

Osteoporotic Pain is Associated with Increased Transient Receptor Vanilloid 4 Expression in the Dorsal Root Ganglia of Ovariectomized Osteoporotic Rats: A Pilot Basic Study

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Abstract:

Introduction: Osteoporosis can produce a persistent state of pain known as osteoporotic pain. One proposed mechanism of this pathology is increased calcitonin gene-related peptide (CGRP; a marker related to inflammatory pain) expression in the dorsal root ganglia (DRG) innervating osteoporotic vertebrae. Alternatively, a previous study revealed that axial loading caused osteoporotic pain in a rodent model of coccygeal vertebrae compression. Because this compression model is associated with trauma, additional mechanistic studies of osteoporotic pain in the absence of trauma are required. The current study aimed to evaluate the expression and relative distribution of transient receptor potential vanilloid 4 (TRPV4), a pain-related mechanoreceptor, in ovariectomized (OVX) osteoporotic rats.

Methods: CGRP-immunoreactive (-ir) and TRPV4-ir DRG neurons innervating the L3 vertebrae of Sprague-Dawley rats were labeled with a neurotracer, FluoroGold. Intravertebral pH was also measured during the neurotracer procedure. TRPV4-ir/CGRP-ir FluoroGold-positive DRG neurons were quantified in sham control and OVX rats ($n = 10$, ea). The threshold for statistical significance was set at $P < 0.05$.

Results: There was no statistical difference in the number of FluoroGold-positive DRG neurons between groups; however, there were significantly more CGRP-ir/TRPV4-ir FluoroGold-positive DRG neurons in the OVX group compared with the sham control group ($P < 0.05$) as well as the significantly increased molecular production of each peptide. Intravertebral pH was also lower in the OVX group compared with the sham control group ($P < 0.05$).

Conclusion: Sensory neurons innervating osteoporotic vertebrae exhibited increased expression of co-localized CGRP and TRPV4 in OVX osteoporotic rats. Additionally, intravertebral pH was low in the vicinity osteoporotic vertebrae. Considering that TRPV4 is a mechanosensitive nociceptor that is activated in acidic environments, its upregulation may be associated with the pathology of osteoporotic pain derived from microinflammation involved in osteoporosis.

Keywords:

osteoporotic pain, ovariectomized rat, transient receptor potential vanilloid 4 (TRPV4), intravertebral pH, dorsal root ganglia (DRG), calcitonin gene-related peptide (CGRP)

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Introduction

Osteoporotic pain is a pain state derived from osteoporosis in the absence of fractures^{1,2)}. This pain state has been

previously identified in preclinical and clinical studies³⁻⁶⁾. Studies using ovariectomized (OVX) rodent models have suggested that the mechanism of osteoporotic pain involves both internal and external factors. Internally, estrogen

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deficiency-derived osteoporosis leads to the increased expression of calcitonin gene-related peptide (CGRP), an inflammatory pain-related neuropeptide, and transient receptor potential vanilloid 1 (TRPV1), a ligand-gated non-selective cation channel activated by stimuli including capsaicin, noxious heat⁷⁾, and low pH⁸⁾, in the dorsal root ganglia (DRGs) innervating osteoporotic vertebrae¹⁾. These changes are thought to decrease nociceptive thresholds, leading to hypersensitivity^{7,9,10)} as a component of osteoporotic pain²⁾. Other TRP families, TRPV4, is a Ca²⁺-permeable cation channel¹¹⁾ that is expressed in DRGs innervating several musculoskeletal tissues including cartilage, synovium, and bone. TRPV4 is known as a mechanoreceptor, and it is activated by osmotic stimulation, heat^{12,13)}, stretch, low pH, and citrate¹¹⁾. TRPV4 activation is associated with increased pain sensitivity, specifically in response to noxious pressure and pH stimuli^{11,14)}. Our previous study using the same pathological OVX models has indicated that continuous axial compression of osteoporotic coccygeal vertebrae leads to increased DRG expression of the nerve injury-related neuropeptide, ATF3, suggesting that continuous external axial loading may also play a role in the generation of osteoporotic pain⁴⁾. However, other pressure-related factors such as TRPV4 has not been evaluated yet.

Thus, we hypothesized that the TRPV4 in the sensory nerves innervating osteoporotic vertebrae would be increased. The goal of this study was to investigate the expression of TRPV4 in the sensory nerve in an OVX rodent model of osteoporotic pain to conform the pathological mechanisms underlying osteoporotic pain.

Materials and Methods

Ethical approval

All animal protocols were reviewed and approved by the ethics committee of our institute and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1996 revision).

Experimental model of osteoporosis

To generate the OVX osteoporotic pain model, female Sprague-Dawley rats were ovariectomized at 5 weeks of age and used in experiments at 35 weeks of age (weight, 300-500 g) (OVX group ($n = 10$); CLEA Japan Inc., Tokyo, Japan) as is described in our previous study^{1,2,4)}. Animals in the sham control group underwent the same surgical procedure without ovariectomy (i.e., the ovaries were only exposed) ($n = 10$). The animals were housed in a semi-barrier system with a controlled environment (lighting, 12 h light/dark cycle; humidity, 45-65%; temperature, 21-23°C). Bone mineral density (BMD) of each animal was measured using L5 vertebrae resected at the time of harvesting DRGs described later to confirm osteoporotic state, using peripheral quantitative computed tomography (XCT Research SA; Stratec Medizintechnik, Pforzheim, Germany).

Retrograde neurotracing and intravertebral pH measurement

The osteoporotic pain model using L3 vertebral body was generated using the OVX rats in a previously published protocol¹⁾. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and prepared for aseptic surgery. A midline abdominal incision was made, and the retroperitoneal space was exposed to access the lumbar spine. After confirmation of the L3 vertebra, the anterior wall was perforated 3 mm proximal to the L3-L4 intervertebral disc with an 18-gauge needle inserted at a depth of 1.5 mm. Then, a pH tester with a fine spear electrode was inserted through the hole to measure intervertebral pH (pH Spear[®], Eutech Instruments Pte Ltd., Crescent, Singapore). Next, crystals of retrograde neurotracer, Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) were inserted into the hole using a 23-gauge needle, followed by immediate sealing with cyanoacrylate-based glue to prevent leakage. After confirming the absence of FG leakage into peripheral tissues, the surface of the abdomen was sutured.

Immunofluorescent DRG labeling

Four weeks after FG labeling, rats were anesthetized, and the intravertebral pH was measured as the same way using L4 vertebra, and then the rats were transcardially perfused with 0.9% saline followed by 500 mL of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Bilateral DRGs from the L1-L2 level (i.e., those predominantly innervating the L3 vertebra) were resected. Bilateral DRGs were harvested together at each level to get enough number of samples, and then the samples were divided into L1 and L2 DRGs cohort and treated respectively. Specimen DRGs were immersed in buffered paraformaldehyde fixative overnight at 4°C, and 1 week for the L5 vertebrae for following BMD measurements, followed by immersion in phosphate-buffered saline (PBS) containing 20% sucrose for 20 h at 4°C. Finally, specimen DRGs were embedded in OCT Tissue Tek (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. Sections of 10 µm in thickness were cut on a cryostat (Leica Microsystems, CM3050S, Wetzlar, Germany), and endogenous tissue peroxidase activities were quenched by soaking in a 0.3% hydrogen peroxide solution in PBS for 30 min. Sections were then treated in a blocking solution (PBS containing 0.3% Triton X-100 and 3% skim milk) for 90 min at room temperature. Next, sections were incubated with primary rabbit antibody against CGRP (1:1000; Immunostar, Hudson, WI, USA) and chicken antibody against TRPV4 (1:500; Santa Cruz, Dallas, TX, USA) diluted in blocking solution for 20 h at 4°C. Sections were rinsed three times in PBS and subsequently incubated with Alexa 488-conjugated donkey anti-rabbit IgG (CGRP immunoreactivity in green; 1:1000; Molecular Probes, Eugene, OR, USA) followed by Alexa 594-conjugated chicken anti-goat IgG (TRPV4 immunoreactivity in red; 1:1000; Molecular Probes, Eugene, OR, USA). Fluorescent signal was observed using a

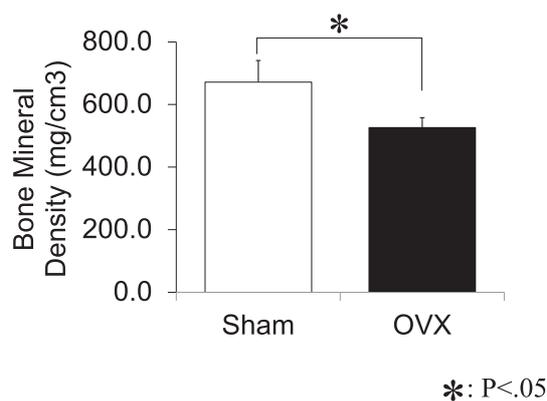


Figure 1. Bone mineral density (BMD) of the experimental animals.

BMD was significantly decreased in the ovariectomized (OVX) animals to show osteoporotic state.

fluorescence microscope (Olympus, Tokyo, Japan) in a blinded fashion. Numbers of FG-labeled, CGRP-immunoreactive (-ir), and TRPV4-ir neurons were counted and expressed as a proportion of the total number of cells and then as a proportion of the FG-labeled neurons. In evaluating the fluorescent images, three observers who were not familiar with the experiment evaluated the images. The DRG neurons were regarded as countable when they have nuclei in them. The criteria to define molecular-positive neurons were as follows: (1) The interest neurons include cross-section of nuclei, and (2) positive area distributes more than half of the cytoplasm. The evaluations of the observers were statistically evaluated after confirming that their standard deviation showed no significance. The neurons were not considered to be FG-labelled if the apparent staining is too thin and to be immersed into the interstitium by slight adjustment of the contrast.

Statistical analysis

Between-group differences in the proportions of immunoreactive DRG neurons, BMD, and intravertebral pH were analyzed using Mann-Whitney *U*-tests. $P < 0.05$ was the threshold for statistical significance (PASW statistics ver. 18 (SPSS Inc. (IBM), Somers, NY, USA)). Data are expressed as the mean \pm standard error unless otherwise indicated.

Results

BMD was significantly decreased in the OVX animals to show osteoporotic state (Fig. 1).

Histologically, there were no differences between the OVX and sham groups such as the proportion of the number of small- or large-sized DRG neurons and their shapes besides immunofluorescent properties. FG-labeled DRG neurons were equally present in the bilateral DRGs at each level of L1 and L2 DRG, respectively, in both groups (Fig. 2(a, b), and Fig. 3(a)). CGRP-ir DRG neurons were signifi-

cantly increased in the OVX group (Fig. 3(b)), and also TRPV4-ir DRG neurons were significantly increased in the OVX group (Fig. 3(c)). Regarding CGRP/TRPV4-ir (double positive) DRG neurons, they were significantly increased in the OVX group (Fig. 2(c, d) and Fig. 3(d)).

Regarding Intravertebral pH, it was significantly lower in the OVX group compared with the sham control group (5.9 ± 0.16 vs. 6.6 ± 0.25 , respectively; $P < 0.05$, Fig. 4) at the time of FG application (baseline). Intravertebral pH in each group showed no difference at the time of sacrifice (4 weeks) compared with the baseline, showing significant decrease in the OVX group ($P < 0.05$, Fig. 4). These results indicate the possible evidence for osteoporosis-related TRPV4 upregulation in accordance with the reduced intravertebral pH as well as decreased BMD in OVX rats.

Discussion

The current study demonstrated a significant increase in TRPV4-ir/CGRP-ir DRG neurons innervating osteoporotic vertebrae as well as significantly decreased intravertebral pH in an OVX rodent model of osteoporotic pain.

TRPV4 is a mechanosensitive and Ca^{2+} -permeable channel expressed in DRG neurons¹¹ that is associated with hyperalgesia through the upregulation of glial-derived neurotrophic factor and cyclooxygenase-2 expression¹⁵ and is reported to be expressed in sensory nerve in the tongue and trigeminal nerve¹¹. Furthermore, CGRP is commonly used as a marker related to inflammatory pain in rodents¹⁶. On this premise, we hypothesized that TRPV4-ir/CGRP-ir DRG neurons would represent pain-generating neurons in our rodent model of osteoporotic pain.

Previous studies have reported the involvement of TRPV4 in pain-related pathology; TRPV4-deficient mice show significantly reduced sensitivity to mechanical and acidic nociceptive stimuli, indicating a role for TRPV4 in detecting mechanical nociceptive signals, especially in low-pH environments¹¹. The responsiveness of TRPV4 to noxious mechanical pressure has been reported in other organs and cells including retinal ganglion cell neurons, where TRPV1 and TRPV4 are co-localized to sense ocular pressure¹⁷, and in joint disease, where TRPV4 plays a key role in transducing noxious, mechanical, and inflammatory signals within joint tissues¹⁸.

We previously demonstrated that the axial compression of osteoporotic vertebrae elicits osteoporotic pain associated with ATF3 upregulation and increased CGRP expression⁴; however, the osteoporotic model used in our previous study included physical compression of the coccygeal vertebrae, which were fused using penetrating k-wires and compressing rubber bands. A major issue in our previous study was thus the traumatic nature of the procedure itself, which may have activated the expression of pain-related factors. The current study provided evidence for the possible involvement of mechanical pressure in the pathology of osteoporotic pain by investigating the expression of TRPV4 and evaluating in-

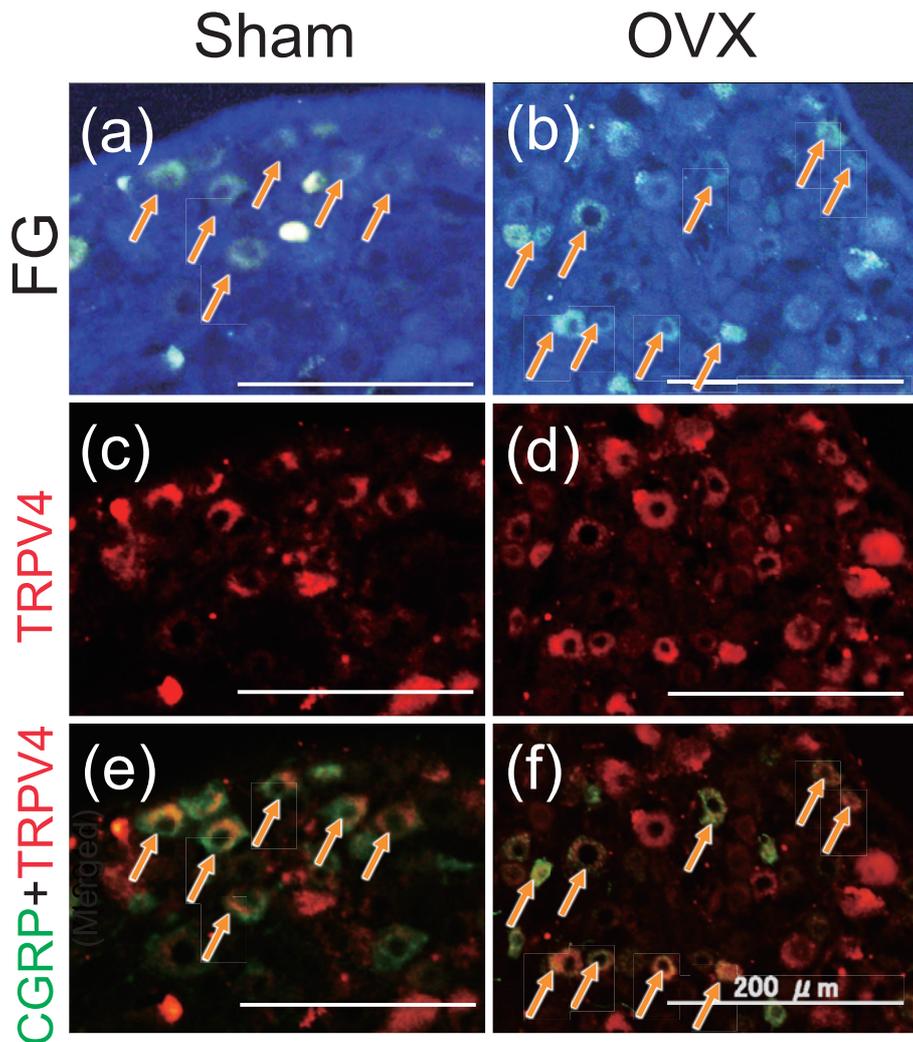


Figure 2. Representative fluorescence photomicrographs of L2 dorsal root ganglia. Images in each group are from the same section. Scale bars=200 μ m. Arrows indicate TRPV4/CGRP double-positive FluoroGold (FG) -labeled dorsal root ganglion neurons (green, CGRP; red, TRPV4; and orange, merged). Sham, sham control group; OVX, ovariectomized osteoporotic pain group. (a, b) FG-labeled DRG neurons are described in yellow with no significant distribution; (c, d) TRPV4 showed significant immunoreactivity in OVX animals (d) compared with Sham animals (c); and (e, f) TRPV4/CGRP double-positive FG-labeled DRG neurons showed significant increase in OVX animals (f) compared with Sham animals (e).

travertebral pH in a model involving minimal trauma. The observation of low intravertebral pH was likely associated with chronic microinflammation elicited by activated osteoclasts¹⁾. These facts indicate that increased mechanical stress on the osteoporotic vertebrae can increase the TRPV4 production via the activation of TRPV4 receptor by the stress as well as the acid circumstance induced by the microinflammation from osteoporotic osteoclasts that can result in osteoporotic pain. Considering the mechanism, the elevation of TRPV4 production and microinflammation can follow a protracted course, which should be confirmed in the future study with multi-time points. Accordingly, the role of osteoclast activation should be examined in future studies of osteoporotic pain.

The current study had some limitations. First, local find-

ings in the vertebrae and bone tissue were not directly and histologically evaluated, as the current study mainly focused on the TRPV4 expression in sensory neurons. The degree of osteoporosis also should be evaluated in the future study in terms of osteoporotic pain-related pathogenesis. Second, we did not directly measure pressure on the osteoporotic vertebrae, which is difficult to measure in an *in vivo* situation; thus, the fact of the TRPV4 increasing is important as it indicates the possible increasing in mechanical pressure as well as osteoporotic acid circumstance. Third, we did not examine the direct relationship among TRPV4 upregulation, increased CGRP, and decreased pH, which may be a critical component of the pathological nociceptive mechanism. Fourth, we did not directly evaluate pain behaviors in our rodent model. The evaluation of pain or nociception was

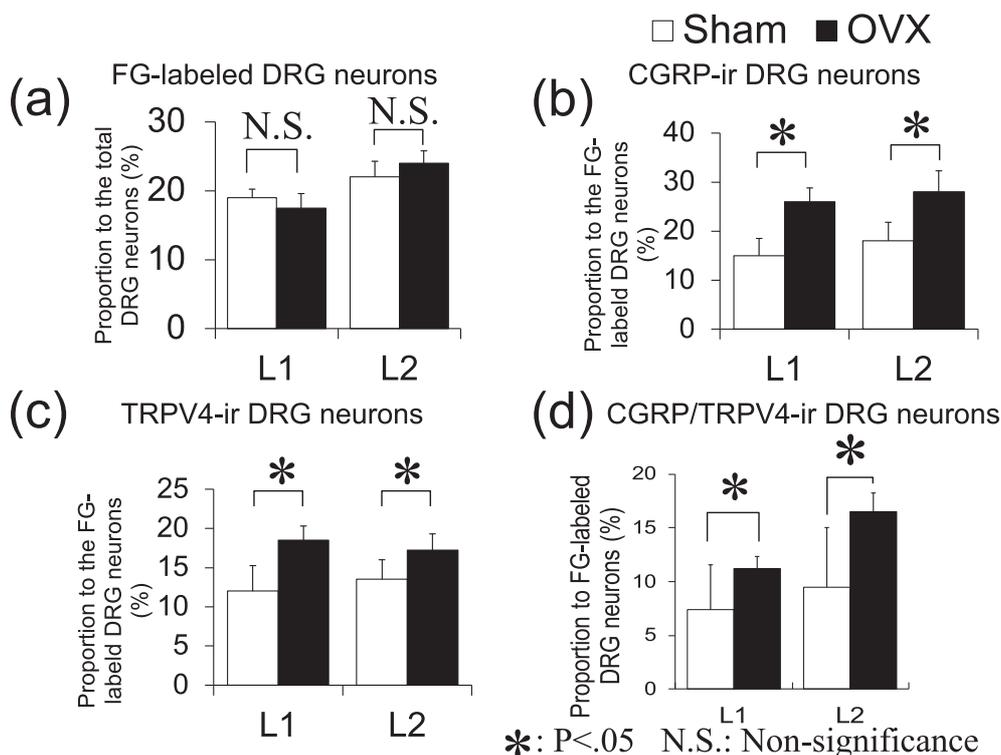


Figure 3. (a) Quantification of FluoroGold-labeled dorsal root ganglion neurons. The proportions are shown to the number of total DRG neurons (b, c) CGRP-immunoreactive (-ir) and TRPV4-ir DRG neurons were significantly increased in the OVX group, respectively. (b: CGRP, c: TRPV4) (d) CGRP/TRPV4-ir (double-positive) DRG neurons were significantly increased in the OVX group. Sham, sham control group; DRG, dorsal root ganglion; OVX, ovariectomized osteoporotic pain group; N.S., non-significant. * $P<0.05$

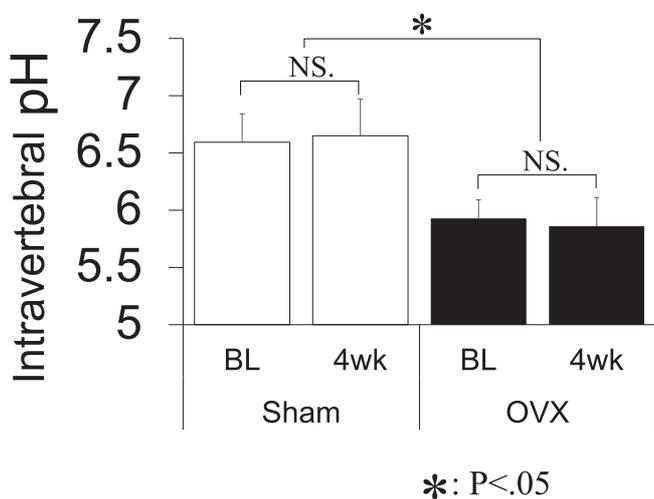


Figure 4. Intravertebral pH measurements. Vertebral pH was measured through the small hole made in the anterior portion of the L3 vertebra using a pH meter at baseline (the time of FG application) and measured using L4 vertebra as the same way in L3 at the time of sacrifice (4 weeks). Intravertebral pH in each group showed no difference at the time of sacrifice compared with the baseline, showing significant decrease in the OVX group. Sham, sham control group; OVX, ovariectomized osteoporotic pain group. * $P<0.05$

complicated in our model compared with other models, as direct stimulation testing such as von Frey testing was not available. To this end, alternative methods such as gait analysis can be useful¹⁹ and should be implemented in further studies. Finally, additional work is required to investigate the nature of osteoporotic bone pain in greater detail, as the mechanism is multifaceted and may involve changes at the level of the spinal cord as well as the DRG^{2,20}.

In conclusion, we demonstrate that TRPV4 and decreased intravertebral pH may play a critical role in the mechanism of osteoporotic pain. Considering the role of TRPV4 as a mechanosensitive nociceptor that is activated under acidic conditions, future studies should examine whether increased TRPV4 expression and decreased pH act in concert to promote osteoporotic pain.

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Author Contributions: SO, MS, KH, KF, and YS performed the experiment. HK, KA, MI, HK, MN, and TU corrected and analyzed the data. SO wrote and prepared the manuscript, and all of the authors participated and approved the study design. All authors have read, reviewed, and approved the manuscript.

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