Expression of TRPV1 and iNOS in the Dorsal Root Ganglion Exposed by Autologous Nucleus Pulposus in the Rat

The Journal Korean Society of Physical Therapy

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Purpose: To determine whether upregulation of inducible nitric oxide synthase (iNOS) transcription and translation is related to radicular pain in a model of lumbar disc herniation. Also, to investigate the temporal changes of mRNA expression of iNOS and the identity of iNOS and transient receptor potential vanilloid (TRPV) 1 channel expression cells in dorsal root ganglion (DRG) of a model of lumbar disc herniation.

Methods: A lumbar disc herniated rat model was developed by implantation of the autologous nucleus pulposus, harvested from the coccygeal vertebra of each tail, on the left L5 nerve root just proximal to the DRG. Rats were tested for mechanical allodynia of the plantar surface of both hind paws 2 days before surgery and 1, 5, 10, 20 and 30 days postoperatively. Reverse transcription polymerase chain reaction (RT-PCR) was used to follow iNOS mRNA expression. To stain iNOS and TRPV1 in DRG, an immunohistochemical study was done 10 days after surgery.

Results: A significant drop in mechanical withdrawal threshold on the ipsilateral and contralateral hind paws was observed 1 day after surgery and was prolonged to 30 days in rats with lumbar disc herniation. The expression of mRNA for iNOS peaked at postoperative day 10 on both sides of the DRG. iNOS-positive sensory neurons in the DRG varied in size from large to small diameter cells. A majority of small and intermediate sensory neurons were TRPV1-positive cells. Double immunofluorescence staining for TRPV1 and iNOS revealed that most intermediate TRPV1-positive sensory neurons co-localized with iNOS-positive neurons.

Conclusion: Nucleus pulposus-induced mechanical allodynia can be generated without mechanical compression. This pain is related to temporal changes in expression of iNOS mRNA in the DRG. Co-localization of TRPV1 and iNOS in intermediate neurons of the DRG is correlated with pain modality and intensity.

Keywords: Inducible nitric oxide synthase, Transient receptor potential vanilloid (TRPV) 1 channel, Lumbar disc herniation, pain

Received: May 13, 2010
Revised: June 10, 2010
Accepted: June 14, 2010
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I. Introduction

Radicular pain caused by disc herniation is mediated by both chemical and mechanical factors with a predominant involvement of inflammatory mediators.[1,2] A number of inflammatory mediators have been implicated in radicular pain due to disc herniation.[3,4] Increased expression of cytokines such as interleukin-1 (IL-1),[5] interleukin-6 (IL-6),[6] interleukin-8 (IL-8),[3,7] tumor necrosis factor-α (TNF-α),[8] cyclooxygenase-2 (COX-2),[9] and inducible nitric oxide synthase (iNOS)[10] have been implicated in nucleus pulposus–induced nerve root injury and the associated radicular pain. Some studies reported that iNOS around the nerve root produced by application of nucleus pulposus may influence the nerve root directly and induce

hyperalgesia resulting from a direct transport to the nerve root or the dorsal root ganglion.[4,11] iNOS is related to the inflammation and also involved in peripheral pathologic changes.[12] Therefore iNOS may play an important role in the pathogenesis of radicular pain due to disc herniation. However, little have been reported that which cells express iNOS and how long do they express iNOS in dorsal root ganglion of a model of lumbar disc herniation.

Recent studies have reported that transient receptor potential vanilloid (TRPV) 1 channel play an important role in nociception.[13,14] Activation of TRPV1 in the peripheral terminals by noxious stimuli initiates action potentials that carry the nociceptive information. TRPV1 was described as a polymodal receptor activated by chemical, thermal and mechanical stimuli, and their function has been investigated in models of acute inflammatory pain[15] and neuropathic pain.[16] These studies suggest that TRPV1 may play a role in the development and maintenance of chronic pain. The contribution of TRPV1 in inflammatory and neuropathic pain has generated a major interest in the development of specific antagonists. These antagonists have been reported to act as analgesics in different models of chronic pain.[17,18] Although TRPV1 is an important contributor to pain, its role and expression is rarely reported in a model of lumbar disc herniation.

Thus, in present study, we want to investigate the identity of iNOS expression cells and temporal changes of mRNA expression of iNOS in DRG. Additionally, immuno histochemical expression of the TRPV1 was investigated in dorsal root ganglion of a model of lumbar disc herniation.

II. Materials and Methods

1. Animals

A total of 81 male Sprague-Dawley rats (200～250 g) were used in this study (n=10 for pain behavioral test, n=9 for real-time PCR for each time course, n=8 for immunohistochemistry). Rats were housed two per cage and had free access to water and food. All animal experiments were conducted in accordance to the guidelines of the Institutional Animal Care and Use Committee at the Yeungnam University, South Korea.

2. Lumbar disc herniation

Rats were anesthetized by Zoletil (Virbac, 50 mg/kg, i.p.) additional doses were used as required to maintain anesthesia throughout the experiment. A midline dorsal incision was made over the lumbar spine, the multifidus muscles were separated along the L4-S1 spinous processes, and the left L5 nerve roots and DRG were exposed through laminectomy. Nucleus pulposus, harvested from the tail disc between the second and third coccygeal vertebrae (Co2-3), was implanted next to the left L5 nerve root just proximal to the DRG.[19] Surgery in control rats was identical, except for the implantation of nucleus pulposus.

3. Pain behavior

Rats were tested for thermal and mechanical sensitivity of the plantar surface of the hindpaw 2 days before surgery, and 1, 5, 10, 20 and 30 days after surgery by an investigator blinded to the experimental group and protocol of each rat.

We tested for mechanical allodynia by measuring the withdrawal response to a mechanical stimulation of the hindpaw with von Frey filaments (North Coast Medical, Inc., USA) that have been calibrated for the force in grams required to elicit a withdrawal response: rats were placed in a clear plastic cage with a metal mesh floor, adapted to the testing environment for 15 minutes, and the plantar surface of each hindpaw was stimulated with von Frey filaments of increasing or decreasing thickness, beginning with 0.1 g probe, until a filament consistently gives withdrawal responses to 5 out of 10 stimuli. Fifty percent probability thresholds of mechanical paw withdrawal were calculated. If no withdrawal response was elicited by the 26 g filament, 26 g was assigned as the mechanical threshold.[20]

4. Real-time PCR

Total RNA was isolated from lumbar spinal cord and DRG tissue, corresponding to L5 root at 1, 5, 10, 20 and 30 days after surgery. Total RNA was isolated from each sample with Trizolreagent and purity was checked with spectrophotometer. Reverse transcription of 1 μg aliquots of total mRNA was carried out at 45℃ using a cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time PCR assays were performed using a 7500 Real Time PCR system (Applied Biosystems, USA). Primers and the TaqMan probe were designed using Probe Finder software (Universal Probe Library (UPL), Roche, Switzerland).

To amplify inducible nitric oxide synthase (iNOS) and hypoxanthine guanine phosphoribosyl-transferase (HPRT) transcripts the following primers were used: sense primer 5′-GCT TTG CCA CGG AAG AGA C -3′ and antisense primer 5′-TTC CAA

TCG TTG TAC TCT GAG G-3′ for iNOS (GenBank accession number: ENSRNOT00000016133) and sense primer 5′-GGT CCA TTC CTA TGA CTG TAG ATT TT-3′ and antisense primer 5′-CAA TCA AGA CGT TCT TTC CAG TT-3′ for HPRT (GenBank accession number: NM012583). The HPRT gene was used as an internal control to adjust for differences between samples. The mastermix consisted of 250 nM of UPL probe, 700 nM of each primer (sense and antisense), 10 μm of 2X TaqMan master and 2 μl of cDNA. All PCR reactions were run in duplicate. After pre-incubation at 95℃ for 10 minutes, PCR was performed using 50 amplification cycles of denaturation at 95℃ for 15 seconds and annealing at 60℃ for 60 seconds.

5. Immunohistochemistry

To assess the expression of TRPV1 and iNOS, we immunostained the sections of DRG of a lumbar disc herniated model. Ten days after surgery, rats were anesthetized with Zoletil (50 mg/kg, i.p.) and perfused with heparinized saline followed by 500 ml of 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4). The DRG on both sides were removed, postfixed for 3 hrs in the fixative used for perfusion, and cryoprotected in 30% sucrose in PB. Twenty micrometer-thick transverse sections were cut on a cryostat, blocked with 10% normal donkey serum (NDS, Jackson Immunolabs, USA) in phosphate-buffered saline (PBS, 0.01 M, pH 7.2) for 10 min, and incubated overnight with primary antibodies. For double immunofluorescence, sections were incubated with a mixture of goat anti-TRPV1 (Neuromics, USA, 1:500) and rabbit anti-iNOS (BD, USA, 1:100). The next day, sections were rinsed and incubated in 2% NDS for 10 min and then incubated with an appropriate combination of anti- goat or anti-rabbit secondary antibodies conjugated to Alexa 488 (1:200, Invitrogen Corporation, Carlsbad, CA, USA) or Cy3 (1:200, Jackson Immunolabs, West Grove, PA, USA) in PBS for 3 hours. After several rinses, sections were mounted on slides, coverslipped with Vectashield (Vector laboratories, Burlingame, CA, USA), and observed on a Leica DMR microscope. Images were acquired with a CCD camera (Fluoview, SIS, Muenster, Germany) attached to the microscope, and contrast and brightness were adjusted with Photoshop (CS2, Adobe Systems Inc, San Jose, CA, USA).

6. Statistical analysis

Data were analyzed with the Wilcoxon signed rank test and Mann Whitney U test using SPSS/PC v. 15.0 and expressed as mean±standard deviation. Significance was set at $p<0.05$.

III. Results

1. Pain behavior

In rats with lumbar disc herniation, the experimental group showed a significant drop in mechanical withdrawal threshold on the ipsilateral and contralateral hindpaw 1 day after surgery, which did not recover for the duration of the experiment ($p<0.01$, Figure 1).

2. Expression of mRNA for iNOS in the DRG

In rats with lumbar disc herniation, the expression of mRNA for iNOS was increased in contralateral side of DRG at postoperative day 5 and then peaked at postoperative day 10 in both of the ipsilateral and the contralateral side of DRG ($p<0.01$, Figure 2).

3. Immunohistochemistry

We have investigated the iNOS-positive cells in the DRG of a rat model of lumbar disc herniation. iNOS-positive cells were observed in ipsilateral and contalateral side of the experimental and sham group (Figure 3). The size of iNOS-positive sensory

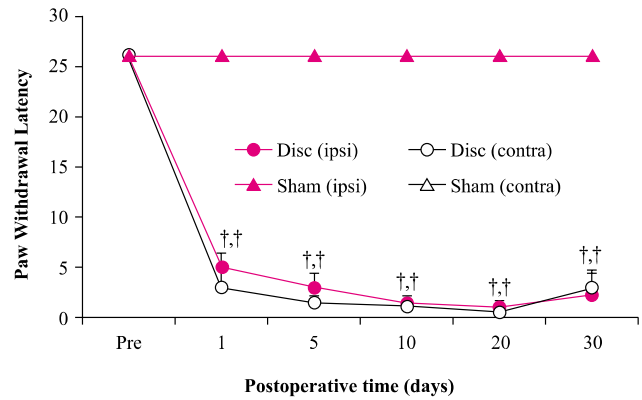

Figure 1. Behavioral responses to mechanical stimulation on the ipsilateral (ipsi) and contralateral (contra) hindpaw. A significant reduction in mechanical withdrawal threshold was seen during 1 day through 30 days in the both hindpaw after surgery (†$p<0.01$).

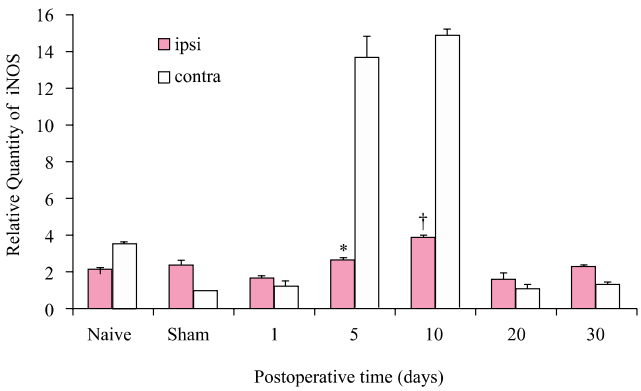

Figure 2. Expression of mRNA for iNOS in the ipsilateral (ipsi) and contralateral (contra) DRG for 30 days after application of nucleus pulposus to the nerve root. *$p<0.05$, †$p<0.01$ compared to contralateral of sham value.

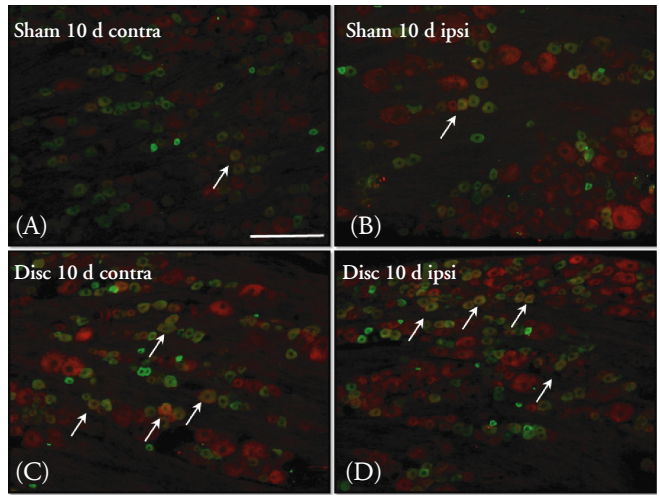

Figure 3. Immunohistochemistry for TRPV1 and iNOS in the contralateral (Contra, A and C) and ipsilateral (Ipsi, B and D) L5 spinal dorsal root ganglion at 10 days after surgery. Scale bar = 200 μm. TRPV1/iNOS positive neurons (white arrow) were showed in both sides at 10 days after surgery. There were more TRPV1/iNOS positive neurons in a lumbar disc herniation model than sham-operated group. The most of small and intermediate TRPV1-positive sensory neurons were co-localized with iNOS-positive neurons.

neurons in the DRG was various from large to small diameter cells. Majority of small and intermediate sensory neurons are TRPV1-positive cells. Double immunofluorescence for TRPV1 and iNOS revealed that the most of small and intermediate TRPV1-positive sensory neurons were co-localized with iNOS-positive neurons.

IV. Discussion

In this study, we have investigated the temporal changes of mRNA expression of iNOS and immunohistochemical localization of iNOS and TRPV1 in DRG of a lumbar disc herniated rat model. A mechanical withdrawal threshold on the ipsilateral and contralateral hindpaw was significantly reduced at 1 day after surgery and prolonged to 30 days in rats with lumbar disc herniation. The expression of mRNA for iNOS was peaked at postoperative day 10 in both sides of DRG. The iNOS-positive sensory neurons in the DRG were various from large to small diameter cells. Majority of small and intermediate sensory neurons are TRPV1-positive cells. Double immunofluorescence for TRPV1 and iNOS revealed that the most of small and intermediate TRPV1-positive sensory neurons were co-localized with iNOS-positive neurons. Previous studies reported that autologous nucleus pulposus application in the rat induced mechanical allodynia during 4 weeks without a mechanical compression.[19,21] Our data indicate that mechanical allodynia developed at 1 day after surgery and developed in the ipsilateral and contralateral hindpaw.

Contralateral hyperalgesia was also reported in other neuropathic pain models,[22,23] and is presumably mediated by neuronal communication between the two dorsal horns.[24]

Inducible NOS is implicated in numerous inflammatory diseases and, more recently, in neuropathic pain states.[12,25] Expression of iNOS in spinal cord,[26] DRG[27] and sciatic nerve[28] of neuropathic pain models has been reported. In a previous study, rats in which nucleus pulposus was implanted showed the expression of iNOS in the nerve root and DRG was peaked at 1 week after surgery, and the mechanical allodynia was increased at the same time.[4] These results demonstrated that the peak time of the expression of iNOS is related to the intensity of pain perception. In our study, expression of mRNA for iNOS in DRG of nucleus pulposus-implanted rats was found during the experimental period. Ten days after surgery, the level of expression was the highest and the mechanical allodynia was also much increased than any other period. While the expression of iNOS in DRG was peaked at 10 days after surgery and then decreased after 10 days postoperatively, the mechanical allodynia was sustained for 30 days. It is that because iNOS may be just one of numerous factors influencing the behavioral response in this study. Therefore we could guess that upregulation of iNOS

could be different with behavioral response.

Several studies reported that the characterization of iNOS-positive cells in spinal dorsal horn and sciatic nerve of the spinal cord injury or the sciatic nerve injury models.[26-28] In the spinal cord injury model, most of the iNOS-positive cells were co-localized with macrophage (ED-1) and neuron (synaptophysin), and they weakly positive to astrocyte (GFAP) and oligodendrocyte in spinal dorsal horn.[26] In the sciatic nerve injury model, immunoreactivity of iNOS were positive to schwann cell and macrophage in sciatic nerve.[28] However, there was few studies on the immunoreactivity for iNOS in the DRG of a lumbar disc herniated model. We were observed that iNOS was stained in the cytoplasm of various diameter of neuron cells in the DRG. The number of iNOS-immunoreactive cells was increased in a rat model of lumbar disc herniation compare to the sham-operated group. To our knowledge, we have firstly found that the localization of iNOS in the DRG of a rat model of lumbar disc herniation using the immunohistochemical method.

We have also found that the most iNOS-positive neurons of small and intermediate size were co-localized with TRPV1-positive cells in the DRG of a lumbar disc herniated model. The expression of TRPV1 signals is found in small to intermediate-sized dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons, which give rise to unmyelinated C and Aδ-fibers conveying various modes of noxious stimuli.[29] TRPV1 is involved in nociception, and analysis of capsaicin receptor lacking mice confirmed that the TRPV1 channel contributes to the detection and integration of painful stimuli.[30] TRPV1 was first identified in DRG and trigeminal ganglion (TG) neurons.[31] TRPV1 has since been described in many other neuronal and non neuronal cells,[32,33] high levels of TRPV1 expression were detected in DRG and relatively lower levels in the CNS, including spinal cord, hippocampus and cerebral cortex.[34]

In summary, the expression of iNOS and TRPV1 in a lumbar disc herniated model may play important roles in pathomechanisms of radicular pain in patients with lumbar disc herniation. The inhibition of iNOS might suppress radicular pain due to disc herniation.

V. Conclusion

We have demonstrated that the nucleus pulposus-induced mechanical allodynia was generated without a mechanical compression and this pain was relatively related to temporal changes of mRNA expression of iNOS in DRG. Also, we suggested that co-localization of TRPV1 and iNOS in small and intermediate neurons of DRG is highly correlated with pain modality or intensity.

Author Contributions

Research design: Kim SJ, Ahn SH
Acquisition of data: Kim SJ, Hwang SJ, Seo JM
Analysis and interpretation of data: Kim SJ, Cho YW, Park HW, Ahn SH, Hwang SJ
Drafting of the manuscript: Kim SJ
Administrative, technical, and material support: Lee JH, Seo JM
Research supervision: Ahn SH, Hwang SJ

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF- 2008-532-E00019).

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