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Pretreatment with midazolam suppresses morphine withdrawal response in mice and rats

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KEY WORDS midazolam; morphine; substance withdrawal syndrome; adenosine cyclic monophosphate; GABA; proto-oncogene proteins c-fos; spinal cord

ABSTRACT

AIM: To investigate the roles of pretreatment with midazolam on morphine withdrawal in mice and rats. **METHODS:** Acute and chronic morphine dependence and naloxone-precipitated withdrawal models were employed in the present study. Cyclic adenosine monophosphate (AMP) content and Fos protein expression were measured by radioimmunoassay and immunocytochemistry, respectively. **RESULTS:** Coadministration of midazolam (2 mg/kg, ip) and morphine prevented the development of both acute and chronic morphine dependence in mice. Compared to saline-morphine group (3.0, 95 % confidence limits: 1.9–4.3 mg/kg), ED_{50} of naloxone-precipiated withdrawal jumping increased significantly in midazolam-morphine group (10.4, 95 % confidence limits: 8.5–12.3 mg/kg) in acute morphine-dependent mice ($P<0.01$). Pretreatment with midazolam lowered the number and incidence of naloxone-precepiated withdrawal jumping and prevented loss in body weight in chronic morphine-dependent mice ($P<0.01$). Midazolam-pretreatment inhibited the increase of Fos protein expression, not cyclic AMP content, in rat spinal cord during morphine withdrawal. **CONCLUSION:** Midazolam suppresses morphine withdrawal response by inhibiting hypersensitization of the spinal cord neurons, and this effect may not be mediated by cAMP pathway.

INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central neuron system(CNS). Several lines of evidence have shown that GABAergic system plays an important role in the development of morphine-induced physical dependence[1,2]. Midazolam, a benzodiazepine-receptor agonist, has been widely used for inducing and maintaining anesthesia by co-administration with opioids or inhaled anesthetic in clinics. Midazolam can occupy the benzodiazepine receptor on

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a benzodiazepine-GABA-Cl^- channel complex and therefore facilitate the inhibitory action of GABA on neuronal transmission. Midazolam could prolong the antinociceptive effect of morphine by delaying the chronic morphine-induced development of tolerance to antinociception in rats[3]. Pretreatment of diazepam suppressed morphine withdrawal signs in rats[4]. However, the mechanisms remain unclear.

Most studies on GABAergic system involving in morphine withdrawal focused on supraspinal level like, ventral tegmental area (VTA), nucleus accumbens, striatum, locus coeruleus (LC) and so on. Our recent study has shown that hypersensitization of the spinal cord neurons participates in mediating morphine withdrawal response[5]. Large quantities of GABA receptor are located on the spinal dorsal cord. Intrathecally administration of midazolam not only potentiated opioids antinociception in the spinal cord, but also produced segmental antinociceptive effect, which were mediated by $GABA_A$-receptor in the spinal dorsal cord[6,7]. We speculated that midazolam could suppress morphine withdrawal response by inhibiting the spinal cord neurons hypersensitization. Many studies have demonstrated that compensatory up-regulation of cyclic AMP (cAMP) level and its overshoot increase could be the biochemical base of morphine dependence and naloxone-precipitated withdrawal[8]. The purpose of the present study was to investigate the roles of pretreatment with midazolam on morphine withdrawal response in mice and rats, and on cAMP level and Fos protein expression in rat spinal cord neurons during morphine withdrawal.

MATERIALS AND METHODS

Animal and drugs Kunming mice (20–24 g) and SD rats (250–300 g) were supplied by Experimental Animal Center of Xuzhou Medical College (Grade Ⅱ, Certificate № SUA95021). Midazolam was produced by Roche (Switzerland). Morphine and naloxone were respectively obtained from Qinghai and Sihuan Pharmaceutical Factory, China. Cyclic AMP CPBA Kit was produced by China Atomic Energy Institute. Fos antibody, ABC-HRP, and DAB were from Vector (USA). All drugs were dissolved in physiology saline. Other chemicals were of analytical grade.

Acute and chronic morphine dependence test in mice In acute dependence test, the degree of morphine physical dependence was assessed by estimating the amount of naloxone required to induce withdrawal jumping. It has been shown that there is an inverse relationship between the degree of dependence and the amount of naloxone required to precipitate withdrawal jumping in mice. In order to set up acute morphine dependence model, mice were injected with a single large dose of morphine (100 mg/kg, sc) 30 min after pretreatment with saline or midazolam (2 mg/kg, ip)[9]. Up and down method was designed in acute dependence test. In brief, 4 h after injection of morphine, a mouse was injected with one of several doses of naloxone, which were assigned according to an equal ratio grade. The dose of naloxone for next mouse was determined by the result of the previous one. The criterion for positive jump response was that the mouse jumped more than 4 times during the first 15 min after injection of naloxone. The ED_{50} of naloxone-precipitated withdrawal jumping was obtained by Dixon-mood method[10].

In chronic dependence test, 30 min after pretreatment with saline or midazolam (2 mg/kg, ip) every day, mice were injected with either saline or morphine (10, 20, 50 mg/kg, sc) for each of the three consecutive days, d 1, 2, and 3, respectively. On d 4, all mice were injected with an identical dose of naloxone (5 mg/kg, sc). We observed the number, the incidence of withdrawal jumping during the first 30 min and loss in body weight within 1 h after injection of naloxone.

Motor function assessment Motor function was evaluated by observation of placing/stepping reflexes and righting reflexes following 15 min after pretreatment with midazolam in mice.

Chronic morphine dependence in rats The following procedures were taken to set up a chronic morphine dependence model. Rats were subcutaneously injected with morphine 30 min after pretreatment with ip midazolam 3 mg/kg (bid, for 5d). The dose of morhine was 10 mg/kg in d 1, and increased by 10 mg/

kg each day. On d 6, 4 h after injection of morphine 50 mg/kg, morphine withdrawal syndrome was precipitated by administration of naloxone (4 mg/kg, ip). An equal volume of saline was injected in control group. Within 1 h after naloxone-precipitated withdrawal, scores of morphine withdrawal response were obtained based on the following morphine withdrawal syndrome: wet dog shaking, teeth chatting, abnormal position, irritability, weight lose, and autonomic nerves symptoms and so on.

Radioimmunoassay for cAMP Ten minutes before and 30 min after injection of naloxone, rats were killed and the spinal cord was dissected at the level of thoracolumbar. The tissues were frozen immediately in liquid nitrogen after dissection and kept in a –80 ℃ freezer before use. Tissues (30–50 mg) were homogenized in cold 2 mL of $HClO_4$ 1 mol/L, then homogenates were centrifuged at 1000×*g* for 10 min. Supernatants were recentrifuged at 700×*g* for 20 min after neutralizing by 20 % KOH. The supernatant fraction was dried in a water bath (70–75 ℃). The last extractions were dissolved in TE buffer for assaying cAMP level. Cyclic AMP content was assayed by protein competition binding analysis as described by Liu *et al*[11].

Fos immunocytochemistry One hour after injection of naloxone, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and underwent sternotomy, transcardia aortic needle cannulation, perfusion with 100 mL saline, followed by 400 mL 4 % ice-cold paraformaldehyde in phosphate buffer (PB) 0.1 mol/L. The spinal cord of thoracolumbar level was removed, postfixed in the same fixative for 3 h, then immersed in 30 % sucrose in PB overnight at 4 ℃. Frozen series sections, thick 30 μm, were cut and collected in PB. Tissue sections were washed in phosphate buffer saline (PBS) and incubated in PBS containing 5 % normal goat serum and 0.3 % Triton X-100 at room temperature for 30 min, followed by rabbit anti-fos serum (1:1000) at 4 ℃ for 48 h. Then sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at 37 ℃ for 1 h and in avidin-biotin-peroxidase complex (1:100) at 37 ℃ for 2 h. Finally, the sections were reacted with DAB for 5–10 min. Then sections were rinsed in PBS 0.01 mol/L to stop reaction, mounted on gelatin-coated slides, air dried, dehydrated with 70 %–100 % alcohol cleared with xylene and coverslipped for microscopic examination.

Five spinal cord sections were selected from each animal to show the greatest number of positive neurons. Total number of positive neurons in bilateral spinal cord was measured in each rat. All positive neurons were counted without considering the intensity of the staining.

Experimental groups In mice and Fos immunocytochemistry test, animals were divided into 4 groups: saline-saline group (sal-sal), midazolam-saline group (mid-sal), saline-morphine group (sal-mor), and midazolam-morphine group (mid-mor). In cAMP content test, as a control, some rats were killed to assay cAMP level 10 min prior to injection of naloxone. So we got 8 groups: sal-sal, mid-sal, sal-mor, mid-mor, sal-sal-nal, mid-sal-nal, sal-mor-nal, and mid-mor-nal.

Statistical analysis Simplified direct probability method[12] and Mann-Whitney test[10] were used to examine the significance of difference in the incidence and the number of morphine withdrawal jumping, respectively. One-way ANOVA was used for other quantitative data. The data were presented as mean±SD.

RESULTS

Effects of pretreatment with midazolam on acute morphine dependence in mice Midazolam did not affect motor tone of mice. In sal-mor group, administration of a low dose of naloxone precipitated withdrawal jumping (ED_{50} 3.0, 95 % confidence limits: 1.9–4.3 mg/kg), suggesting that mice exhibited acute dependence a few hours after injection of morphine 100 mg/kg. The mice pretreated with midazolam required about 3 times more than naloxone to precipitate withdrawal jumping (ED_{50} 10.4, 95 % confidence limits: 8.5–12.3 mg/kg) ($P<0.01$) (Tab1). It indicated that coadministration of midazolam with morphine prevented the development of acute physical dependence to morphine.

Effects of pretreatment with midazolam on chronic morphine dependence in mice With the same

Tab 1. Effects of pretreatment with midazolam on naloxone ED_{50} for precipitating withdrawal jumping in acute morphine-dependent mice. *n*=11 mice. Mean± SD. $^cP<0.01$ *vs* sal-mor group.

Groups	ED_{50} of naloxone/mg·kg^{-1}	95 % Confidence limits
Sal-sal	>30	–
Mid-sal	>30	–
Sal-mor	3.0	1.9–4.3
Mid-mor	10.4	8.5–12.3^c

dose of naloxone (5 mg/kg, sc), pretreatment with midazolam significantly decreased the number of withdrawal jumping and the incidence of jumping in the first

Tab 2. Effects of pretreatment with midazolam on precipitating withdrawal jumping and loss in body weight in chronic morphine-dependent mice. *n*=10 mice. Mean±SD. $^cP<0.01$ *vs* sal-mor group.

Groups	Incidence of jumping/%	Number of jumping	Loss in body weight/g
Sal-sal	0	0	0
Mid-sal	0	0	0
Sal-mor	100	37±14	1.6 ± 0.8
Mid-mor	40^c	9 ±12^c	0.6 ± 0.3^c

15 min after administration of naloxone and inhibited the loss in body weight ($P<0.01$) (Tab 2).

Effects of pretreatment with midazolam on scores of morphine withdrawal response in rats Pretreatment with midazolam could significantly decrease scores of morphine withdrawal symptoms in rats ($P<0.05$) (Fig 1).

Effects of pretreatment with midazolam on cAMP level in rat spinal cord during morphine withdrawal Cyclic AMP content of rats spinal cord had no difference in each group before injection of naloxone. Naloxone itself did not influence cAMP level. Naloxone-precipiated withdrawal markedly increased cAMP level in rats spinal cord ($P<0.01$), but pretreatment with

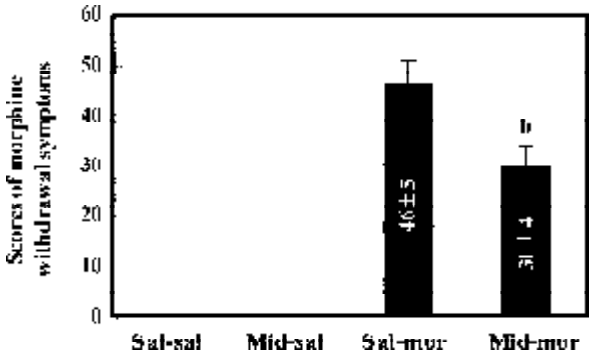

Fig 1. Effects of pretreatment with midazolam on scores of morphine withdrawal symptoms in rats. *n*=8 rats. Mean±SD. $^bP<0.05$ *vs* sal-mor group.

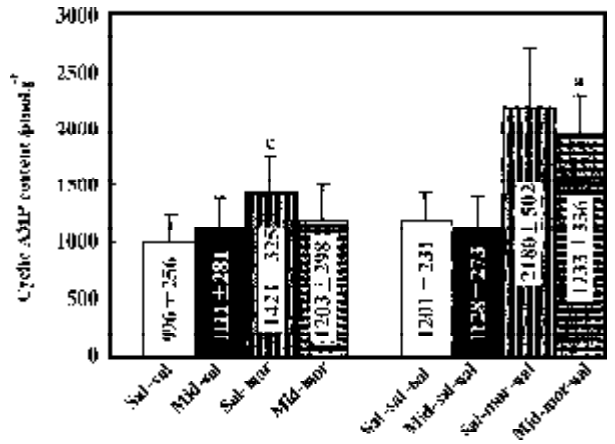

Fig 2. Effects of pretreatment with midazolam on cAMP content in the rat spinal cord during morphine withdrawal. *n*=10 rats. Mean± SD. $^aP>0.05$, $^cP<0.01$ *vs* sal-mor-nal group.

midazolam couldn't inhibit this increase ($P>0.05$) (Fig 2).

Effects of midazolam on Fos protein expression in rat spinal cord during morphine withdrawal Compared to nondependent rats, chronic administration of morphine did not influence Fos protein expression in rats spinal cord (data not shown). Midazolam and naloxone itself did not affect Fos protein expression. Fos protein expression was significantly increased and distributed in total laminae of bilateral spinal cord of rats in sal-mor group. Pretreatment with midazolam significantly inhibited Fos protein expression during morphine withdrawal ($P<0.05$) (Fig 3, 4).

DISCUSSION

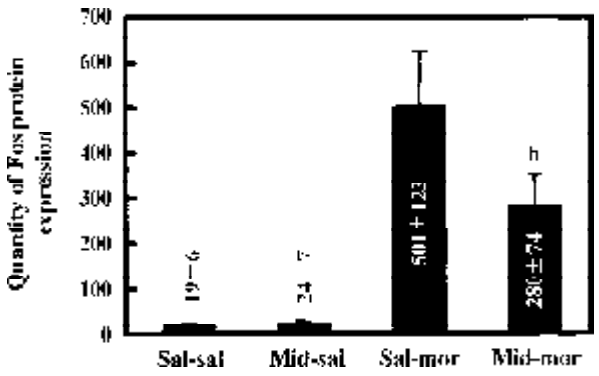

Fig 3. Effects of pretreatment with midazolam on Fos protein expression in rat spinal cord during morphine withdrawal. *n*=6 rats. Mean± SD. $^{b}P<0.05$ *vs* sal-mor group.

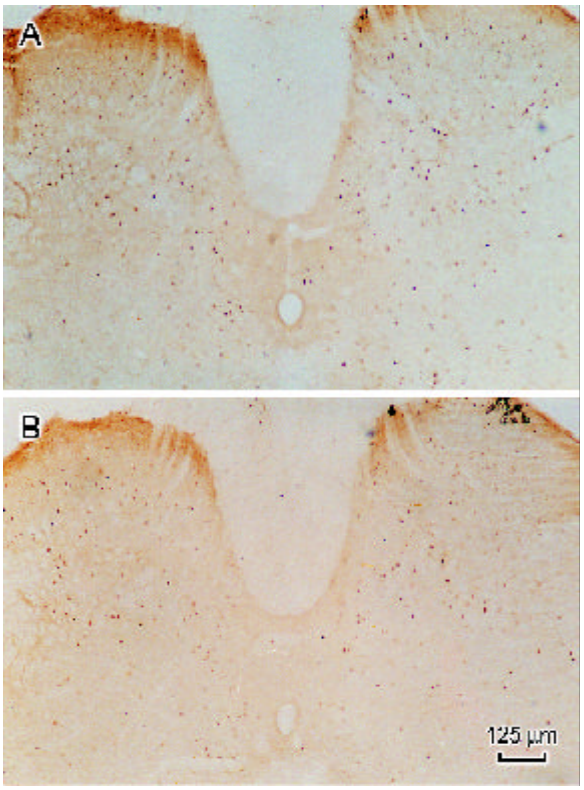

Fig 4. Micrographs of Fos protein expression in rat spinal cord during morphine withdrawal. (A) sal-mor group; (B) mid-mor group. ×40.

Midazolam is a water-soluble, short-acting benzodiazepine with potency that is 2–3 times greater than diazepam. In agreement with previous findings[3,4], to different animal strain and dependence model, our present study showed that pretreatment with midazolam significantly suppressed morphine-induced physical dependence and naloxone-precipitated withdrawal. Fos protein, the product of c-fos immediate early gene (IEG), has been used as a maker for neuronal activation in CNS. The present results showed that Fos protein expression was significantly increased and distributed in total laminae of bilateral spinal cord of rats during morphine withdrawal. This suggested that a state of hypersensitization have developed in the spinal cord neurons of morphine withdrawal rats, which may be the reason of resulting in morphine withdrawal response. Pretreatment with midazolam decreased Fos protein expression in spinal cord of morphine withdrawal rats, indicating that inhibition of hypersensitization of the spinal cord neuron played an important role in suppressing morphine withdrawal response by midazolam. It also indicated that spinal cord is a critical site for inhibiting morphine withdrawal response by midazolam.

Fos protein, as a transcription factor, forms an AP-1 (activating protein-1) complex with another IEG protein product called Jun, which then subsequently binds to the AP-1 binding site of DNA to modulate the transcription of target gene relevant to extracellur specific stimulation. There is evidence to suggest that c-*fos* participates in the regulation of mRNA encoding various peptides like, enkephalin, dynorphin, *et al*, in the rat spinal cord[13]. Some studies demonstrated that morphine dependence and withdrawal increased immunoreactivity of met-enkephalin, beta-endorphin in spinal cord and pretreatment with midazolam could antagonize the increase[14,15]. Our results further proved that c-*fos* gene might mediate antagonistic effect of midazolam to increasing enkephalin immunoreactivity during morphine withdrawal.

Cyclic AMP pathway is one of most important intracellular signal transduction systems in CNS. Activation of cAMP pathway could result in increased excitation and sensitization of neurons and induced Fos protein expression. In present study, pretreatment with midazolam inhibited Fos protein expression, but could not inhibit the increase of cAMP level during morphine withdrawal. It suggested that other intracellular messenger participated in mediating effects of inhibiting Fos protein expression by midazolam during morphine withdrawal. Our previous study indicated that nitric

oxide (NO) played an important role in inducing Fos protein expression in spinal cord, especially in the deeper laminae, in morphine withdrawal rats. In other words, NO participated in the spinal cord neuron sensitization in morphine-withdrawal rats[5]. It is unknown if NO mediates inhibition of Fos protein expression by midazolam during morphine withdrawal.

In conclusion, midazolam suppresses morphine withdrawal response by inhibiting hypersensitization of the spinal cord neuron, and this effect may not mediated by cAMP pathway.

REFERENCES

1 Heikkila AT, Echenko O, Uusi-Oukari M, Sinkkonen ST, Korpi ER. Morphine withdrawal increases expression of GABA(A) receptor epsilon subunit mRNA in locus coeruleus neurons. Neuroreport 2001; 17: 2981-5.

2 Bonci A, Williams JT. A common mechanism mediates long-term changes in synaptic transmission after chronic cocaine and morphine. Neuron 1996; 16: 631-9.

3 Tejwani GA, Rattan AK, Pongrak S, Shen MJ, Znniya J, MacDonald JS. Inhibition of morphine-induced tolerance and dependence by a benzodiazepine receptor agonist midazolam in rat. Anesth Analg 1993; 76: 1052-60.

4 Suzuki T, Tsuda M, Narita M, Funada M. Mizoguchi H, Misawa M. Diazepam pretreatment suppresses morphine withdrawal signs. Life Sci 1996; 58: 447-54.

5 Cao JL, Zeng YM, Zhang LC, Gu J, Liu HF, Zhou WH, *et al*. NO mediated the increase of Fos protein and $NMDA_{1A}R$ mRNA expression in the rat spinal cord during morphine withdrawal. Acta Pharmacol Sin 2001; 22: 505-11.

6 Nadeson R, Guo Z, Porter V,Gent JP, Goodchild CS. γ-Aminobutyric $acid_A$ receptor and spinally mediated antinociception in rat. J Pharmacol Exp Ther 1996; 278: 620-6.

7 Thomas JL, Ingo HL, Christina GW, Takahiko H. Effect of the NMDA-antagonist, MK 801, on benzodiazepine-opioid interactions at the spinal and supraspinal level in rats. Br J Pharmacol 1995; 114: 1097-103.

8 Nestler EJ, Aghajanian GK. Molecular and cellular basis of addition. Science 1997; 278: 58-63.

9 Abdelhamid EE, Suctana M, Portoghese PS, Takemor AE. Selective backage delta opioid receptor prevents the development of morphine tolerance and dependence in mice. J Pharmacol Exp Ther 1991; 258: 299-303.

10 Tallarida RJ, Murray RB. Manual of pharmacologic calculations with computer programs. 1st ed. New York: Spring-Verlag; 1981.

11 Liu JS, Wang XJ, Zeng SP, Cheng JX, Wang ZG. Measurement of clyclic nucleotide. In: Xu SY, Bian RL, Chen S, editors. Methodology of pharmacological experimental. Beijing: People' s Medical Publishing House; 1994. p 415-23.

12 Sun RY. Quantitative Pharmacology. 1st ed. Beijing: People' s Medical Publishing House; 1987.

13 Coderre TJ, Katz J, Vaccarino AL, Melzack R. Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain 1993; 52: 259-85.

14 Tejwani GA, Rattan AK. Met-enkephalin alteration in the rat during chronic injection of morphine and/or midazolam. Brain Res 1997; 775: 119-26.

15 Rattan AK, Tejwani GA. Effect of chronic treatment with morphine, midazolam and both together on dynorphin (1-13) levels in the rat. Brain Res 1997; 754: 239-44.

预先应用咪哒唑仑抑制小鼠和大鼠吗啡戒断反应

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关键词　咪哒唑仑；吗啡；物质禁断综合征；腺苷环一磷酸；GABA；原癌基因蛋白质 c-fos 类；脊髓

目的：研究咪哒唑仑对小鼠和大鼠吗啡戒断反应的影响．方法：实验中采用急性和慢性吗啡依赖和纳洛酮催促戒断模型．使用放免法测定cAMP含量，免疫组织化学方法观察Fos蛋白表达变化．结果：合用咪哒唑仑和吗啡可抑制小鼠急性和慢性吗啡依赖的发展．在急性吗啡依赖小鼠，咪哒唑仑-吗啡组纳络酮催促跳跃的ED_{50} (10.4，8.5-12.3 mg/kg)明显大于生理盐水-吗啡组(3.0，1.9-4.3 mg/kg) ($P<0.01$)．在慢性吗啡依赖小鼠，咪哒唑仑-吗啡组纳络酮催促跳跃的发生率和跳跃次数明显低于生理盐水-吗啡组($P<0.01$)．预先使用咪哒唑仑抑制吗啡戒断大鼠脊髓Fos蛋白表达，但不能抑制脊髓cAMP含量的增加．结论：咪哒唑仑通过抑制脊髓神经元敏感化减轻吗啡戒断反应，cAMP信号转导通路不参与介导这一效应．

(责任编辑　吕　静)