Possible Involvement of the Total Amount of Morphine Infused in the Development of Acute Morphine Dependence in Rats

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ABSTRACT—The severity of naloxone-precipitated withdrawal in rats infused intravenously with morphine at the rates of 2.5, 5 and 10 mg/kg/hr over various time periods was investigated. Plasma morphine concentration reached a constant and rate-dependent level at 1 hr after the start of morphine infusion, and this level was maintained until the termination of infusion. Naloxone (2.0 mg/kg, s.c.) was challenged 18 hr after infusion was stopped, and the withdrawal was evaluated by plasma corticosterone (PCS) increase, diarrhea and body weight loss. The incidence of naloxone-precipitated withdrawal signs was related to both the infusion rate and duration of morphine infusion. The duration of morphine infusion (ET50) needed to elicit naloxone-precipitated PCS increase and diarrhea in 50% of the rats was inversely related to the morphine infusion rates, but the total amount of infused morphine (EA50) that elicited naloxone-precipitated withdrawals in 50% of rats was the same at all infusion rates. These results suggest that the total amount of morphine infused may play an important role in the development of acute physical dependence on morphine rendered by continuous intravenous morphine infusion for 1–8 hr.

Keywords: Intravenous morphine infusion, Naloxone-precipitated withdrawal, Total amount of morphine infused, Plasma morphine concentration, Duration of morphine infusion

Morphine has been used widely for relieving severe pain, and the possibility of developing physical dependence is one of the major limitations of its clinical use. It is thus instructive for the understanding of the therapeutic use of morphine to clarify the factors influencing morphine dependence liability.

The intensity of physical dependence on morphine is assessed by the severity of morphine withdrawal induced by the administration of an opioid antagonist (opioid antagonist-precipitated withdrawal) or by the discontinuance of morphine exposure (spontaneous withdrawal). It is well-known that the severities of both kinds of withdrawals are correlated with the dose and the duration of morphine exposure, but there is little information on the direct relationship between the extent of morphine dependence and the morphine concentration at the acting sites and/or the duration of morphine exposure at a given concentration of morphine.

Several lines of evidence show that opioid antagonist-induced morphine withdrawal is manifested after several hours of exposure to morphine via morphine pellet implantation in rats (1); morphine infusion in mice (2) and dogs (3); and after a single injection of morphine in mice (4), rats (5) and humans (6). In all of these studies, acute physical dependence developed after brief exposure to morphine. However, it is not clear whether the level of morphine at its acting sites, the duration of morphine exposure or both of these conditions are the most important of the factors influencing the development of acute morphine dependence.

In the present study, we estimated the severity of naloxone-precipitated withdrawal in rats infused i.v. with morphine at the rates of 2.5, 5 and 10 mg/kg/hr over various time periods and investigated the relationship between the severity of withdrawal and the infusion rate of morphine, the duration of morphine infusion and the total amount of morphine infused.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Nihon Clea Co., Tokyo) weighing 250–300 g were used. The animals were kept in a room with controlled temperature (23–24°C), humidity (60%) and lighting (7:00–19:00), and they were provided
free access to tap water and commercial chow (MF; Oriental Yeast Co., Osaka).

Rats were cannulated in the right external jugular vein under pentobarbital anesthesia (50 mg/kg, i.p.) according to the method of Upton (7). The cannulated rats were housed individually to prevent chewing of the exposed part of the catheter and were allowed a recovery period of 3 days after the operation. Morphine was infused i.v. through the jugular cannula at constant rates of 2.5, 5 and 10 mg/kg/hr, for 0.5–8 hr in a volume of 1 ml/hr/rat (Roller pump; Furue Science, Tokyo). Plasma morphine concentrations during the infusion were measured. Blood samples (0.4 ml) were obtained via the cannula for the determination of plasma morphine concentrations immediately after the termination of morphine infusion in freely moving animals. Immediately after the blood sampling, an equal volume of saline was transferred to the jugular vein through the cannula. Plasma morphine content was evaluated by the HPLC-ECD method (8) as follows: The mixture of plasma samples (0.1 ml), 1% bovine albumin solution (0.9 ml) and 40% K2HPO4 solution (1 ml) was shaken with 5 ml of ethylacetate for 5 min and then centrifuged at 2000× g for 5 min at 4°C. The organic layer was collected, and the aqueous layer was re-extracted with 5 ml of ethylacetate. Then, the morphine content was evaluated by the HPLC-ECD system. The conditions of the HPLC-ECD system were as follows: Column, Eicom Pak MA-ODS (Eicom, Kyoto); mobile phase, 0.1 M citric-acetate buffer (pH 3.9) / methanol (82/18) containing 3 mg/liter of EDTA and 150 mg/liter of sodium octanesulfonate; flow rate, 1 ml/min; detector, ECD-100 (Eicom) 750 mV Ag/AgCl; temperature, 25°C. In this assay, a level of 40 ng/ml of plasma was the lower limit of detection.

In all paradigms, morphine infusion was finished at 15:30 and naloxone (2.0 mg/kg, s.c.) was challenged at 9:30 the next day; that is, 18 hr after terminating the i.v. infusion of morphine. Plasma corticosterone (PCS) increase, diarrhea and body weight loss were assessed as indicators of naloxone-precipitated withdrawal.

The blood samples (0.4 ml) for PCS estimation were obtained at 9:30 and 10:00 (i.e., immediately before and 0.5 hr after naloxone, respectively) through the cannula in freely moving animals in order to avoid circadian variation in circulating PCS. After the blood sampling, an equal volume of saline was injected into the jugular vein through the cannula. The collected blood samples were centrifuged at 2000× g for 15 min at 4°C, and the plasma was stored at −20°C until assayed. The PCS was estimated fluorometrically according to the method of Zenker and Bernstein (9). The presence of diarrhea was checked during the 1.5-hr period after naloxone. Body weight loss (%) was calculated as follows: [(body weight at 1 hr before naloxone − body weight at 1.5 hr after naloxone) / body weight at 1 hr before naloxone] × 100.

The incidence (%) of naloxone-induced PCS increase, body weight loss and diarrhea were determined. Higher PCS and lower body weight than the mean plus 3 × S.D. of the respective saline-treated control rats were defined as PCS increase and body weight loss, respectively. The infusion time of morphine (ET50, hr) and total amount of morphine infused (EA50, mg/kg) required to elicit naloxone (2 mg/kg)-precipitated PCS increase, diarrhea and body weight loss in one-half of the morphine-infused rats were calculated.

Morphine (morphine hydrochloride; Takeda Chem. Industries, Ltd., Osaka) and naloxone (naloxone hydrochloride; Endo Labo., Inc., Garden City, NY, USA) were freshly dissolved in saline before use. Other chemicals were purchased from the following sources: sodium pentobarbital (Nembutal; Dinabot, IL, USA); bovine albumin (special grade; Katayama Chemicals, Osaka); sodium acetate, citric acid monohydrate, EDTA-2Na (special grade, Katayama Chemicals); sodium octanesulfonate (HPLC grade; Nacalai Tesque, Kyoto) and methanol (HPLC grade; Kanto Chemicals, Tokyo).

Values are each expressed as a mean±S.E.M. Data were analyzed by a one way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparison and unpaired Student’s t-test for comparison between two groups. The ET50 and EA50 value, potency ratios and their 95% confidence limits were calculated according to Litchfield and Wilcoxon. Analyses of data were done using the computer programs described by Tallarida and Murray (10).

RESULTS

Plasma morphine concentrations during morphine i.v. infusion

Plasma morphine concentrations at the termination of morphine infusion with 2.5, 5 and 10 mg/kg/hr for various time periods are presented in Fig. 1. In rats infused with either 2.5 mg/kg/hr of morphine for 1–8 hr or 5 mg/kg/hr for 1–4 hr, there was no significant difference in plasma morphine concentrations at any infusion time, indicating that the plasma morphine concentration reached a steady state at 1 hr after the start of morphine infusion with 2.5 or 5 mg/kg/hr, and these levels were maintained throughout the infusion period. With 10 mg/kg/hr, the morphine concentrations at 2 and 3 hr were significantly higher (P<0.05) than that at 0.5 hr
after the start of morphine infusion, but no significant difference in plasma morphine concentrations at 1−4 hr after the start of morphine infusion were observed, i.e., plasma morphine concentration also reached a steady state at 1 hr after the start of morphine infusion with 10 mg/kg/hr, and this was maintained until the termination of dosing. These steady state levels of plasma morphine during morphine infusion were related to the infusion rates of morphine.

**Plasma corticosterone increase induced by naloxone**

PCS changes induced by naloxone in rats infused with morphine at the infusion rates of 2.5, 5 and 10 mg/kg/hr for various time periods are shown in Fig. 2. PCS levels at 18 hr after terminating i.v. infusion of morphine (just before saline or naloxone) were as low as the resting PCS levels in naive rats (11); i.e., increase in PCS was not induced by discontinuance of morphine infusion under the present experimental conditions.

In rats infused with 2.5 mg/kg/hr of morphine for 6 and 8 hr, a PCS increase was induced by naloxone. Likewise, significant increases in PCS were induced by naloxone in rats infused with either 5 or 10 mg/kg/hr of morphine for 2, 3 and 4 hr.

**Diarrhea induced by naloxone**

In all rats infused with morphine, diarrhea was not observed at 18 hr after terminating infusion of morphine (just before saline or naloxone); i.e., diarrhea was not induced by discontinuance of morphine infusion under the present experimental conditions.

Incidence of naloxone-induced diarrhea in rats infused with 2.5, 5 or 10 mg/kg/hr of morphine for various periods of time is presented in Table 1. In rats infused with 2.5 mg/kg/hr of morphine for 3−8 hr, diarrhea was induced by naloxone, and the incidence was dependent on the duration of morphine infusion. Diarrhea was also induced by naloxone in rats infused with 5 or 10 mg/kg/hr of morphine for 1−4 or 2−4 hr, respectively, and its incidence was proportional to the duration of morphine infusion. Thus, in a given infusion time of morphine, the incidence of naloxone-induced diarrhea increased in relation to the dose of infused morphine.

**Body weight loss induced by naloxone**

Body weight change after naloxone is presented in Fig. 3. The body weight of naive rats decreased during the observed period of 2.5 hr (8:30−11:00). Although the body weight also decreased after saline in rats infused with morphine at various rates over various time periods, the degree of body weight loss was the same as that in naive rats.

In rats infused with 2.5 mg/kg/hr of morphine for 6 or 8 hr, body weight was significantly decreased by naloxone, compared with that of the respective saline control, although the decreases were not greater than

Fig. 1. Plasma morphine concentration immediately after terminating i.v. infusion of morphine at various rates. Open column: 2.5 mg/kg/hr morphine, lightly hatched column: 5 mg/kg/hr morphine, heavily hatched column: 10 mg/kg/hr morphine. Each column represents the mean and vertical bars indicate the S.E.M. of 11−15 rats. Differs from the respective open column (2.5 mg/kg/hr), **P<0.01. Differs from the respective lightly hatched column (5 mg/kg/hr), ##P<0.01.
those in naive rats. After the i.v. infusion of 5 mg/kg/hr of morphine for 2 hr, body weight was significantly decreased by naloxone, compared with that of both naive rats and the respective saline control. However, significant body weight loss was not induced by naloxone in rats infused with 5 mg/kg/hr of morphine for 3 or 4 hr. In rats infused with 10 mg/kg/hr of morphine for 4 hr, a significant decrease in body weight was induced by naloxone, compared with that of both naive rats and the respective saline control. Thus, naloxone-precipitated body weight loss that is greater than the body weight decrease in naive rats and dependent on the duration of morphine infusion was only observed in rats infused with 10 mg/kg/hr of morphine for 4 hr.

Correlation between the incidence of morphine withdrawal and the duration of morphine infusion or the total amount of morphine infused

The incidence percent of naloxone-precipitated withdrawal was plotted versus the duration of morphine

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Table 1. Diarrhea induced by naloxone in rats infused with 2.5, 5 and 10 mg/kg/hr of morphine for various time periods

| Treatment    | Infusion time (hr) | 0.5 | 1 | 2 | 3 | 4 | 6 | 8 |
|--------------|-------------------|-----|---|---|---|---|---|---|
| 2.5 mg/kg/hr | Saline            | 0/6 | 0/6 | 0/6 | 0/7 | 0/6 | 0/6 |
|              | Naloxone          | 0/6 | 0/6 | 1/6 | 2/7 | 2/6 | 3/6 |
| 5 mg/kg/hr   | Saline            | 0/6 | 0/6 | 0/6 | 0/6 |
|              | Naloxone          | 1/6 | 1/6 | 2/6 | 4/6 |
| 10 mg/kg/hr  | Saline            | 0/5 | 0/6 | 0/7 | 0/6 | 0/8 |
|              | Naloxone          | 0/6 | 0/6 | 4/8 | 3/6 | 8/8 |

Naloxone (2.0 mg/kg, s.c.) was injected 18 hr after terminating i.v. infusion of morphine. The presence of diarrhea was checked during the 1.5-hr period after naloxone. Each data value represents "number of rats with diarrhea / total rats used".
infusion and the total amount of morphine infused by each infusion rate (Fig. 4). Both the duration of morphine infusion—incidence of PCS increase and diarrhea curves shifted to the right when the infusion rate of morphine was increased (Fig. 4A). The ET_{50} for the incidence of PCS increase and diarrhea were inversely proportional to the infusion rate of morphine; and the potency ratios of 2.5 vs 5 mg/kg/hr and 2.5 vs 10 mg/kg/hr for the PCS increase and that of 2.5 vs 10 mg/kg/hr for the incidence of diarrhea differed significantly (Table 2). However, the total amount of morphine infused—incidence of PCS increase and diarrhea curves were located in the same position irrespective of the infusion rate of morphine (Fig. 4B). In both PCS increase and diarrhea, there was no significant difference in the EA_{50} and the potency ratio between the various infusion rates of morphine (Table 2). Naloxone-induced body weight loss was only observed in rats infused with 10 mg/kg/hr and not found in rats infused with 2.5 or 5 mg/kg/hr of morphine.

With an infusion rate of 10 mg/kg/hr of morphine, both ET_{50} and EA_{50} in body weight loss, diarrhea and PCS increase were in the order of duration and quantity (Table 2), suggesting the order of sensitivity of naloxone-precipitated withdrawal signs: PCS increase > diarrhea > body weight loss.

**DISCUSSION**

In the present study, male Sprague-Dawley rats were rendered acutely morphine-dependent by intravenous infusion of morphine at the constant rates of 2.5, 5 and 10 mg/kg/hr for 0.5 – 8 hr, and withdrawal was induced by naloxone injected 18 hr after terminating infusion of morphine. Then we investigated the relationship between the severity of the naloxone-precipitated morphine withdrawal and the infusion rate of morphine, the duration of morphine infusion and the total amount of morphine infused.

Constant levels of plasma morphine were maintained
Fig. 4. Incidence of naloxone-precipitated withdrawal and the duration of i.v. morphine infusion (A) or the total amount of morphine infused (B) in rats infused with morphine at the rates of 2.5, 5 and 10 mg/kg/hr. In the determination of the incidence of withdrawal (%), the definitions of plasma corticosterone increase and body weight loss were described in "Materials and Methods". Circle: 2.5 mg/kg/hr, triangle: 5 mg/kg/hr, square: 10 mg/kg/hr. As naloxone-induced body weight loss was not detected in rats infused with 2.5 or 5 mg/kg/hr of morphine, these curves are represented by dashed lines.

Table 2. Infusion time (ET<sub>50</sub>) and total amount of morphine infused (EA<sub>50</sub>) to elicit naloxone-precipitated withdrawal in one-half of the morphine-treated rats

| Withdrawal signs | Infusion dose (mg/kg/hr) | ET<sub>50</sub> (hr) (95% C.L.) | Potency ratio (95% C.L.) | EA<sub>50</sub> (mg/kg) (95% C.L.) | Potency ratio (95% C.L.) |
|------------------|--------------------------|-------------------------------|------------------------|-------------------------------|------------------------|
| Corticosterone increase | 10 | 1.29 (0.74–2.26) | 1.0 | 12.92 (7.40–22.56) | 1.0 |
| | 5 | 1.93 (1.22–3.05) | 1.5 (0.7–6.1) | 9.61 (6.10–15.23) | 0.7 (0.4–1.5) |
| | 2.5 | 4.27 (2.78–6.55) | 3.3 (1.6–6.7)* | 2.2 (1.2–4.1)* | 10.67 (6.95–16.38) | 0.8 (0.4–1.7) |
| Diarrhea | 10 | 2.05 (1.29–3.25) | 1.0 | 20.52 (12.95–32.50) | 1.0 |
| | 5 | 3.76 (1.85–7.65) | 1.8 (0.8–4.3) | 18.79 (9.23–38.25) | 0.9 (0.4–2.1) |
| | 2.5 | 7.84 (4.54–13.52) | 3.8 (1.9–7.8)* | 2.1 (0.9–5.1) | 19.59 (11.35–33.80) | 1.0 (0.5–1.9) |
| Body weight loss | 10 | 8.42 (3.73–19.00) | 1.6 | 84.24 (37.34–190.01) | 1.0 |
| | 5 | Not elicited | Not elicited | Not elicited | |
| | 2.5 | Not elicited | Not elicited | Not elicited | |

Data are from Fig. 4. "Not elicited" means naloxone (2 mg/kg)-induced body weight loss was not elicited. The ET<sub>50</sub> and EA<sub>50</sub> value, potency ratios and their 95% confidence limits (95% C.L.) were calculated according to Litchfield and Wilcoxon (10). *Value is greater than 1.0 (P<0.05).
during morphine infusion of 2.5, 5 and 10 mg/kg/hr from 1 hr after the start of infusion and were related to the infusion rates (Fig. 1), indicating that i.v. infusion of morphine at a constant rate provided a constant level of morphine exposure for a given duration of morphine exposure.

The severity of naloxone-precipitated withdrawal was related to the dose of naloxone (5, 11) and pretreatment with morphine inhibited the naloxone-precipitated withdrawal dose-dependently (12, 13), indicating that naloxone-precipitated withdrawal is elicited by the replacement of morphine by naloxone at the binding sites and that the intensity of the naloxone-precipitated withdrawal may be affected by the residual morphine at the acting sites at the time of naloxone challenge. We have previously shown the following: 1) The plasma morphine concentration decreased sharply; i.e., a 98.0% decline, during the initial 12 hr after morphine infusion of 10 mg/kg/hr for 4 hr. From 12 hr onward, the plasma morphine concentration decreased slowly, showing a 58.8% decline during the later 12 hr. There was a significant difference between the plasma morphine concentrations at 12 and 24 hr after infusion, but no difference between the plasma morphine concentrations at 18 and 24 hr after infusion. 2) The extent of body weight loss induced by a small dose (0.5 mg/kg) of naloxone at 12 hr after terminating i.v. infusion (10 mg/kg/hr for 4 hr) of morphine was less than that at 24 hr, while the body weight losses induced by a large dose (2.0 mg/kg) of naloxone at 12, 18 and 24 hr after the termination of the infusion were of the same degree in spite of the higher plasma morphine concentration at 12 hr than that at 24 hr after the end of infusion (14). These results indicate that 2 mg/kg of naloxone is all that is needed to replace morphine at its binding site at 18 hr after ending morphine infusion with 10 mg/kg/hr. Thus, in this experiment, we employed a large dose (2.0 mg/kg) of naloxone to precipitate the morphine withdrawal at 18 hr after ending morphine infusion at infusion rates of 2.5–10 mg/kg/hr.

As indicators of morphine withdrawal, we employed the PCS increase, diarrhea and body weight loss, all of which have been used to estimate morphine withdrawal (5, 11, 15–17). Body weight loss is known as a reliable and quantitative indicator of morphine withdrawal, correlating quantitatively with the dose of naloxone used to induce body weight loss in morphine-dependent rats (15). Nakaki et al. (17) reported that naloxone (5 mg/kg)-precipitated body weight loss after i.v. infusion (2 and 4 mg/kg/hr) of morphine for 24 or 48 hr was available as an index of physical dependence. However, in the present study, naloxone-precipitated body weight loss was only observed in rats infused with 10 mg/kg/hr of morphine for 4 hr, and no body weight loss was induced by naloxone in rats infused with 10 mg/kg/hr of morphine for less than 3 hr, 5 mg/kg/hr for 1–4 hr or 2.5 mg/kg/hr for 1–8 hr. These results suggest that the producibility of naloxone-induced body weight loss is related to both the duration of morphine infusion and its infusion rate.

PCS increase and diarrhea were induced by naloxone in rats infused with various doses of morphine over various time periods, and the incidence of PCS increase and diarrhea were well-correlated with the duration of morphine infusion at each rate (2.5, 5 and 10 mg/kg/hr) and the total amount of morphine infused, irrespective of the rate of morphine infusion (Fig. 4). The duration of morphine infusion—the incidence of PCS increase and diarrhea curves with the infusion rate of 2.5 mg/kg/hr were apparently shifted to the right compared to the respective curves for 10 mg/kg/hr (Fig. 4A). However, the total amount of morphine infused—the incidence of PCS increase and diarrhea curves were located in the same position irrespective of the morphine infusion rate (Fig. 4B). To elucidate these relationships, the ET50 and the EA50 were calculated (Table 2), and it was clarified that although the ET50s were inversely proportional to the infusion rates of morphine, the EA50s were constant for the PCS increase and incidence of diarrhea regardless of the morphine infusion rates.

There was a clear difference in the ET50s and the EA50s of PCS increase, diarrhea and body weight loss even in rats infused with morphine at the same rate (Table 2). According to the order of the ET50 and the EA50 in PCS increase, diarrhea and body weight loss in rats infused with 10 mg/kg/hr of morphine (Table 2), the rank order of sensitivity of naloxone-precipitated withdrawal signs was defined to be: PCS increase > diarrhea > body weight loss. This was consistent with our previous finding (11) that PCS increase was a sensitive indicator of morphine withdrawal.

It is thought that morphine withdrawal induced-diarrhea may result in body weight loss. However, in the present study, naloxone-induced diarrhea was observed without any body weight loss in rats infused with 2.5 or 5 mg/kg/hr of morphine (Table 2). This discrepancy may be due to the estimation method of these withdrawal signs; i.e., the body weight loss was calculated according to the body weight change during 2.5 hr (from 1 hr before naloxone to 1.5 hr after naloxone) and the presence of diarrhea was determined within 1.5 hr after naloxone. However, the degree of body weight loss may be related to the amount of body water lost by diarrhea that is dependent on the duration of diarrhea.

As mentioned above, the incidence of naloxone-precipitated withdrawal was correlated to the total amount of morphine infused irrespective of the morphine...
infusion rate, and it was suggested that the total amount of morphine infused may be involved in the development of the acute physical dependence on morphine within 8 hr of morphine infusion. Nakaki et al. (17) also demonstrated a correlation between the maximum % loss of body weight and the total amount of morphine infused intravenously in rats given 2 and 4 mg/kg/hr of morphine for 24 and 48 hr. The total amount of morphine infused to elicit naloxone-induced body weight loss could be calculated as 48 – 192 mg/kg for perfusion rates of 2 and 4 mg/kg/hr of morphine for 24 and 48 hr. In the present study, naloxone-induced body weight loss was elicited in rats infused with 10 mg/kg/hr of morphine for 4 hr and the EA50 was 84 mg/kg (95% confidence limits: 37 – 190 mg/kg), being consistent with the previous report (17). These results led us to presume that morphine dependence can be induced even by a single administration of morphine if the dose is high enough. In fact, it was reported that morphine withdrawal was induced by naloxone after treatment with a single large dose of morphine in mice (4), rats (5) and humans (6).

Clinically, in critically ill infants and children, continuous i.v. infusions of opiate narcotics are used to provide a constant level of analgesia and sedation (18–20). Katz et al. (20) evaluated prospectively the occurrence of spontaneous withdrawal syndrome in critically ill children who received fentanyl by continuous i.v. infusion for 3 days and concluded that the occurrence of withdrawal was related to the total amount of fentanyl received. These and our results suggest that the total amount of opiate administered determines the intensity of physical dependence on the opiate, which develops within 3 days by intravenous infusion and can be assessed by opioid antagonist-precipitated and spontaneous withdrawal.

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