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The spinal nitric oxide involved in the inhibitory effect of midazolam on morphine-induced analgesia tolerance

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Abstract

Previous studies had shown that pretreatment with midazolam inhibited morphine-induced tolerance and dependence. The present study was to investigate the role of spinal nitric oxide (NO) in the inhibitory effect of midazolam on the development of morphine-induced analgesia tolerance. Subcutaneous injection of 100 mg/kg morphine to mice caused an acute morphine-induced analgesia tolerance model. To develop chronic morphine tolerance in mice, morphine was injected for three consecutive days (10, 20, 50 mg/kg sc on Day 1, 2, 3, respectively). In order to develop chronic tolerance model in rats, 10 mg/kg of morphine was given twice daily at 12 h intervals for 10 days. Midazolam was intraperitoneally injected 30 min prior to administration of morphine. Tail-flick test, hot-plate and formalin test were conducted to assess the nociceptive response. Immunocytochemistry, histochemistry and western blot were performed to determine the effect of midazolam on formalin-induced expression of Fos protein, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and nitric oxide synthase (NOS) in chronic morphine-tolerant rats, respectively. The results showed that pretreatment with midazolam significantly inhibited the development of acute and chronic morphine tolerance in mice, which could be partially reversed by intrathecal injection of NO precursor L-arginine (L-Arg). In chronic morphine-tolerant rats, pretreatment with midazolam significantly decreased the formalin-induced expression of Fos and Fos/NADPH-d double-labeled neurons in the contralateral spinal cord and NADPH-d positive neurons in the bilateral spinal cord. Both inducible NOS (iNOS) and neuronal NOS (nNOS) protein levels in the spinal cord were significantly increased after injection of formalin, which could be inhibited by pretreatment with midazolam. The above results suggested that the decrease of the activity and expression of NOS contributed to the inhibitory effect of midazolam on the development of morphine tolerance.

Keywords: Midazolam; Morphine tolerance; Spinal cord; Nitric oxide

1. Introduction

The development of tolerance to the antinociceptive effects of morphine and other opioids continues to be a significant clinical problem in the treatment of conditions associated with chronic, severe pain. Likewise, the biochemical mechanisms underlying the development of opioid tolerance have been elusive (McNally, 1999; Law et al., 2004; Waldhoer et al., 2004). Elucidation of the various mechanisms involved in this phenomenon is requisite to the development of treatment strategies to attenuate or circumvent tolerance. Previous studies have shown that coadministration of midazolam inhibited morphine-induced tolerance and dependence (Tejwani et al., 1993; Rattan and Tejwani, 1996, 1997; Tejwani and Rattan, 1997; Cao et al., 2002), but its mechanism remains unclear.

Midazolam, a benzodiazepine-receptor agonist, has been widely used for inducing and maintaining anesthesia state by

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coadministration with opioids or inhaled anesthetics in clinics. Midazolam can occupy the benzodiazepine-receptor on a benzodiazepine-gamma amino butyric acid (GABA)-Cl^- channel complex and therefore facilitate the inhibitory action of GABA on neuronal transmission (Ticku, 1983; Saano, 1987). Thomas et al demonstrated that intrathecal administration of midazolam not only potentiated the antinociceptive effect of opioids in the spinal cord, but also produced segmental antinociceptive effect, which were mediated by $GABA_A$ receptor in the spinal dorsal cord (Thomas et al., 1995; Nadeson et al., 1996). Rattan et al reported that coadministration with midazolam abolished chronic treatment with morphine-induced the increase of β-endorphin level in the spinal cord of rats (Rattan and Tejwani, 1996). Our recent study also showed that midazolam suppressed morphine withdrawal response by inhibiting the hypersensitization of the spinal cord neurons (Cao et al., 2002). In clinic, long-term intrathecal infusion of morphine combined with midazolam achieved sufficient analgesia without major adverse effects and without rapid development of tolerance towards morphine in patients with chronic nonmalignant back and leg pain due to degenerative spinal disease and multiple spinal surgeries (Rainov et al., 2001). These studies indicate that spinal cord is an important site for inhibiting morphine tolerance and dependence by midazolam.

Nitric oxide (NO), which produces from the conversion of the L-arginine (L-Arg) catalyzed by nitric oxide synthase (NOS), is an important intra- and inter-cellular messenger and plays a crucial role in a number of physiological and pathological processes within the nervous system. A growing body of evidences suggests that the spinal NO participates in the initiation and development of morphine tolerance and dependence (Kumar and Bhargava, 1997; Machelska et al., 1997; Ozek et al., 2003; Cao et al., 2000, 2001). Three different types of NOS, called neuronal, endothelial and inducible NOS, have been identified in the brain and spinal cord. Moreover, histochemical mapping of NOS revealed that NOS-positive neurons are co-localized with GABA or GABA receptor in lamina I-II of rat spinal cord (Valthschanoff et al., 1992, 1993; Spike et al., 1993; Laing et al., 1994; Heinke et al., 2004). In vivo and in vitro studies suggest that NO modulates either release or uptake of GABA and the activity of $GABA_A$ receptor or acts directly on $GABA_A$ receptor (Lonart et al., 1992; Lipton et al., 1993; Guevara-Guzman et al., 1994; Segovia et al., 1994; Zarri et al., 1994; Kano et al., 1998). Furthermore, there are evidences that the interaction between NO and GABA or GABA receptor is involved in nociceptive information modulation of the spinal cord level (Lin et al., 1999a,b; Bie and Zhao, 2001). Galley et al reported that midazolam could inhibit the activity of nNOS in rat brain (Galley and Webster, 1996). The benzodiazepine-induced antinociception is intensified by coadministration with N-nitro-L-arginine methyl ester hydrochloride (L-NAME), a non-selective NOS inhibitor, as well as 7-nitroindazole (7-NI), a neuronal NOS inhibitor, and is decreased by L-Arg in mice (Talarek and Fidecka, 2002).

Taken together, these data suggest that NO may be involved in the inhibitory effect of midazolam on the development of morphine tolerance and dependence. The aim of this study was to investigate the role of the spinal NO in the effect of midazolam on acute and chronic morphine tolerance in mice by intrathecal administration of L-Arg and the effects of midazolam on formalin-induced sensitization of the spinal cord neurons and the activity and protein expression of NOS in chronic morphine tolerance rats.

2. Materials and methods

2.1. Animals

Male Kunming mice (20–24 g) and male adult Sprague–Dawley rats (200–250 g) were provided by Experimental Animal Center of Xuzhou Medical College. The animals were housed at room temperature (22±2 °C) under a natural light–dark cycle conditions (12 h: 12 h) with food and water available ad libitum. All experimental protocols were approved by the Animal Care and Use Committee of Xuzhou Medical College and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Nociceptive response and motor function assessment

The nociceptive thresholds of mice were assessed by tail-flick test. The nociceptive endpoint in tail-flick test was the characteristic withdrawal the tail from warm water (52±0.1 °C). To avoid tissue damage, a cut-off time was established at 10 s. Basal tail flick latency (TFL) was measured prior to any treatment. Mice were excluded from study if the basal TFL exceeded 5 s. TFL for each mouse was expressed as the percentage of maximum possible effect (MPE %). MPE%= (latency after medication - baseline latency)/(10 - baseline latency)×100%.

Hot-plate test was conducted to assess the nociceptive response of rats. The metal plate surface was maintained at 54±0.1 °C. Licking the hind paw was considered as nociceptive endpoint and a cut-off time was established at 30 s.

In the formalin test, 5% formalin 100 μl was injected into the plantar surface of the left hind paw of rat. Within 1 h after formalin injection, the amount of time that each rat spent displaying one of the three following behaviors was continuously recorded according to the remodified method of Dubuisson and Dennis (1977). Behavior was recorded as a '2' if the rat licked or bit the injected paw, as a '1' if the rat elevated the paw from the floor, and as a '0' if any part of the paw other than the

tips of the digits was in contact with any surface of the box. A weighted formalin pain score for each rat at the 12 5-min test intervals was calculated using the following formula: pain score=[1×(time spent in *s* with inflamed paw elevated)+2×(time spent in *s* with licking inflamed paw)]/300 s (Watson et al., 1997).

Motor function was evaluated by observing placing/stepping reflexes and righting reflexes and conducted 5 min before assessment of nociceptive response.

2.3. Intrathecally injection

L-Arg was intrathecally injected according to the method of Hylden and Wilcox (1980). The 30-gauge needle was inserted from the side of the L_5 or L_6 spine process. The injection volume was 5 μl per mouse.

2.4. Acute morphine tolerance test in mice

Thirty minutes after pretreatment with midazolam (2 mg/kg ip) or saline, acute dependence of mice was induced by a single subcutaneous injection of morphine at 100 mg/kg. For mice in control conditions, saline was substituted for morphine. Four hours after morphine injection, mice were challenged with various probe doses of morphine (0.5–60 mg/kg sc). We chose the 4-h time interval in this experiment because it has been previously shown to yield maximal morphine analgesia tolerance (Yano and Takemori, 1977). To investigate the role of NO in the effect of midazolam on acute morphine tolerance, some mice were intrathecally injected with 30 μg L-Arg 30 min prior to injection of the probe dose of morphine followed by the tail-flick test 45 min later. To observe the effect of L-Arg on nociceptive response of mice, the same dose of L-Arg was intrathecally injected in non-tolerance mice. ED_{50} value for probe morphine analgesia and 95% confidence limits were calculated according to Tallarida and Murray (1981). At least 4 doses for each group and 10 mice for each dose were used to determine each ED_{50} value.

2.5. Chronic morphine tolerance test in mice

In the chronic tolerance conditions, mice received morphine once daily for three consecutive days (10, 20, 50 mg/kg sc on Days 1, 2, and 3, respectively) following administration of midazolam (2 mg/kg ip) or saline 30 min ago. For mice in control conditions, saline was substituted for morphine. On the test day (Day 4), mice were injected with the probe dose of morphine (5 mg/kg sc) or saline after measuring baseline TFL. In order to study the role of NO in the effect of midazolam on chronic morphine tolerance, parts of mice were intrathecally injected with 30 μg L-Arg 30 min prior to the probe dose of morphine injection. TFL of mice was measured 45 min after the probe dose of morphine injection.

2.6. Chronic morphine tolerance test in rats

To develop a chronic morphine tolerance model, rats were subcutaneously injected with 10 mg/kg morphine (twice daily at 12 h intervals for 10 days) or saline following administration of midazolam (3 mg/kg ip) or saline 30 min ago. Forty-five minutes later, the anti-nociceptive response to morphine was measured by the hot-plate test on the odd days (Days 1, 3, 5, 7, and 9). To investigate morphine tolerance-induced hyperalgesia, some rats were measured hot-plate latency (HPL) without any injection on Day 11, others were performed formalin test. Rats received 5 mg/kg morphine via tail vein 20 min prior to formalin injection. Within one hour after formalin injection, a weighted formalin pain score for each rat was calculated according to the above mentioned method. One hour after injection of formalin, the rats were performed Fos immunocytochemistry, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, Fos/NADPH-d double-labeling test or western blot technique.

2.7. Fos immunocytochemistry, NADPH-d histochemistry and double-labeling test

Rats were anesthetized with sodium pentobarbital (60 mg/kg ip) and undergone sternotomy, intracardially perfused with 100 ml saline followed by 400 ml 4% ice-cold paraformaldehyde in 0.1 mol/L phosphate buffer (PB). The spinal cord of $L_{4\text{-}5}$ was removed, post-fixed in 4% paraformaldehyde for 3 h, and subsequently allowed to equilibrate in 30% sucrose in PB overnight at 4 °C. Thirty micrometers transverse series sections were cut on a cryostat and stored in PB. Tissue sections were performed NADPH-d histochemistry, Fos immunocytochemistry and Fos/NADPH-d double labeling test, respectively.

Tissue sections for Fos immunocytochemistry were washed and incubated in phosphate buffer saline (PBS) containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min, followed by primary rabbit anti-c-Fos antibody (1:1000) at 4 °C for 48 h. Then the sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at 37 °C for 1 h, then in avidin–biotin–peroxidase complex (1:100) at 37 °C for 2 h. Finally, the sections were reacted with diaminobenzidine (DAB) for 5–10 min and then rinsed in 0.01 mol/L PBS to stop reaction, mounted onto gelatin-coated slides, air dried, dehydrated with 70–100% alcohol, cleared with xylene and cover slipped for microscopic observation.

Tissue sections for NADPH-d histochemistry were rinsed with 0.1 mol/L PB and then incubated in PB (pH 8.0) containing 0.3% Triton X-100, 0.6 mg/ml β-nicotinamide adenine dinucleotide phosphate (β-NADPH) and 0.5 mg/ml nitrotetrazolium blue (NBT) at 37°C for 40–60 min. Tissue sections were then rinsed with 0.01 mol/L PBS to stop reaction.

Tissue sections for double-labeling were processed with NADPH-d histochemistry followed by Fos immunocytochemistry. Other procedures were similar to the above.

2.8. Counting of positive neurons

To observe the distribution of positive neurons, the spinal cord was divided into four regions: superficial laminae (I-II laminae), nucleus proprious (III-IV laminae), neck of dorsal horn (V-VI laminae) and ventral laminae (VII-X laminae). We selected 5 sections per rat to count the greatest number of positive neurons. To every rat, two parameters were recorded: (1) total number of positive neurons in the ipsilateral spinal cord; (2) total number of positive neurons in the contralateral spinal cord. All positive neurons were counted without considering the intensity of the staining.

2.9. Western blot analysis

One hour after formalin injection, rats were anesthetized with sodium pentobarbital (60 mg/kg ip). L_4–L_5 spinal cord was dissected and frozen in liquid nitrogen. Tissue samples were homogenized in lysis buffer containing (in mM): Tris 20.0, sucrose 250.0, Na_3VO_4 0.03, $MgCl_2$ 2.0, EDTA 2.0, EGTA 2.0, phenylmethylsulfonyl fluoride (PMSF) 2.0, dithiothreitol (DTT) 1.0, protease inhibitor cocktail 0.02% (v/v), pH 7.4. The homogenates were centrifuged at 5000 g for 30 min at 4 °C. The supernatant was collected and protein concentration was performed according to Bradford (1976) using BSA as standard. Proteins were dissolved in 4× sample buffer containing (in mM): Tris-HCl 250.0, Coomassie brilliant blue-G 0.01%, sodium dodecyl sulfate (SDS) 8%, sucrose 200.0, and DTT 300.0, pH 6.8. After boiling for 5 min, equivalent amounts of protein (30 μg) were separated by 8% denaturing SDS polyacrylamide gel electrophoresis, electroblotted onto a nitrocellulose membrane and immunoreacted with nNOS and iNOS antibodies overnight at 4 °C, followed by 1 h incubation at room temperature with the second antibody (Sigma, USA) conjugated with horse radish peroxidase. The nNOS and iNOS were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Inc.) and each nNOS or iNOS signal was normalized to a selected control standard signal. The scanned image was imported into Adobe Photoshop software and scanning densitometry was used for quantitative analysis of the data.

2.10. Drugs and chemicals

Midazolam was purchased from Roche Pharmaceuticals (Switzerland). The timing and doses for midazolam used in the present study were shown to inhibit the morphine-induced tolerance and dependence in the rats and mice (Tejwani et al., 1993; Cao et al., 2002). Morphine and naloxone were obtained from Qinghai and Sihuan Pharmaceutical Factory. All drugs were dissolved in saline solution. β-NADPH, NBT, L-Arg, protease inhibitor cocktail and primary rabbit anti-c-Fos antibody were purchased from Sigma (USA). Primary rabbit anti-nNOS and iNOS antibody were purchased from Santa Cruz (USA). ABC kit and DAB were the productions of Vector (USA).

2.11. Statistical analysis

Data are presented as the mean±SD. Bliss program was used to determine the difference ED_{50} of probe dose morphine-induced analgesia (Bliss, 1967). Among groups, comparisons were performed by a one-way analysis of variance (ANOVA) with multiple comparisons or Student's *t* test when appropriate. Differences were considered as statistically significant at $P<0.05$.

3. Results

3.1. Acute and chronic morphine tolerance test in mice

Treatment with midazolam, L-Arg or midazolam plus L-Arg in non-tolerant mice had no significant effects on ED_{50} for morphine analgesia. In acute morphine tolerance test of mice, 4 h after coadministration of midazolam (2 mg/kg ip) and morphine (100 mg/kg sc), ED_{50} for morphine-induced analgesia significantly decreased in midazolam-morphine group (6.1, 95% confidence limits: 4.8–7.9 mg/kg) compared to saline-morphine group (19.6, 95% confidence limits: 15.2–25.2 mg/kg), which could be partially reversed by intrathecal administration of L-Arg (10.7, 95% confidence limits: 8.3–14.8 mg/kg) (Table 1).

Multiple injections with saline, midazolam or saline plus midazolam and single intrathecal injection of L-Arg or

Table 1
The changes of morphine analgesia ED_{50} in acute morphine-tolerant mice

Groups	Injection route	ED_{50} of morphine ($mg.kg^{-1}$)	95% CL
Saline-saline	ip+sc	3.8	2.1~6.7
Midazolam-saline	ip+sc	4.0	2.3~6.8
Saline-morphine	ip+sc	19.6	15.2~25.2
Midazolam-morphine	ip+sc+it	6.1	4.8~7.9**
Saline-L-Arg	ip+it	4.3	3.0~7.1
Midazolam-L-Arg	ip+it	3.9	2.5~7.0
Saline-morphine-L-Arg	ip+sc+it	20.3	15.9~26.3
Midazolam-morphine-L-Arg	ip+sc+it	10.7	8.3~14.8*

Acute morphine tolerance was induced by injecting a single dose of morphine (100 mg/kg sc). Tail-flick test was used to assess the nociceptive threshold of mice. At least 4 doses for each group, 10 mice for each dose were used in the present study. Results are expressed as mean±SD. Bliss program was used for comparing the difference of ED_{50}. CL=confidence limits.

* $P<0.05$ compared with Midazolam-morphine group.

** $P<0.01$ compared with Saline-morphine group.

midazolam plus L-Arg in non-tolerant mice had no effects on the baseline TFL. Chronic treatment with morphine in mice produced significant analgesia tolerance as evidence that saline-morphine group produced less analgesic effect in tail-flick test after injection with morphine on the fourth day. However, treatment with midazolam and morphine produced a significant analgesia effect. The inhibitory effect of midazolam on chronic morphine tolerance was also partially reversed by intrathecally injection of L-Arg (Table 2).

3.2. Chronic morphine tolerance in rats

In chronic tolerance experiment of rats, three days after treatment with morphine, there was little analgesic effect of morphine in saline-morphine group; moreover, HPL was less than basal HPL from Day 7 to Day 11, which suggested that a state of morphine tolerance-related hyperalgesia had developed in saline-morphine group rats. In midazolam-morphine group, from Day 1 to Day 7, morphine analgesia effect was maintained and there was no difference between HPL of Day 11 and basal HPL (Fig. 1). In formalin test, pretreatment with midazolam significantly reduced nociceptive scores at every time point (Fig. 2). The above results suggested that coadministration of midazolam inhibited the development of chronic morphine tolerance and tolerance-induced hyperalgesia in rats.

3.3. Fos immunocytochemistry, NADPH-d histochemistry and double-labeling test

In non-tolerant rats, formalin-induced Fos-like immunoreactivity (Fos-LI) mainly located in superficial laminae (I–II laminae) and neck (V–VI laminae) of ipsilateral spinal cord, a few Fos-LI were distributed in VII–X laminae, very few Fos-LI was present in nucleus proprious (VII–X laminae) and the contralateral spinal cord. Injection with morphine (5 mg/kg iv) could significantly reduce the expression of Fos-LI neurons (data not shown). In morphine-tolerant rats, the expression of formalin-induced Fos-LI was markedly increased and distributed not only in total laminae of the ipsilateral spinal cord but also in the contralateral spinal cord. Injection with morphine (5 mg/kg iv) 20 min prior to injection with formalin could not inhibit the expression of formalin-induced Fos-LI. Compared to saline-morphine group, injection of morphine could inhibit the expression of formalin-induced Fos-LI in the contralateral, but not in the ipsilateral spinal cord in midazolam-morphine group (Table 3, Fig. 3A,B).

NADPH-d positive neurons were distributed in the superficial laminae of spinal dorsal horn and the gray matter surrounding central canal of spinal cord. Stimulation with formalin increased the expression of NADPH-d positive neurons bilaterally in the spinal dorsal horn and acute administration of morphine at 5 mg/kg decreased the expression of NADPH-d positive neurons (data not

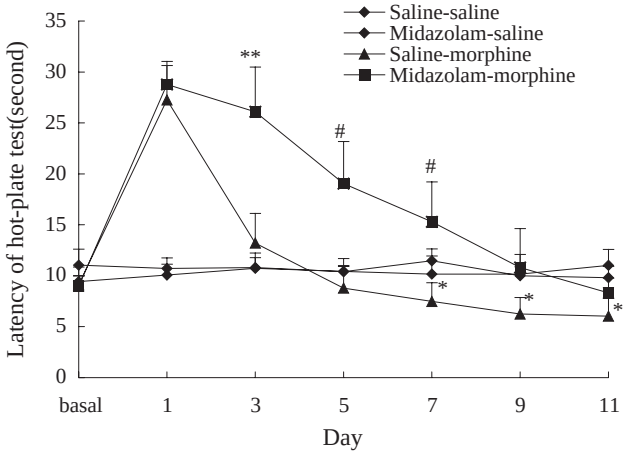

Fig. 1. Coadministration of midazolam inhibited chronic morphine analgesia tolerance and tolerance-related hyperalgesia in rats. n=8 rats. $^{**}P<0.01$, $^{\#}P<0.05$ compared with Saline-morphine group, $^{*}P<0.05$, compared with Saline-saline group, Student's t test.

Table 2
The effect of midazolam on chronic morphine analgesia tolerance

Groups	Injection route	Basal TFL	TFL after s.c.morphine
Saline-saline	ip+sc	1.89±0.62	6.20±1.87
Midazolam-saline	ip+sc	1.98±1.45	6.52±1.75
Saline-morphine	ip+sc	1.97±0.58	2.98±1.67
Midazolam-morphine	ip+sc	2.18±0.64	5.21±2.07**
Saline-L-Arg	ip+it	1.83±0.66	5.87±1.63
Midazolam-L-Arg	ip+it	1.90±0.79	6.02±1.68
Saline-morphine-L-Arg	ip+sc+it	1.78±0.45	2.33±0.96
Midazolam-morphine-L-Arg	ip+sc+it	1.85±0.42	3.21±0.74*

Chronic morphine tolerance was produced by injecting of morphine for three consecutive days (10, 20, 50 mg/kg sc on Days 1, 2, and 3, respectively). Probe dose of morphine (5 mg/kg sc) was injected on the fourth day. Tail-flick test was used to assess the nociceptive threshold of mice. Basal TFL was got 10 min before injection of probe dose of morphine. n=10 mice. Data are expressed as mean±SD.

* $P<0.05$ compared with Midazolam-morphine group.

** $P<0.01$ compared with Saline-morphine group.

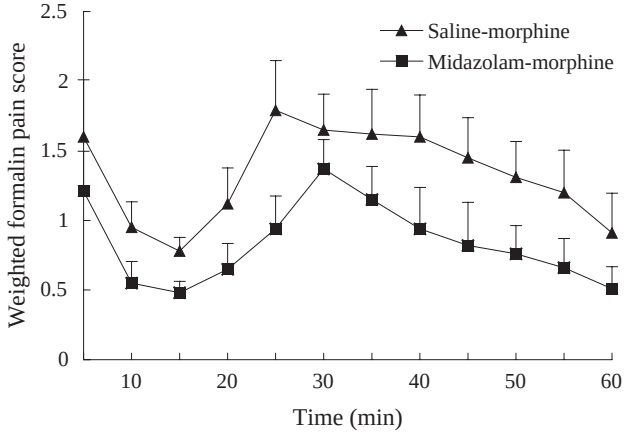

Fig. 2. Coadministration of midazolam decreased weighted formalin pain score in rats. There is significant difference between the two groups at each time point. n=8 rats.

Table 3
The effect of midazolam on formalin-induced Fos, NADPH-d and double-labeled positive neurons in the spinal cord of chronic morphine-tolerant rats

Groups	Number of positive neurons of ipsilateral spinal cord			Number of positive neurons of contralateral spinal cord		
	Fos-LI	NADPH-d	Double-labeled	Fos-LI	NADPH-d	Double-labeled
Saline-saline	34±12	35±7	1.8±4.4	8±7	27±8	0
Midazolam-saline	31±8	29±9	1.1±3.7	6±4	24±5	0
Saline-morphine	395±67	98±21	15.4±4.6	198±63	90±32	9.7±2.8
Midazolam-morphine	379±47	58±17*	13.4±5.3	108±35*	54±15*	4.1±0.9**

Data are expressed as mean±SD. n=4~6 rats.
* $P<0.05$ compared with Saline-morphine group (Student's t test).
** $P<0.01$ compared with Saline-morphine group (Student's t test).

shown). Morphine tolerance further enhanced the expression of formalin-induced NADPH-d positive neurons and injection of morphine (5 mg/kg iv) 20 min prior to injection with formalin could not inhibit the increase in saline-morphine group, but could in midazolam-morphine group (Table 3, Fig. 3C,D).

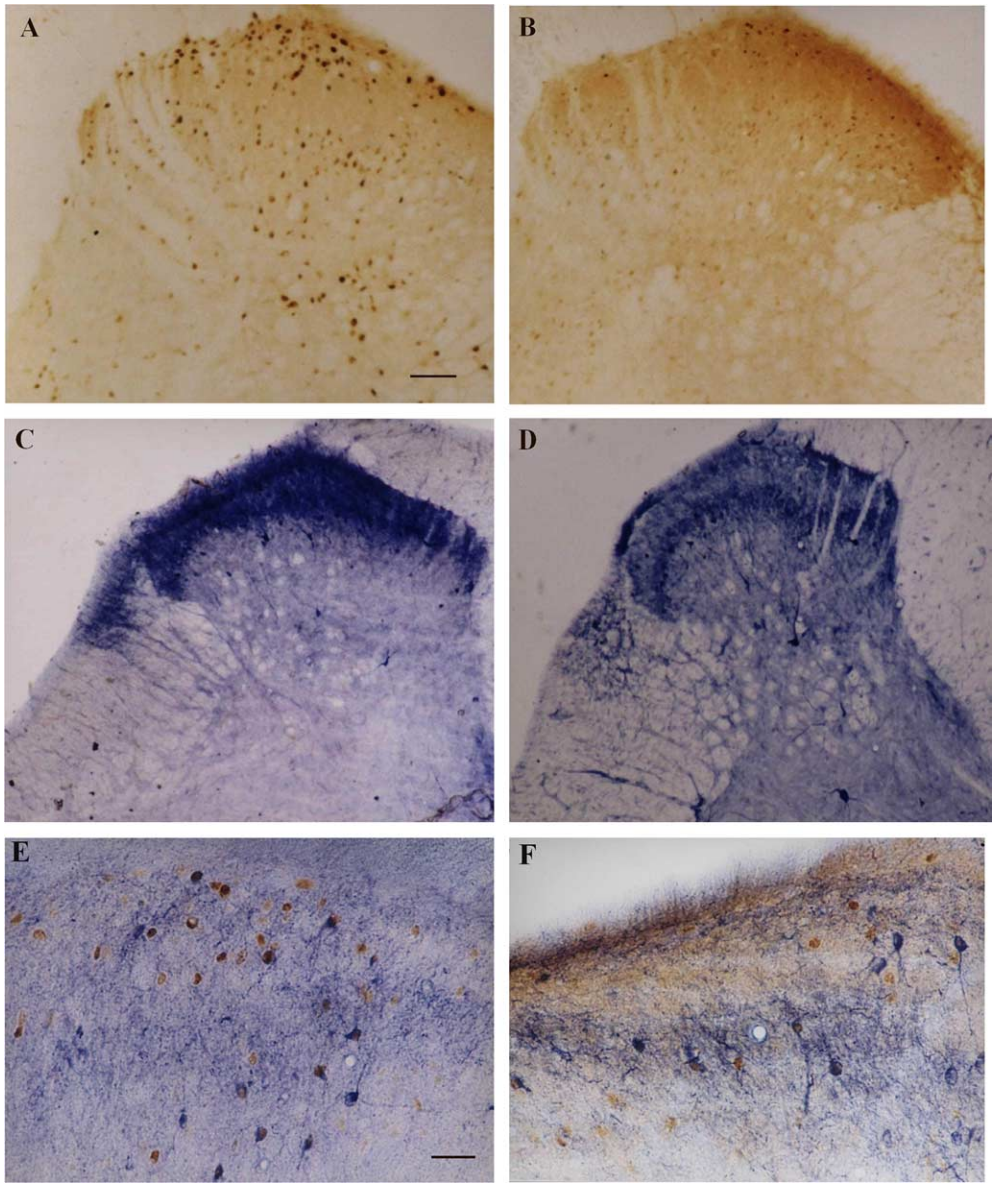

Fig. 3. Photomicrograph showing the effect of coadministration of midazolam on formalin-induced Fos (A, B), NADPH-d (C, D) and Fos/NADPH-d double-labeled (E, F) neurons in the contralateral spinal cord in morphine-tolerant rats. There was a significant decrease in Fos, NADPH-d and Fos/NADPH-d double-labeled neurons expression in the contralateral spinal cord of midazolam-morphine group rats. *A, C, E*=Saline-morphine group, B, D, F=Midazolam-morphine group. n=4~6 rats. (A, B, C, D) Bar=100 μm, (E, F) Bar=25 μm.

Formalin-induced double-labeled neurons were mainly located in the superficial laminae of the ipsilateral spinal dorsal horn in non-tolerant rats, and other regions had few positive neurons (data not shown). In morphine-tolerant rats, formalin-induced double-labeled neurons were significantly increased in the superficial laminae of the ipsilateral spinal dorsal horn. Furthermore, some double-labeled neurons were expressed in nucleus proprious (III-IV laminae), the gray matter surrounding central canal of spinal cord and the contralateral spinal cord and injection with morphine could not inhibit its expression. Compared to saline-morphine group, in midazolam-morphine group, injection of morphine could inhibit the expression of formalin-induced Fos/NADPH-d double-labeled neurons in the contralateral, but not in the ipsilateral spinal cord (Table 3, Fig. 3E,F).

3.4. Western blot

Chronic morphine treatment increased the expression of the spinal iNOS and had no significant effect on the spinal nNOS expression. No difference in the iNOS and nNOS level was detected after coadministration of midazolam with morphine. Both iNOS and nNOS levels were significantly enhanced after injection formalin into the plantar surface of hindpaw of rats and the increasing expression could inhibited by coadministration of midazolam (Fig 4).

3.5. Motor function assessment

In motor function assessment test, midazolam at 2 mg/kg for mice and 3 mg/kg for rats did not affect motor tone of animals. No animals were excluded in this study.

4. Discussion

In agreement with previous findings (Tejwani et al., 1993; Rattan and Tejwani, 1996, 1997; Tejwani and Rattan, 1997), the present investigation showed that pretreatment with midazolam significantly suppressed morphine-induced analgesia tolerance in the diverse animal strain and tolerance model. Furthermore, we demonstrated that the spinal NO involved in the inhibitory effect of midazolam on morphine-induced analgesia tolerance.

4.1. Methods consideration

Fos protein, the product of c-*fos* immediate early gene (*IEG*), has been used as a maker for neuronal activation in the central nervous system (CNS). The expression of Fos protein also may be a useful tool to examine the effectiveness of different analgesic regimens (Munglani et al., 1996). There is a positive correlation between the quantity of expression of Fos protein and the degree of sensitization induced by nociceptive stimuli in spinal cord neurons. In the present study, the expression of Fos protein was used to detect the degree of sensitization of spinal cord neurons. In the CNS, there is a remarkable correspondence between neurons containing NADPH-d and those containing NOS. NADPH-d histochemistry has often been used for mapping NOS-containing neurons in the brain and the spinal cord (Bredt et al., 1991; Dawson et al., 1991; Hope et al., 1991; Vincent and Kimura, 1992). However, in the superficial dorsal horn and around the central canal in the rat, the number of NADPH-d expressing neurons is higher than the number of the NOS immunoreactive somata (Lee et al., 1993; Vizzard et al., 1994). Therefore, NADPH-d histochemistry was used to map and detect the changes of NOS activity in the

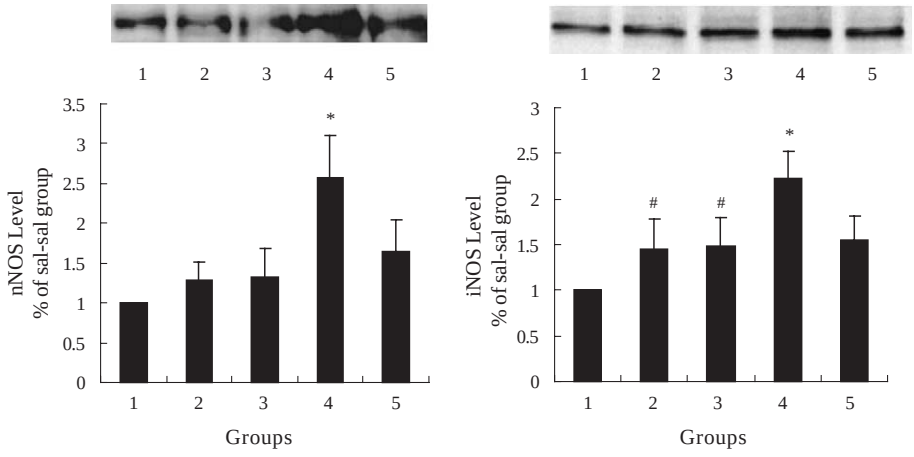

Fig. 4. Coadministration of midazolam inhibits the increase of formalin-induce nNOS and iNOS expression in spinal cord of morphine-tolerant rats. The immunoreactive bands of nNOS and iNOS were detected with Western blotting. Lane 1=Saline-saline group, lane 2=Saline-morphine group, lane 3=Midazoalm-morphine group, lane 4=Saline-morphine-formalin group, lane 5=Midazolam-morphine-formalin group. Values in the *graphs* are the mean±SD of the fold change over sal-sal group (lane 1). The expression of nNOS and iNOS in sal-sal group is expressed as 100%. n=3~4 rats.*P<0.05 compared with lane 2, 3 and lane 5, $^{\#}P$<0.05 compared with lane 1.

spinal dorsal horn of rats in the present study. To better understand the involvement of NO in formalin-induced Fos protein expression in the rats spinal cord, we used the double-labeling staining method to determine whether Fos immunoreactivity co-localized with NADPH-d in the neurons of the spinal activated areas.

4.2. Morphine tolerance and latent sensitization of spinal cord neurons

Compared to non-tolerant rats, formalin-induced expression of Fos protein was greatly enhanced in morphine-tolerant rats. Furthermore, the deeper laminae of the ipsilateral spinal cord, which is the site of termination of large diameter non-nociceptive myelinated afferent, and the contralateral spinal cord, which does not receive noxious stimulation inputs, had also expressed a large number of Fos protein. These results suggested that latent sensitization of spinal cord neurons had developed in morphine tolerant rats. Noxious stimuli (injection formalin into hind paw or noxious thermal stimuli in present experiment) could unmask the latent sensitization of the spinal neurons and lead to more intense hyperalgesia and allodynia states in behavior. Pretreatment with midazolam suppressed thermal and formalin-induced hyperalgesia and decreased formalin-induced Fos protein expression in the spinal cord of morphine-tolerant rats, indicating that inhibition of latent sensitization of the spinal cord neurons played an important role in suppressing development of morphine analgesia tolerance by midazolam. It also indicated that the spinal cord is an important site for inhibiting morphine analgesia tolerance by midazolam.

4.3. NO and sensitization of spinal cord neurons

Formalin-induced noxious stimuli could make primary afferent terminals release nociceptive neurotransmitter as glutamate, substance P, which induces Fos protein expression in the ipsilateral superficial laminae of spinal dorsal horn. But how to interpret Fos protein expression in other laminae of the ipsilateral spinal cord and the contralateral spinal cord, which have not received noxious inputs? Numerous lines of evidence have shown that the unilateral noxious stimuli could produce the bilateral hyperalgesia in the animal model of bee venom or carrageenin-induced peripheral inflammation, sciatic inflammatory neuritis (SIN) and chronic constriction injury (CCI) of the sciatic nerve (Chen et al., 2000; Chacur et al., 2001; Radhakrishnan et al., 2003). The immune activation may indeed be a critical factor in the initiation and maintenance of the ipsilateral and contralateral (mirror-image) hyperalgesia (Schafer, 2003; Watkins et al., 2003). Furthermore, chronic morphine treatment led to glial activation and enhanced proinflammatory cytokines expression in rat spinal cord (Raghavendra et al., 2002). In spinal cord, immune-like glial cells (astrocytes and microglia), which could be activated in response to diverse noxious stimuli, released proinflammatory cytokines (TNF, IL-1, IL-6). Proinflammatory cytokines act in a paracrine fashion to affect cells far beyond their site of release and also can enhance the release of pain-related transmitters or substances (NO, prostaglandins, and excitatory amino acids) from primary afferent terminals. Glia form positive-feedback circuits whereby substances they release further activate these cells, creating perseverative responses. Glia-neuron interaction may contribute to the development of hyperalgesia (Watkins and Maier, 2002). In accord with previous reports, NO in the spinal cord participated in the development and maintenance of morphine tolerance and hyperalgesia (Mayer et al., 1999; Ozek et al., 2003; Watanabe et al., 2003; Heinzen and Pollack, 2004). In the present study, NADPH-d positive neurons, fibers and terminals were significantly increased in the superficial laminae of bilateral spinal dorsal horn and the gray matter surrounding central canal and the levels of nNOS and iNOS also were upregulated by formalin-induced noxious stimuli in morphine-tolerant rats. Importantly, Fos/NADPH-d double-labeled neurons were expressed in bilateral spinal cord. It suggested that NO played an important role in fomalin-induced Fos protein expression in the spinal cord, especially in the deeper laminae of ipsilateral spinal cord and contralateral spinal cord during morphine tolerance. We considered that the increasing expression of NOS by chronic morphine administration produced a background of latent NOS hyperactivation in the spinal cord neurons of morphine tolerant rats. Latent NOS hyperactivation could be revealed by noxious inputs and then resulted in NO burst. NO, through NO/cGMP pathway, sensitized the spinal cord neurons, induced Fos protein expression and created exaggerated pain states.

4.4. NO and the inhibitory effect of midazolam on morphine tolerance

Treatment with L-Arg or midazolam plus L-Arg had no significant effects on the baseline TFL and ED_{50} for morphine analgesia in non-tolerant mice. But intrathecally injection of L-Arg could reverse in part the inhibitory effect of midazolam on acute and chronic morphine tolerance. Moreover, coadministration of midazolam inhibited formalin-induced the increase of the expression of NADPH-d positive and Fos/NADPH-d double-labeled neurons, and decreased the levels of nNOS and iNOS in the spinal cord of morphine-tolerant rats. These data suggested that NO, at least in part, involved in the inhibitory effect of midazolam on morphine tolerance. Some previous studies also supported our conclusion. Galley et al reported that midazolam could directly inhibit the activity of nNOS in rat brain (Galley and Webster, 1996). The benzodiazepines-induced antinociception is intensified by coadministration with L-NAME and 7-NI, and is decreased by L-Arg in mice (Talarek

and Fidecka, 2002). Wilms reported that midazolam suppressed LPS-induced the release of NO and TNF-α from activated microglia in vitro (Wilms et al., 2003). There also are some controversial results about the effect of midazolam on the activity of NOS in rat brain. Tobin et al reported that some intravenous anesthetics including midazolam, not volatile agents, had no effect on NOS activity in rat cerebellum by the method of in vitro conversion of ^{14}C-arginine to ^{14}C-citrulline (Tobin et al., 1994). A subsequent study, however, found no effect of the volatile anesthetics on rat brain NOS activity using an identical technique (Rengasamy et al., 1997). With a modified method of measuring NOS activity, adding superoxide dismutase and catalase in their assay mixture, Galley et al demonstrated a significant decrease of NOS activity by intravenous anesthetics from brain tissue. They considered that superoxide dismutase removed superoxide, which inactivates nitric oxide, and catalase removes hydrogen peroxide. Purified brain NOS generates superoxide. The inclusion of these enzymes in the assay mixture prevents reduction of NOS activity (Galley and Webster, 1996). However, the present results could not interpret why midazolam only inhibited the Fos, NADPH-d and Fos/NADPH-d double-labeled neurons expression in the contralateral spinal cord, not in the ipsilateral. We speculated that the received different signal strength between the ipsilateral and contralateral spinal cord contributed to this phenomenon.

Earlier studies demonstrated that midazolam or diazolam, another benzodiazepine-receptor agonist, could inhibit morphine tolerance and dependence by reversing the changes of opioid peptides, such as met-enkephalin, β-endorphin and dynorphin$_{1\text{-}13}$, induced by chronic morphine treatment in brain and spinal cord (Sribanditmongkol et al., 1994; Tejwani et al., 1994; Rattan and Tejwani, 1996, 1997; Tejwani and Rattan, 1997). A lot of studies had shown that NO participated in modulating the release of opioid peptides in brain and spinal cord underlining many physiological and pathological processes (Xu and Tseng, 1993, 1994; Johnston and Morris, 1994; Xu et al., 1995; Hu et al., 1996; Tseng et al., 1997; Boyadjieva et al., 2003). It is possible that NO mediated the effect of midazolam on the levels of opioid peptides in brain and spinal cord in morphine tolerance and dependence rats.

In conclusion, the results of these studies confirm the inhibitory effect of midazolam on morphine-induced analgesia tolerance. Importantly, this study provides new information that endogenous NO in the spinal cord mediates the above effect.

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