Original Article

DOI: 10.5582/ddt.2011.v5.2.71

Availability of serum corticosterone level for quantitative evaluation of morphine withdrawal in mice

Keiko Ueno[1], Takehiko Maeda[1], Norikazu Kiguchi[1], Yuka Kobayashi[1], Masanobu Ozaki[2], Shiroh Kishioka[1,*]

[1] *Department of Pharmacology, Wakayama Medical University, Wakayama, Japan;*
[2] *Department of Toxicology, Niigata University of Pharmacy and Applied Life Science, Niigata, Japan.*

ABSTRACT: Physical dependence on morphine is evidenced by the withdrawal syndromes, including body weight loss, which are induced by the discontinuation of morphine exposure or by the treatment with naloxone, an opioid receptor antagonist. The present study was designed to examine whether the elevation of serum corticosterone (SCS) level induced by naloxone-precipitated morphine withdrawal was a useful index to quantify the physical dependence on morphine in mice, which was compared with body weight loss induced by naloxone-precipitated morphine withdrawal. The SCS level was dependent on the dosage and the number of dosing of morphine and challenging dosage of naloxone. Intraplantar injection of formalin, potentially producing inflammatory pain, inhibited both body weight loss and SCS increase induced by naloxone challenge in mice receiving repeated exposure of morphine, indicating that formalin-induced pain attenuated the development of physical dependence on morphine. The magnitude of body weight loss in morphine withdrawal was significantly correlated with the magnitude of naloxone challenge-induced SCS increase. These results suggest that the naloxone-induced increase in SCS level is a quantitative index of the magnitude of physical dependence on morphine in mice.

Keywords: Body weight loss, corticosterone, inflammatory pain, morphine withdrawal, physical dependence

**Address correspondence to:*
Dr. Shiroh Kishioka, Department of Pharmacology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 6410012, Japan.
e-mail: kishioka@wakayama-med.ac.jp

1. Introduction

Repeated intake of opiate develops its physical dependence and subsequently abrupt discontinuation of its intake or administration of opiate receptor antagonist produces withdrawal symptom in human. The magnitude of physical opiate dependence is positively correlated with the magnitude of opiate withdrawal. Morphine withdrawal symptoms in rodents have been evidenced by piloerection, salivation, diarrhea and body weight loss. However, there were not any more quantitative and objective indexes for morphine withdrawal than body weight loss. We have reported the elevated concentration of plasma corticosterone (CS) for useful index as physical dependence on morphine in rats, which is induced putatively by activation of hypothalamo-pituitary-adrenal (HPA) axis (*1*). Therefore an increment of CS level in blood induced by naloxone (μ-opiate receptor antagonist) in rodents repeatedly treated with morphine could be an objective index as physical dependence. The change in the level of CS might be compared to the magnitude of body weight loss which concurrently occurred in mice. Moreover, it is known that psychological dependence on morphine is inhibited by prior loading of persistent inflammatory pain elicited by intraplantar injection of formalin (*2*). Therefore, we examined the influence of pain induced by formalin, an inflammatory, pungent compound, on naloxone-induced body weight loss and increments of serum CS (SCS) in mice with morphine dependence.

2. Materials and Methods

2.1. *Animals*

Male ICR mice (20-22 g; SLC, Hamamatsu, Shizuoka, Japan) were housed five per cage in animal room with controlled temperature (23-24°C, 60% humidity) and light-dark cycle (on 8:00-20:00). Feed (Oriental, Tokyo, Japan) and water were available *ad libitum*. All experiments were conducted on the basis of Guiding Principles for Care and Use of Laboratory Animals

approved by the Japanese Pharmacological Society and the Guidelines for Animal Experiments in Wakayama Medical University.

2.2. *Drug administration*

All drugs were administered subcutaneously. Morphine hydrochloride (Takeda, Osaka, Japan) was injected to mice twice a day (10:00 and 16:00) for 3-7 consecutive days to develop morphine dependence. Naloxone (1 mg/kg; Sigma-Aldrich, MO, USA) was injected on the next day of the last morphine injection to induce morphine withdrawal. Twenty μL of 2% formalin (Wako, Tokyo, Japan) was injected into the planter surface of the mice hind paw 2 h before the first morphine injection. The thickness of the hind paw in mice after formalin injection was measured to evaluate the consecutive effect of formalin-induced inflammation. Morphine was injected to the formalin-treated mice twice a day for 6 days with the dose-escalating regimen; mice received 50 mg/kg morphine for the first two days, 100 mg/kg for the second two days, and 200 mg/kg for final two days. On day 7, mice received naloxone (1 mg/kg), followed by subsequent decapitation for collecting trunk blood 1 h after naloxone injection.

2.3. *Estimation of SCS level and body weight*

Because the level of SCS with circadian rhythm was most stable before noon in a day, the blood was collected for SCS determination at 10:00 AM. Mice were killed by decapitation and trunk blood was collected 30 min or 1 h after naloxone injection. The serum was separated by centrifugation and stored at –20°C until the fluorometric assay of SCS level according to the method of Zenkar and Bernstein (*3*). Change in body weight (BW) was defined as follows: % change of BW = [(BW at 1 h after naloxone injection) – (BW before naloxone injection)]/(BW before naloxone injection) × 100.

2.4. *Statistical analysis*

All values represent the mean ± S.E.M. Statistical significance was assessed using one-way ANOVA for multiple group comparisons, followed by Dunnett's multiple comparison test (Figures 1, 3, and 4) or Tukey multiple comparison test (Figures 2, 4, and 5). A correlation between SCS level and % change of BW was estimated, and the regression line was calculated by the least squares method (Figure 6). Statistically significant difference was set at $p < 0.05$.

3. Results

3.1. *SCS level in morphine withdrawal*

The levels of SCS were measured in mice with morphine dependence followed by naloxone challenge (Figure 1). Morphine (10, 20, or 40 mg/kg) was injected twice a day for consecutive 4 days, followed by naloxone challenge (5 mg/kg) on day 5. Naloxone challenge induced the increments of the SCS level in a dose-dependent manner of repeatedly administered morphine. The SCS level was significantly larger in mice treated with 40 mg/kg morphine than in mice treated with saline. We examined the influence of repeated dosing period of morphine (10 mg/kg) on naloxone-induced elevation of SCS (Figure 2). Naloxone challenge on the day after final administration of morphine increased the SCS level in a manner dependent on dosing period of morphine. The SCS levels after naloxone challenge were significantly higher in mice treated with morphine for 5 and 7 days than in mice treated with saline. However, saline challenge, a single injection of saline, did not induce significant increases in SCS level in repeatedly treated mice with 10 mg/kg

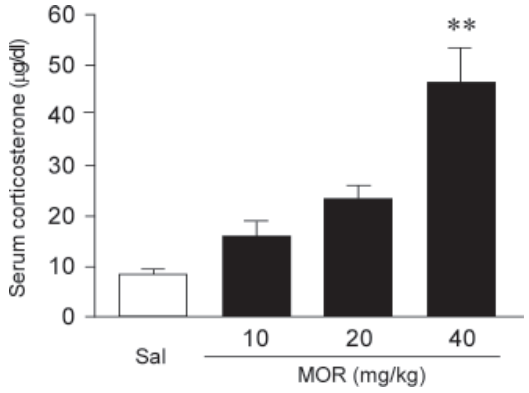

Figure 1. Naloxone-induced increase in serum corticosterone level in mice repeatedly treated with morphine. Morphine (MOR) was administered twice a day for 4 days. On day 5, naloxone (5 mg/kg) or saline (Sal) was injected and 30 min later, the trunk blood was collected. Each column represents the mean and vertical bar indicates the S.E.M. of 4-6 mice. *vs.* Sal, ** $p < 0.01$.

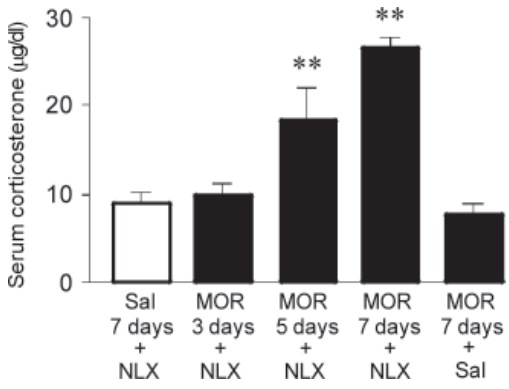

Figure 2. Naloxone-induced increase in serum corticosterone level depends on the period of days of morphine treatment. Morphine 10 mg/kg (MOR) or saline (Sal) was administered twice a day for consecutive 3, 5, or 7 days. On the day after final administration of morphine, naloxone 5 mg/kg (NLX) or Sal was administered. Each column represents the mean and vertical bar indicates the S.E.M. of 6 mice. *vs.* Sal, ** $p < 0.01$.

morphine twice a day for 7 days. Then, the relationship between doses of naloxone challenge and elevation of SCS levels was tested (Figure 3). Morphine 40 mg/kg was administered twice a day for 4 days, followed by a single administration of naloxone on day 5. Naloxone challenge raised the SCS level in a manner dependent on naloxone doses, of which 0.1-10 mg/kg produced significant increases in the SCS level, compared to saline challenge.

3.2. *Influence of pain on morphine withdrawal*

To test whether inflammatory pain has effects on the development of physical dependence on morphine, the influence of formalin-induced pain on naloxone-induced SCS elevation and body weight loss in morphine dependence was examined (Figure 4). We injected saline to the plantar surface of hind paw in mice, resulting in significant, but transient slight increase in paw thickness, compared to that before its injection; the increased thickness was returned to that before the saline injection 1 day later. Intraplantar injection of formalin induced remarkable increases in paw thickness, which reached maximum level 120 min later and lasted for at least 4 days. This result indicates that the formalin injection produces inflammatory edema, most likely in association with inflammatory pain in the affected plantar surface of hind paw in mice.

Then, we examined effects of intraplantar injection of formalin on morphine withdrawal (Figure 5). Mice

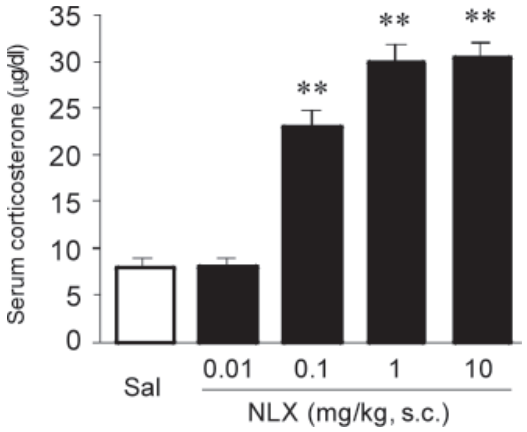

Figure 3. Effects of several doses of naloxone (NLX) on serum corticosterone level in mice repeatedly treated with morphine. Morphine 40 mg/kg was administered twice a day for 4 days. On day 5, NLX (0.01-10 mg/kg) or saline (Sal) was injected, and 30 min later blood was collected. Each column represents the mean and vertical bar indicates the S.E.M. of 6 mice. *vs.* Sal, ** $p < 0.01$.

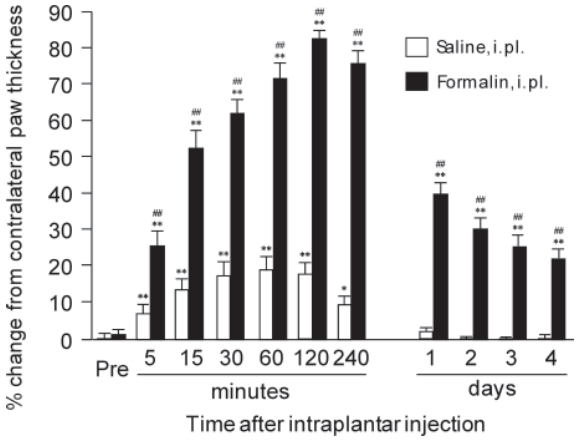

Figure 4. Effects of intraplantar injection (i.pl.) of formalin (2%, 20 μL) on the thickness of the hind paw in mice. Each column represents the mean and vertical bar indicates the S.E.M. of 4-5 mice. *vs.* pre-injection (Pre), * $p < 0.05$, ** $p < 0.01$. *vs.* saline i.pl., ## $p < 0.01$.

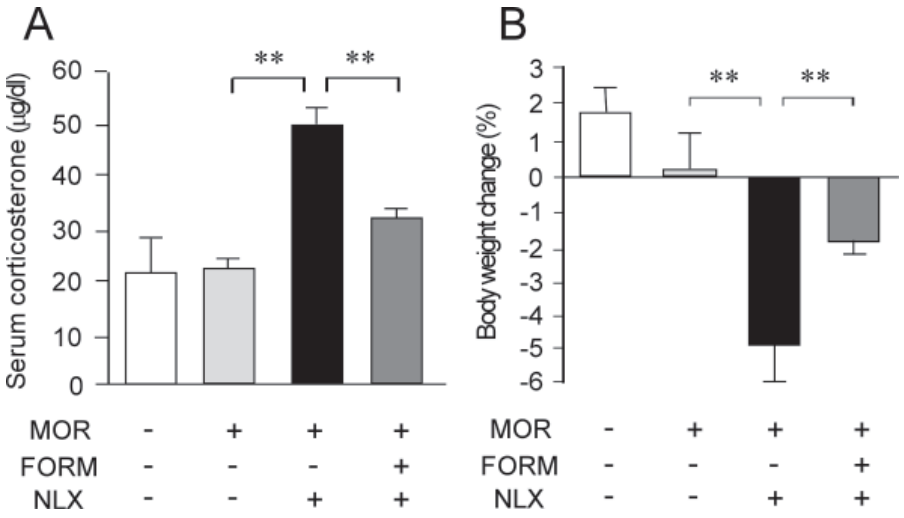

Figure 5. Formalin (FORM) attenuates naloxone (NLX)-precipitated withdrawal of morphine (MOR) dependence. Mice were treated with MOR or saline (Sal), represented by "-", for 6 days (50, 100, and 200 mg/kg, twice a day for each 2 days), followed by NLX (5 mg/kg) or Sal challenge on the next day of final treatment with MOR. A single intraplantar injection of FORM or Sal was given 120 min before the first administration of MOR. **A**: Effects of FORM on NLX-induced elevation of serum level of corticosterone in MOR dependence. **B**: Effects of FORM on NLX-induced change in body weight in MOR dependence. Each column represents the mean and vertical bar indicates the S.E.M. of 4-5 mice. ** $p < 0.01$.

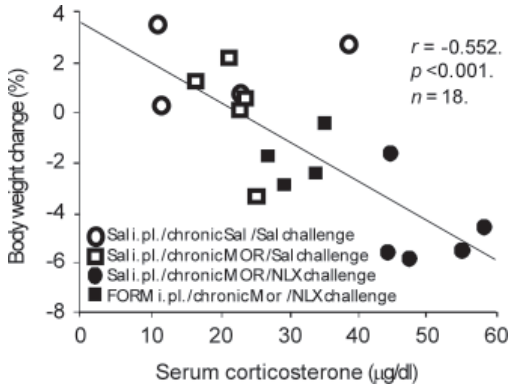

Figure 6. Correlation between naloxone (NLX)-induced increase in serum corticosterone level and body weight loss. Each plot is derived from the data on individual mice treated with morphine (MOR) shown in Figure 5. *r*, Pearson's correlation coefficient. The regression lines were calculated by the least squares method. The Pearson's correlation coefficient was significantly different from zero ($p < 0.001$).

received a single intraplantar injection of formalin or saline. Two hours later, morphine was administered twice a day for 6 days with dose-escalating regimen, as described in "Materials and Methods", followed by 5 mg/kg naloxone challenge on day 7. In mice with preemptive intraplantar injection of saline, naloxone challenge after repeated administration of morphine induced significant increases in SCS levels, compared to saline challenge (Figure 5A). The naloxone-induced increase in SCS level was significantly attenuated by the preemptive injection of formalin. Naloxone challenge after repeated administration of morphine also induced body weight loss in formalin-naïve mice; % change in body weight was significantly different between in saline challenge group and naloxone challenge group with preemptive intraplantar injection of saline followed by repeated administration of morphine (Figure 5B). The preemptive intraplantar injection of formalin significantly attenuated body weight loss induced by naloxone challenge after repeated administration of morphine. To test the correlation between the elevation of SCS level and body weight loss by naloxone-induced morphine withdrawal, we utilized data in all examined individuals in Figure 5 to make graph relating SCS levels to the change in body weight (Figure 6). There was significant correlation between SCS levels and % change in body weight.

4. Discussion

The increases in SCS level precipitated by morphine withdrawal have been reported already in rodents (*1*,*4*). However, it has not been reported that the degree of SCS elevation could be a quantitative index as magnitude of morphine withdrawal in mice. In mice, we therefore examined the relation between the naloxone-induced SCS elevation and dosing conditions of morphine and naloxone. The naloxone-induced SCS elevations were dependent on the regimen of morphine treatment (Figures 1 and 2). Moreover, the degree of increase in SCS level was dependent on the dose of naloxone (Figure 3) and thus, was regarded as an index to reflect the magnitude of morphine withdrawal. The magnitude of morphine withdrawal symptoms correlates with the degree of development of physical dependence (*5*,*6*). Therefore, it is suggested that degree of naloxone-induced SCS elevation correlates with the degree of development of physical dependence.

It is thought that body weight loss in morphine withdrawal is elicited by hyper tonicity of sympathetic nervous system, diarrhea, slobber and breakdown activity of white adipose cell (*7*). It has been reported that diltiazem, a calcium antagonist, inhibits body weight loss in morphine withdrawal (*8*), though the detail of mechanism has not been clear. It is well known that the increases in SCS level induced by morphine withdrawal are due to activity in HPA axis function (*9*,*10*). Morphine withdrawal activates A2 cell (adrenergic nerve axis) in nuclei of solitary tract, which stimulates adrenergic receptor in paraventricular nucleus to release corticotropin-releasing hormone (CRH). CRH acts at the pituitary gland in the way of humoral transmission to release adrenocorticotrophic hormone (ACTH), which in turn releases corticosteroid from the adrenal cortex (*11*). This conception accords with the reports that adrenergic blocking agent inhibits ACTH secretion induced by morphine withdrawal (*12*). Moreover, the transcription factor cAMP response element binding protein (CREB), which has been implicated in the actions of drugs of abuse, is reportedly phosphorylated in the nucleus tractus solitarius (NTS)-A2 catecholaminergic cell group, one of the key regions of the brain stress system, in morphine withdrawal (*11*). Thus, the mechanism as discussed above most likely works in SCS elevation observed in the present study.

We used body weight loss with increase in SCS level by naloxone-induced morphine withdrawal as a marker for morphine withdrawal to examine influence of formalin-induced pain on development of physical dependence on morphine. When formalin was injected into the plantar surface of the hind paw of mice, the remarkable increase in paw thickness lasted more than 4 days (Figure 4). It is reported that hyperalgesia elicited by the formalin-induced increase in rat paw thickness lasts for 9 days (*13*). In consequence, it is inferred that formalin-induced pain lasted for at least 6 days during repeatedly morphine treatment in the present study. When naloxone was administered to mice repeatedly treated with morphine without exposure of the pain stimulation, both the increase in SCS level and the body weight loss were significantly precipitated by naloxone. Formalin-induced inflammatory edema attenuated significantly both the naloxone-induced increase in SCS level and bodyweight loss in mice

(Figure 5). Therefore, it is suggested that pain loaded beforehand prevents repeated morphine treatment for producing physical dependence. This may support clinical evidence that patients are difficult to lapse into abuse, when narcotic analgesics are prescribed to them for chronic pain relief. In addition, we found the significantly negative correlation between the body weight and the level of SCS (Figure 6). These results suggest that a degree of increase in SCS level in morphine withdrawal is a quantitative index of magnitude of morphine withdrawal (*14*). It is known that pain-induced activation of the endogenous kappa opioid nervous system in rewarding system inhibits development of morphine psychological dependence, as revealed by conditioned place preference tests (*2*). In this context, it is reported that administrations of combination of a kappa opioid agonist with morphine inhibit development of morphine physical dependence (*15,16*). Considering this fact, the activation of endogenous kappa opioid by formalin-induced pain might inhibit the development of morphine physical dependence.

In conclusion, we demonstrated that the SCS level may be a quantitative and objective index of morphine withdrawal in mice repeatedly treated with morphine. Furthermore those indexes revealed that preexisting inflammatory pain attenuates the development of morphine physical dependence. Transgenic animal technology and achievement in mice is accumulated more in comparison with other laboratory animals such as rats. It is important that the present study revealed that naloxone-induced SCS elevation in mice repeatedly treated with morphine is allowed as index of degree of development of morphine physical dependence, because the quantitative evaluation is necessary for elucidation of molecular mechanisms underlying development of morphine dependence.

Acknowledgements

This work was supported by MEXT KAKENHI (20591376).

References

1. Kishioka S, Nishida S, Fukunaga Y, Yamamoto H. Quantitative properties of plasma corticosterone elevation induced by naloxone-precipitated withdrawal in morphine-dependent rats. Jpn J Pharmacol. 1994; 66:257-263.
2. Suzuki T, Kishimoto Y, Misawa M, Nagase H, Takeda F. The role of the kappa-opioid system in the attenuation of the morphine-induced place preference under chronic pain. Life Sci. 1999; 64:PL1-7.
3. Zenkar N, Bernstein DE. The estimation of small amount of corticosterone in rat plasma. J Biol Chem. 1958; 231:695-701.
4. Yamamoto H, Mikita S, Yano I, Masuda Y, Murano T. Studies on the physical dependence liability of analgesics. 2nd report: Relationship between transformation of intramitochondrial structures in adrenocortical cells and corticosterone biosynthesis in morphine addicted rats. Jpn J Pharmacol. 1973; 23:217-225.
5. Eisenberg RM. Further studies on the acute dependence produced by morphine in opiate naïve rats. Life Sci. 1982; 31:1531-1540.
6. Antonio Martínez J, Vargas ML, Fuente T, Del Rio Garcia J, Milanés MV. Plasma-β-endorphin and cortisol levels in morphine-tolerant rats and in naloxone-induced withdrawal. Eur J Pharmacol. 1990; 182:117-123.
7. Kovács GL, Telegdy G. Hypothalamo-neurohypophseal neuropeptides and experimental drug addiction. Brain Res Bull. 1988; 20:893-895.
8. Kishioka S, Inoue N, Nishida S, Fukunaga Y, Yamamoto H. Diltiazem inhibits naloxone-precipitated and spontaneous morphine withdrawal in rats. Eur J Pharmacol. 1996; 316:7-14.
9. Laorden ML, Castells MT, Milanés MV. Effects of morphine and morphine withdrawal on brainstem neurons innervating hypothalamic nuclei that control the pituitary-adrenocortical axis in rats. Br J Pharmacol. 2002; 136:67-75.
10. Nunez C, Földes A, Laorden ML, Milanes MV, Kovács KJ. Activation of stress-related hypothalamic neuropeptide gene expression during morphine withdrawal. J Neurochem. 2007; 101:1060-1071.
11. Martín F, Laorden ML, Milanés MV. Morphine withdrawal regulates phosphorylation of cAMP response element binding protein (CREB) through PKC in the nucleus tractus solitaries-A2 catecholaminergic neurons. J Neurochem. 2009; 110:1422-1432.
12. Laorden ML, Fuertes G, González-Cuello A, Milanés MV. Changes in catecholaminergic pathways innervating paraventricular nucleus and pituitary-adrenal axis response during morphine dependence: Implication of α1-and α2-adrenoceptors. J Pharmacol Exp Ther. 2000; 293:578-584.
13. Suzuki T, Kishimoto Y, Misawa M. Formalin- and carrageenan-induced inflammation attenuates place preferences produced by morphine, methamphetamine and cocaine. Life Sci. 1996; 59:1667-1674.
14. Akera T, Brody TM. The addiction cycle to narcotics in the rats and its relation to catecholamines. Biochem Pharmacol. 1968; 17:675-688.
15. Tao PL, Hwang CL, Chen CY. U-50,488 blocks the development of morphine tolerance and dependence at a very low dose in guinea pigs. Eur J Pharmacol. 1994; 256:281-286.
16. Tsuji M, Takeda H, Matsumiya T, Nagase H, Yamazaki M, Narita M, Suzuki T. A novel kappa-opioid receptor agonist, TRK-820, blocks the development of physical dependence on morphine in mice. Life Sci. 2000; 66: PL353-358.

(Received March 26, 2011; Accepted April 12, 2011)