ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8): 737 - 740
http://www.simm.ac.cn; www.chinainfo.gov.cn periodical

Intrathecal injection of corticotropin inhibited nitric-oxide synthase-positive neuron increase in rat spinal cord after formalin-induced hyperalgesia

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KEY WORDS corticotropin; nitric-oxide synthase; spinal cord; hyperalgesia; formaldehyde; pain measurement; histocytochemistry; proto-oncogene proteins c-*fos*; immunohistochemistry; neurons

ABSTRACT

AIM: To study the effects of corticotropin (Cor) on formalin-induced hyperalgesia and the change of nitric-oxide synthase (NOS)-positive neurons in spinal dorsal horn in rats. **METHODS**: Measurement of pain intensity rating (PIR). NADPH-d histochemistry, and Fos immunohistochemistry were adopted. **RESULTS**: The increases of NOS-positive neurons, Fos, NOS/Fos double labelling neurons of the spinal dorsal horn and the PIR after formalin injection were markedly inhibited by intrathecal injecting (ith) Cor (0.5 - 1.5 U), which were obviously attenuated by *L*-arginine (Arg, 5 - 15 nmol, ith), the substrate of NOS. **CONCLUSION**: Cor inhibits formalin-induced hyperalgesia by the decrease of NOS-positive neurons in the spinal dorsal horn of rats.

INTRODUCTION

The corticotropin (Cor) plays a role in nociceptive modulation in central nervous system in addition to its endocrine effects. Antinociceptive effects have been observed when injections were confined to the periaqueductal gray area[1] and hippocampus[2]. Cor administrated directly onto the spinal cord produced the inhibition of nociceptive discharges of neurons in dorsal horn of rats[3]. Recent reports have begun to define the role of nitric oxide (NO) in spinal nociceptive processing[4]. The NO inhibitor, nitro-*L*-arginine methyl ester (*L*-NAME) reduced the For-induced nociceptive discharges of dorsal horn neurons[5]. But it was not clear whether Cor could affect NO production to depress centrally induced hyperalgesia. The present study was designed to observe the effects of intrathecal injecting (ith) Cor on formalin-induced nitric-oxide synthase (NOS), Fos, NOS/Fos-positive neurons and hyperalgesia.

MATERIALS AND METHODS

Rats Wistar rats ($n = 98$, weighing 180 - 230 g, Grade Ⅱ, Certificate № 24301050) of either sex were provided by the Experimental Animal Center of Third Military Medical University. Rats were implanted with retained intrathecal catheters[6].

Drug and reagents Cor (Tianjin Biochemistry Pharmaceutical Factory), nicotinamide adenine dinucleotide phosphate (β-NADPH, Boehringer Mannheim GmbH), Fos antiserum (Cambridge Research Biochemicals), Arg (Shanghai Kangda Amino Acid Factory), Nitroblue tetrazolium (NBT, Shanghai Qianjin Chemical Factory), ABC reagent kit (Vector Labs), 3,3'-diaminobenzidine (DAB, Sigma).

Histochemistry and immunohistochemistry After being anesthetized with intraperitoneal injection of sodium pentobarbital 65 mg · kg^{-1}, the rats were perfused transcardially with phosphate-buffered saline (PBS) 0.1 mol · L^{-1}, pH 7.2, followed by 4 % paraformaldehyde. The spinal cord was removed and placed in 30 % sucrose for 24 h prior to sectioning. Transverse frozen sections (40 μm) of the lumbar

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Received 1998-09-10 Accepted 1999-02-10

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8)
E-mail aps@server shcnc.ac.cn Phn/Fax 86-21-6474-2629

spinal cord were cut and every third section was collected in PBS 0.1 mol·L^{-1}, pH 7.2. They were incubated in PB (0.1 mol·L^{-1}, pH 7.4) containing β-NADPH 1.2 mmol·L^{-1}, NBT 1 mmol·L^{-1}, and 0.3 % Triton X-100, at 37 ℃ for 1 h. After incubation, the sections were rinsed in PBS, put on gelatin-coated glass slides, dried in air, dehydrated, and coverslipped for examination under a light microscope. Meanwhile, the sections were processed for Fos and NOS/Fos double labelling[2]. For each rat the mean number of cell counts in superficial lamina (Ⅰ - Ⅲ) of spinal cord was calculated by averaging the number of stained cells from 5 to 8 maximally stained sections. The measurement of pain intensity rating (PIR) was done[7].

Statistic analysis Data were expressed as $\bar{x} \pm s$ and analyzed by t-test and correlation.

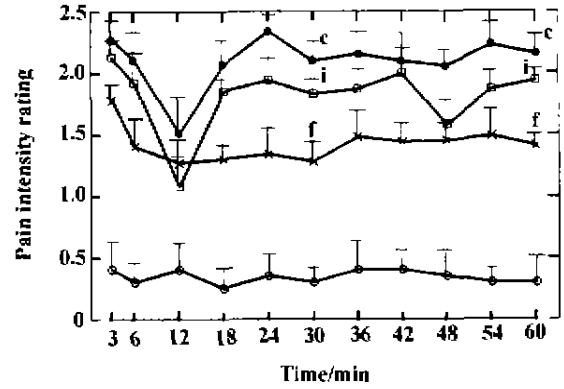

Fig 1. Effect of intrathecal injection of Cor and Cor + Arg on For-induced pain response. (○) Control, (●) For, (×) Cor 1.0 U + For, (□) Cor 1.0 U + Arg 10 nmol + For. $n = 6$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* For. $^iP < 0.01$ *vs* Cor 1.0 U + For.

RESULTS

For induced NOS-positive neurons increasing and hyperalgesia In tissue from control rats (not injected with For), NOS-positive neurons were concentrated in the superficial dorsal horn (lamina Ⅰ - Ⅲ). NOS-stained cell bodies appeared round and small. They had relatively large nuclei and a narrow ring of cytoplasm. The NOS-positive neurons were increased in the lamina Ⅰ - Ⅲ of ipsilateral dorsal horn at 10, 30, 60, 120 min following the subcutaneous injection of For into one hindpaw of rats. The maximal increase of NOS-positive neurons occurred at 30 min after For injection. Meanwhile, the PIR was markedly enhanced by For injection as compared with control rats. (Tab 1, Fig 1).

Effects of ith Cor on For-induced NOS-positive neurons and hyperalgesia Seven groups of rats were injected ith with 0.9 % saline, Cor (0.5, 1.0, 1.5 U), Cor (1.0 U) + Arg (5, 10, 15 nmol) 15 min before For injection. (Tab 2)

Tab 1. NOS-positive neurons in superficial laminae of rat spinal cord induced by formalin. $n = 6$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Groups		Number of NOS-positive neurons
Control		20 ± 2
Formalin	10 min	30 ± 2^c
	30 min	37 ± 2^c
	60 min	31 ± 2^c
	120 min	24 ± 1^c

Tab 2. Effect of intrathecal injection (ith) of corticotropin (Cor) and Cor + *L*-Arginine (Arg) on For-induced NOS-positive neurons in the superficial laminae of rat spinal cord. $n = 6$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* For + saline. $^fP < 0.01$ *vs* Cor 1.0 U + For.

Groups	Number of NOS-positive neurons
Saline + For	37 ± 1
Cor 0.5 U + For	28 ± 1^c
Cor 1.0 U + For	19 ± 1^c
Cor 1.5 U + For	17 ± 2^c
Cor 1.0 U + For + Arg 5 nmol	24 ± 2^f
Cor 1.0 U + For + Arg 10 nmol	30 ± 2^f
Cor 1.0 U + For + Arg 15 nmol	31 ± 3^f

The number of NOS-positive neurons and PIR had no obvious change in 0.9 % saline group. Cor (0.5 - 1.5, ith) produced a dose-related inhibition of For-induced NOS-positive neurons increasing in the ipsilateral superficial dorsal horn at 30 min following For injection. The PIR was markedly decreased by the pretreatment with Cor (1.0 U, ith). But Cor ith

alone had few effect on the number of NOS-positive neurons in rats not injected with For ($n = 6$, 19.6 ± 1.4). The inhibitory actions of Cor (1.0 U, ith) on the increase of For-induced NOS-positive neurons and PIR were obviously attenuated by coadministration of the substrate of NOS, Arg (5, 10, 15 nmol, ith). (Tab 2, Fig 1).

Effects of ith Cor on For-induced Fos and NOS/Fos double-labelling neurons Rats were given ith injection with 0.9 % saline 20 μL, Cor 1.0 U, and Cor 1.0 U + Arg 10 nmol at 15 min before For injection into one hindpaw. In 0.9 % saline group, the Fos-positive neurons showed marked increases in the ipsilateral superficial dorsal horn at 90 min following For injection, as compared with rats not injected with For. Cor (1.0 U, ith) produced marked decrease of Fos-positive neurons induced by For injection into one hindpaw of rat. The inhibitory effect of Cor on Fos-positive neurons was partly attenuated when Cor (1.0 U) was injected ith together with Arg 10 nmol. Between treatment groups, a significant positive relationship existed for ranks of NOS-positive neurons and ranks of Fos-positive neurons ($r = 0.7542$, $P < 0.01$). The increase of NOS-positive neurons exhibited a greater number of Fos-positive neurons. Double staining revealed colocalization of NOS with Fos, and they showed the similar change as NOS- or Fos-positive neurons in control, Cor, and Cor + Arg groups. (Tab 3).

Tab 3. Effects of ith Cor on formalin-induced Fos-, NOS/Fos-positive neurons in the superficial laminae of rat spinal cord. $n = 6$ **rats.** $\bar{x} \pm s$.
[c] $P < 0.01$ *vs* **saline + For.** [f] $P < 0.01$ *vs* **Cor 1.0 U + For.**

Groups	Fos-positive neurons	NOS/Fos-positive neurons
Saline + For	41 ± 3	5.8 ± 0.8
Cor 1 U + For	29 ± 4[c]	1.7 ± 0.4[c]
Cor 1 U + For + Arg 10 nmol	38 ± 3[f]	5.3 ± 1.0[f]

DISCUSSION

Our results showed that Cor (ith) produced a dose-related decrease of For-induced Nos-positive neurons in the superficial dorsal horn and For nociceptive behaviors (hyperalgesia), which was attenuated by pretreatment with the substrate of NO synthase, Arg. This hints that Cor might inhibit the For-induced activation of NOS in the superficial dorsal horn. We also found that the increase of NOS-positive neurons occurred at 10 min and maximum appeared at 30 min after For injection, which was kept the level above that of the control and significant at 2 h. This profile of neuronal change was very similar to that of behavior observed in rats after the injection of For into a paw. These results suggested that the analgesic effect of Cor in rats of hyperalgesia appear to be attributable to inhibition of *L*-Arg-NO pathway in the superficial dorsal horn of spinal cord. However, the detailed mechanism needs further investigation.

Fos expression is a marker of neuronal activity following noxious stimulation[8]. Our studies showed that the Fos-positive neurons obviously increased in the superficial dorsal horn after For injection into a paw. Cor (ith) inhibited the For-induced Fos expression in spinal cord, which further exhibited the antinociceptive activity of Cor in the spinal cord. Fos expression induced by noxious stimuli was mediated by a guanylate cyclase, a known target of NO[9]. Our results found that a significant positive relationship existed between the ranks of NOS and Fos-positive neurons. NOS colocalised with Fos in spinal cord neurons following noxious stimulation of rats. The inhibitory effect of Cor on For-induced increase of Fos-, Nos/Fos-positive neurons in dorsal horn was attenuated by the precursor of NO, Arg. These results suggest that the production of NO and Fos are associated. Yet, the relationship between NO and Fos needs to be further studied.

Cumulatively our results indicated that ith injection of Cor inhibited the increase of NOS-positive neurons and produced an analgesic effect in rat spinal cord in response to peripheral noxious stimuli.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

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鞘内注射促皮质素抑制甲醛痛敏大鼠脊髓 NOS 阳性神经元增多

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关键词 促皮质素；一氧化氮合酶；脊髓；痛敏；甲醛；痛测定；组织细胞化学；原癌基因蛋白 c-*fos*；免疫组织化学；神经元

目的：研究鞘内注射促皮质素（Cor）对甲醛痛敏大鼠脊髓背角一氧化氮合酶（NOS）阳性神经元增多的影响。**方法**：采用痛级均数（PIR）测定、NADPH-d 组织化学法、Fos 免疫组织化学法染色，观察鞘内注射（ith）Cor 对甲醛痛敏大鼠脊髓背角 NOS 阳性神经元、Fos 免疫反应神经元、NOS/Fos 双标记神经元及痛敏的影响。**结果**：ith Cor（0.5 - 1.5 U）均能显著抑制甲醛引起的大鼠脊髓背角 NOS、Fos、NOS/Fos 阳性神经元的增多和痛敏反应，其作用为 ith NOS 底物左旋精氨酸（Arg，5 - 15 nmol）部分翻转。**结论**：Cor 通过抑制大鼠脊髓背角 NOS 阳性神经元的增多抑制痛敏。

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书 讯

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