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Research article

Urinary trypsin inhibitor attenuates the development of neuropathic pain following spinal nerve ligation

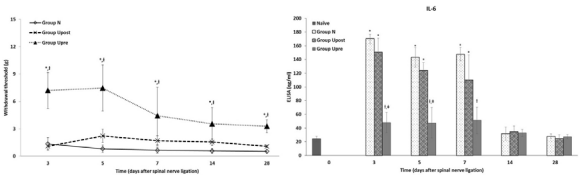
Seon Hee Oh[a], Hyung Jin So[b], Hyun Young Lee[c], Kyung Joon Lim[c], Myung Ha Yoon[d], Ki Tae Jung[c,*]

[a] The Division of Natural Medical Sciences, College of Health Science, Chosun University, Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea
[b] Department of Anesthesiology and Pain Medicine, Chosun University Hospital, 365 Pilmun-daero, Dong-gu, Gwangju 501-717, South Korea
[c] Department of Anesthesiology and Pain Medicine, School of Medicine, Chosun University, Chosun University Hostpital, 365 Pilmun-daero, Dong-gu, Gwangju 501-717, South Korea
[d] Department of Anesthesiology and Pain Medicine, Medical School, Chonnam National University, 42 Jebongro, Donggu, Gwangju 501-757, South Korea

HIGHLIGHTS

- Mechanical allodynia was made by spinal nerve ligation.
- The levels of TNF-α, IL-1β, and IL-6 were increased afterspinal nerve ligation.
- Intravenous UTI before development of neurophatic pain increased the withdrawal threshold.
- Intravenous UTI before development of neurophatic pain only decreased the level of IL-6.

GRAPHICAL ABSTRACT



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ABSTRACT

Following nerve injury, inflammatory and algesic mediators are released. This neuroinflammatory outbreak causes neuronal damage and chronic neuropathic pain. Urinary trypsin inhibitor (ulinastatin, UTI), which has anti-inflammatory properties and neuroprotective effects, is used to lower the levels of inflammatory factors during surgery. This study evaluated the effect of ulinastatin in a rat model of spinal nerve ligation (SNL). Neuropathic pain was induced by L5 and L6 SNL in male Sprague–Dawley rats. Rats were divided into three groups: group N (control) received normal saline through the tail vein for three days immediately following SNL, group U_{pre} received ulinastatin (50,000 U/kg) intravenously for three days immediately following SNL, and group U_{post} received ulinastatin (50,000 U/kg) intravenously for three days from the 3rd day following SNL. The paw withdrawal threshold was examined and the development of mechanical allodynia was confirmed with a behavioral test using a von Frey filament three days following SNL. On the 3rd, 5th, 7th, 14th, and 28th day following SNL, the paw withdrawal threshold was examined and the levels of inflammatory mediators (i.e., tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], and interleukin-6 [IL-6]) were measured by ELISA. The paw withdrawal threshold was significantly increased in the rats from group U_{pre} compared with the rats from groups N and U_{post} until the 7th post-SNL day ($P < 0.05$). The levels of IL-6 also were significantly decreased in the rats from group U_{pre} compared with the rats from group N and U_{post} until the 7th post-SNL day ($P < 0.05$). Ulinastatin increased the paw withdrawal threshold following SNL when it was administered before the development of neuropathic pain, and may attenuate the development of neuropathic pain.

* Corresponding author. Tel.: +82 62 220 3223; fax: +82 62 223 2333.
E-mail addresses: seonh@chosun.ac.kr (S.H. Oh), lsopp@naver.com (H.J. So), lhy109@chosun.ac.kr (H.Y. Lee), kjlim@chosun.ac.kr (K.J. Lim), mhyoon@chonnam.ac.kr (M.H. Yoon), mdmole@chosun.ac.kr (K.T. Jung).

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1. Introduction

Neuropathic pain syndrome (NPS) following a nerve injury is characterized by a reduced nociceptive threshold and sensitization to innocuous stimuli. The development of NPS is associated with not only changes in the activity of neuronal systems or inflammatory immune and immune-like glial cells but also with immune cell-derived inflammatory cytokines [1]. Recent studies on altered nociceptive processing following nerve injury have provided evidence for the roles of inflammatory mechanisms in the production of neuropathic pain [1,2]. Pro-inflammatory mediators such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) are known to play important roles in the progress of pain [3,4]. Inhibition of these cytokines reduces or attenuates nerve injury-induced mechanical allodynia [5–7].

Ulinastatin, or urinary trypsin inhibitor (UTI), is a serine protease inhibitor [8] that can be used not only in inflammatory diseases such as pancreatitis or sepsis due to its anti-inflammatory effect but also in patients with shock, trauma, organ injury, or undergoing surgery due to its cytoprotective effects [8,9]. Moreover, ulinastatin is also effective in the protection of the nervous system after an injury [10,11]. A recent study has demonstrated the possibility of using ulinastatin to reduce pain in a rat model following spinal nerve ligation (SNL) [12].

This study examined the impact of ulinastatin on mechanical allodynia and the levels of inflammatory mediators (TNF-α, IL-1β, and IL-6) in rats following neuropathic pain evoked by SNL.

2. Material and methods

2.1. Animal preparation

This study was approved by the Institutional Animal Care and Use Committee of Chonnam National University. Male Sprague–Dawley rats weighing 100–120 g were used in all experiments. The animals were raised in cages housed in a room with the temperature maintained between 20 and 23 °C under a 12:12 light:dark cycle with free access to food and water.

2.2. Introduction of neuropathic pain

Segmental SNL was performed to introduce neuropathic pain according to the experimental model used by Chung [13,14]. All procedures were conducted under anesthesia. Once the rat was anesthetized with sevoflurane, a skin incision was made in the midline of the L5–S2 spine. First, a dissection to separate the left paraspinal muscles from the spinous process was made to expose the spine. Then, the transverse process of the L6 spine was removed with a small rongeur. The left L5 and L6 spinal nerves were exposed and tightly ligated distally to the dorsal root ganglia using 6–0 silk. The wound then was closed and the rats were allowed to recover from the anesthesia. After the procedure, the rats with signs of L4 spinal nerve damage were excluded from the study. After three days, the paw withdrawal threshold was measured using von Frey filaments to confirm the development of neuropathic pain. Animals were considered to have developed neuropathic pain when they exhibited mechanical allodynia, which was defined as a paw flinching response upon the application of a bending force of less than 4 g.

2.3. Groups and drug administration

The rats were divided into three groups with $n = 30$ in each group (Fig. 1). The rats in group N, the control group, were administered normal saline for three days following the SNL procedure. The rats in group U_{pre} were administered 50,000 U/kg of UTI (Ulistin®, Hanlim Pharmaceutical Co. Ltd., Seoul, Korea) through the tail vein for three days immediately following the SNL procedure, before the development of neuropathic pain was confirmed. The rats in group U_{post} were administered 50,000 U/kg of UTI through the tail vein for three days from the 3rd day following the SNL procedure, after the development of neuropathic pain was confirmed [12].

2.4. Assessment of mechanical allodynia

Mechanical allodynia was assessed by the paw withdrawal threshold using von Frey filaments (Stoelting; Wood Dale, IL, USA). The rats were transported to a plastic cage with a mesh floor and acclimated to the laboratory environment for 30 min before the test. Mechanical stimulation was applied to the plantar surface of the hind paw vertically for 5 s with a series of eight von Frey filaments (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, and 15 g). A positive response was recorded when an abrupt withdrawal or flinching of the hind paw was observed. The cut-off value was a negative response to 15 g. The withdrawal threshold was calculated using the up and down method [15]. A series of tests (on the 3rd, 5th, 7th, 14th, and 28th days following SNL) was conducted to measure the change in mechanical allodynia across time.

2.5. Enzyme-linked immunosorbent assays

Inflammatory mediators (IL-1β, IL-6, and TNF-α) were measured by enzyme-linked immunosorbent assay (ELISA) on the 3rd, 5th, 7th, 14th, and 28th days following SNL for the experiment groups and before SNL for the control (naïve) group. The rats were sacrificed and decapitated under sevoflurane anesthesia, and the spinal cord was isolated by inserting an 18 gauge needle into the caudal end of the vertebral column and flushing the spinal cord out with ice-cold phosphate-buffered saline (PBS). The ipsilateral dorsal spinal cord at L4–L6 was obtained by a cut in the spinal cord at the midline. The tissue was immediately stored at −70 °C by liquid nitrogen until homogenization. A 1 cm section of the lumbar spinal cord at L4–L6 was removed from the intact frozen cord and weighed. The spinal cord was homogenized in a radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific; Waltham, MA, USA) with a protease inhibitor cocktail (Calbiochem, Germany), and the homogenate was centrifuged (13,000 rpm for 10 min at 4 °C). The protein concentration in the supernatant was measured using the BCA Protein Assay kit (Pierce, WI, USA) standardized to the BSA according to the manufacturer's protocol.

The levels of IL-1β, IL-6, and TNF-α were determined using a Quantikine ELISA kit (R&D Systems; Minneapolis, MN, USA) according to the manufacturer's directions. The inflammatory mediators were quantified by comparing the samples to the standard curves generated from the respective kits.

2.6. Statistical analysis

The data are expressed as the mean ± SEM. The results of the behavioral experiments were analyzed by a one-way analysis of variance (ANOVA) and Scheffe's post-hoc test. The samples obtained from ELISA were non-parametrically distributed, and therefore, were analyzed by the Kruskal–Wallis test followed by Scheffe's post-hoc test. Results with $P < 0.05$ were considered statistically significant.

3. Results

Mechanical allodynia was confirmed in all rats three days after the SNL procedure. The withdrawal threshold of the left hind paw