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Role of spinal cholecystokinin in neuropathic pain after spinal cord hemisection in rats

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ABSTRACT

In the present study we determined whether spinal cholecystokinin (CCK) or the cholecystokinin receptor is involved in below-level neuropathic pain of spinal cord injury (SCI). The effect of the CCK_B receptor antagonist, CI-988 on mechanical allodynia and the expression level of CCK and CCK_B receptor were investigated. Spinal hemisection was done at the T13 level in rats under enflurane anesthesia. CI-988 was administered intraperitoneally and intrathecally and behavioral tests were conducted. After systemic injection, mechanical allodynia was reduced by higher doses of CI-988 (10 and 20 mg/kg). Intrathecal CI-988 (100, 200 and 500 μg) dose-dependently increased the paw withdrawal threshold in both paws. Following spinal hemisection, CCK mRNA expression increased on the ipsilateral side at the spinal segments caudal to the injury and both sides of the spinal L4-5 segments without any significant changes in CCK_B receptor mRNA levels. These results suggest that up-regulation of spinal CCK may contribute to maintenance of mechanical allodynia following SCI and that clinical application of CI-988 or similar drugs may be useful therapeutic agents for management of central neuropathic pain.

Neuropathic pain following spinal cord injury (SCI) includes spontaneous pain syndromes, hyperalgesia and allodynia, which occur above, at or below the level of the lesion [17] in about half of patients with SCI [18]. These pain syndromes are usually chronic and may cause persistent problems for SCI patients in rehabilitation. The underlying mechanism is not fully understood.

Injury to the spinal cord leads to neuroplastic changes including anatomical, physiological and molecular changes within the spinal cord and brain. Previous studies suggest that alterations in neuropeptides such as substance P, calcitonin gene-related peptide and cholecystokinin are involved in generation of neuropathic pain [10,25]. Of these peptides, cholecystokinin (CCK) is well known as an antagonist of endogenous opioids in the central nervous system [3,22]. Experimental evidence has shown that CCK mRNA increases after injury to the nervous system [2,25]. Xu et al. [25] reported up-regulation of CCK and CCK receptor mRNA in dorsal root ganglia after sciatic nerve injury in rats. Brewer et al. [2] reported an increase in CCK mRNA in the cortex of painful rats compared to painless rats in an excitotoxic SCI model. Previous reports clearly show that neuropathic pain can be controlled by blocking the actions of CCK in rats [28]. Xu et al. [24] reported, using an ischemic SCI model, that systemic opioids did not effectively reduce mechanical allodynia but systemic CI-988, an antagonist of the CCK_B receptor alleviated mechanical allodynia. Kovelowski et al. [12] demonstrated blockade of the action of CCK by injection of L365,260, a CCK_B receptor antagonist, into the rostroventromedial medulla (RVM) reversed tactile allodynia and thermal hyperalgesia after spinal nerve injury, suggesting that endogenous CCK may contribute to maintenance of neuropathic pain following nerve injury. However, most studies are limited to peripheral nerve injury or certain types of SCI, especially ischemic SCI [23,24]. Thus, in the present study, we investigated the role of CCK in the maintenance of pain below the level of thoracic spinal hemisection.

All experiment procedures were done in accordance with guidelines set by the Korea University College of Medicine Animal Research Policies Committee. Male Sprague–Dawley rats ($n = 68$, 150–200 g, at the time of operation) were used for this experiment. The animals were kept in a 12-h light/12-h dark cycle with light on 7:00 A.M.

Under enflurane anesthesia (by mixture of 4% enflurane and 95% O_2), a longitudinal incision was made exposing several segments, a laminectomy was performed, and the spinal cord was hemisected at T13 on the left side with a no. 11 scalpel blade. The wound was closed in anatomical layers, the skin with stainless steel wound clips.

Behavioral tests for motor function and mechanical allodynia were performed preoperatively and postoperatively for the hindpaws. The tests were performed on each rat 1 day prior to surgery

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and 1, 4, 7 and 14 days postoperatively (PO), before the study of drug effects. Rats that showed a little mechanical allodynia or displayed contralateral hindlimb motor deficits were excluded.

Mechanical allodynia was assessed by measuring the threshold of brisk paw withdrawal response to graded mechanical stimulus with a series of von Frey filaments (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g, Stoelting, Wood Dale, IL, USA). To do this, the rat was placed under a transparent plastic dome (28 cm × 28 cm × 10 cm) on a metal mesh floor and a von Frey filament was applied to the plantar surface of the foot. A von Frey filament was applied for 3–4 s to each hind paw while the filament was bent. The 50% withdrawal threshold was determined using the up–down method [4], starting with a 2.0 g (4.31 mN) strength of filament. A withdrawal response was cause to present the next weaker stimulus, and lack of withdrawal led to presentation of the next stronger stimulus. Stimuli were presented at intervals of several seconds. A brisk foot withdrawal to application of a von Frey filament was regarded as a positive response. Interpolation of the 50% threshold was carried out according to the method of Dixon [6].

The investigator conducting the behavioral tests was blinded about the injected drug. Tests were carried out 3 weeks after spinal hemisection. During this period, motor function in hemisected rats fully recovered and signs of mechanical allodynia were well established as described in our previous report [11]. More than one test was performed on some rats. Intervals between repeated tests were at least 3 days. CI-988 (5, 10 and 20 mg/kg) or vehicle (saline) was injected intraperitoneally. Rats were randomly assigned to different treatment groups. Behavioral signs of mechanical allodynia were measured 30 min before and 15, 30, 45, 60, 90 and 120 min after injection of a drug.

In order to determine the effect of intrathecally (IT) administered drugs on mechanical allodynia, rats for IT drug administration were implanted with catheters 2 weeks after hemisection. Under enflurane anesthesia (by mixture of 4% enflurane and 95% O_2), the occipital muscles were separated from their attachment point and retracted caudally to expose the cisternal membrane at the base of the skull. Sterilized PE-10 tubing was threaded through an incision in the atlanto-occipital membrane to the 1 or 2 segments rostral to the hemisection site (5.5 cm). Animals showing evidence of neuromuscular dysfunction were excluded from further tests. After the experiment, the position of the IT catheter was confirmed by injection of Evans Blue dye following laminectomy. Drug was injected IT in a volume of 10 μl followed by 10 μl saline to flush the catheter. Behavioral signs of mechanical allodynia were measured 30 min before and 15, 30, 60, 90, 120 and 180 min after the injection. CI-988 was a generous gift from Pfizer Inc. (Groton, CT, USA).

To exclude the possibility that motor impairment might contribute to changes in withdrawal threshold during testing, a modification of the combined behavioral score (CBS) of Gale et al. [8] was performed at the time of the behavioral test. The CBS assigns a weight to each of the tests and combines them into one total score that represents the degree of motor impairment. Tests were as follows: motor scores, toe spread, righting reflex, extension withdrawal reflex, placing reflex and inclined plane. Neurological function was evaluated by a scoring system that ranged from 0 for a normal rat to 90 for a completely paralyzed rat.

To determine whether there were changes in expression levels of CCK and CCK_B receptor mRNA, segments of the spinal cord at T11-12, L1-2 and L4-5 levels were removed and dissected into ipsilateral and contralateral dorsal quadrants from SCI rats ($n = 8$) and sham rats ($n = 6$). T11-12 and L1-2 levels of the spinal cord were segments just rostral and caudal, respectively, to the level of injury. The L4-5 spinal cord segment was also examined because it receives mainly sensory inputs from the foot, pain responses from which were measured in the present study. The tissues were immediately frozen in liquid nitrogen and stored at −70 °C. Total RNA extraction was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using 50 pM oligo $(dT)_{20}$ primer with Moloney-Mouse Leukemia Virus reverse transcriptase (Invitrogen, #28025-013, Carlsbad, CA, USA) at 37 °C for 50 min. Total RNA and cDNA concentrations were determined at 260 nm by UV spectrophotometer (GeneQuant, Pharmacia, USA). We amplified 500 ng of cDNA for cholecystokinin (CCK), CCK_B receptor (CCKBR) and 375 ng of cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with i-star taq DNA polymerase (Intron, #25162, Sungnam, Korea). PCR primer and conditions are presented in Table 1. PCR products were detected on a 1% agarose gel. A picture was taken using a digital camera (Canon A620) and we measured the density of the PCR bands using a computer-assisted image analysis system (NIH image software).

All values are expressed as mean ± SEM. The Friedman repeated measures of analysis of variance followed by multiple comparison tests were used to compare behavioral test results before and after drug administration with the same animal. The absorbencies of the PCR bands were measured using Scion image software. Normal and injured group comparisons were performed using a one-way ANOVA with Scheffe's post hoc test.

Rats with SCI showed significant decrease in withdrawal threshold to von Frey stimulation with maximal motor recovery 3 weeks after spinal hemisection, as in our previous report [11]. The effects of systemic CI-988 on the hindlimb withdrawal threshold to mechanical stimulation applied to the plantar surface of the right foot and the left foot are shown in Fig. 1A. CI-988 at 5 mg/kg had no significant effects on paw withdrawal threshold, whereas doses of 10 and 20 mg/kg significantly increased paw withdrawal threshold without change of motor function tested using a CBS scale (data not shown). This anti-allodynic effect was significant at 15 min and persisted up to 60 min after injection in both feet compared to pretreatment values. There were no significant differences in threshold changes induced by CI-988 between ipsilateral and contralateral hind paws. Saline had no effect on the responsiveness to mechanical stimuli with von Frey stimulation.

Intrathecally injected CI-988 (100, 200 and 500 μg) significantly increased paw withdrawal threshold to mechanical stimulation applied to the plantar surface of the foot in a dose-dependent manner (Fig. 1B). Even a lower dose of CI-988 (100 μg, $n = 12$) significantly increased withdrawal threshold from 15 to 60 min. The effect was peak at 15–30 min after injection, gradually diminished, and persisted for 90 min after higher doses of CI-988 (200 and 500 μg). Saline ($n = 9$) did not change the withdrawal threshold throughout the test period. No changes in the CBS scale were observed after injection of any of the doses that were tested in this study (data not shown).

We attempted to determine whether there were changes in expression level of CCK and CCK_B receptor mRNA in the spinal cord after hemisection. The expression of CCK mRNA was assessed for both ipsilateral and contralateral sides at T11-12, L1-2 and L4-5 spinal cord segments and was compared to levels in normal rats (Fig. 2). CCK mRNA expression was significantly increased in both ipsilateral and contralateral sides at the L4-5 level compared to control ($p < 0.05$). At the L1-2 spinal cord level, CCK mRNA expression was significantly increased on the ipsilateral side ($p < 0.05$). On the contralateral side, CCK mRNA expression increased slightly, but there was no significant difference. At the T11-12 spinal cord level, CCK mRNA expression was similar to that of control on both ipsilateral and contralateral sides. In contrast to expression of CCK mRNA, CCK_B receptor mRNA expression in the spinal cord did not show significant differences between hemisected rats and normal rats at all of the sites tested in this study.