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Formalin Pretreatment Attenuates Tail-Flick Inhibition Induced by β-Endorphin Administered Intracerebroventricularly or Intrathecally in Mice

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We examined the effect of the subcutaneous (s.c.) pretreatment of formalin into both hind paws of mice on the antinociception induced by the intracerebroventricularly (i.c.v.) or intrathecally (i.t.) administration of β-endorphin using the tail-flick test. Pretreatment with formalin (5%) for 5 h had no affect on the i.c.v. administered β-endorphin-induced tail-flick response. However, pretreatment with formalin for 40 h attenuated the tail-flick inhibition induced by i.c.v. administered β-endorphin. This antinociceptive tolerance to i.c.v. β-endorphin continued up to 1 week, but to a lesser extent. Pretreatment with formalin for 5 and 40 h significantly reduced the i.t. β-endorphin-induced inhibition of the tail-flick response, which continued up to 1 week. The s.c. formalin treatment increased the hypothalamic pro-opiomelanocortin (POMC) mRNA level at 2 h, but this returned to the basal level after 40 h. Our results suggest that the increase in the POMC mRNA level in the hypothalamus appears to be involved in the supraspinal or spinal β-endorphin-induced antinociceptive tolerance in formalin-induced inflammatory pain.

Key words: β-Endorphin, Pro-opiomelanocortin mRNA, Hypothalamus, Formalin, Antinociceptive tolerance

INTRODUCTION

Formalin subcutaneously (s.c.) injected into the hind paws of mice induces nociceptive behaviors, such as licking, shaking/flinching or biting of the injured hind paw, which has been used as a model of pain induced by tonic inflammation (Hunskaar and Hole, 1987; Wheeler-Aceto *et al.*, 1990). Opioids produce antinociception in the formalin test. For example, opioids administered systemically, supraspinally or spinally produce antinociception in the formalin test (Abdollahi *et al.*, 2000; Chung and Suh, 2001; Hammond *et al.*, 1998; Yang *et al.*, 2002). Furthermore, repeated administration or infusion of morphine causes the development of antinociceptive tolerance when the formalin test is used as an antinociceptive test (Connell *et al.*, 1994; Detweiler *et al.*, 1995). In addition, the endogenous opioid system appears to be involved in the regulation of antinociception, as revealed in the formalin test. For example, repeated exposure to forced walking stress produced antinociception in the formalin test. This forced walking stress-induced antinociception was also reversed by naloxone, an opioid receptor antagonist (Onodera *et al.*, 2000). Furthermore, Kamei *et al.* (1998) previously reported that spinal delta-opioid receptors were involved in the production of antinociception induced by cold-water swimming stress in the formalin test.

Formalin s.c. injected into the plantar surface of the hind paw causes changes in the endogenous opioid system. For example, Noguchi *et al.* (1989) and Crosby *et al.* (1994) demonstrated that a formalin injection increased the proenkephalin mRNA level in the spinal cord. In addition, the spinal prodynorphin mRNA expression was increased by formalin injection (Tanimoto *et al.*, 1998). Furthermore, a formalin injection increases the plasma β-endorphin level (Aloisi *et al.*, 1995). It can be speculated that the endogenous opioid system, when activated by formalin, may cause desensitization or tolerance against opioid-induced antinociception. In the present study; therefore, we investigated whether a s.c. injection of for-

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malin into the hind paw inhibited the production of antinociception induced by supraspinally or spinally administered β-endorphin in mice using the tail-flick test. Furthermore, to assess the possible involvement of pro-opiomelanocortin (POMC) in the regulation of nociceptive behaviors, the effects of formalin pretreatment on the POMC mRNA expressions in the hypothalamus and pituitary gland were also examined.

MATERIALS AND METHODS

Experimental animals

These experiments were approved by the University of Hallym Animal Care and Use Committee. All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals', published by the National Institutes of Health, and the ethical guidelines of the International Association for the Study of Pain. Male ICR mice (Daehan Lab., Korea), weighting 23-25 g, were used for all the experiments. Animals were housed 5 per cage, in a room maintained at 22 ± 0.5 °C, with an alternating 12 hour light-dark cycle; food and water were available ad libitum. Each animal was used only once. All the experiments were performed between 10:00 and 17:00 h.

Intracerebroventricular (i.c.v.) injections

Intracerebroventricular (i.c.v.) injections were made according to the procedure of Haley and McCormick. Intrathecal (i.t.) administration was performed following the method described by Hylden and Wilcox (Hylden and Wilcox, 1981; Hylden and Wilcox, 1980), using a 30-gauge needle connected by polyethylene tubing to a 25 μL Hamilton. The i.c.v. and i.t. injection volumes were both 5 μL, and the injection sites verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the ventricular space or spinal cord. The i.c.v. injected dye was found to be distributed throughout the ventricular spaces, reaching the ventral surface of the brain and upper cervical portion of the spinal cord. The i.t. injected dye was distributed both rostrally and caudally, but only over a short distance (about 0.5 cm from the injection site), with no dye found visually in the brain. The success rate for the injections prior to the experiments was consistently found to be over 95%.

Tail-flick test

Antinociception was determined by the tail-flick test (D'Amour and D.L. Smith, 1941). For measurement of the latency of the tail-flick response, mice were gently held by one hand, with the tail positioned in the apparatus (EMDIE Instrument Co., Maidens, VA., USA, Model TF6) for radiant heat stimulation. The tail-flick response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of the heat stimulus in the tail-flick test was adjusted so that the animal flicked its tail within 3 to 5 s. The tail-flick latency was measured before (T_0) and 30 min after (T_1) the injection of opioid agonists. The inhibition of the tail-flick response was expressed as the "percent maximal possible effect (% MPE)", which was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where the cut-off time (T_2) was set at 10 s.

Isolation of total RNA and Northern blot

Total cellular RNAs were extracted from pooled mice tissues (n=7/group), using rapid guanidine thiocyanate-water saturated phenol/chloroform extraction, with subsequent acidic sodium acetate precipitation (Chomczynski and Sacchi, 1987). Total cellular RNAs in the aqueous phase were precipitated with cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 and 280 nm.

The cRNA probes for POMC (Uhler and Herbert, 1983) and cyclophilin (Danielson *et al.*, 1988) were synthesized *in vitro* from linearized expression vectors, using DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim, Germany).

Ten microgram of total RNA were denatured, subjected to electrophoresis on 1% agarose-formaldehyde gels (Kopchick *et al.*, 1981) and transferred to nylon Hybond-*N* hybridization membrane sheets (Amersham, Buckinghamshire, England). After baking for 1-2 h at 80 °C, the membranes were prehybridized at 68 °C for at least 1 hr in prehybridization buffer (5 X SSC, 50% formamide, 0.02% SDS, 0.1% sodium *N*-lauroyl sarcosine and 2% blocking reagent). The Dig-labeled POMC probes were added to prehybridization buffer, containing 50% formamide. The membranes were incubated overnight at 68 °C in a shaking water bath, and washed twice, for 10 min per wash, in 2 X wash solution (2 X SSC, 0.1% SDS) at room temperature. The membranes were then washed twice, for 15 min per wash, in 0.1 X wash solution (0.1 X SSC and 0.1% SDS). After equilibrating in buffer I (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 1 min, the membranes were gently agitated in buffer II (1% blocking reagent in buffer I) for 30-60 min. The membranes were hybridized with anti-DIG-alkaline phosphatase, diluted 1:10,000 (75 mU/mL) in buffer II, for 30 min. After washing twice, for 15 min per wash, in 0.3% Tween 20 (in buffer I), the membranes were equilibrated in buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for 2 min. Approximately 0.5 mL (per 100 cm^2) CSPD (Boehringer Mannheim, Germany) was spread over the surface of the membrane. After incubation at 37 °C for 15-20 min, the membranes were exposed to Hyperfilm-MP (Amersham, Buckinghamshire, England) for detection of the chemilumi-

nescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterile Millipore water, then further washed, overnight, at 65 °C in 50 mM Tris-HCl, pH 8.0, 50% dimethylformamide and 1% SDS, to remove the hybridized probe, and then rehybridized to a Dig-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl *cis-trans* isomerase, which is constitutively expressed in most mammalian tissues, with the exception of skeletal muscle (D'Amour and Smith, 1941; Takahashi *et al.*, 1983).

Statistics

The data were presented as the mean ± S.E.M. Statistical analysis was carried out using the Student's *t*-test. P values less than 0.05 were considered statistically significant.

Drugs

The β-Endorphin was purchased from Peninsula Laboratory Inc. (Belmont, CA, USA). 5% of formalin (Sigma Chemical Co., St. Louis, MO, USA) was made by dissolving in sterile saline (0.9% NaCl solution).

RESULTS

In the saline-pretreated control mice, i.c.v. or i.t. administered β-endorphin (2 μg) produced inhibition of the tail-flick response. As shown in Fig. 1A; however, the s.c. treatment with formalin (5%) 5 h before the tail-flick test did not affect the i.c.v. administered β-endorphin-induced tail-flick response. Conversely, the i.t. administered β-endorphin-induced tail-flick response was inhibited by pretreatment with formalin 5 h prior to the tail flick test (Fig. 1B). The s.c. treatment with formalin 40 h prior to tail-flick test attenuated the tail-flick inhibition induced by i.c.v. or i.t administered β-endorphin. These antinociceptive effects to the i.c.v. or i.t. administered β-endorphin continued up to 1 week.

To examine the role of endogenous opioid peptide in the inhibition of β-endorphin-induced antinociceptive effects in the formalin-pretreated group, we examined the

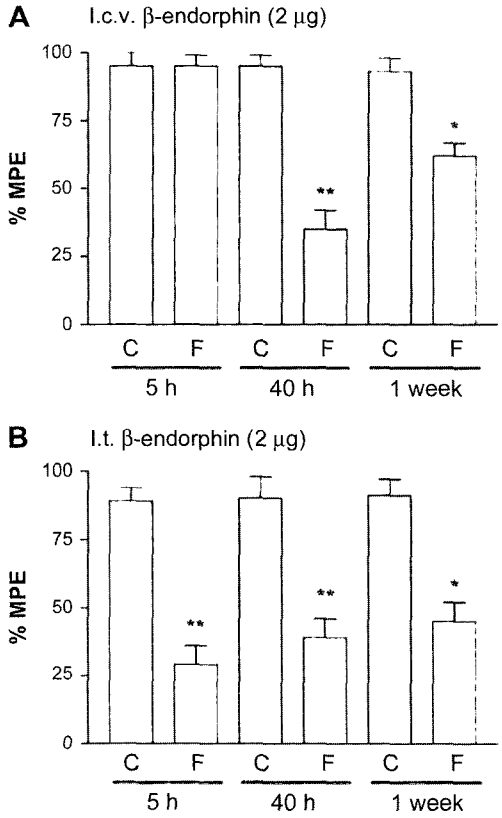

Fig. 1. Effect of formalin on intracerebroventricularly (i.c.v.; A) or intrathecally (i.t.; B) administered β-endorphin-induced antinociception. Mice were pretreated subcutaneously in both hind paws with saline (C; 10 μL) and formalin (F; 5%/10 μL) for 5 h, 40 h and 1week. The tail-flick response was measured 30 min after the β-endorphin (i.c.v.; 2 ug/ 5 μL) injection. The vertical bars indicated the standard error of the mean. N=10 mice for each group. *P<0.05, **P<0.01 compared to the control (C) group mice.

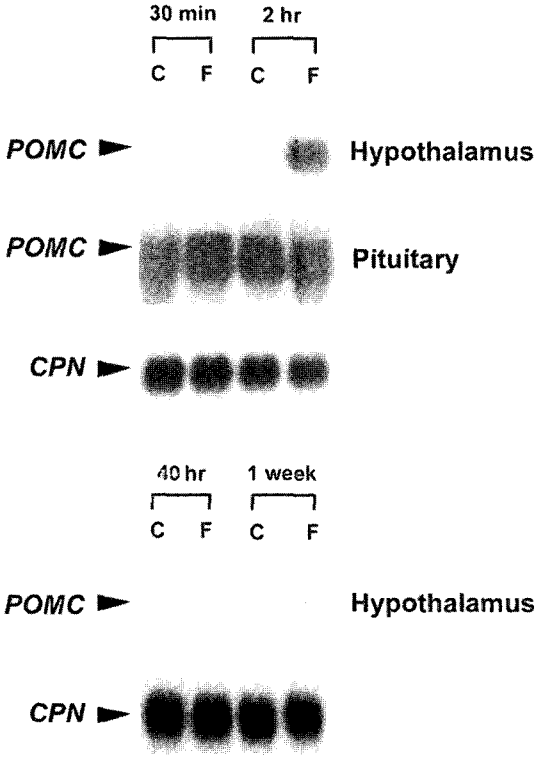

Fig. 2. Effect of formalin treatment on the pro-opiomelanocortin (POMC) mRNA expression in the hypothalamus and pituitary gland. Mice were treated subcutaneously in both hind paws with saline (C; 10 μL) and formalin (F; 5%/10 μL) for 30 min, 2 h and 40 h. Northern blot analyses for POMC mRNA were performed using 10 μg of total mRNAs extracted from the hypothalamus and pituitary gland of mice. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. N=7 per group.

expression of the POMC gene in the hypothalamus and pituitary gland. As revealed in Fig. 2, the s.c. administered formalin treatment did not affect the POMC mRNA level for up to 30 min, but increased the POMC mRNA level in the hypothalamus 2 h after treatment. The formalin-induced POMC mRNA level returned to the control level 40 h after formalin treatment in hypothalamus. However, the POMC mRNA level in the pituitary gland was unaltered by the formalin treatment.

DISCUSSION

In the present study, attenuation of the antinociceptive effect with supraspinally or spinally administered β-endorphin developed in formalin-pretreated mice when the tail-flick response was measured as an analgesic test. The attenuation of the antinociceptive effect of β-endorphin administration was maximal at 40 h after the s.c. formalin pretreatment. After 1 week, the diminution of the β-endorphin-induced antinociceptive effects began to disappear. Our findings indicate that persistent formalin-induced inflammatory pain may cause inhibition of the antinociceptive effects of supraspinally or spinally administered β-endorphin.

In the present study, we found that formalin treatment increased the POMC mRNA level in the hypothalamus, which the arcuate nucleus is located. However, the s.c. administration of formalin did not affect the POMC mRNA expression in the pituitary gland. The neurons containing β-endorphin in the hypothalamus are known to project to various regions, such as the periaqueductal gray (PAG), nucleus raphe magnus (RMg) or nucleus raphe obscurus (ROb) (Tseng, 2001). Furthermore, a microinjection of β-endorphin into these regions has shown a pronounced antinociceptive effect (Narita and Tseng, 1998). According to the secretion-transcription coupling mechanism, it can be speculated that β-endorphin is secreted into the PAG, RMg or ROb during persistent formalin-induced pain. Thus, it is suggested that one of the mechanisms underlying antinociceptive tolerance to supraspinally administered β-endorphin may, at least in part, be due to the continuous stimulation of opioid receptor induced by endogenous β-endorphin release into the PAG, RMg or ROb brain regions during persistent formalin-induced pain.

Previously, others, and ourselves, have demonstrated that supraspinally administered β-endorphin produces antinociception by activation of the spinal Met-enkephalin system (Suh and Tseng, 1990; Tseng, 1995). Thus, it can be speculated that continuous secretion of Met-enkephalin, which is derived from descending enkephalinergic neurons or spinal interneurons, at the spinal cord level, may cause tolerance development in supraspinally or spinally administered β-endorphin-induced antinociception. Although some studies have reported no observed changes in the proenkephalin mRNA expression in formalin-treated animals, Noguchi *et al.* (1989) and Crosby *et al.* (1994) demonstrated that formalin injections increased the proenkephalin mRNA level in the spinal cord. In addition, the spinal prodynorphin mRNA expression has been shown to be increased by formalin injections (Choi *et al.*, 2001; Tanimoto *et al.*, 1998; Zarrindast *et al.*, 2000). Thus, it is suggested that the endogenous spinal cord opioid system, when activated by formalin, may be responsible for the antinociceptive tolerance against spinally administered β-endorphin-induced antinociception.

In conclusion, our results suggest that the increase in the POMC mRNA level in the hypothalamus appears to be one of the major reasons for the development of antinociceptive tolerance of supraspinally or spinally administered β-endorphin in persistent formalin-induced inflammatory pain.

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