

Capsaicin Treatment Inhibits Osteopenia and Heat Hyperalgesia Induced by Chronic Constriction Injury to the Sciatic Nerve in Rats

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ABSTRACT

Chronic constriction injury (CCI) to the rat sciatic nerve results in osteopenia in the affected hind limb. One possible mechanism for this osteopenia is neurogenic inflammation, in which neuropeptides, represented by substance P (SP), are involved. We attempted to determine whether capsaicin treatment, which can deplete SP from nerve terminals, is effective in inhibiting osteopenia induced by CCI. Capsaicin (total dose, 125 mg) or the vehicle alone was given intraperitoneally to adult rats 2 days before (Experiment 1) and 7 days after (Experiment 2) CCI surgery. Paw withdrawal latency (PWL) was measured prior to and every week for 5 weeks after surgery. Bone mineral density (BMD) and the number of osteoclasts in tibial bones were determined 5 weeks after surgery. In rats treated with the vehicle, BMD on the CCI side was decreased significantly, while the number of osteoclasts was significantly increased in both experiments. Capsaicin treatment either before or 1 week after surgery inhibited the decreases in BMD as well as the increase in the number of osteoclasts on the CCI side. PWL for the CCI side in the vehicle group was significantly shorter than for the sham side in both experiments. However, capsaicin treatment before surgery resolved heat hyperalgesia in Experiment 1, while in Experiment 2, even though heat hyperalgesia developed on the CCI side, it was resolved by capsaicin treatment. The results of the present study show that capsaicin inhibits the development of osteopenia as well as heat hyperalgesia induced by CCI. They also support our hypothesis that neurogenic SP release is involved in the pathogenesis of bony changes induced by CCI.

Key words: Neuropathic pain, Osteopenia, Substance P, Capsaicin

Although the clinical criteria of complex regional pain syndrome (CRPS) do not include osteopenia as an essential sign⁴⁵⁾, abundant clinical evidence has demonstrated that bone pathology is one of the typical signs of CRPS^{15,22,31,37,40,44,46)}. In our previous experiments using rats with chronic constriction injury (CCI), an animal model of CRPS type 2^{30,39)}, we found that loose ligation of the sciatic nerve with chromic gut resulted in osteopenia in the affected limb⁴⁷⁾, which included decreases in bone mineral density (BMD) as well as an increase in the number of osteoclasts in the affected tibial bone.

Substance P (SP) and other neuropeptides have been reported to be released from the terminals of primary sensory fibers after loose ligation of the

sciatic nerve, which could cause neurogenic inflammation in the peripheral tissues^{9,50)}. Further, sensory fibers distributed densely in bone, the periosteum and bone marrow are known to contain SP^{3,23,27,28)}, and the expression of neurokinin-1 receptor (NK1-R), a specific receptor for SP, has been found in rat bone cells¹⁶⁻¹⁹⁾. Meanwhile, *in vitro* studies using rat or rabbit osteoclasts have indicated that SP increases bone resorption by the activation of osteoclasts³⁶⁾ and stimulates osteoclast formation¹⁷⁾.

These findings suggest that SP is released from primary sensory nerves and influences local bone metabolic processes. Thus, we hypothesized that SP plays an important role in the pathogenesis of nerve injury-induced osteopenia in rats with CCI.

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We attempted to determine whether capsaicin treatment, which can deplete the sensory neuropeptide SP from primary sensory neurons, is effective in inhibiting osteopenia induced by CCI.

MATERIALS AND METHODS

Animals

All experimental procedures used in this study were approved by our institutional Animal Care Committee and carried out in accordance with the International Association for the Study of Pain (IASP) guidelines for investigation of experimental pain in animals⁵¹. We used adult male Sprague-Dawley rats (Charles River, Japan) that were approximately 7 weeks old and weighed 250–300 g. The rats were housed individually under controlled temperature and lighting conditions, with food and water *ad libitum* for at least 1 week prior to and throughout the experiments.

CCI surgery

Under anesthesia with a dose of 50 mg/kg of sodium pentobarbital, injected intraperitoneally (i.p.) and repeated if necessary, the right sciatic nerve of each rat was exposed at the mid-thigh level by blunt dissection. Four ligatures using 4–0 chromic gut were loosely tied around the nerve at a spacing of approximately 1 mm, as described in detail elsewhere². The left limb sciatic nerve was also exposed through identical dissection, but it was not ligated (sham surgery). The incisions were then closed in layers with sutures using 4–0 silk.

Capsaicin and vehicle treatment

According to the method of previous studies^{4,7,33}, capsaicin (Sigma Chemical Co., USA) was administered i.p. 3 times over 36 hours. Briefly, the rats were premedicated with atropine sulfate (Sigma Chemical Co., USA) (2 mg/kg i.p.) and theophylline (Nacalai Tesque Inc., Kyoto) (5 mg/kg i.p.) 30 min before capsaicin treatment, and then kept under ketamine anesthesia (80 mg/kg i.p.). Capsaicin at 25 mg/kg was dissolved in a vehicle solution that contained 50% saline and 50% dimethyl sulfoxide solution (DMSO, Sigma Chemical Co., USA), and then injected i.p. 5 min after administering ketamine. Thereafter, 50 mg/kg of capsaicin was given at 18 and 36 hours after the initial injection, also under ketamine anesthesia. The concentration of the capsaicin solution for all injections was 25 mg/ml. In the vehicle group, an equal volume of the vehicle solution was injected at the same times. After injection of capsaicin or the vehicle, all rats received a dose of ketamine, capable of providing anesthesia for 2 hours. Rats that experienced respiratory distress or cardiovascular problems during the capsaicin treatment were excluded from our study. Only rats that showed blunting of the corneal reflex, an indication of SP depletion from the

peripheral sensory nerves, were used for the study.

Measurement of heat hyperalgesia

Heat hyperalgesia was measured using a Plantar Test device (Model 7370, Ugo Basile, Italy), in a manner similar to that described by Hargreaves et al²¹. Heat stimulation was then applied after the rats had been allowed to habituate for about 10 min. When a clear pain-like paw withdrawal response was observed, the time was recorded and referred to as paw withdrawal latency (PWL). Both hind paws were tested 8 times at 10 min intervals, and the last 5 measurements were averaged and used for statistical analysis. If PWL for the CCI side was not 1 standard deviation or more shorter than that for the sham side, the rat was excluded from the experiment. The PWL score for the sham side was subtracted from that on the CCI side and a negative value of this difference indicated a shorter latency on the CCI side as compared to that on the sham side.

Tissue fixation

Each rat was deeply anesthetized with sodium pentobarbital and perfused via the aorta with 400 ml of heparinized saline followed by 400 ml of a 3.7% formaldehyde in neutral buffer solution at pH 7.2. Both left and right hind paws were then immediately excised at the knee and calcaneal joints, and, after all the surrounding skin, muscle, soft tissue elements and the fibula were cut away, the tibia was fixed in 3.7% formaldehyde for 2 days.

Measurement of Bone Mineral Density (BMD)

The bone mineral content (BMC) of the tibial bone of each rat was measured using dual-energy X-ray absorptiometry (DEXA, DCS-600EX, Aloka, Tokyo) 2 days after fixation. The mineralization profiles of each specimen as well as the monitoring images were stored in a data processor, and then BMD (in milligrams per square centimeter) for the whole tibial bone was calculated as BMC divided by projected bone area.

Bone histology

After undergoing decalcification in 10% sodium ethylenediamine tetraacetic acid (EDTA) solution in Tris Buffer at pH 7.4 for 4 weeks, the tibial bones were sectioned at 5 μ m and then stained for tartrate-resistant acid phosphatase (TRAP) activity to visualize the osteoclasts, according to the method of Cole and Walters⁸. The number of osteoclasts per unit area (mm²) was counted in all areas within 1 mm of the distal end of the metaphysis under a light microscope by a counter who was blinded to the intervention. TRAP-positive cells with more than 3 nuclei were counted.

Experimental protocols

1. Experiment 1:

The effects of capsaicin treatment before CCI surgery on osteopenia and heat hyperalgesia were studied in Experiment 1. Briefly, capsaicin or the vehicle alone was given i.p. 3 times within 36 hours to the rats, starting at 2 days before CCI surgery. PWL was measured for each hind paw 3 days before and every week until 5 weeks after CCI surgery.

2. Experiment 2:

The effects of capsaicin treatment on osteopenia and heat hyperalgesia 1 week after CCI surgery were studied in Experiment 2. Briefly, capsaicin or the vehicle alone was given i.p. 3 times within 36 hours, starting at 7 days after CCI surgery. PWL was measured for each hind paw 1 day before and every week until 5 weeks after CCI surgery.

Data analysis

Data are presented as mean \pm standard deviation (SD), unless otherwise indicated. A Wilcoxon signed-rank test or Mann-Whitney U test was

used for comparison of BMD, the number of osteoclasts per area between the CCI and sham sides, percent reduction in BMD and percent increase in osteoclasts between vehicle and capsaicin in all experimental groups. Differences in latency scores between groups were analyzed using the Friedmann test, followed by the Mann-Whitney U test. Differences were considered statistically significant if p values were less than 0.05.

RESULTS

General observations

Rats which underwent CCI surgery without capsaicin treatment showed typical neuropathic signs, including guarding behavior of the affected hind paw, ventroflexed toes, holding the paw in an elevated position, and increased sensitivity to light touch. Furthermore, affected hind paws appeared to be edematous in rats receiving the vehicle only as well as in those in the capsaicin group before treatment with it. However, this was no longer the case after receiving capsaicin.

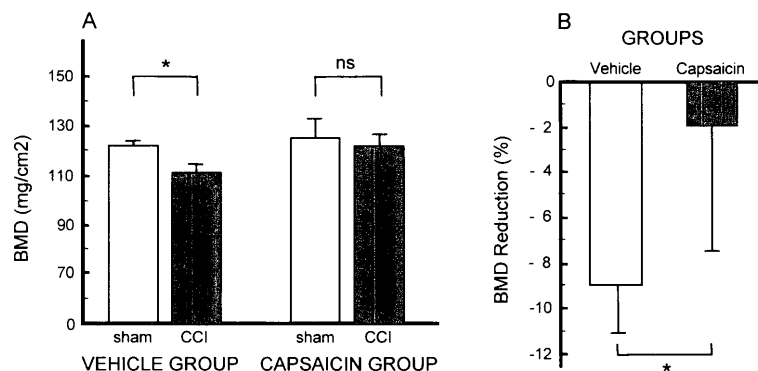


Fig. 1. A: Comparison of bone mineral density (BMD) between the CCI and sham sides in the vehicle treatment group (left) and the capsaicin group (right) 5 weeks after CCI surgery. B: Comparison of BMD on the CCI side between the capsaicin and vehicle groups. The BMD reduction in the capsaicin group was significantly smaller than that in the vehicle group. * $p < 0.05$, ns: not significant.

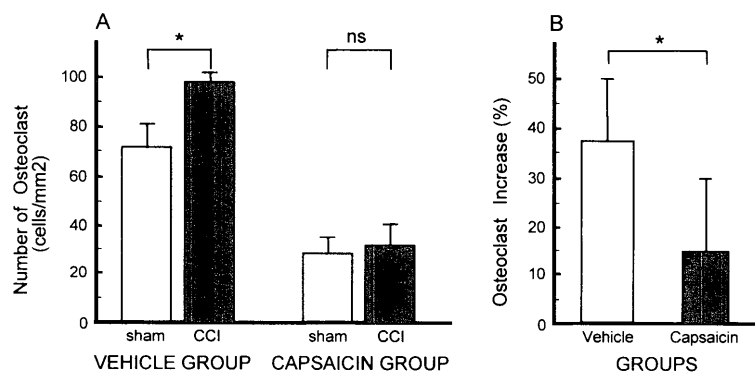


Fig. 2. A: Comparison of the number of osteoclasts in each area between the CCI and sham sides in the vehicle group (left) and the capsaicin group (right) 5 weeks after CCI surgery. B: Comparison of percent osteoclast increases between the vehicle and capsaicin groups. The increase in the number of osteoclasts in the capsaicin group was significantly inhibited as compared with that in the vehicle group. *, ns: see legend of Fig. 1.

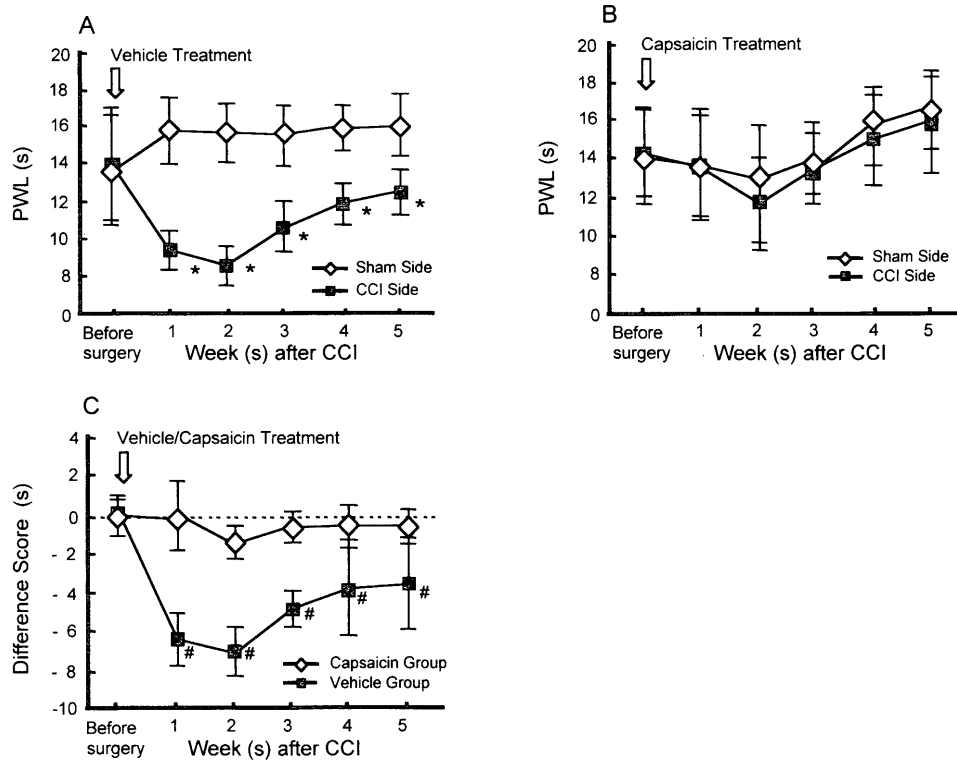


Fig. 3. Change in PWL after CCI surgery in the vehicle group (A) and the capsaicin group (B). Comparison of difference in latency scores between the vehicle and capsaicin groups (C). The significant shortening of PWL on the CCI side in the vehicle group, which was demonstrated by a large negative difference in scores, was almost completely absent in the capsaicin group. *: $p < 0.005$ vs. PWL on sham side, #: $p < 0.005$ vs. difference in scores in vehicle group.

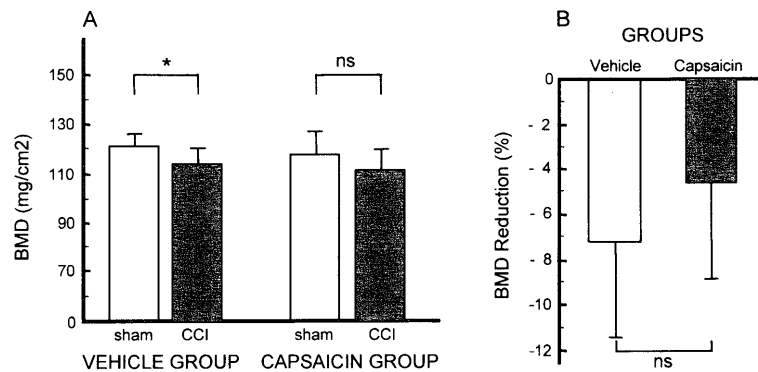


Fig. 4. A: Comparison of BMD between CCI and sham sides in the vehicle group (left) and the capsaicin group (right) 5 weeks after CCI surgery. B: Comparison of percent BMD reductions between the vehicle and capsaicin groups. *, ns: see legend of Fig. 1.

Experiment 1: Capsaicin treatment before CCI surgery

1. Effect of capsaicin on BMD and the number of osteoclasts

In the vehicle group ($n = 6$), BMD of the tibial bone on the CCI side (111 ± 4 mg/cm²) was significantly less than on the sham side (122 ± 2 mg/cm²) 5 weeks after CCI surgery ($p < 0.05$, Fig. 1A, left). However, when rats were treated with capsaicin ($n = 6$) the difference in BMD between the CCI (122 ± 5 mg/cm²) and sham (125 ± 8 mg/cm²) sides was not significant (Fig. 1A, right). Further, the percent reduction in BMD (relative to the sham

side as the control) in rats with capsaicin treatment was significantly less than with the vehicle alone ($p < 0.05$, Fig. 1B).

The number of osteoclasts per counting area in the metaphyseal regions was significantly higher on the CCI side (98 ± 4 cells/mm²) than on the sham side (72 ± 9 cells/mm²) in rats which received the vehicle alone ($p < 0.05$, Fig. 2A, left). However, in rats treated with capsaicin, the number on the CCI side (32 ± 9 cells/mm²) was not significantly different from that on the sham side (28 (7 cells/mm²) (Fig. 2A, right). Further, the percent increase in the number of osteoclasts (relative to

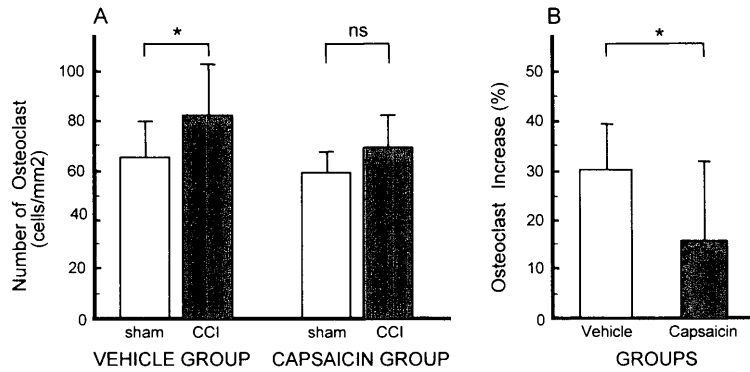


Fig. 5. A: Comparison of the number of osteoclasts per area between CCI and sham sides in the vehicle group (left) and the capsaicin group (right) 5 weeks after CCI surgery. B: Comparison of percent osteoclast increases between the vehicle and capsaicin groups. The increase in the number of osteoclasts in the capsaicin group was significantly inhibited as compared with the vehicle group. *, ns: see legend of Fig. 1.

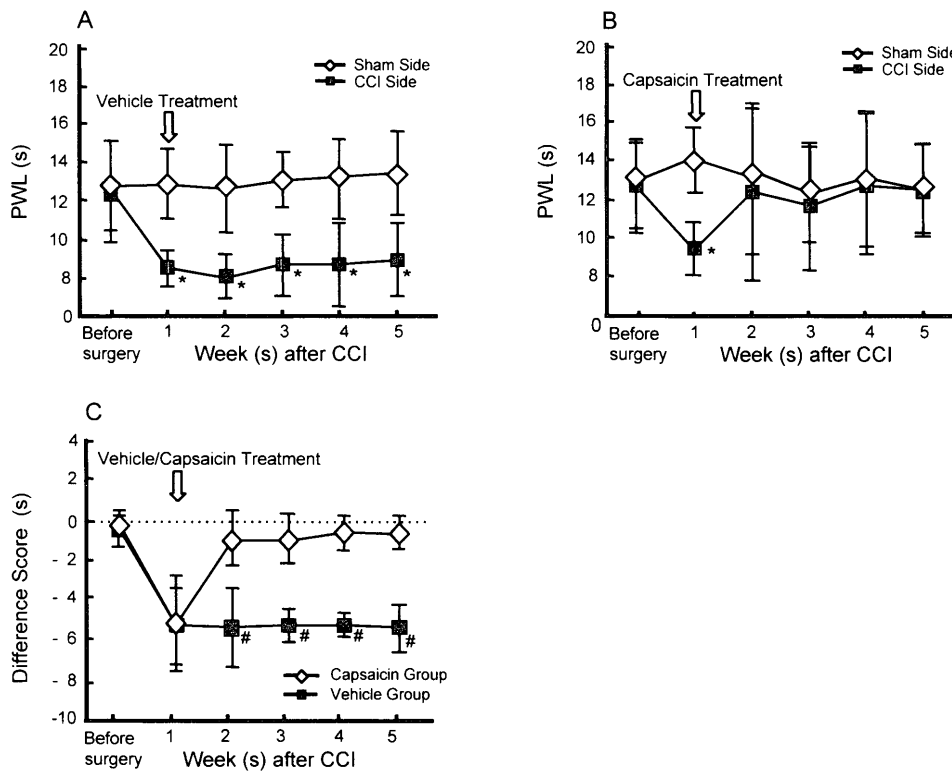


Fig. 6. Changes in PWL with time in the vehicle group (A) and the capsaicin group (B). Comparison of changes in difference in latency scores for both groups (C). Rats that underwent CCI surgery developed heat hyperalgesia 1 week after CCI surgery. However, this was completely resolved after treatment with capsaicin. *, #: see legend of Fig. 3.

the sham side as the control) in the capsaicin group was significantly less than in the vehicle group ($p < 0.05$, Fig. 2B).

2. Effect of capsaicin on heat hyperalgesia

In the vehicle group, PWL for the CCI side was significantly shorter than before CCI surgery and significantly different from the sham side each week for 5 weeks after CCI surgery ($p < 0.05$, Fig. 3A). However, in the capsaicin group, PWL for the CCI side was not significantly different from the sham side (Fig. 3B). The differences in latencies between the CCI and sham sides were significant-

ly smaller in the capsaicin group than for the vehicle group each week after CCI surgery ($p < 0.005$, Fig. 3C).

Experiment 2: Capsaicin treatment one week after CCI surgery

1. Effect of capsaicin on BMD and the number of osteoclasts

The BMD on the CCI side ($114 \pm 6 \text{ mg/cm}^2$) in the vehicle group was significantly different from that on the sham side ($121 \pm 5 \text{ mg/cm}^2$) ($p < 0.05$, Fig. 4A, left). However, the BMD on the CCI side

(111 ± 8 mg/cm²) in the capsaicin group was not significantly lower than that on the sham side (117 ± 9 mg/cm²) (Fig. 4A, right). Further, the percent reduction in BMD (relative to the sham side as the control) was not significantly different between the capsaicin and vehicle group (Fig. 4B).

The number of osteoclasts in each counting area was significantly higher on the CCI side (82 ± 20 cells/mm²) than on the sham side (65 ± 14 cells/mm²) in the vehicle group ($p < 0.05$, Fig. 5A, left). However, that on the CCI side (69 ± 13 cells/mm²) was not significantly different from the sham side (60 ± 7 cells/mm²) in the capsaicin group (Fig. 5A, right). Further, the percent increase in the number of osteoclasts (relative to the sham side as the control) in the capsaicin group was significantly lower than in the vehicle group ($p < 0.05$, Fig. 5B).

2. Effect of capsaicin on heat hyperalgesia

In the vehicle group, PWL for the CCI side was significantly shorter than before CCI surgery and significantly different from the sham side each week for 5 weeks after CCI surgery ($p < 0.05$, Fig. 6A). In the capsaicin group, PWL for the CCI side was significantly shorter than before CCI surgery and significantly different from the sham side 1 week after CCI surgery. However, from 2 to 5 weeks following surgery, PWL for the CCI side was not different from the sham side in this group (Fig. 6B). In the capsaicin group, differences in latency scores between the sham and CCI sides were not significant 1 week after CCI surgery, but they were significantly smaller than those in the vehicle group from 2 to 5 weeks after CCI surgery ($p < 0.05$, Fig. 6C).

DISCUSSION

A. Inhibition of CCI-induced osteopenia by capsaicin treatment

We found that a high intraperitoneal dose of capsaicin before CCI surgery inhibited decreases in BMD as well as an increase in the number of osteoclasts induced by CCI in tibial bones (Experiment 1). We also showed that, in the case of capsaicin treatment 1 week after CCI surgery, differences in BMD between the CCI and sham side disappeared, and an increase in the number of osteoclasts in the tibial bones was inhibited (Experiment 2). The results of these two experiments clearly supported our hypothesis that neurogenic SP release from the terminals of primary sensory nerves is involved in the pathogenesis of osteopenia induced by CCI.

This finding is supported by the following studies. SP release from the peripheral nerve terminals after CCI surgery was inferred from an increase in SP noted in the skin 7 days after surgery⁵⁰, and the expression of preprotachykinin mRNA, which encodes SP, was increased in the dorsal root ganglia (DRG) 5 days after CCI

surgery³⁴. Capsaicin, an active ingredient of spicy peppers known to be a vanilloid receptor 1 (VR1) agonist⁶, can cause depletion of SP content in DRG neurons over time and reaches a maximum at 10 days after treatment^{5,13,26}. Therefore, it is probable that the capsaicin used in the present study inhibited SP release following CCI surgery.

There is ample evidence suggesting that SP released from the peripheral nerve terminals is closely involved in bone metabolism. Mineralized bone, the periosteum, and bone marrow are abundantly innervated by sensory nerves containing SP^{23,27,28,41}. SP has also been implicated as a mediator of bone resorption²⁴, and reported to stimulate differentiation of osteoclasts from their progenitor macrophages^{1,41,42}, which may be followed by an increase in the number of osteoclasts. Further evidence supporting the involvement of SP in the differentiation of osteoclasts comes from the *in vitro* study on rat osteoclasts by Goto et al¹⁷, who demonstrated the activating effect of SP on osteoclast formation. They also demonstrated that the addition of the SP receptor antagonist [D-Pro², D-Trp^{7,9}]SP inhibited the increase in the number of multinucleated TRAP(+) osteoclasts¹⁷. Since the SP secreted from sensory nerve terminals is directly associated with osteoclastogenesis, depletion of SP from these terminals by capsaicin treatment may result in inhibiting the increase in the number of osteoclasts. Our results support this speculation (Fig. 2, 5). Further, SP receptors (NK1-R) have been reported to be abundant in osteoclasts¹⁶⁻¹⁹ and administration of micromolar concentrations of SP were found to stimulate the activity of cultured rabbit osteoclasts³⁶ and rat osteoclasts¹⁷; while spantide, an SP receptor antagonist, significantly inhibited the enhancement of bone resorption activity in osteoclasts induced by SP³⁶. Taken together with such evidence, our results indicate that SP release from sensory nerve terminals after nerve injury might cause osteopenia in the affected limb.

SP is probably the main cause of osteopenia induced by CCI. However, the inhibitory effect of capsaicin on this osteopenia may not be confined to SP, as treatment with it has also been reported to cause a marked reduction in the levels of several other neuropeptides from DRG and the dorsal horn of the spinal cord, such as calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), neurokinin A, and somatostatin^{11,13,29}. Capsaicin treatment could, therefore, reduce the content of these neuropeptides, among which, VIP is known to stimulate osteoclast activity and increase bone resorption^{25,32}. Further study is needed to determine whether VIP may also be involved in the underlying mechanism of CCI-induced osteopenia.

B. Inhibition of CCI-induced heat hyperalgesia by capsaicin treatment

In the present study, we found that capsaicin treatment resolved CCI-induced heat hyperalgesia. Two other studies have reported similar results. Meller et al³⁵⁾ reported that capsaicin treatment during the neonatal period completely prevented the development of the heat hyperalgesia induced by CCI in rats. Further, resiniferatoxin, a potent capsaicin analogue, was also reported to resolve heat hyperalgesia induced by CCI in adult rats²⁰⁾. The SP contents of dorsal horn of the spinal cord^{14,26)}, dorsal roots^{12,38)} and DRGs^{5,13)} have been reported to decrease after systemic capsaicin treatment. Increases in the SP content of the spinal cord and DRG are therefore involved in the pathogenesis of heat hyperalgesia that develops after nerve injury.

Capsaicin treatment resolved heat hyperalgesia induced by CCI almost completely, and PWL with heat stimuli was not prolonged (Fig. 3B and 6B), indicating preservation of the normal response to noxious heat. This may also indicate that adequate numbers of VR1, which contribute to the detection of noxious heat stimuli⁶⁾, still exist and that their normal function is not affected by capsaicin treatment. Meller et al³⁵⁾ suggested that the central processing mechanisms for the reflex response to noxious heat and heat hyperalgesia may be different. They cited the fact that the reflex withdrawal response to a heat stimulus was unchanged after administration of antagonists of NMDA receptors with MK-801, but that the heat hyperalgesia induced by CCI was completely resolved after administration of MK-801^{10,49)}. VR1 may also be involved in the central processing of heat hyperalgesia. Thus, further study is needed to elucidate the underlying molecular mechanism by which capsaicin resolves CCI-induced heat hyperalgesia.

C. Relationship between osteopenia and heat hyperalgesia induced by CCI

In the present study, capsaicin treatment inhibited both heat hyperalgesia and osteopenia induced by CCI. There are two possible relationships between the inhibition of CCI-induced osteopenia and that of heat hyperalgesia. First, both heat hyperalgesia and osteopenia are dependent on SP being released from the nerve terminals, and capsaicin treatment inhibits both by depleting SP from the terminals. Second, the protective behavior of the affected hind paw associated with heat hyperalgesia, such as elevation and avoidance of bearing weight, may result in osteopenia. Immobilization has been reported to be one of the possible causes of osteopenia associated with an increased number of osteoclasts^{43,48)}. If so, normalization of the protective pain behavior following capsaicin treatment should result in

remission of the osteopenia. However, in another experiment that we performed, the oral administration of 10 mg/kg etodolac (a cyclooxygenase-2 inhibitor) to CCI rats completely prevented the development of heat hyperalgesia, but failed to inhibit osteopenia (Suyama et al, 2000. Effect of COX2 inhibitor [Etodolac] on neuropathic pain in a rat model. Abstract A-931: ASA Annual Meeting, San Francisco, California). This result indicates that osteopenia induced by CCI is independent from protective pain behavior or immobilization. Therefore, we suggest that both the osteopenia and heat hyperalgesia are dependent on SP released from primary sensory nerves as a result of CCI.

CONCLUSION

In conclusion, we found that treatment with capsaicin inhibited the development of the osteopenia and heat hyperalgesia induced by CCI in rats. Although further confirmation is needed, taken together with the findings of previous studies, our results seem to support the hypothesis that SP plays an important role in the pathogenesis of both osteopenia and heat hyperalgesia induced by CCI. The present findings may also indicate that neurogenic inflammation, in which SP plays an important role, underlies the osteopenia and heat hyperalgesia seen in patients with CRPS.

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