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Original Research

Effect of tetrahydropalmatine analogs on Fos expression induced by formalin-pain[1]

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KEY WORDS tetrahydropalmatine; tetrahydroprotoberberines; stepholidine; proto-oncogene proteins c-*fos*; formaldehyde; immunohistochemistry; quinpirole; spiperone

ABSTRACT

AIM: To study the effect of tetrahydropalmatine (THP) analogs on Fos protein expression induced by formalin-pain and elucidate analgesic mechanism of THP analogs. **METHODS**: The pain response to Sprague Dawley rats was induced with formalin injected sc into the plantar surface of the right hindpaw. Fos protein expression in brain and spinal cord was investigated with immunohistochemistry. The numbers of Fos-like immunoreactive (FLI) neurons were counted with Leica Q570 image analyzer. **RESULTS**: In the groups of THP analogs and D_2 antagonist spiperone, FLI neurons induced by intraperitoneal (ip) injection of THP analogs and spiperone were mainly located in the striatum and accumbens nucleus, and a few FLI neurons were also in sensorimotor cortex. In the D_1 antagonist, D_1 agonist, D_2 agonist, saline and vehicle groups, FLI neurons were seldom seen in the striatum and accumbens nucleus. Moreover, the Fos protein expression induced by *l*-THP and spiperone could be prevented by the pretreatment of the D_2 agonist quinpirole but not D_1 agonist SKF38393. In the formalin-pain group, FLI neurons were mainly distributed in ascending pain afferent system (APAS) and descending pain modulation system (DPMS). Following ip THP analogs, however, the numbers of FLI neurons induced by formalin-pain in the APAS, such as dorsal horn (mainly laminae Ⅰ, Ⅱ, Ⅳ - Ⅵ) were markedly decreased, while the numbers of FLI neurons in the DPMS, such as periaqueductal gray (PAG) and reticular paragigantocellular lateral nucleus (RPLN) were significantly increased. **CONCLUSION**: THP analogs enhanced the activity of brainstem DPMS by the blockade of D_2 receptors in the striatum and accumbens nucleus, and sequentially inhibited the inputs of peripheral pain afferent message in spinal cord level.

INTRODUCTION

Tetrahydropalmatine (THP) is the main active ingredient of the *Corydalis ambigua* Cham et Sch, a famous analgesic of Chinese traditional medicine. Its levo-enatiomer (*l*-THP) possesses the analgesic action with remarkable sedative tranquilizing effect[1]. *l*-THP is used as an remedy for analgesic or sedation listed in the Chinese Pharmacopoeia. The pharmacological action of *l*-THP has been cited in the textbooks of pharmacology in China. However, the analgesic mechanism of *l*-THP still remains unclear. Interestingly, *l*-THP has been verified as a dopamine (DA) receptor antagonist, and it has no affinity for opiate receptors[2-5].

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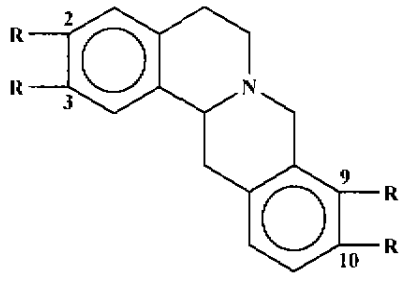
Moreover, we have found a lot of THP analogs, such as *l*-SPD, THB, and THPB-18. They belong to tetrahydroprotoberberines (THPB), sharing the common structure of isoquinoline ring and methoxyl groups or hydroxyl groups at position C_2, C_3, C_9, and C_{10}. *l*-Stepholidine (SPD), an alkaloid isolated from Chinese herb *Stephania intermedia* Lo, is a novel DA receptor antagonist. THPB-18, a synthetic compound, is also a potent antagonist of DA receptors. Although THP analogs have analgesic actions, the analgesic mechanism of them relevant to their DA receptor antagonistic effect is unclear too.



	2	3	9	10
THB	$O\text{-}CH_2\text{-}O$		OCH_3	OCH_3
THP	OCH_3	OCH_3	OCH_3	OCH_3
SPD	OH	OCH_3	OCH_3	OH

Tetrahydropalmatine analogs

Immediate early gene c-*fos* is a primary response gene which is characterized with the stimulus-mediated, rapid induction of expression in neuronal cells[6]. Fos protein, the product of c-*fos* gene, is the marker of neuronal activity and regulates the expression of target genes involved in cellular differential long-term plastic changes[7]. Fos protein can be rapidly expressed in relevant neurons following a peripheral noxious stimuli. It has well been established that formalin stimuli is an activator of c-*fos* gene in the ascending pain afferent system, and its patterns and intensities of Fos protein expression in the ascending pain afferent system can reflect directly the nature and intensity of the formalin-pain[8-9].

The present study was attempted to observe Fos protein expression in ascending pain afferent system (APAS) and descending pain modulation system(DPMS) by immunohistochemistry, and to investigate the effects of THP analogs on Fos protein expression induced by formalin-pain, so as to get the better knowledge of analgesic mechanism of THP analogs.

MATERIALS AND METHODS

Materials Sprague-Dawley rats (♂, 180 - 200 g) were supplied by Shanghai Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Certificate № 005). *dl*-THP (mp 218 - 220 ℃), *l*-THP (mp 141 - 142 ℃, $[\alpha]_D$ -289°), *l*-SPD (mp 161 - 162 ℃, $[\alpha]_D$ -440°), tetrahydroberberine (THB, mp 162 - 165 ℃), and THPB-18, isolated or synthesized by Shanghai Institute of Materia Medica, were dissolved in H_2SO_4 0.1 mol·L^{-1}, then diluted with NaOH 0.1 mol·L^{-1} and adjusted to pH 5.0. SKF38393, Sch23390, quinpirole, and spiperone were purchased from RBI (USA). Fos antibody and avidin-biotin-peroxidase complex immunostain system were purchased from Santa Cruz Biotechnology Inc (USA).

Formalin-pain Formalin-pain was induced with formalin (5 %, 50 μL) injected subcutaneously into the plantar surface of right hindpaw of rats.

Drugs All drugs were given by (ip) injection 10 min after formalin stimuli: *dl*-THP 60, *l*-THP 60, *l*-SPD 60, THB 60, THPB-18 60, SKF38393 3, Sch23390 3, quinpirole 2, and spiperone 2 mg·kg^{-1}. Quinpirole was injected 10 min before *l*-THP or spiperone. SKF38393 was injected 10 min before *l*-THP or spiperone.

Immunohistochemistry Two hours after the formalin stimulus or drug injection, the rats were killed under sodium pentobarbitone (40 mg·

kg^{-1}, ip) and then perfused intracardially for 25 min with a 4 % paraformaldehyde solution preceded by rapid saline flush. The lower lumbar portions (L_3-L_6) of the spinal cord and brain were fixed for 12 h in 20 % sucrose fixative, and sunk in 30 % sucrose at 4 ℃ overnight. The segments of spinal cord and brain were cut into 30-μm thick transverse sections in a cryostat. Sections were pre-incubated in 10 % normal goat serum for 1 h, and incubated at 4 ℃ for 48 h in Fos antibody at a dilution of 1∶1000 in phosphate buffer 0.01 $mol \cdot L^{-1}$ (pH 7.4) with 1 % goat serum and 0.3 % triton X-100. Sections were washed with phosphate buffer saline (PBS) 0.01 $mol \cdot L^{-1}$ and incubated at 37 ℃ for 1 h in biotinylated goat anti-rabbit immunoglobulin G solution (1∶200). Sections were washed with PBS and incubated at 37 ℃ for 1 h in avidin-biotin-peroxidase complex solution (1∶200). After 3-washes with PBS, the sections were washed with Tris-HCl buffer (pH 7.4). The immune product was stained with 0.03 % H_2O_2 in a solution containing 3,3′-diaminobenzidine (0.05 %). The sections were mounted, dehydrated, and coverslipped with neutral balsam.

Quantification and statistical analysis The numbers of Fos-like immunoreactive (FLI) neurons were counted with Leica Q570 image analyzer (Germany). Data ($\bar{x} \pm s$) were analyzed by ANOVA followed by Bonferroni t-test.

RESULTS

Fos protein expression induced by ip THP analogs, DA receptor agonists and antagonists Fos protein expression was induced by ip drugs as shown in Tab 1.

In the THP analogs (*dl*-THP, *l*-THP, *l*-SPD, THB, THPB-18), and D_2 antagonist spiperone groups, FLI neurons were mainly found in the striatum and accumbens nucleus, and there were also a few FLI neurons in the sensorimotor cortex (Fig 1).

Tab 1. Number of FLI neurons induced by ip THP analogs, DA receptor agonists and antagonists. $n = 3$ rats. $x \pm s$. $^cP < 0.01$ *vs* vehicle. $^dP > 0.05$, $^fP < 0.01$ *vs* saline. $^gP > 0.05$, $^iP < 0.01$ *vs* *l*-THP. $^jP > 0.05$, $^lP < 0.01$ *vs* spiperone.

Drug	Number of FLI neuron·mm^2	
	Striatum	Accumbens nucleus
dl-THP 60 $mg \cdot kg^{-1}$	432 ± 15^c	465 ± 22^c
l-THP 60 $mg \cdot kg^{-1}$	412 ± 17^c	424 ± 23^c
THB 60 $mg \cdot kg^{-1}$	155 ± 16^c	149 ± 19^c
l-SPD 60 $mg \cdot kg^{-1}$	245 ± 22^c	257 ± 19^c
THPB-18 60 $mg \cdot kg^{-1}$	365 ± 15^c	373 ± 16^c
Vehicle	9 ± 4	7 ± 3
SKF38393 3 $mg \cdot kg^{-1}$	15 ± 6^d	11 ± 5^d
Sch23390 3 $mg \cdot kg^{-1}$	11 ± 5^d	8 ± 4^d
Quinpirole 2 $mg \cdot kg^{-1}$	12 ± 7^d	9 ± 6^d
Spiperone 2 $mg \cdot kg^{-1}$	451 ± 21^f	481 ± 10^f
Saline	8 ± 5	6 ± 4
Quinpirole + *l*-THP	82 ± 24^i	79 ± 22^i
SKF38393 + *l*-THP	397 ± 17^g	386 ± 27^g
Quinpirole + spiperone	43 ± 12^l	46 ± 18^l
SKF38393 + spiperone	421 ± 28^j	441 ± 30^j

In the D_1 antagonist Sch23390, D_1 agonist SKF38393, D_2 agonist quinpirole, saline and vehicle groups, FLI neurons were seldom seen in the striatum and accumbens nucleus. The Fos protein expression induced by *l*-THP as well as D_2 antagonist spiperone, however, prevented by the pretreatment of the D_2 agonist quinpirole ($P < 0.01$, Tab 1, Fig 1), while their Fos protein expression was not affected by pretreatment of D_1 agonist SKF38393 ($P > 0.05$).

Fos protein expression induced by formalin-pain The experiments were performed in the groups of vehicle, formalin-pain, and saline. In formalin-pain group, FLI neurons were found in the dorsal horn of the lower lumbar cord (L_3-L_6), and mainly distributed in the superficial layers (laminae Ⅰ and Ⅱ) and deep layers (laminae Ⅳ – Ⅵ) on the side of the injected paw. FLI neurons were rarely found in

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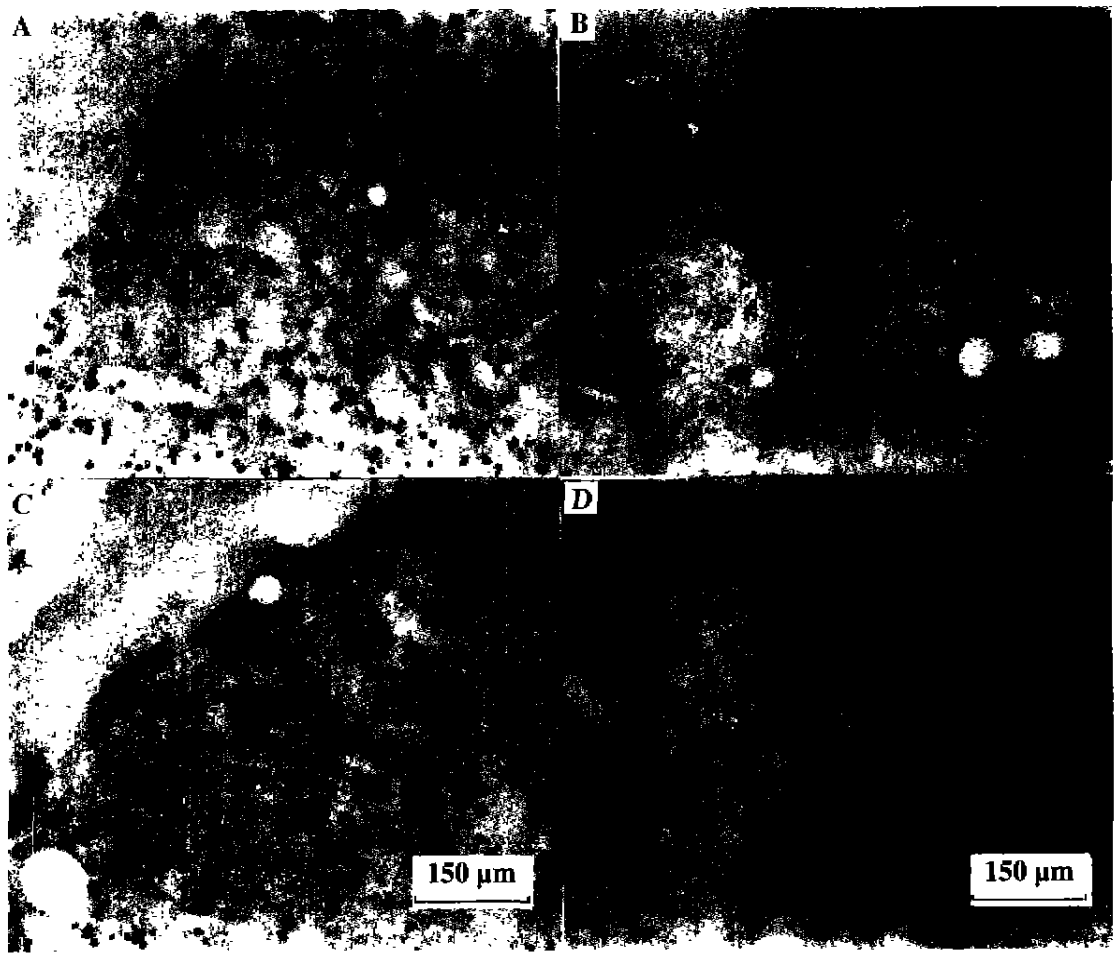

Fig 1. Fos-like immunoreactive neurons in striatum of rats after ip spiperone (A), quinpirole (B), *l*-THP (C), and quinpirole + *l*-THP (D). × 100.

spinal cord on the controllateral side to the injected paw. FLI neurons were still found in reticular paragigantocellular lateral nucleus (RPLN), periaqueductal gray (PAG), ventroposterior thalamic nucleus (VTN), sensorimotor cortex (SC), and a few in the arcuate nucleus (AN), periventricular hypothalamic nucleus (PHN), *etc* (Tab 2, Fig 2). In contrast, in the groups of vehicle and saline, FLI neuron was rarely found in the above mentioned spinal cord and brain areas.

Effect of ip THP analogs on Fos protein expression induced by formalin-pain The experiments were carried on the groups of pain,

Tab 2. Number of FLI neurons induced by formalin-pain. $n = 5$ rats. $\bar{x} \pm s$.
$^cP < 0.01$ *vs* vehicle or saline.

Brain regions	Formalin-pain	Vehicle	Saline
Laminae Ⅰ, Ⅱ	86 ± 6^c	6.2 ± 1.7	4 ± 4
Laminae Ⅳ – Ⅵ	123 ± 7^c	6 ± 3	4 ± 4
RPLN	64 ± 3^c	8 ± 3	3.3 ± 2.2
PAG	166 ± 9^c	10 ± 5	5 ± 3
VTN	128 ± 12^c	8 ± 7	4 ± 3
SC	143 ± 11^c	7 ± 5	5 ± 3
AN	36 ± 8^c	5 ± 4	2.9 ± 1.7
PHN	32 ± 7^c	4 ± 4	3.7 ± 2.1

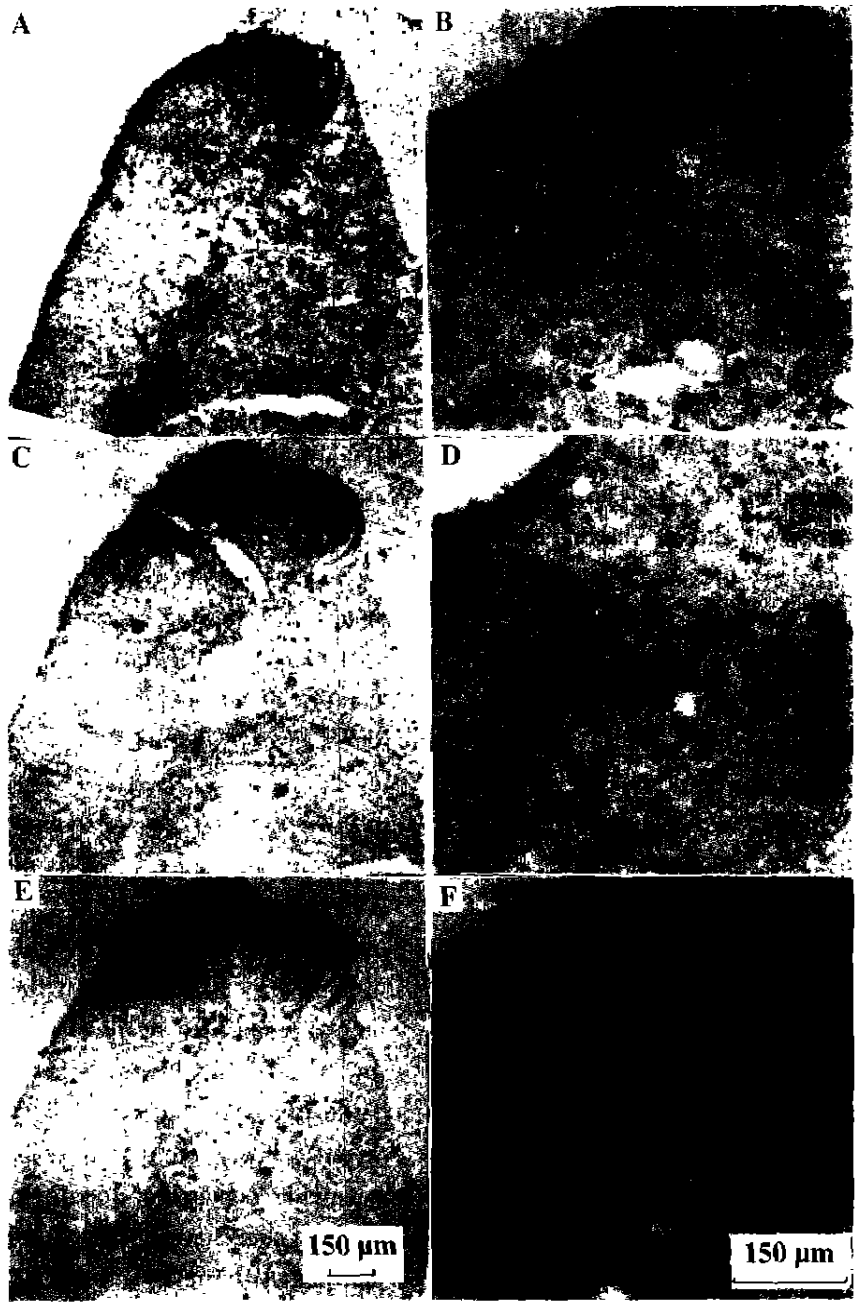

Fig 2. Effect of ip THP on c-*fos* expression induced by formalin-pain in dorsal horn (A – C, ×40) and PAG (D – F, ×100). A, D) formalin-pain; B, E) formalin-pain + *dl*-THP; C, F) formalin-pain + *l*-THP.

pain + *dl*-THP, pain + *l*-THP, pain + *l*-SPD, pain + THB, pain + THPB-18 and pain + vehicle. In the pain + THP analogs groups, although the distribution pattern of FLI neurons in the brain areas and spinal cord was similar to that seen in pain group (Fig 2), the densities of FLI neurons were found attractive changes in the brain areas of RPLN, ventrolateral part of PAG (VL – PAG), and dorsal horn of spinal cord.

After ip THP analogs, the numbers of FLI

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neuron induced by formalin-pain were decreased in the dorsal horn (mainly laminae Ⅰ, Ⅱ, Ⅳ - Ⅵ, $P < 0.05$ or 0.01), while the numbers of FLI neurons in the RPLN and VL-PAG of pain + *dl*-THP, pain + *l*-THP, pain + *l*-SPD, pain + THPB-18 groups were increased against the pain group ($P < 0.05$ or 0.01, Tab 3, Fig 2). In the pain + vehicle group, vehicle did not affect Fos protein expression induced by formalin-pain ($P > 0.05$).

DISCUSSION

The formalin-pain test is considered as a good model for tonic or chronic inflammatory pain in clinic [10]. It has become a popular useful experimental test for evaluating analgesics in general[11]. Fos protein expression has widely been used to investigate the nervous pathway or neuronal activity since it was established in 1987[8]. In the present study, Fos protein was used as a marker of neuronal activity to characterize the DA antagonistic effect of THP analogs on pain processing. Thus, it is possible to find out the relevance between analgesic effect of THP analogs and their DA antagonistic effect.

In the present study, the results of Fos protein expression induced by DA receptor agonists and antagonists showed that FLI neurons induced by THP as well as D_2 antagonist spiperone were mainly located in the striatum and accumbens nucleus, while FLI neurons were seldom seen in the same nucleus by Sch23390, SKF38393, and quinpirole which had no analgesic effect. Similarly, THP analogs also induced Fos protein expression in the striatum and accumbens nucleus. Furthermore, Fos protein expression induced by *l*-THP as well as D_2 antagonist spiperone could be prevented by the pretreatment of the D_2 agonist quinpirole but not D_1 agonist SKF38393. These results suggested that THP analogs acted as D_2 receptor antagonists to induce the Fos protein expression by their blockage of D_2 receptors. Thus, it is presumed that the analgesic action of THP analogs mainly resulted from the blockage of D_2 receptors in the striatum and accumbens nucleus.

The present study still showed that FLI neurons following formalin-pain were mainly distributed in the typical APAS, including the superficial layers (laminae Ⅰ and Ⅱ) and the deep layers (laminae Ⅳ - Ⅵ) of the spinal cord, *ventroposterior thalamic nucleus*, periventricular hypothalamic nucleus and sensorimotor cortex, and also distributed in DPMS, including PAG and RPLN. The distribution was consistent with previous report[12]. Fos protein in the superficial layers is expressed as a result of postsynaptic activation by C-afferent fibers innervating the plantar surface of the hindpaw[13], while in the deep layers, Fos protein expression is the result of receiving convergent inputs from the superficial layers, suggesting that Fos protein expression in the dorsal horn is closely related to inputs

Tab 3. Effects of ip THP analogs on number of FLI neuron induced by formalin pain. $n = 5$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* pain.

Brain regions	Pain	Pain + vehicle	Pain + *dl*-THP	Pain + *l*-THP	Pain + THB	Pain + *l*-SPD	Pain + THPB-18
Laminae Ⅰ, Ⅱ	86 ± 6	87 ± 3^a	48 ± 5^c	62 ± 3^c	83 ± 4	66 ± 4^c	79.2 ± 2.5
Laminae Ⅳ - Ⅵ	123 ± 7	124 ± 8^a	54 ± 4^c	65 ± 4^c	73 ± 3^c	73 ± 8^c	67 ± 6^c
RPLN	64.2 ± 2.6	63 ± 5^a	82 ± 5^c	83 ± 6^c	70 ± 4	78 ± 5^b	79 ± 5^b
VL-PGA	116 ± 9	115 ± 11^a	144 ± 7^c	137 ± 8^b	117 ± 12	131 ± 16	139 ± 11^b

of peripheral pain messages. The reduction of Fos protein expression in the dorsal horn reflects a reduction of peripheral pain afferent message. After ip injected THP analogs, a reduction of Fos protein expression in the dorsal horn of formalin stimulus rats was apparent. It implied that THP analogs could inhibit the inputs of peripheral pain afferent message in spinal cord level. Neurons in laminae Ⅰ and in the neck of the spinal cord (laminae Ⅳ – Ⅵ) are the projecting neurons to the brainstem and thalamic targets[14], which transmit pain message. So, the reduction of Fos protein expression by THP analogs in the dorsal horn also demonstrated that THP analogs could block the ascending transmission of pain message. However, THP analogs significantly increased the numbers of FLI neurons in the VL-PAG and RPLN in the DPMS of formalin stimulus rats, and these opposite effects could be reasonably understood by the analgesic mechanism of THP analogs that they could enhance the activity of brainstem descending pain modulation system (endogenous pain inhibitory system), which powerfully inhibits the responses of dorsal horn to peripheral pain afferent message mainly going through the PAG-RPLN-dorsal horn pathway[14], and sequently blocks the pain message transmission at the spinal cord level, and finally exerts the analgesic effect.

In conclusion, THP analogs enhanced activity of DPMS, such as PAG and RPLN, mediated via the blockade of D_2 receptor in the striatum and accumbens nucleus, and sequentially inhibited the inputs of peripheral pain afferent message in spinal cord level; and finally exerted the analgesic effect.

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四氢巴马汀同类物对福尔马林致痛诱导 Fos 蛋白表达的影响

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关键词 四氢巴马汀；四氢原小檗碱类；千金藤立定；原癌基因蛋白 c-*fos*；甲醛；免疫组织化学；喹吡罗；螺哌隆

目的：研究四氢巴马汀（THP）同类物对福尔马林致痛诱导的 Fos 蛋白表达的影响，以阐明 THP 同类物的镇痛机制。**方法**：在右后肢脚掌皮下注射 5 %福尔马林 50 μL，诱发炎性疼痛，用免疫组织化学方法观察 Fos 蛋白表达。**结果**：腹腔注射 THP 同类物和 D_2 受体拮抗剂螺哌隆诱导的 Fos 蛋白表达主要位于纹状体和伏膈核。D_2 受体激动剂喹吡罗可阻滞 *l*-THP 和螺哌隆诱导的 Fos 蛋白表达。THP 同类物明显增加脑干下行痛觉调制系统的 Fos 蛋白表达，并能明显抑制福尔马林诱导的脊髓背角浅层和深层的 Fos 蛋白表达。**结论**：THP 同类物通过阻滞纹状体和伏膈核的 D_2 受体，加强脑干下行痛觉调制系统的功能，抑制外周痛觉信息在脊髓水平的传入，达到它们的镇痛作用。

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