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Topical capsaicin treatment suppresses formalin-induced *fos* expression in rat spinal cord[1]

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ABSTRACT Capsaicin (Cap) is a pharmacological tool to inactivate nociceptive afferents. The present study was undertaken to investigate the effects of topical application of Cap to sciatic nerve on the formalin-induced expression of proto-oncogene proteins c-*fos* in the rat spinal cord using immunohistochemical display of *fos*-like protein. In rats subjected to formalin injection into the hind paw, numerous *fos*-like immunoreactivity (FLI) neurons were found in the spinal dorsal horn, with heavy labeling in laminae I—II and V—VI. Following pretreatment of Cap, formalin-induced FLI expression was significantly abolished. It was suggested that activation of Cap-sensitive unmyelinated nociceptive afferents following formalin injection was primarily responsible for the activation of c-*fos* gene. Our data also provided further evidence supporting that topical application was an effective way to block the transmission of noxious primary afferents.

KEY WORDS capsaicin; proto-oncogene proteins c-*fos*; formaldehyde; spinal cord; immunohistochemistry

Capsaicin (Cap) (8-methyl-*N*-vanilyl-6-nonenamide), a constitute of red peppers, is a neurotoxin that appears to affect selectively primary nociceptive afferents (C-fibers).

The topical application of Cap solution onto a peripheral nerve in adult animals led to (a) the depletion of substance P and other neuropeptides from somatic and visceral afferent nerves and (b) a reduction in response to noxious stimulation[1-4]. Locally applied Cap results in the conduction block of C-fibers[3,4].

The expression of proto-oncogene c-*fos* can be regarded as a marker for neuronal activity following noxious stimulation[5,6]. It would be meaningful to examine the effects of Cap treatment on c-*fos* expression induced by noxious stimulus. It has been shown that following noxious stimuli, the expression of c-*fos* gene was transient starting at 30 min, reaching climax after 1—2 h, and declining to basal level after 24 h[7,8]. In the present study, the effect of Cap treatment on the c-*fos* expression in the rat spinal cord in response to formalin injection in the rat paw were investigated using immunohistochemical detection of *fos* protein, a protein product of c-*fos* protooncogene.

MATERIALS AND METHODS

Experiments were performed on 18 female Wistar rats weighing 219 ± *s* 19 g (Animal Center of Beijing Medical University). As c-*fos* expression is sensitive to sensory stimuli, rats were handled as gently as possible.

Rats were divided into 6 groups: A) normal control group received no treatment or specific stimuli, B) saline control group, C) vehicle control group with vehicle of 20% Tween 80 in paraffin, D) Cap control group, E) formalin-vehicle group, and F) formalin-Cap group. Prior to surgical operation, rats were anaesthetized with chloral hydrate 300 $mg \cdot kg^{-1}$, ip. One femoral nerve was severed so that sensory stimuli from that hind limb were transmitted predominantly via the sciatic nerve. The sciatic nerve in the ipsilateral side was then isolated. For Cap treatment, a small piece of cotton wool of 3 cm long was soaked in 0.05 ml of 1% Cap (Sigma) solution in the vehicle (20% Tween 80 in paraffin), and were gently wrapped around an 1-cm-long nerve. In saline control and

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vehicle control rats, Cap was replaced by saline and vehicle, respectively.

After 48 h the effect of Cap was examined by nociceptive test on the hindpaw. The rat was put in a holder with 2 hindlegs sticking out. Noxious radiant heat (a light of 5 mm in diameter) was focused on the plantar surface of the hind paw and the latency of the withdrawing reaction was determined. The latency of the vehicle control rats was 4.4±1.4 s. In Cap treated rats, the latencies were over 15 s, which was set as the cut-off level, illustrating a significant reduction in response to noxious stimulation. In groups E and F, 0.15 ml of 5% formalin was injected subcutaneously into the planta of the hindpaw. After 2 h, rats were deeply anesthetized with sodium pentobarbital 70 $mg \cdot kg^{-1}$, ip. The rats were perfused transcardially with 100 ml saline followed oy 200 ml 4% paraformaldehyde PB solution. The removed lumbar enlargements of spinal cord were postfixed in the same fixative for 8—10 h, and then transferred to a 30% sucrose solution at 4 C until the sample sank down to the bottom of the container.

For immunohistochemistry, tissue sections were cut coronally in a cryostat at 40 um. Free-floated sections were processed according to the ABC method[11] using a polyclonal *fos* antibody (Oncogene Science, Inc, USA) and ABC Kit (Vecror Labs, USA). The sections were preincubated in 0.5% Triton X-100 for 10 min and rhen in 1.5% goat serum for 1 h at 22 C. The sections were incubated for 48 h at 4 C in the primary antibody, and for 1 h at 37 C in the secondary antibody and ABC Kit. The sections were incubated with 0.05% DAB/0.001% H_2O_2/acetate buffer 0.1 $mol \cdot L^{-1}$ containing 2% ammonium nickel sulfate for 2—5 min, and rhen rinsed in acetate buffer, mounted onto slides, dehydrated, and coverslipped.

Immunohistochemically stained tissue sections were examined under light microscope (100×). Neurons with stained nuclei were plotted on camera lucida drawings and counted. Three sections with the greatest number of labeled cells at L5 level were selected from each rat. The average number of plotted cells in these 3 sections was recorded as the number of *fos*-like immunoreactivity (FLI) neurons in the subregion in that rat. All data were analyzed by multifactorial ANOVA.

RESULTS

The nuclei of immunoreactive cells appeared as dark, round to oval structure, leaving the nucleoli unlabeled. The spinal dorsal horn was divided into 3 subregions, the superficial layers (laminae I—II), the nucleus proprius (laminae III—IV), and the neck (laminae V—VI)[12,13]. In normal controls, the level of FLI expression was extremely low. In the saline and vehicle control groups, no significant increase in FLI expression was seen in each subregion. In Cap control group, there was a moderate (58—70%) increase in *fos* expression, which may result from the release of substance P and other neuropeptides/neurotransmitters at the central terminals of the primary afferents in the dorsal horn. In rats subjected to formalin injection, a large number of FLI cells were seen in the dorsal spinal cord with ipsilateral predominance with heavier labeling in laminae I — II and V — VI, which were 21 and 8.4 fold that of the normal

Tab 1. Counts of *fos*-like immunoreactive neurons in laminae I—II, II—IV, and V—VI in dorsal horn of spinal cord. $n=3$ **rats,** $\bar{x}\pm s$. [a] $P>0.05$ *vs* **normal control group.** [c] $P<0.01$ *vs* **every control group.** [f] $P<0.01$ *vs* **formalin-vehicle group.**

	Laminae I—II	Laminae III—IV	Laminae V—VI
Normal control	4.3±0.3	8.5±6.6	9.8±8.3
Saline control	7.4±1.9[a]	12.2±4.0[a]	12.6±5.7[a]
Vehicle control	4.7±1.2[a]	11.9±3.6[a]	11.0±1.2[a]
Capsaicin control	6.8±2.3[a]	13.6±1.6[a]	16.7±5.7[a]
Formalin-vehicle	91.1±15.8[c] (100%)	47.9±7.1[c] (100%)	82.0±15.4[c] (100%)
Formalin-capsaicin	14.9±6.8[f] (16.4%)	24.1±5.2[f] (50.3%)	23.6±13.9[f] (28.8%)

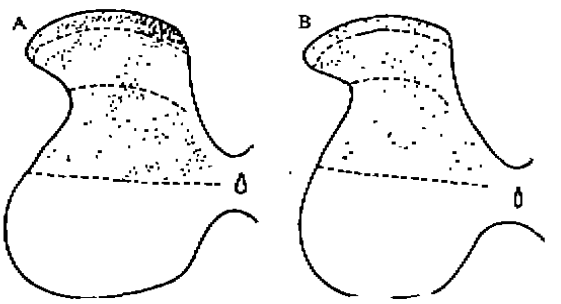

Fig 1. Camera lucida drawings showing the distribution of *fos*-like immunoreactive neurons in laminae I—II, III—IV, and VI—VI of a rat spinal cord. A) formalin-vehicle; B) Formalin-capsaicin. Each dot represented one labeled neuron.

control group, respectively. The FLI expression in laminae III — IV was comparatively low, which was 5.6 fold that of the normal control (Tab 1, Fig 1A, 2A, Plate 1).

In rats receiving Cap pretreatment prior to formalin injection, formalin-induced expression of *fos*-like immunoreactive neurons was significantly suppressed by capsaicin treatment as compared with formalin vehicle group. The expression of FLI in laminae I—II, III—IV, and V — VI was suppressed by 83.6%, 49.7%, and 71.2%, respectively ($P<0.01$, Tab 1).

DISCUSSION

In order to avoid the influence induced by surgical operation and nerve manipulation, we injected formalin 48 h after the Cap treatment. The animals were perfused 2 h after formalin injection when *fos* expression may increase to its maximum.

Subcutaneous formalin injection into the paw has been considered as a useful pain model. This stimulus produced a stereotyped behavioral syndrome thought to be indicative of pain. Rats may lick the paw injected, though usually do not vocalize. Formalin stimulus induced a lasting, opiate sensitive excitation of dorsal horn nociceptive neurons, the time course of which paralleled with that of the formalin behavioral syndrome[11,12]. It was shown that noxious thermal, chemical and mechanical cutaneous stimuli evoked *fos* expression in neurons located predominately in laminae I—II, and V—VI[5,6,7], where the majority of nociceptive primary afferents terminate, and where dorsal horn nociceptive neurons predominate[13,14]. Our results of formalin study were in line with these findings[5,6,7,13].

What was new in the pressent study was that formalin induced protooncogene proteins c-*fos* expression could be suppressed by Cap treatment to the sciatic nerve, implying that *fos* expression in the spinal dorsal horn following formalin stimulus depended to a large extent on the input from Cap-sensitive primary afferents. In other words, Cap-sensitive unmyelinated nociceptive afferents were primarily responsible for formalin induced activation of the cellular immediate early gene c-*fos* in the rat dorsal horn neurons.

Cap has been used to inactivate C afferents either by neonatal peripheral injection or by topical application on peripheral nerve in adult animals. The latter had some advantages such as safety (never fatal, because of low dose), easy comparison between pre- and post-treatment conditions and between the treated and the control sides in the same animal.

Expression of *fos* in dorsal horn neurons was a sensitive parameter for noxious input. Cap treatment produced a profound suppression of formalin-induced *fos* expression in laminae I—II (—83.6%) where nocispecific neurons resided and laminae V—VI (—71.2%) where wide-dynamic neurons aggregated, and a milder suppression in laminae III—IV (—43.95%) in which the large myelinated afferent fibers ended. The results were compat-

ible to the notion that C-fiber conduction was more seriously affected by topical application of Cap. These findings fitted very well with the results of behavioral observation that the latency of noxious heat induced withdrawal was increased to a cutoff limit after Cap treatment on the sciatic nerve.

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局部辣椒素处理抑制福尔马林诱导的脊髓 *fos* 表达

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摘要 辣椒素是一种阻断伤害性传入(C-纤维)的工具药. 利用免疫组织化学技术，本工作发现1%辣椒素包埋坐骨神经可抑制福尔马林（5%，150 μl，后肢脚掌注射）诱导的脊髓背角 I—II，III—IV，V—VI 层 *fos* 蛋白表达($P<0.01$). 本结果提示 C-纤维的激活参与 c-*fos* 基因的诱导，同时也说明外周辣椒素处理是阻断伤害性初级传入的有效途径.

关键词 辣椒素；原癌基因蛋白 c-*fos*；甲醛；脊髓；免疫组织化学