Quantitative Properties of Plasma Corticosterone Elevation Induced by Naloxone-Precipitated Withdrawal in Morphine-Dependent Rats

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ABSTRACT—Elevation of plasma corticosterone (PCS) has been used as an indicator of morphine withdrawal, but it is not clear whether the magnitude of elevation is related to the intensity of the dependence. The dose-dependent effects of naloxone on PCS and body weight were studied in male Sprague-Dawley rats rendered physically dependent on morphine by injecting increasing doses of 40–120 mg/kg/day, s.c. twice daily for 1–6 days. Naloxone (0.01–2.0 mg/kg, s.c.) was administered 3 hr after the last morphine administration. Naloxone elevated PCS levels in a dose-dependent manner in all groups treated with morphine, and the elevation was correlated with the number of days of morphine treatment. Naloxone also reduced dose-dependently the body weight in all groups treated with morphine; in this case, a reverse correlation was obtained between the body weight changes and the PCS levels. It was confirmed that PCS elevation is a quantitative sign of naloxone-precipitated morphine withdrawal and that the elevation is indicative of the degree of morphine physical dependence.

Keywords: Morphine dependence, Plasma corticosterone, Body weight loss, Naloxone withdrawal

Plasma corticosterone (PCS) has been shown to be elevated during both spontaneous and naloxone-precipitated withdrawal from acute and chronic morphine exposure, and the elevation of PCS has been used as a measure of morphine dependence (1–4). Interestingly, the elevation of PCS is also induced by acute morphine administration (1, 5) and the development of tolerance to this hormone effect follows chronic exposure to morphine (6–8).

Intrahypothalamic and intraventricular morphine injections elevate PCS (9, 10), and the increase in PCS induced by acute morphine administered systemically is blocked by hypophysectomy or lesions of the median eminence (11, 12). Responses of PCS to exogenous corticotropin releasing factor (CRF) and adrenocorticotropic hormone (ACTH) are not attenuated in morphine-tolerant rats, showing that an attenuated PCS response to morphine, i.e., tolerance to the PCS effect, does not occur at the pituitary and adrenal levels (8). Secretion of CRF is stimulated during the periods of both acute morphine administration (13) and morphine withdrawal (14). These results suggest that the stimulation of PCS secretion and the development of tolerance to the PCS effect of morphine both occurred in the region of the central nervous system involved in the neural control of the hypothalamo-pituitary-adrenal (HPA) axis. Additionally, the effect of morphine on PCS is mediated selectively through mu opioid receptor, which in turn is involved in the development of tolerance to the PCS effect of morphine and morphine withdrawal (8, 15).

Accordingly, if the increase in PCS is a quantitative sign of morphine withdrawal, the understanding of the neural mechanism of PCS elevation during morphine withdrawal would contribute to the elucidation of the underlying neural mechanism of morphine tolerance and/or dependence. However, little is known about whether the increase in PCS is indicative of the severity of morphine dependence.

On the other hand, the body weight loss has been known as a reliable and quantitative indicator of morphine withdrawal, being indicated to correlate quantitatively to the dose of naloxone used to induce body weight loss in morphine-dependent rats (16).

To clarify whether the magnitude of the PCS elevation is related to the period of morphine treatment and to the dose of naloxone used to precipitate the response, we studied the effects of various doses of naloxone on PCS levels in rats treated with morphine for 1–6 days and compared the effects with those on body weight.
MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Clea, Tokyo), weighing 300–380 g, were housed two per wire cage in a room with controlled temperature (23 – 24°C), humidity (60 – 70%), and light cycle (7:00 – 19:00) during the experiments. Food (MF, Clea) and water were available ad libitum.

Induction of morphine dependence and withdrawal
Rats were made dependent on opioid by twice daily (10:00 and 16:00) s.c. injections of morphine for 1–6 days. Doses of morphine were progressively increased every second day during 6 days, i.e., 20 mg/kg × 2/day (day 1–2), 40 mg/kg × 2/day (day 3–4) and 60 mg/kg × 2/day (day 5–6). The same dose of morphine as the last of the repeated doses was injected additionally on the next day (6:00) as the final administration of morphine. Morphine withdrawal was induced by various doses of naloxone (0.01–2.0 mg/kg, s.c.) administered at 9:00, that is, 3 hr after the final morphine injection.

Body weight was measured just before the final morphine injection and 1 hr after naloxone administration. The naloxone-induced change (%) of body weight was calculated as follows:

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\text{[(body weight at final morphine – body weight 1 hr after naloxone)/body weight at final morphine}] \times 100
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Blood samples for PCS assay were obtained 1 hr after naloxone by cardiopuncture (1–2 min after 600–900 mg/kg, i.p. of pentobarbital) and were centrifuged at 2000 x g for 15 min at 4°C. The plasma was stored at −20°C until the fluorometric assay of PCS according to the method of Zenker and Bernstein (17).

Drugs
Drugs used were morphine hydrochloride (Takeda Chemical Industries, Ltd., Osaka); naloxone hydrochloride (Endo Laboratories, Inc., Garden City, NY, USA); sodium pentobarbital (Somnopentyl; Pitman Moore, Inc., Washington Crossing, NJ, USA); heparin sodium (heparin sodium inj.; Fuso Pharmaceutical Industries, Ltd., Osaka); chloroform (special grade), sulfuric acid, sodium hydroxide (Katayama Chemical, Osaka); and ethanol (special grade; Wako Pure Chemical Industries, Ltd., Osaka). Morphine and naloxone were dissolved in saline; the solutions were freshly prepared immediately before use. The injection volume for s.c.-administration was 0.2 ml/100 g body weight. Doses of drugs were given in terms of their salts.

Statistical analyses
All values represent the mean ± S.E.M. Data were analyzed by a one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. Regression line was calculated by the least squares method.

RESULTS

Plasma corticosterone increase induced by naloxone
In rats treated with saline for 1–6 days, PCS after saline and naloxone (2 mg/kg) injection were 3.3 – 6.4 μg/dl and 3.9 – 7.3 μg/dl, respectively; and 2 mg/kg of naloxone did not affect PCS in morphine naive rats (Fig. 1A).

PCS levels after saline in rats treated with morphine for 1–6 days (4 hr after final morphine, 1 hr after saline) were similar to those in rats treated with saline for 1–6 days (Fig. 1A). In rats treated with morphine for 1 day, PCS was not increased by 0.01–0.1 mg/kg of naloxone, but an increase was induced by 1.0–2.0 mg/kg of naloxone. In rats treated with morphine for 2–6 days, PCS was not increased by 0.01 mg/kg of naloxone, but an increase was induced by 0.1–2.0 mg/kg of naloxone. These results indicate that the naloxone-induced increase in PCS was dose-dependent in all groups treated with morphine (Fig. 1A).

The dose of 0.1 mg/kg of naloxone did not increase PCS in rats treated with morphine for 1 day, but increased PCS in rats treated with morphine for 2–6 days (Fig. 1A); that is, the dose of naloxone needed to elicit PCS elevation in rats treated with morphine for 2–6 days was smaller than that in rats treated with morphine for 1 day.

The increase in PCS induced by 0.1 mg/kg of naloxone was correlated with the days of morphine treatment (Fig. 1B), and this indicates that the naloxone-induced increase in PCS was dependent on the length of morphine treatment.

Body weight changes induced by naloxone
The changes of body weight induced by naloxone (0.01 – 2.0 mg/kg) in rats treated with saline or morphine for 1–6 days are shown in Fig. 2A. In rats treated with saline for 1–6 days, 0.62–1.25% body weight loss was observed after saline and 0.11–2.25% loss after naloxone (2.0 mg/kg), with no appreciable effect of naloxone on body weight.

In rats treated with morphine for 1–6 days, an increase in body weight was observed after saline injection, and the magnitude of the increase was correlated with the days of morphine treatment (Fig. 2, A and B). In all groups treated with morphine for 1–6 days, the increase in body weight was suppressed by a small dose of naloxone (0.01 mg/kg), with a further decrease of body weight gain turning to body weight loss as the dose of naloxone (0.1–2.0 mg/kg) was increased; i.e., naloxone reduced the body weight of rats treated with morphine for
Fig. 1. Changes in plasma corticosterone levels induced by various doses of naloxone in rats treated with saline or morphine for 1–6 days. A: Plasma corticosterone and the dose of naloxone at each day of morphine treatment. Open and hatched columns represent repeated treatment with saline and morphine, respectively. B: Plasma corticosterone and the days of morphine treatment for each dose of naloxone. Dashed lines indicate the mean plasma corticosterone levels in morphine naive and saline-injected rats. The regression line drawn in the figure indicates a significant correlation, when calculated by the least squares method. Abbreviations: 1 DAY, morphine or saline treatment for 1 day; 2 DAYS, morphine or saline treatment for 2 days; 3 DAYS, morphine or saline treatment for 3 days; 5–6 DAYS, morphine or saline treatment for 5–6 days; SAL, saline injection 1 hr before sacrifice; NLX, naloxone injection 1 hr before sacrifice; r, coefficients of correlation. Numbers in parentheses are the number of rats. Numbers under "NLX" indicate the dose of naloxone (mg/kg, s.c.). All values are presented as the mean; vertical bars indicate S.E.M. Differs from repeated saline + SAL (Newman-Keuls test), **P<0.01, *P<0.05. Differs from repeated morphine + SAL (Newman-Keuls test), ††P<0.01, †P<0.05.
Fig. 2. Percent changes in body weight induced by various doses of naloxone in rats treated with saline or morphine for 1–6 days. A: Body weight change and the dose of naloxone at each day of morphine treatment. Open and hatched columns represent repeated treatment with saline and morphine, respectively. B: Body weight change and the days of morphine treatment for each dose of naloxone. Dashed lines indicate the mean body weight change (%) during 4 hr in morphine naive and saline-injected rats. The regression line drawn in the figures indicates a significant correlation, when calculated by the least squares method. Abbreviations: 1 DAY, morphine or saline treatment for 1 day; 2 DAYS, morphine or saline treatment for 2 days; 3 DAYS, morphine or saline treatment for 3 days; 5–6 DAYS, morphine or saline treatment for 5–6 days; SAL, saline injection 1 hr before sacrifice; NLX, naloxone injection 1 hr before sacrifice; r, coefficients of correlation. Numbers in parentheses are the number of rats. Numbers under "NLX" indicate the dose of naloxone (mg/kg, s.c.). All values are presented as the mean; vertical bars indicate S.E.M. Differs from repeated saline + SAL (Newman-Keuls test), **P<0.01, *P<0.05. Differs from repeated morphine + SAL (Newman-Keuls test), ††P<0.01, †P<0.05.
1-6 days in a dose dependent manner (Fig. 2A), but this was not dependent on the number of days of morphine treatment (Fig. 2B).

Correlation between body weight changes and plasma corticosterone levels

The correlation between body weight change and PCS level in rats treated with morphine for 1-6 days is shown in Fig. 3. A negative correlation was obtained in all groups with higher coefficients of correlation in the rats treated with morphine for 3 and 5-6 days.

DISCUSSION

Physical dependence on morphine is indicated by specific signs after the injection of an opioid antagonist or cessation of morphine administration; i.e., morphine withdrawal signs. Quantitative withdrawal responses are used for the quantitative assessment of morphine dependence. The hyperactivity of the HPA axis during morphine withdrawal has been shown to be a reliable indicator of withdrawal in experimental animals (1-4). However, few studies have systematically examined whether the hyperactivity of HPA is a quantitative withdrawal response indicative of the degree of dependence. In this respect, it has been reported that the magnitude of PCS elevation induced by naloxone is correlated to the priming dose of morphine or to the dose of naloxone used to precipitate the response in rats exposed to a single dose of morphine (3).

In the present study, we estimated the PCS levels after various doses of naloxone in rats treated with morphine for 1-6 days and compared them with body weight change as a quantitative morphine withdrawal response (16).

The highest dose of naloxone used in these experiments (2.0 mg/kg) did not show any effect on PCS in rats treated with saline for 1-6 days (Fig. 1A), nor were the PCS levels after saline in rats treated with morphine for 1-6 days significantly different from those in the respective
control saline-treated rats. The lowest dose of naloxone used in this experiment (0.01 mg/kg) did not affect PCS levels in rats treated with morphine for 1–6 days; and although 0.1 mg/kg of naloxone did not increase PCS in rats treated with morphine for 1 day, this dose did significantly increase PCS in rats treated with morphine for more than 2 days (Fig. 1A). Moreover, the magnitude of increase in PCS induced by this dose of naloxone was significantly correlated with the number of days of morphine treatment (Fig. 1B). At more than 1 mg/kg, naloxone elevated PCS in all rats treated with morphine, even those for 1 day; and the increase in PCS was not dependent on the number of days of morphine treatment. This may indicate that the maximal response of PCS to naloxone-precipitated morphine withdrawal has been induced already in rats treated with morphine for 1 day by 1.0–2.0 mg/kg of naloxone. These results indicate that 1) Naloxone-induced elevation of PCS was dose-dependent in all groups treated with morphine for 1–6 days, 2) The magnitude of PCS elevation induced by naloxone at 0.1 mg/kg was dependent on the number of days of morphine treatment, and 3) The longer the treatment with morphine, the smaller was the dose of naloxone needed to elicit PCS elevation. The selected signs of naloxone-precipitated withdrawal syndrome had been reported to be dose-dependent and related to the period of morphine exposure, and the sensitivity to naloxone has been used as a measure of the degree of physical dependence on morphine (16, 18, 19).

As shown in Figs. 2A and B, there was a slight decrease in body weight after saline injection to rats treated with saline for 1–6 days, whereas body weight after saline injection to rats treated with morphine for 1–6 days increased as estimated by the change of body weight during 4 hr from the last morphine injection (6:00) to 1 hr after saline (9:00). It is well known that body weight loss is induced by spontaneous morphine withdrawal (20) and morphine was injected twice (10:00, 16:00) daily in the present study. Therefore, it is possible to assume that the increase in body weight after saline in morphine-treated rats might be the result of recovery from morphine withdrawal-induced body weight loss after final morphine injection at 6:00. However, the change of body weight from 16:00 to 10:00 of the next day during morphine treatment was −2.9–4.0%, and body weight loss was not dependent on the number of days of morphine treatment. That is, body weight loss due to cessation of morphine administration was not induced in the schedule of morphine administration employed in the present study, and it is unlikely that the increase in body weight in morphine-treated rats is the result of recovery from morphine withdrawal-induced body weight loss after injection of morphine at 6:00. Recently it was reported that a single dose of morphine evoked a dose-dependent change in feeding (21, 22): a brief (1 hr) anorexia was followed by hyperphagia (3 hr). Therefore, the body weight increases after saline in the rats treated with morphine for 1–6 days might be the result of the hyperphagic effect of morphine. Furthermore, these increases in body weight were correlated with the number of days of morphine treatment (Fig. 2B). The last dose of morphine treatment for both 1 and 2 days was the same, but the increase in body weight of rats treated with morphine for 2 days was greater than that of rats treated with morphine for 1 day; i.e., the body weight change in rats treated with morphine for 2 days was significantly different from that in the corresponding saline-treated rats, but treatment for 1 day induced no significant change (Fig. 2A). Although the doses of morphine were increased gradually from 3 days onward, the increase in body weight of rats treated with morphine for 3–6 days was more prominent than that of rats treated for 2 days. This result suggests the development of a reversed tolerance to the hyperphagic effect of morphine as has been observed for the stimulating effect of morphine on locomotor activity (23). The body weight of rats treated with morphine was reduced by naloxone (0.01–2.0 mg/kg) in a dose-dependent manner (Fig. 2A), but the loss was not dependent on the number of days of morphine treatment (Fig. 2B). In the present experiment, body weight was assessed at the final morphine administration and 1 hr after naloxone was injected 3 hr after the final morphine administration to avoid PCS elevation due to handling for body weight measurement, and naloxone-induced body weight change was estimated by the difference between the body weight at the final morphine treatment and that at 1 hr after naloxone; that is, body weight was measured 1 hr after naloxone under pentobarbital anesthesia, followed by blood sampling. However, an increase in body weight was observed after the final morphine administration followed by saline, and the change was dependent on the number of days of morphine treatment (Fig. 2B). This morphine-induced increase in body weight was not contained in the estimation of naloxone-induced body weight change, and this might be the reason why naloxone-induced body weight loss was not dependent on the number of days of morphine treatment.

As mentioned above, the body weight loss has been used as a quantitative morphine withdrawal sign but was not dependent on the number of days of morphine treatment in the present experiment. The increase in PCS was dependent on the number of days of morphine treatment only in the withdrawal precipitated by 0.1 mg/kg of naloxone. However, the naloxone-induced changes of PCS and body weight were dose-dependent in all groups treated with morphine (Figs. 1A and 2A), and negative correlations were observed between the body weight
changes and PCS levels in all groups treated with morphine (Fig. 3). These results indicate that both the body weight loss and the increase in PCS induced by naloxone-precipitated morphine withdrawal are similarly quantitative.

In conclusion, we have clarified, at least in rats rendered morphine-dependent within 1–6 days, that the naloxone-induced PCS increase is a reliable and quantitative morphine withdrawal sign, being indicative of the severity of morphine dependence. Furthermore, naloxone-induced PCS increases correlate with the naloxone-induced body weight change that has been used as a reliable and quantitative morphine withdrawal sign.

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