptive tolerance to morphine might not be developed, even if a low dose of morphine was administered 1 week before.

Surprisingly, there was more than a 10-fold difference in the amount of morphine (3 mg/kg, s.c.) analgesia among the 26 male ICR mice tested (Fig. 2). However, we did not observe remarkable differences (i.e., maximally 2.4-fold difference) in the amount of analgesia when 7 mg/kg of morphine was administered (data not shown). The difference between the high (7 mg/kg) and low (3 mg/kg) dose of morphine might be due to the saturation of morphine–P-gp coupling, leading to the increment of nonspecific permeation of morphine across the BBB. On the other hand, the expression levels of P-gp in the cortex also showed over a 10-fold difference among the mice, and there was a significant negative correlation between individual P-gp expression levels and P-gp ATPase activity as described above, there was not any correlation between the time required to reach the peak of morphine analgesia and P-gp expression levels (r = −0.094, n = 26, data not shown). The correlation between the time required to reach the peak of morphine analgesia and basal P-gp ATPase activity remains to be determined.

Although individual differences in analgesic response related to genetic or environmental factors have been reported (23, 24), there are very few reports on the individual differences in isogenic mice reared under certain conditions. Recently, Kalinichev et al. reported individual differences in morphine-induced locomotor sensitization and analgesia in male Sprague-Dawley rats, although the details remain unclear (25, 26). The present study is the first report showing that central P-gp expression levels correlate with interindividual differences in morphine analgesia. An increasing number of reports indicate that P-gp expression levels in kidney and placenta are affected by P-gp gene polymorphism, suggesting the possibility of involvement of inherited parameters in the P-gp expression variability seen in this study. Although it is empirically known that there is interindividual variability in morphine analgesia in isogenic mice, the mechanism of these differences is not clear. In this study, we found a significant negative correlation between P-gp expression levels in the brain and morphine analgesia. In addition, in the clinic, it is known that the doses of opioids needed for pain relief clinically vary between individuals (4). For example, a large (differences of up to 10-fold) interpatient variability in patient-controlled analgesia morphine doses has been reported (27). Traditionally, this variation has been explained by variable bioavailability; differences in intensities of pain stimuli, and interindividual differences in pain perception; or differences in pharmacodynamics at the µ-opioid receptor, in drug metabolism, and in transport of opioids across the BBB; however the exact reasons remain controversial (28–33). The present results suggest that the variation of the P-gp expression levels or its function in the BBB may be contributed to the interindividual validity of analgesic effects of opioids. Furthermore, this may help in the development of an effective strategy for customization of opioid therapy.
In conclusion, we have shown that individual differences in morphine analgesia are highly negatively correlated with individual differences in P-gp expression levels and basal P-gp ATPase activity in the mouse brain. These results suggest that the individual differences of the antinociceptive effects of morphine might be due to the intrinsic individual differences of brain P-gp expression levels and/or the basal P-gp ATPase activities. The present findings support the clinical observation of individual differences in morphine effects in pain therapy.

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