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Alterations of *c*-Fos mRNA Expression in Hypothalamic-Pituitary-Adrenal Axis and Various Brain Regions Induced by Intrathecal Single and Repeated Substance P Administrations in Mice

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The effect of substance P (Sub P) injected intrathecally (i.t.) on *c*-fos mRNA expression in various tissues was examined in the present study. We found that a single administration of Sub P (0.5 nM) caused an increase of the *c*-fos mRNA level in the hypothalamic-pituitary-adrenal (HPA) axis, hippocampus, and spinal cord. The time-course study showed that *c*-fos mRNA level was maximal at 10 min and began to decrease 30 min after the Sub P injection in all tissues, and the Sub P-induced increase of the *c*-fos mRNA level was returned to the control level 1 h after the injection. The kinetics of the *c*-fos mRNA expression in mice that were repeatedly injected with Sub P (every 30 min interval up to 4 times) were different in the HPA axis, hippocampus, and spinal cord. The increased *c*-fos mRNA level in the hypothalamus and the spinal cord induced by i.t. injected Sub P remained at a high level. In the pituitary gland, adrenal gland, and hippocampus, the increased level of *c*-fos mRNA expression gradually returned to the control level during the repeated substance P injections up to 4 times. Our results suggest that spinally injected Sub P-induced pain stress increases *c*-fos mRNA expression in the spinal cord, hippocampus, and HPA axis. In mice repeatedly injected with Sub P, the kinetics of *c*-fos mRNA appear to be different varied from tissue to tissue.

Key words: Hypothalamic-pituitary-adrenal axis, Substance P, *c*-Fos, Intrathecal

INTRODUCTION

It has been well known that a proto-oncogene product, such as *c*-Fos protein, serves as the third messenger in the regulation of various types of genes. Indeed, several families of the proto-oncoproteins, such as Fos (*c*-fos, fra-1, fra-2, and fos B) and Jun (*c*-jun, jun B, and jun D) family proteins, serve as transcriptional factors in the expressions of various genes, such as proenkephalin, prodynorphin, nerve growth factor, vasoactive intestinal polypeptide (VIP), and tyrosine hydroxylase, through the interaction with AP-1 and AP-1-like DNA domains in their promoter regions (Arenander and de Vellis, 1994; Sheng and Greenberg, 1990). Several lines of evidence have revealed that *c*-fos mRNA or *c*-Fos proteins appear to be important participants in the regulation of nociception in various types of pain models. For example, *c*-fos mRNA expression is increased during formalin-induced inflammatory pain (Heughan *et al.*, 2002; Li and Clark, 2001; Siegan *et al.*, 2002), neuropathic pain (Lee *et al.*, 2002), noxious stimuli (Huang *et al.*, 1999; Todd *et al.*, 2002), and capsaicin-induced pain (Zou *et al.*, 2001).

Substance P (Sub P) located in the primary nerve ending has been known as an important neurotransmitter for the pain transmission. Although the pain reflex induced by Sub P that was injected intrathecally (i.t.) has been used as a pain model (Chung *et al.*, 2001; Hunskaar *et al.*, 1986), the signal molecule, especially *c*-fos mRNA, activated by Sub P that was administered spinally has not been well characterized yet. Thus, the present study was designed to assess the expression of *c*-fos mRNA expression by a single and repeated injection with Sub P i.t. in the spinal cord, hippocampus, and hypothalamic-pituitary-adrenal (HPA) axis.

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MATERIALS AND METHODS

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 also in accordance with the ethical guidelines of the International Association for the Study of Pain.

Experimental animals

Male ICR mice (MJ LTD., Seoul, Korea) weighing 23-25 g were used for all the experiments. Five animals were housed per cage in a room maintained at 22 ± 0.5 °C with an alternating 12 h light-dark cycle for at least 5 days before the initial procedures of the experiment, and 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.

Intrathecal (i.t.) injection

Intrathecal administration was performed in conscious mice following the method of Hylden and Wilcox (Hylden and Wilcox, 1980) using a 30-gauge needle connected to a 25 μL Hamilton syringe with polyethylene tubing. The i.t. injection volume was 5 μL, and the injection site was verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the spinal cord. The dye injected i.t. was distributed both rostrally and caudally but in short distances (about 0.5 cm), and no dye was found in the brain. Before the experiments were performed, the success rate for the injections was consistently found to be over 95%.

Total RNA isolation and Northern blot analysis

Total cellular RNAs were extracted from pooled mice tissues (n=5/group) through rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate (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 nm and 280 nm.

Ten micrograms of total RNA were denatured and electrophoresed on 1% agarose-formaldehyde gels (Kopchik *et al.*, 1981) and were 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 h in a prehybridization buffer (5X SSC, 50% formamide, 0.02% SDS, 0.1% sodium *N*-lauroyl sarcosine, 2% blocking reagent). The Dig-labelled *c*-fos probes were added to the prehybridization buffer containing 50% formamide. The membranes were incubated overnight at 68°C in a shaking water bath, and were washed twice for 10 min per wash in 2X wash solution (2X SSC, 0.1% SDS) at room temperature. Then, the membranes were washed twice for 15 min per wash in 0.1X wash solution (0.1X SSC, 0.1% SDS). After equilibrating in buffer I (100 mM maleic acid, 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 the diluted anti-DIG-alkaline phosphatase [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, 50 mM $MgCl_2$) for 2 min. Approximately, 0.5 mL (per 100 cm^2) of CSPD (Boehringer Mannheim, Germany) was spread over the surface of the membranes. After incubation at 37°C for 15-20 min, the membranes were exposed to Hyperfilm-MP (Amersham, Buckinghamshire, England) in order to detect the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterile millipore water, and was 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 rehybridized to Dig-labelled 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 (Danielson *et al.*, 1988; Takahashi *et al.*, 1989).

The cRNA probes for *c*-fos (Curran *et al.*, 1987) and cyclophilin (Danielson *et al.*, 1988) were synthesized *in vitro* from a linearized expression vector by using DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim, Germany).

RESULTS

The effect of Sub P injected i.t. on *c*-fos mRNA expression in various tissues

In this study, the effect of Sub P injecting i.t. once or repeatedly on *c*-fos mRNA expression in various tissues were examined. As shown in Fig. 1, a single administration of Sub P (0.5 nM) caused an increase of *c*-fos mRNA level in the HPA axis, the hippocampus, and the spinal cord. The time-course study showed that the *c*-fos mRNA level was maximal at 10 min and began to decrease 30 min after the Sub P injection in all tissues, and Sub P-induced increase of *c*-fos mRNA level was returned to the control level 1 h after the injection. As shown in Fig. 2, the kinetics of *c*-fos mRNA expression in mice injected with Sub P repeatedly (every 30 min interval up to 4 times) were different in the HPA axis, hippocampus, and spinal cord. The increased *c*-fos mRNA level in the hypothalamus and

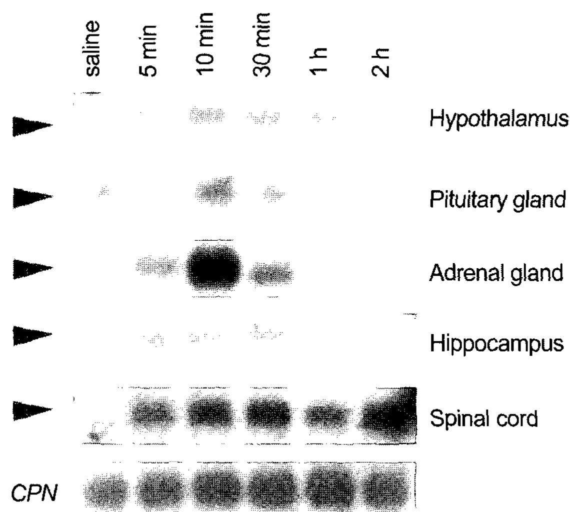

Fig. 1. Effect of a single administration of substance P (Sub P) on the c-fos mRNA level in the hypothalamic-pituitary-adrenal (HPA) axis, hippocampus, and the spinal cord. Animals were treated with Sub P (0.5 nM) intrathecally (i.t.). At the indexed time points (5 min, 10 min, 30 min, 1 h and 2 h) after i.t. Sub P injection, the level of c-fos mRNA in the hypothalamus, pituitary gland, adrenal gland, hippocampus, and spinal cord was examined using the Northern blot analysis. The number of animals used for each group was 5. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control.

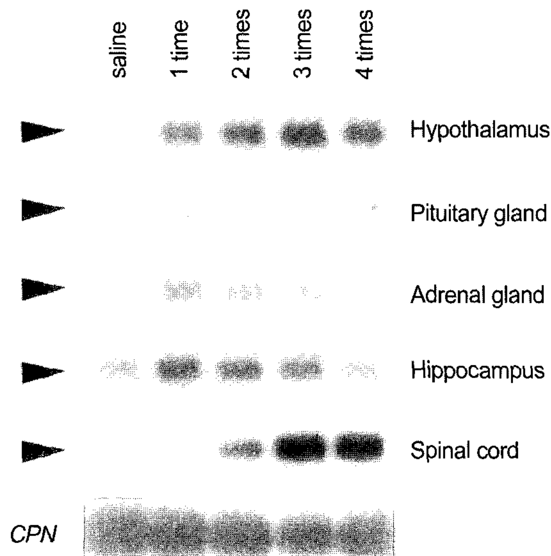

Fig. 2. Effect of repeated administration of substance P (Sub P) on the c-fos mRNA level in the hypothalamic-pituitary-adrenal (HPA) axis, hippocampus, and the spinal cord. Animals were treated with Sub P (0.5 nM) intrathecally up to 4 times in 30 min intervals. Mice were sacrificed 30 min after the last injection of Sub P, and then, the level of c-fos mRNA in the hypothalamus, pituitary gland, adrenal gland, hippocampus, and spinal cord was examined using the Northern blot analysis. The number of animals used for each group was 5. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control.

the spinal cord induced by i.t. injected Sub P was remained at a high level. In the pituitary, adrenal gland, and hippocampus, increased c-fos mRNA expression gradually returned to the control level during the repeated Sub P injections up to 4 times.

DISCUSSION

We found in the present study that spinally injected Sub P-induced pain stress increases c-fos mRNA expression in the spinal cord, hippocampus, and HPA axis. Furthermore, in mice that were repeatedly injected with Sub P, the kinetics of c-fos mRNA appear to be different from tissue to tissue, which suggests that the regulation of an immediate early gene expression appears to be different during the persistent pain transmission by repeated i.t. injection of Sub P.

In various types of pain models, the c-fos mRNA level or c-Fos protein expressions are increased at the spinal cord level. For example, c-fos mRNA expression is increased during formalin-induced inflammatory pain (Heughan *et al.*, 2002; Li and Clark, 2001; Siegan *et al.*, 2002), neuropathic pain (Lee *et al.*, 2002), noxious stimuli (Huang *et al.*, 1999; Todd *et al.*, 2002), and capsaicin-induced pain (Zou *et al.*, 2001). In this study, we found that a single spinal injection with Sub P rapidly (within 5 to 10 min) increases the c-fos mRNA level and started to decrease 1 h after the Sub P injection. Interestingly, repeated spinal Sub P injections caused a continuous increase of c-fos mRNA expression in the spinal cord, suggesting that spinal c-fos mRNA expression remained at a high level during the persistent state of pain. Although the exact reason for this observation is not clear, it can be speculated that the c-fos gene product may function as a transcriptional regulator for certain target genes, which are closely related to the persistent pain.

Early studies have shown that the signal molecule such as c-Fos in the hippocampus is induced during pain transmission that was stimulated by the subcutaneous formalin injection into the hind paw (Aloisi *et al.*, 2000; Aloisi *et al.*, 1997; Ceccarelli *et al.*, 1999), suggesting that the c-Fos protein is the pyramidal cell layer in the hippocampus can be increased during the inflammation-induced pain transmission. In the present study, we found that c-fos mRNA level was also increased in the hippocampus up to 30 min by a single i.t. administration of Sub P. However, the increased c-fos mRNA level in the hippocampus remained high up to three repeated injections of Sub P and began to decrease on the fourth injection of Sub P.

The increase of the c-fos mRNA level induced by substance P injected i.t. was also found in the hypothalamus, pituitary gland, and adrenal gland. This finding suggests

that the HPA axis plays an important role in the regulation of pain-related stress induced by Sub P that was spinally injected. In particular, *c*-fos mRNA levels in the adrenal and pituitary glands were dramatically increased. In addition to this finding, it was discovered that the spinal injection of Sub P also increases pERK, pCaMK II, and pCREB levels in the hypothalamus and the adrenal gland (unpublished data). Thus, in the HPA axis, several signal molecules are actively increased during the pain stress induced by spinally administered Sub P. Furthermore, in mice injected i.t. and repeatedly with Sub P, which is analogous to a state of a persistent pain transmission, *c*-fos mRNA kinetics appear to be different in the HPA axis. We also found that the increased *c*-fos mRNA level in the hypothalamus induced by i.t. injected Sub P remained at a high level. In the pituitary gland, adrenal gland, and hippocampus, the increased *c*-fos mRNA expression gradually returned to the control level during the repeated substance P injections up to 4 times, which suggests that *c*-fos expression was differentially regulated in the HPA axis during the persistent pain stress induced by repeated injections of Sub P.

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