Bone 52 (2013) 39–47

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Experimental tooth movement-induced osteoclast activation is regulated by sympathetic signaling

Mayo Kondo [a,b], Hisataka Kondo [a], Ken Miyazawa [b], Shigemi Goto [b], Akifumi Togari [a,*]

[a] Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan
[b] Department of Orthodontics, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan

ARTICLE INFO

Article history:
Received 28 May 2012
Revised 17 August 2012
Accepted 7 September 2012
Available online 18 September 2012

Edited by: David Fyhrie

Keywords:
Experimental tooth movement
Mechano-adaptive response
Sympathetic nervous system

ABSTRACT

Experimental tooth movement (ETM) changes the distribution of sensory nerve fibers in periodontal ligament and the bone architecture through the stimulation of bone remodeling. As the sympathetic nervous system is involved in bone remodeling, we examined whether ETM is controlled by sympathetic signaling or not. In male mice, elastic rubber was inserted between upper left first molar (M1) and second molar (M2) for 3 or 5 days. Nerve fibers immunoreactive for not only sensory neuromarkers, such as calcitonin gene-related peptide (CGRP), but also sympathetic neuromarkers, such as tyrosine hydroxylase (TH) and neuropeptide Y (NPY) were increased in the periodontal ligament during ETM. To elucidate the effect of the sympathetic signal mediated by ETM, mice were intraperitoneally injected with a β-antagonist, propranolol (PRO: 20 μg/g/day), or a β-agonist, isoproterenol (ISO: 5 μg/g/day) from 7 days before ETM. PRO treatment suppressed the amount of tooth movement by 12.9% in 3-day ETM and by 32.2% in 5-day ETM compared with vehicle treatment. On the other hand, ISO treatment increased it. Furthermore, ETM remarkably increased the osteoclast number on the bone surface (alveolar socket) (Oc.N/BS) in all drug treatments. PRO treatment suppressed Oc.N/BS by 39.4% in 3-day ETM, while ISO treatment increased it by 32.1% in 3-day ETM compared with vehicle treatment. Chemical sympathectomy using 6-hydroxydopamine (6-OHDA: 250 μg/g) showed results similar to those for PRO treatment in terms of both the amount of tooth movement and osteoclast parameters. Our data showed that blockade of sympathetic signaling inhibited the tooth movement and osteoclast increase induced by ETM, and stimulation of sympathetic signaling accelerated these responses. These data suggest that the mechano-adaptive response induced by ETM is controlled by sympathetic signaling through osteoclast activation.

Introduction

Orthodontic tooth movement changes the bone architecture through the stimulation of bone remodeling because bone is a dynamic tissue that can adapt its mass and architecture to mechanical loading [1–8]. The periodontal ligament is highly innervated by nerves, and experimental tooth movement (ETM) was shown to increase the number of nerve fibers containing neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP) [9–12]. Alteration of these nerve fibers is considered to be involved in pain transduction, inflammatory response, and periodontal ligament remodeling [13,14]. These nerve fibers are also considered to be involved in bone remodeling. When a force was applied to a tooth, osteoclasts predominantly appeared in the alveolar bone within a few days [15,16]. Inferior alveolar nerve transection suppressed an increase in osteoclast appearance during ETM. This suggests that sensory nerves play an important role in bone resorptive activity during ETM [17].

The sympathetic nervous system regulates bone remodeling through the β2-adrenergic receptor [3,4,4,18–28]. These studies have indicated that β2-adrenergic receptor mediates signaling in osteoblasts, which inhibits bone formation and increases osteoclastogenesis via receptor activator of nuclear factor kappa-B ligand (RANKL) expression [19,26]. Kondo et al. [4] reported that bone loss induced by mechanical unloading is regulated by the sympathetic nervous system. In this reported study, mice were intraperitoneally injected with propranolol (PRO), a non-specific β-adrenergic antagonist, once a day at 20 μg/g/day for 14 days to block sympathetic signaling. To stimulate sympathetic signaling, isoproterenol (ISO), a non-specific β-adrenergic agonist, is administered at 6 μg/g/day for 10 days. PRO treatment blocked unloading-induced bone loss and ISO treatment enhanced unloading-induced bone loss due to osteoblastic suppression and osteoclastic activation. These results suggested that the sympathetic nervous system mediates hindlimb unloading-induced bone loss through suppression of bone formation by osteoblasts and enhancement of resorption by osteoclasts. However, traditionally, bone adaptation to mechanical loading has been considered not to be centrally controlled. Marenzana et al. [5,29] reported

* Corresponding author at: Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan. Fax: +81 52 751 2588.
E-mail address: togariaf@dpc.aichi-gakuin.ac.jp (A. Togari).

8756-3282/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bone.2012.09.007

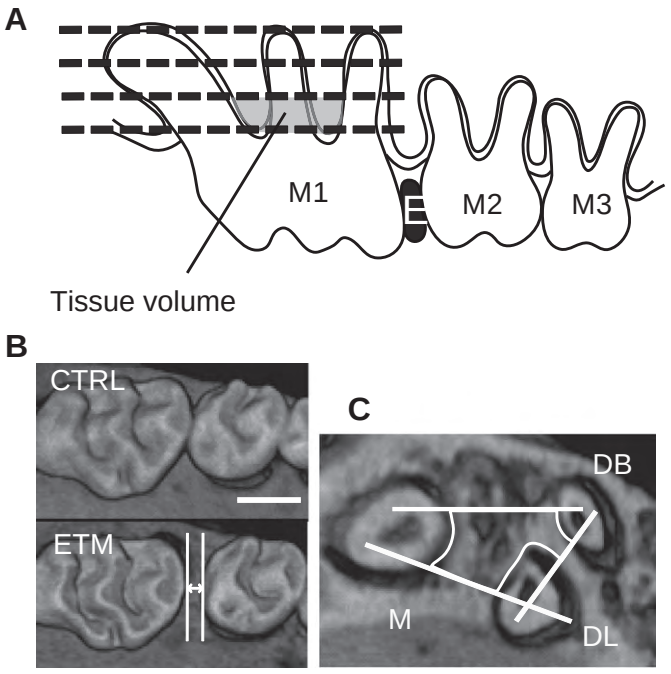


Fig. 1. Tooth movement system in mice. (A) An elastic module was inserted between the left upper first (M1) and second (M2) molars. The root was divided equally into thirds parallel to the occlusal plane. The region of the alveolar bone volume/tissue volume (A.BV/TV) is defined in the coronal region surrounded by the three roots of the first maxillary molar, as described in Materials and methods (gray area). E: elastic module. (B) To measure the amount of tooth movement, three-dimensional images were rotated to an occlusal view. Scale bars = 500 μm. ETM: experimental tooth movement. CTRL: control (before starting ETM). (C) The region of the alveolar bone volume/tissue volume (A.BV/TV) area defined as surrounded by the three tangents of each root canal of the first maxillary molar, as described in Materials and methods. M: mesial root. DL: distal lingual. DP: distal palatal.

that the sympathetic nervous system does not modulate the cortical bone gain induced by external loading. In this report, chemical sympathetic nervous system inactivation was achieved by prolonged daily treatment with guanethidine sulfate or by the addition of propranolol to drinking water. In general, chemical sympathectomy was performed using guanethidine sulfate or 6-hydroxydopamine (6-OHDA). As Kondo et al. [30] reported, the treatment of mice with the neurotoxin 6-OHDA inhibited sympathetic nervous signal-induced expression of interleukin (IL)-6 mRNA in their calvaria. The 6-OHDA destroys noradrenergic nerve terminals in the peripheral nervous system, and does not cross the blood–brain barrier in adult rodents [31,32]. ETM changes the bone architecture through the stimulation of bone remodeling. However, the mechanism of ETM is still unknown. Therefore, we tested whether the sympathetic nervous system is involved in bone remodeling induced by ETM.

Materials and methods

Animals

Male, eight-week-old C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were randomized by weight, assigned to groups and acclimated to their cages for 1 week prior to the experiment. They were treated in accordance with the Guidelines for Animal Experiments at the School of Dentistry, Aichi-Gakuin University. Food and water were available ad libitum. These mice of each group were housed together under automatically controlled conditions of temperature (23 ± 1 °C) and humidity (50 ± 10%). Body weight was measured every other day during the experimental period and recorded in grams (Sartorius BJ 600, Sartorius Corporation, Edgewood, NY, USA).

Experimental tooth movement

Mice were deeply anesthetized with pentobarbital sodium (32.4 mg/kg, i.p.), and a piece of orthodontic elastic band was inserted between the upper first and second molars (M1 and M2) by the method of Waldo [33], as shown in Fig. 1A. On day 3 and day 5 after the insertion, the mice were sacrificed by ether inhalation.

Drug treatment

The mice were subjected to daily intraperitoneal injections of PRO (20 μg/g of body weight/day), ISO (5 μg/g of body weight/day) or vehicle (VEH: 0.9% saline) from 7 days before ETM or one intraperitoneal injection of 6-hydroxydopamine (6-OHDA: 250 μg/g of body weight) or VEH 5 days before ETM.

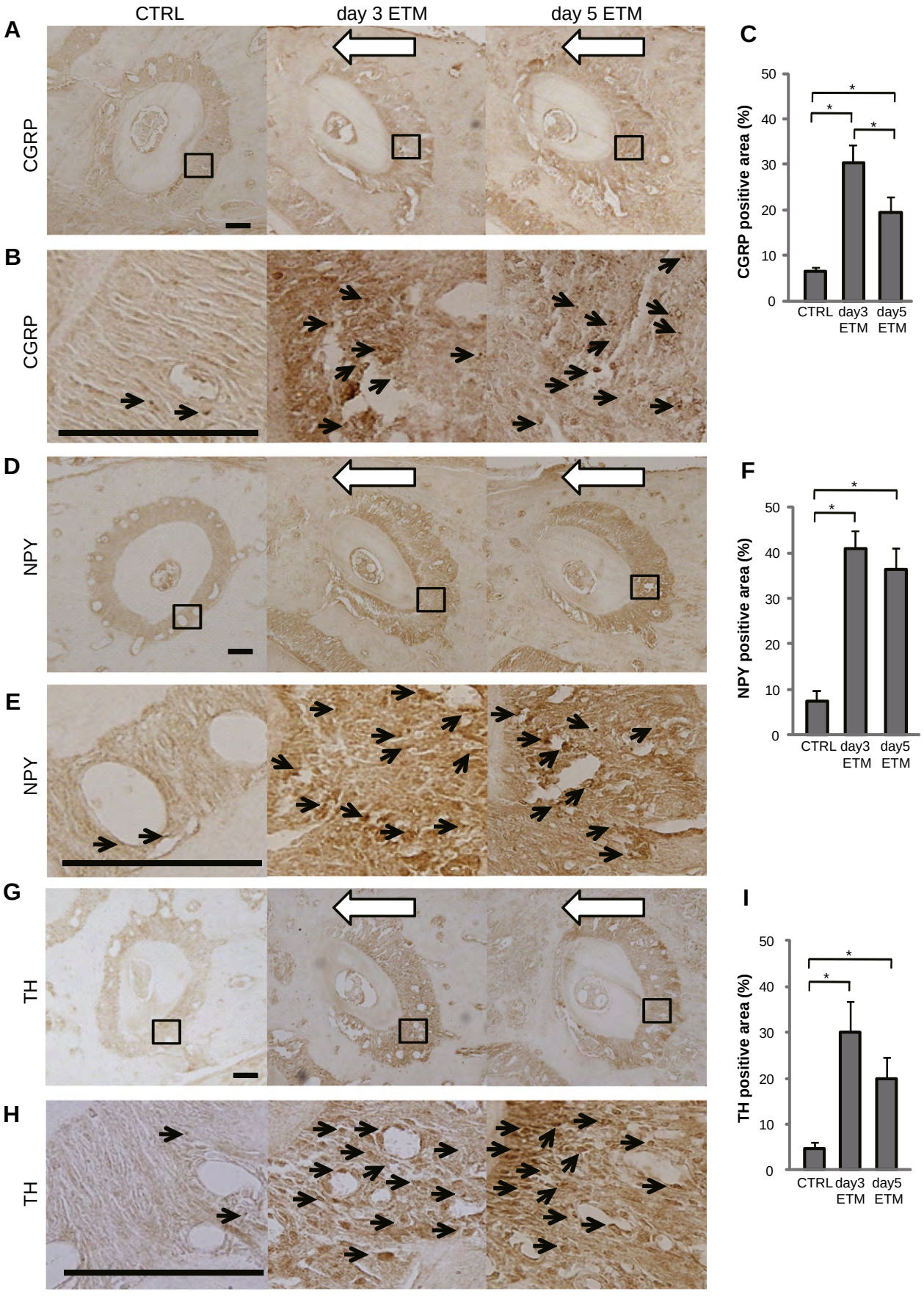
Bone histomorphometry

For the decalcified sections, the left mandibles of the mice were dissected and fixed in 4% paraformaldehyde and then decalcified in 20% EDTA for 2 weeks. Five-μm-thick occlusal sections were made as decalcified sections. For osteoclast analysis, these sections were stained with tartrate-resistant acid phosphatase (TRAP) and the numbers of osteoclasts on the alveolar socket (bone surface) (Oc.N/BS) and the osteoclast surface on the alveolar socket (bone surface) (Oc.S/BS) were evaluated by scoring the TRAP-positive multinucleated cells attached to the bone surface as defined by Parfitt et al. [34]. For immunohistochemistry, tyrosine hydroxylase (TH)-, neuropeptide Y (NPY)- and (CGRP)-immunoreactive nerve fibers were visualized by the ABC (avidin–biotin–horseradish peroxidase complex) method. Briefly, sections were incubated with primary antibody, such as TH (1:1000; Peninsula Lab., Belmont, CA, USA), NPY (1:1000; Yanagihara Institute Inc., Shizuoka, Japan) and CGRP (1:1000; Enzo Life Sciences, Plymouth Meeting, PA, USA) overnight at 4 °C followed by 1 h of incubation with blocking buffer (DS Pharma Biomedical, Osaka, Japan) and 1 h with ImmPACT DAB substrate (VECTOR Labs, Burlingame, CA, USA) at room temperature followed by 1 h of incubation with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Danvers, MA. USA). For light microscopic analysis, sections were dehydrated in a graded series of alcohol, cleared in xylene, and cover-slipped with Permount (Fisher Scientific Inc., Waltham, MA, USA). To quantified immunohistochemistry data, NIH image J software was used.

μCT analysis

The mandibles were subjected to three-dimensional μCT analysis using an R-mCT μCT scanner (RIGAKU, Tokyo, Japan). TRI/3D-BON (Ratoc, Tokyo, Japan) software was used to analyze the distance that

Fig. 2. CGRP, NPY and TH-immunoreactivity were increased by ETM. Representative pictures of immunohistochemical photomicrographs of the M1 distobuccal root. Calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and tyrosine hydroxylase (TH) were immunohistochemically stained. ETM: experimental tooth movement. CTRL: control (before starting ETM). In the mice, an elastic band was inserted between M1 and M2 for 3 days (3-day ETM) and 5 days (5-day ETM). Bar = 100 μm. (A) CGRP-immunoreactive nerve fibers. (B) Higher magnification of boxed area in A. CGRP-immunoreactive cells are indicated by arrows. (C) For CGRP immunostaining, density of positive area relative to periodontal ligament area. (D) NPY-immunoreactive nerve fibers. (E) Higher magnification of boxed area in C. NPY-immunoreactive cells are indicated by arrows. (F) For TH immunostaining, density of positive area relative to periodontal ligament area. (G) TH-immunoreactive nerve fibers. (H) Higher magnification of boxed area in E. TH-immunoreactive cells are indicated by arrows. (I) For NPY immunostaining, density of positive area relative to periodontal ligament area. Similar results were obtained from mesial and distolingual roots (data not shown). Arrows in A, D and G show the direction of force applied. Bars represent the mean ± SEM of 5 mice per group. *Indicates $p<0.05$.


CTRL
day 3 ETM
day 5 ETM
A
CGRP
B
CGRP
C
CGRP positive area (%)
50
40
30
20
10
0
CTRL
day3 ETM
day5 ETM
D
NPY
E
NPY
F
NPY positive area (%)
G
TH
H
TH
I
TH positive area (%)

each tooth had moved and a cancellous parameter: bone volume/total volume (BV/TV). For measurement of the amount of tooth movement, images were rotated and adjusted to ensure that the occlusal view with the narrowest gap between M1 and M2 was observed (Fig. 1B). The region of the alveolar bone volume/tissue volume (A.BV/TV) was defined in the coronal region surrounded by three roots of the first maxillary molar (Figs. 1AC). The coronal extent of the root was demarcated by the adjacent alveolar crest. For the femoral BV/TV, the scanning was initiated 1.0 mm above the distal femoral growth plate, and 1.5 mm length of the secondary spongiosa was analyzed. The measured volume of interest in the femora was obtained by selecting the cancellous bone.

Data analysis and statistics

All results were analyzed statistically by ANOVA using Stat View software (SAS Institute Inc.). Post hoc analysis, using the Tukey-Kramer test, was performed on values exhibiting an interaction effect. A p-value of less than or equal to 0.05 was considered to be statistically significant. All results are shown as the mean and standard error.

Results

The effect of ETM on sympathetic nerve distribution in periodontal ligament

First, we investigated the distribution of neurons in the periodontal ligament (distobuccal roots) during ETM in mice. The periodontal ligament contained a few CGRP-immunoreactive neurons in the control group (before starting ETM) (Figs. 2ABC). In contrast, a marked increase of CGRP-immunoreactive neurons was observed in the periodontal ligament by both 3 days and 5 days after ETM, as reported previously (Figs. 2ABC) [17]. NPY and TH-immunoreactive nerves were also rare in the control group and were increased by both 3-day and 5-day ETM (Figs. 2DEFGHI). Similar results were obtained from mesial and distolingual roots (data not shown). These findings suggested that sympathetic nerve activity may influence orthodontic tooth movement.

The effect of sympathetic signaling on ETM

Next, we tested whether the sympathetic nervous system affects ETM or not. To do this, mice were injected with a β-antagonist, PRO, or a β-agonist, ISO from 7 days before ETM and compared with vehicle treatment. The insertion of an elastic band caused tooth separation between M1 and M2, as shown in Fig. 1B. In the vehicle treatment, 3-day ETM involved separation of about 122 ± 5.8 μm and 5-day ETM involved that of about 142 ± 13.1 μm between M1 and M2 (Figs. 3AB). In the group treated with PRO infusion, the distance between M1 and M2 was suppressed 12.9% by 3-day ETM and 32.2% by 5-day ETM, when compared with that of the vehicle treatment (Figs. 3AB). In contrast, in the group treated with ISO infusion, the distance between the M1 and M2 was accelerated 12.3% by 3-day ETM when compared with that of the vehicle treatment (Figs. 3AB).

The effect of sympathetic signaling on ETM-induced osteoclast activity

Next, we investigated the osteoclast parameters, such as Oc.N/BS and Oc.S/BS in the periodontal ligament during ETM in mice. TRAP-positive cells were rare on the alveolar socket (bone) surface in the control group (before starting ETM) and PRO and ISO infusion did not affect Oc.N/BS and Oc.S/BS (Figs. 4AB). In contrast, marked increases of TRAP-positive cells were observed on the alveolar socket (bone) surface by both 3-day and 5-day ETM in all drug treatment groups (Figs. 4C–F, Tables 1,2). In the vehicle treatment, Oc.S/BS on the tooth separation side increased 8.4-fold by 3-day ETM (Figs. 4CD) and 14.5-fold by 5-day ETM, compared with that on the contralateral side (Figs. 4EF). The Oc.N/BS on the tooth separation side also increased 4.3-fold by 3-day ETM (Table 1) and 4.6-fold by 5-day ETM compared with that on the contralateral side (Table 2).

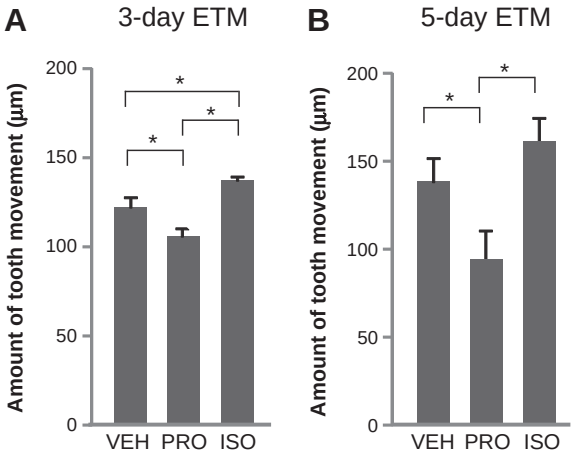


Fig. 3. ETM was suppressed by β-antagonist and increased by β-agonist. In the mice, an elastic band was inserted between M1 and M2 for 3 days (3-day ETM) and 5 days (5-day ETM). ETM: experimental tooth movement. VEH: vehicle-treated group. PRO: propranolol (20 μg/g b.w./day)-treated group. ISO: isoproterenol (10 μg/g b.w./day)-treated group. Bars represent the mean ± SEM of n = 8 mice per group. *Indicates $p<0.05$.

In the group treated with PRO, the increase of Oc.S/BS was suppressed 22.3% by 3-day ETM (Figs. 4CD) when compared with that of the vehicle infusion group. In contrast, in the group treated with ISO, the increase of Oc.S/BS was accelerated 38.5% by 3-day ETM (Figs. 4CD) when compared with that of the vehicle treatment. The Oc.N/BS, the other parameter of bone resorption, showed results similar to Oc.S/BS (Table 1). PRO treatment significantly suppressed Oc.N/BS on the alveolar socket for 3-day ETM, while ISO treatment significantly increased Oc.N/BS in 3-day ETM (Table 1). By 5-day ETM PRO treatment showed further suppression 39.2% compared with vehicle treatment (Figs. 4EF). In contrast, ISO treatment did not show significant increase by 5-day ETM (Figs. 4EF). We also examined the effect of ETM on the bone formation side. There were no significant differences in osteoblast number (Ob.N/BS) among these drug treatments (Fig. 4G). These bone resorption and formation parameters in mesial and distolingual roots showed similar with distobuccal roots we showed in figures and tables.

The effect of sympathectomy on ETM-induced bone remodeling

If ETM increased sympathetic tone, not only the actions of the blockers for sympathetic signaling, which act at the receptor levels, but also the destruction of norepinephrine nerve endings should affect the ETM. Therefore, 6-hydroxydopamine (6-OHDA) was administered to destroy the norepinephrine nerve terminals. To assess the effectiveness of 6-OHDA, we examined TH immunoreactivity in periodontal ligament using immunohistochemistry. Chemical sympathectomy remarkably decreased the TH-immunoreactive neurons in periodontal ligament (Fig. 5A). 6-OHDA administration also resulted in ptosis, as described previously (Fig. 5B) [35,36]. In the group treated with 6-OHDA infusion at 250 μg/g of body weight/day, the amount of tooth movement was suppressed 13.1% by 3-day ETM when compared with that of the vehicle infusion group (Fig. 5C). The osteoclast surface on the tooth separation side was suppressed 26.2% by 6-OHDA infusion compared with that in the vehicle infusion group (Figs. 5DE).

The effect of sympathetic signaling on alveolar bone volume

Finally, we examined the alveolar and femoral bone volume. The alveolar bone volume/total volume (A.BV/TV) was for the area

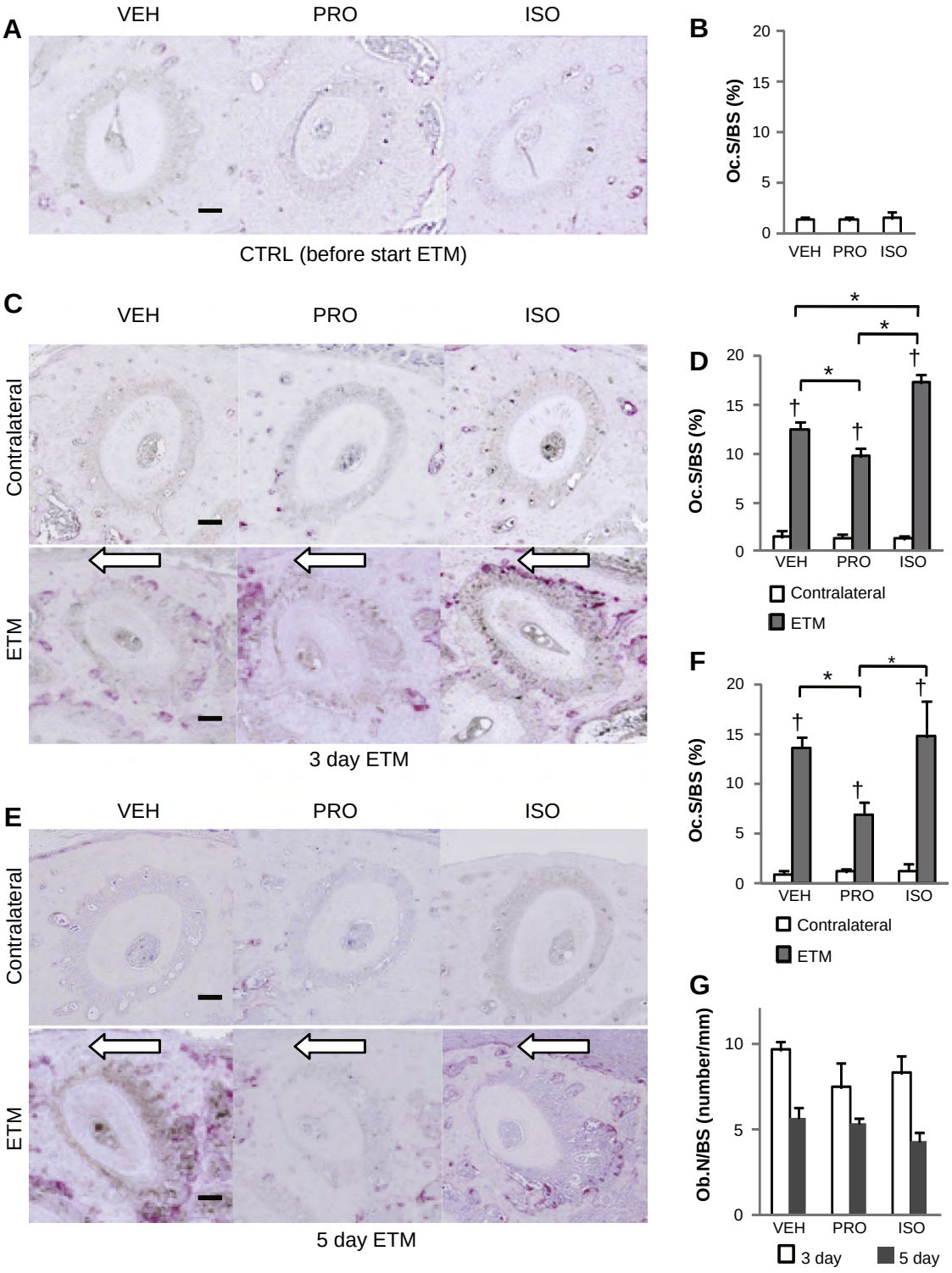


Fig. 4. Osteoclast activity induced by ETM was suppressed by β-antagonist and increased by β-agonist. Osteoclast staining of the M1 distobuccal root. ETM: experimental tooth movement. In the mice, an elastic band was inserted between M1 and M2 for 3 days (3-day ETM) and 5 days (5-day ETM). (AB) Few osteoclasts were observed in the control (CTRL: before starting ETM). Propranolol (PRO) treatment suppressed the osteoclast surface/bone surface on the alveolar socket (Oc.S/BS) with 3-day ETM (CD) and 5-day ETM (EF), while isoproterenol (ISO) treatment increased the osteoclast surface/bone surface (Oc.S/BS) on the alveolar socket with 3-day ETM (CD) and 5-day ETM (EF). (G) PRO and ISO did not affect the osteoblast number/bone surface (Ob.N/BS). Similar results were obtained from mesial and distolingual roots (data not shown). Arrows in C and E show the direction of force applied. Bars represent the mean ± SEM of n = 8 mice per group. *Indicates statistically significant difference between vehicle and each drug treatment ($p<0.05$). †Indicates statistically significant difference between contralateral side and ETM ($p<0.05$). Bar = 100 μm.

defined by the three roots of the first maxillary molar, as mentioned in Materials and methods (Figs. 1AC). Both 3-day and 5-day ETM decreased A.BV/TV in all drug treatments (Fig. 6A, Tables 1, 2). In the vehicle treatment, ETM decreased A.BV/TV (region C) by 18.3% for 3-day ETM (Table 1) and by 17.1% for 5-day ETM (Table 2) compared with that on the contralateral side. In the PRO treatment, ETM decreased A.BV/TV (region C) by 13.3% for 3-day ETM (Table 1) and by 15.8% for 5-day ETM (Table 2). In the ISO treatment, ETM decreased A.BV/

Table 1
3-day ETM.

		VEH		PRO		ISO		6-OHDA	
		Contralateral	ETM	Contralateral	ETM	Contralateral	ETM	Contralateral	ETM
Oc.N/mm		0.3 ± 0.08	1.3 ± 0.1*,**	0.3 ± 0.1	0.8 ± 0.1*,**	0.4 ± 0.1	1.8 ± 0.1*,**	0.3 ± 0.02	0.6 ± 0.0*,**
Region									
A.BV/TV(%)	A	43.1 ± 1.4	31.1 ± 2.5**	42.1 ± 2.9	32.2 ± 2.6**	43.5 ± 1.8	34.9 ± 0.9**	44.3 ± 3.1	33.1 ± 3.1**
(Alveolar bone)	I	52.9 ± 1.9	44.9 ± 3.5**	54.6 ± 1.9	44.9 ± 3.5**	53.7 ± 2.4	46.5 ± 3.7**	50.5 ± 3.3	45.4 ± 3.5**
	C	81.5 ± 1.7	66.6 ± 4.6**	86.7 ± 1.8	75.2 ± 3.1**	86.5 ± 2.7	72.7 ± 3.7**	72.9 ± 3.1	61.6 ± 2.9**
M.BV/TV(%)(Metaphysical region in femur)		16.6 ± 0.8		17.3 ± 0.7		10.5 ± 2.1		16.6 ± 1.3	
Body weight (g)		20.9 ± 0.5		20.4 ± 0.5		21.3 ± 0.4		20.8 ± 0.8	

Vehicle (VEH), proplanolol (PRO), isoproterenol (ISO) and 6-hydroxydopamine (6-OHDA).
A = apical, I = intermediate, C = coronal.
* $p<0.05$ vs. VEH.
** $p<0.05$ vs. contrateral.

TV (region C) by 15.9% for 3-day ETM (Table 1) and by 21.1% for 5-day ETM (Table 2). In the 6-OHDA treatment, ETM decreased A.BV/TV (region C) by 15.5% for 3-day ETM (Table 1). However, there were no significant differences in A.BV/TV between the drug treatments (Tables 1, 2). Region I and A showed similar results (Fig. 6A, Table 1). We also examined the bone volume/total volume in the metaphyseal region of long bone (M.BV/TV). As reported previously, ISO treatment significantly reduced M.BV/TV (Fig. 6B, Table 1) [4,28,37,38]. PRO and 6-OHDA did not have an effect for the 10 days of drug treatments (Fig. 6B, Table 1). To assess general health, body weight was measured every other day during the experimental period. No drug treatments caused a significant difference when compared with vehicle-treated animals (Fig. 6B, Tables 1, 2).

Discussion

Orthodontic tooth movement causes various histological changes, such as neuropeptide and osteoclast distribution in the periodontal ligament [39,40]. The periodontal ligament is highly innervated and the number of nerve fibers containing neuropeptides such as CGRP in the PDL was shown to increase during ETM [9,10,41,42]. CGRP is known to have nociceptive functions in the spinal cord and enhance nociceptive inputs to secondary neurons [43,44]. Little attention has been paid to the alteration of the sprouting of sympathetic neuropeptide induced by orthodontic force because these alterations of nerve fibers induced by orthodontic force are considered to be associated with pain [13]. Recently, many studies have shown that the sympathetic nervous system controls bone mass [3,4,18–24,26–28]. Some studies have supported the involvement of sympathetic signaling in the bone mechano-adaptive response [4,45]. Therefore, we examined the effect of ETM on sympathetic neuromarker such as neuropeptide Y (NPY) and tyrosine hydroxylase (TH). Our data demonstrated that ETM dramatically increased not only CGRP but also TH and NPY in the periodontal ligament (Fig. 2). These findings suggested that sympathetic signal may influence orthodontic tooth movement.

Involvement of sympathetic signal in the ETM was further supported by the analysis on the amount of tooth movement. β-antagonist PRO treatment suppressed tooth movement by 12.9% in 3-day ETM, and further suppression (32.2%) was shown in 5-day ETM (Figs. 3AB). On the other hand, β-agonist ISO treatment significantly increased the amount of tooth movement in both 3-day ETM (Fig. 3A). Since ISO resulted in faster tooth movement, the rate of tooth movement may be associated with accelerated bone remodeling. As ETM was shown to increase osteoclast appearance within a few days [15,16], we analyzed Oc.S/BS and Oc.N/BS in the periodontal ligament during ETM. There were few osteoclasts in the periodontal ligament and no significant difference between each drug treatment and the control (before starting ETM) (Figs. 4AB). However, ETM remarkably increased Oc.S/BS and Oc.N/BS. β-antagonist PRO treatment suppressed the Oc.S/BS by 29.7% in 3-day ETM and further suppression (39.2%) was shown in 5-day ETM (Figs. 4C–F). On the other hand, β-agonist ISO treatment significantly increased the Oc.S/BS in 3-day ETM (Figs. 4CD). No further increase in Oc.S/BS in 5-day ETM was found, with the responses reaching a plateau (Figs. 4EF). In this study, there was no significant difference between each drug treatment and the control (before starting ETM). It was reported previously that ISO treatment by itself increased Oc.N/BS and Oc.S/BS [4]. The different ISO effects on bone volume between the present and previous studies reflect differences in the region of measurement in bone. In this study, we measured the alveolar bone volume within the area defined by the three roots of the first maxillary molar, as shown in Figs. 1AB, while in the previous study, the metaphyseal region of long bone were measured [4,18,37]. To confirm this, we measured femoral BV/TV and found that ISO reduced M.BV/TV, as reported previously. Bataille et al. [46] also suggested that different sympathetic pathways exist on rat femur and mandible envelopes. These observations indicate that orthodontic force activates bone remodeling around teeth and the sympathetic nervous system is involved in ETM-induced osteoclast activity. These observations are consistent with the previous study, which suggests the involvement of sympathetic tone in the induction of bone resorption after unloading [4]. Sympathetic nervous signaling increases osteoclastogenesis via RANKL expression [19,26]; however, Arai et al.

Table 2
5-day ETM.

		VEH		PRO		ISO	
		Contralateral	ETM	Contralateral	ETM	Contralateral	ETM
Oc.N/mm		0.3 ± 0.2	1.4 ± 0.1**	0.3 ± 0.01	0.9 ± 0.1**	0.2 ± 0.06	2.2 ± 0.4**
A.BV/TV(%)(alveolar bone)	Region C	84.4 ± 1.7	70.0 ± 4.9	89.4 ± 2.4	75.2 ± 3.2	83.1 ± 2.1	65.5 ± 2.1
Body weight (g)		22.2 ± 0.08		22.9 ± 0.2		21.8 ± 0.6	

Vehicle (VEH), proplanolol (PRO), isoproterenol (ISO) and 6-hydroxydopamine (6-OHDA).
** $p<0.05$ vs. contrateral.

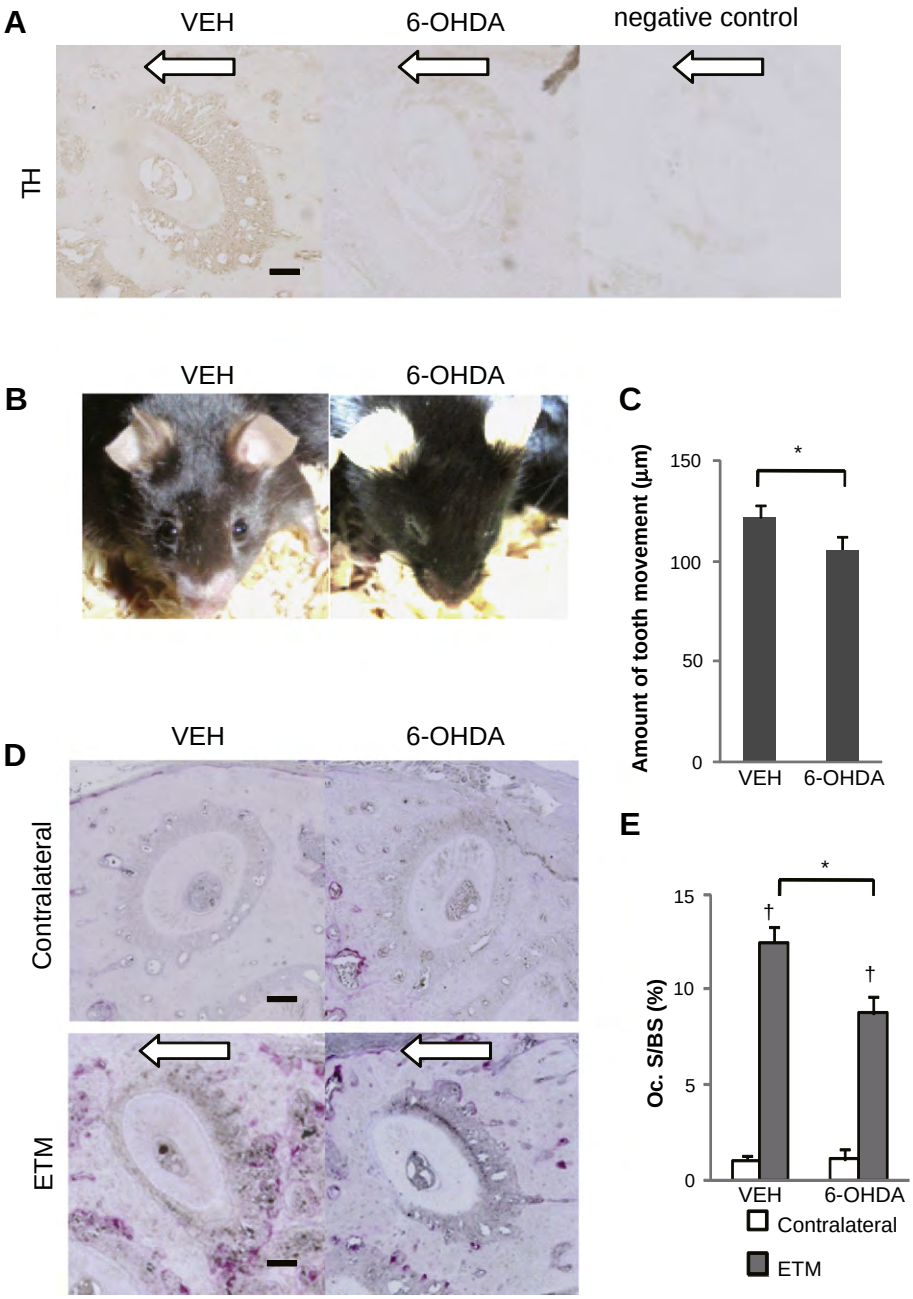


Fig. 5. Chemical sympathectomy showed a similar result to propranolol treatment. (A) TH-immunostainable sthenia was not found using 6-OHDA. (B) Blepharoptosis was found by 6-OHDA administration. (C) ETM was significantly inhibited by 6-OHDA. *Indicates statistically significant difference between vehicle and each drug treatment ($p<0.05$). (DE) 6-OHDA inhibited osteoclast increase induced by ETM. Arrows in A and D show the direction of force applied. Bar = 100 μm. Bars represent the mean ± SEM of n = 8 mice per group. *Indicates statistically significant difference between vehicle and each drug treatment ($p<0.05$). †Indicates statistically significant difference between contralateral side and ETM ($p<0.05$).

[47] reported previously that osteoclast-like cells constitutively expressed mRNA for β2-adrenergic receptor and isoprenaline increased osteoclastic resorbing activity. These data suggest that ETM-increased sympathetic nerve activity indirectly or directly stimulates osteoclasts.

We next analyzed a bone formation parameter: the number of osteoblasts on the alveolar socket (bone) surface (Ob.N/BS). However, there were no significant differences in Ob.N/BS among these drug treatments (Tables 1, 2). These results suggested that the sympathetic nervous system may not influence ETM-induced bone formation change. Marenzana et al. [29] reported that the osteogenic response of bone to external loading is not modulated by the sympathetic nervous system. They used the tibia external axial loading model as an anabolic mechanical stimulus. PRO treatment did not block loading-induced cortical and trabecular bone mass increase. Other studies also suggested that the sympathetic signaling does not involve the bone osteogenic response to mechanical loading [48,49]. The sympathetic nervous system may not influence an anabolic mechanical stimulus, which is the bone gain due to loading. However, the sympathetic nervous system may influence a catabolic mechanical stimulus, which is the bone loss due to unloading. These discrepancies may reflect the different kinds of mechanical stress. Further research on this issue is required. It is possible that five days is not enough time for osteoblast activity to increase. Van et al. [50] reported the cellular kinetics of the bone remodeling sequence in the rat. In their report osteoclasts appeared 3 days after the

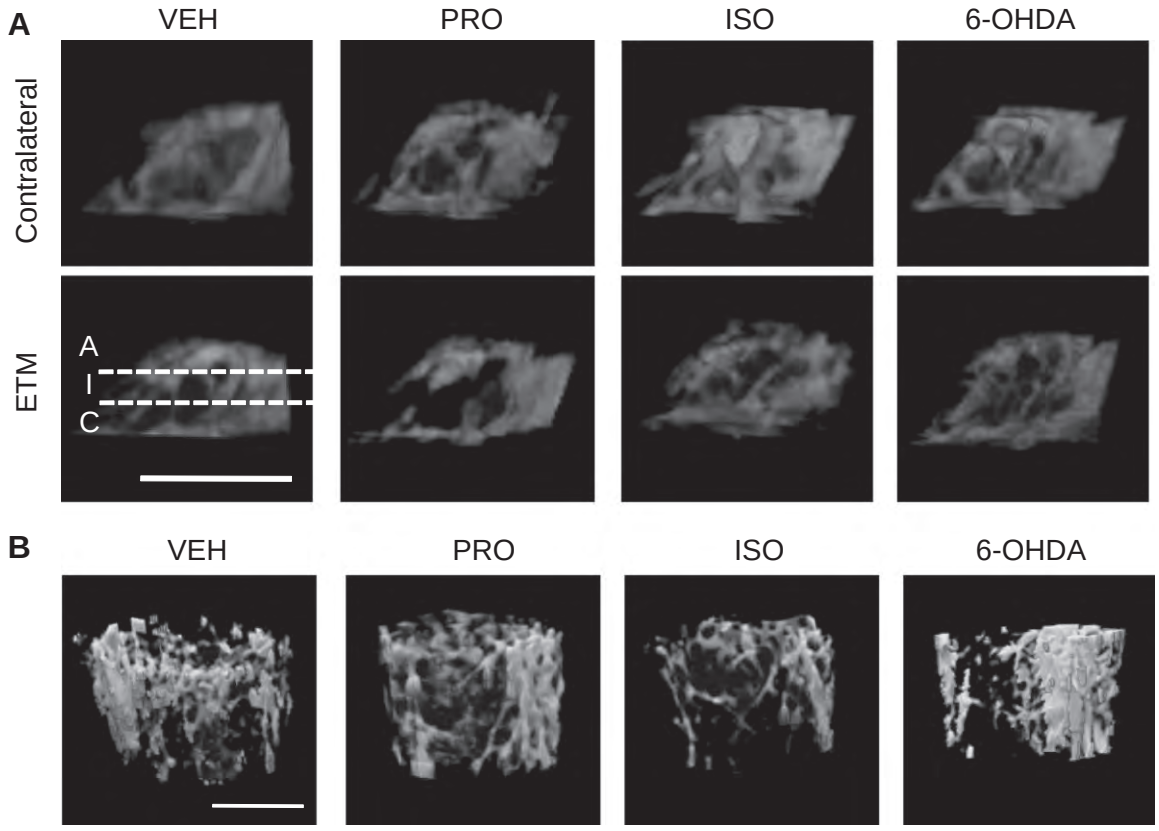


Fig. 6. Bone volume of alveolar bone and metaphyseal region in femur. (A) Representative images of alveolar bone BV/TV. (B) Representative images of metaphyseal region in femur BV/TV. Bar = 100 μm. A = apical, I = intermediate, C = coronal, VEH = Vehicle, PRO = propranolol, ISO = isoproterenol, 6-OHDA = 6-hydroxydopamine.

induction of bone remodeling and peaked at 4–5 days, and reversal activity was then followed by osteoblast formation starting 6 days after the induction of bone remodeling.

β-antagonist, PRO, plays a role not only as a β-blocker but also as a membrane stabilizer. The membrane-stabilizing or anti-oxidant action of PRO might be involved in controlling bone metabolism. Dietrich et al. [51] reported that PRO and membrane-stabilizing local anesthetics have been shown to inhibit parathyroid hormone-induced bone resorption. Therefore, we used 6-OHDA to destroy norepinephrine nerve terminals. Consistent with β-blocker PRO, chemical sympathectomy inhibited ETM and decreased osteoclast appearance during ETM. These findings suggest that the effects of PRO involve suppression of the amount of tooth movement and osteoclast increase induced by ETM due to blockade of sympathetic nervous signaling. This supports the hypothesis that nerve fibers, which are abundant in both periosteum and trabecular surfaces, could act as mechanoreceptors and transmit mechanical loading in bone [52,53]. ETM increases sympathetic nerve activity, which influences ETM-induced osteoclast activity.

We demonstrated that ETM markedly increased CGRP, TH and NPY in the periodontal ligament (Fig. 2). These neuropeptides may influence orthodontic tooth movement. Bjurholm et al. [54] reported that neuroendocrine influences bone physiology. In their report, receptors to CGRP, noradrenaline and NPY were demonstrated by analysis of cyclic AMP formation in osteoblasts and NPY inhibited the effects of noradrenalin and parathyroid hormone. Amano et al. [55] also reported that NPY inhibited isoprenaline-induced osteoclastogenesis in mouse bone marrow. Ishizuka et al. [56] reported the inhibitory effect of CGRP on osteoclast formation by mouse bone marrow cells treated with isoproterenol. These in vitro studies supported that ETM-induced CGRP and NPY may suppress sympathetic signal-induced osteoclast activity.

In summary, the combined effect of the ETM and blockade or stimulation of β-adrenergic signaling has been studied for the first time; our data clearly showed that ETM induced osteoclast activation in periodontal ligament through stimulation of the sympathetic nervous system. These findings suggest that sympathetic nervous signaling contributes to the orthodontic force-induced tooth movement through osteoclast activation.

References

[1] Ehrlich PJ, Lanyon LE. Mechanical strain and bone cell function: a review. Osteoporos Int 2002;13:688-700.
[2] Bikle DD, Sakata T, Halloran BP. The impact of skeletal unloading on bone formation, Gravit Space. Biol Bull 2003;16:45-54.
[3] Hino K, Nifuji A, Morinobu M, Tsuji K, Ezura Y, Nakashima K, et al. Unloading-induced bone loss was suppressed in gold-thioglucose treated mice. J Cell Biochem 2006;99:845-52.
[4] Kondo H, Nifuji A, Takeda S, Ezura Y, Rittling SR, Denhardt DT, et al. Unloading induces osteoblastic cell suppression and osteoclastic cell activation to lead to bone loss via sympathetic nervous system. J Biol Chem 2005;280:30192-200.
[5] Marenzana M, Chenu C. Sympathetic nervous system and bone adaptive response to its mechanical environment. J Musculoskelet Neuronal Interact 2008;8:111-20.
[6] Lanyon L, Skerry T. Postmenopausal osteoporosis as a failure of bone's adaptation to functional loading: a hypothesis. J Bone Miner Res 2001;16:1937-47.
[7] Kondo H, Ezura Y, Nakamoto T, Hayata T, Notomi T, Sorimachi H, et al. MURF1 deficiency suppresses unloading-induced effects on osteoblasts and osteoclasts to lead to bone loss. J Cell Biochem 2011;112:3525-30.
[8] Ishijima M, Tsuji K, Rittling SR, Yamashita T, Kurosawa H, Denhardt DT, et al. Resistance to unloading-induced three-dimensional bone loss in osteopontin-deficient mice. J Bone Miner Res 2002;17:661-7.
[9] Kato J, Wakisaka S, Kurisu K. Immunohistochemical changes in the distribution of nerve fibers in the periodontal ligament during an experimental tooth movement of the rat molar. Acta Anat (Basel) 1996;157:53-62.
[10] Norevall LI, Forsgren S, Matsson L. Expression of neuropeptides (CGRP, substance P) during and after orthodontic tooth movement in the rat. Eur J Orthod 1995;17:311-25.
[11] Saito I, Ishii K, Hanada K, Sato O, Maeda T. Responses of calcitonin gene-related peptide-immunopositive nerve fibres in the periodontal ligament of rat molars to experimental tooth movement. Arch Oral Biol 1991;36:689-92.
[12] Vandevska-Radunovic V, Kvinnsland S, Kvinnsland IH. Effect of experimental tooth movement on nerve fibres immunoreactive to calcitonin gene-related peptide,

protein gene product 9.5, and blood vessel density and distribution in rats. Eur J Orthod 1997;19:517-29.
[13] Brown DF, Moerenhout RG. The pain experience and psychological adjustment to orthodontic treatment of preadolescents, adolescents, and adults. Am J Orthod Dentofacial Orthop 1991;100:349-56.
[14] Deguchi T, Yabuuchi T, Ando R, Ichikawa H, Sugimoto T, Takano-Yamamoto T. Increase of galanin in trigeminal ganglion during tooth movement. J Dent Res 2006;85:658-63.
[15] Rygh P. Ultrastructural cellular reactions in pressure zones of rat molar periodontium incident to orthodontic tooth movement. Acta Odontol Scand 1972;30:575-93.
[16] Yokoya K, Sasaki T, Shibasaki Y. Distributional changes of osteoclasts and pre-osteoclastic cells in periodontal tissues during experimental tooth movement as revealed by quantitative immunohistochemistry of H(+)-ATPase. J Dent Res 1997;76:580-7.
[17] Yamashiro T, Fujiyama K, Fujiyoshi Y, Inaguma N, Takano-Yamamoto T. Inferior alveolar nerve transection inhibits increase in osteoclast appearance during experimental tooth movement. Bone 2000;26:663-9.
[18] Takeda S, Karsenty G. Central control of bone formation. J Bone Miner Metab 2001;19:195-8.
[19] Takeuchi T, Tsuboi T, Arai M, Togari A. Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differentiation factor in MC3T3-E1 osteoblast-like cells. Biochem Pharmacol 2001;61:579-86.
[20] Togari A, Arai M, Mizutani S, Mizutani S, Koshihara Y, Nagatsu T. Expression of mRNAs for neuropeptide receptors and beta-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. Neurosci Lett 1997;233:125-8.
[21] Noda M, Nagao M, Hanyu R, Mizoguchi F, Notomi T, Hayata T, et al. Control of bone remodeling by nervous system. Nervous system and bone. Clin Calcium 2010;20:1801-5.
[22] Bonnet N, Benhamou CL, Brunet-Imbault B, Arlettaz A, Horcajada MN, Richard O, et al. Severe bone alterations under beta2 agonist treatments: bone mass, microarchitecture and strength analyses in female rats. Bone 2005;37:622-33.
[23] Bonnet N, Brunet-Imbault B, Arlettaz A, Horcajada MN, Collomp K, Benhamou CL, et al. Alteration of trabecular bone under chronic beta2 agonists treatment. Med Sci Sports Exerc 2005;37:1493-501.
[24] Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 2000:197-207.
[25] Hino K, Nifuji A, Morinobu M, Tsuji K, Ezura Y, Nakashima K, et al. Unloading-induced bone loss was suppressed in gold-thioglucose treated mice. J Cell Biochem 2006;99:845-52.
[26] Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, et al. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 2005;434:514-20.
[27] Togari A. Adrenergic regulation of bone metabolism: possible involvement of sympathetic innervation of osteoblastic and osteoclastic cells. Microsc Res Tech 2002;58:77-84.
[28] Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, Parker KL, et al. Leptin regulates bone formation via the sympathetic nervous system. Cell 2002;111:305-17.
[29] Marenzana M, De Souza RL, Chenu C. Blockade of beta-adrenergic signaling does not influence the bone mechano-adaptive response in mice. Bone 2007;41:206-15.
[30] Kondo A, Togari A. In vivo stimulation of sympathetic nervous system modulates osteoblastic activity in mouse calvaria. Am J Physiol Endocrinol Metab 2003;285:E661-7.
[31] Williams JM, Peterson RG, Shea PA, Schmedtje JF, Bauer DC, Felten DL. Sympathetic innervation of murine thymus and spleen: evidence for a functional link between the nervous and immune systems. Brain Res Bull 1981;6:83-94.
[32] de Champlain J. Degeneration and regrowth of adrenergic nerve fibers in the rat peripheral tissues after 6-hydroxydopamine. Can J Physiol Pharmacol 1971;49:345-55.
[33] Waldo CM, Rothblatt JM. Histologic response to tooth movement in the laboratory rat; procedure and preliminary observations. J Dent Res 1954;33:481-6.
[34] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. report of the ASBMR histomorphometry nomenclature committee. J Bone Miner Res 1987;2:595-610.
[35] Flach AJ, Peterson JS, Roizen MF, Wood IS. Ptosis in the rat following topically administered 2% epinephrine. Invest Ophthalmol Vis Sci 1983;24:766-71.
[36] Purpura P, Westman L, Will P, Eidelman A, Kagan VE, Osipov AN, et al. Adjunctive treatment of murine neuroblastoma with 6-hydroxydopamine and tempol. Cancer Res 1996;56:2336-42.
[37] Kondo H, Togari A. Continuous treatment with a low-dose beta-agonist reduces bone mass by increasing bone resorption without suppressing bone formation. Calcif Tissue Int 2011;88:23-32.
[38] Nagao M, Feinstein TN, Ezura Y, Hayata T, Notomi T, Saita Y, et al. Sympathetic control of bone mass regulated by osteopontin. Proc Natl Acad Sci U S A 2011;108:17767-72.
[39] Rygh P. Ultrastructural changes in pressure zones of human periodontium incident to orthodontic tooth movement. Acta Odontol Scand 1973;31:109-22.
[40] Takano-Yamamoto T, Kawakami M, Kobayashi Y, Yamashiro T, Sakuda M. The effect of local application of 1,25-dihydroxycholecalciferol on osteoclast numbers in orthodontically treated rats. J Dent Res 1992;71:53-9.
[41] Kvinnsland I, Kvinnsland S. Changes in CGRP-immunoreactive nerve fibres during experimental tooth movement in rats. Eur J Orthod 1990;12:320-9.
[42] Vandevska-Radunovic V, Kvinnsland S, Kvinnsland IH. Effect of experimental tooth movement on nerve fibres immunoreactive to calcitonin gene-related peptide, protein gene product 9.5, and blood vessel density and distribution in rats. Eur J Orthod 1997;19:517-29.
[43] Cridland RA, Henry JL. Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. Neuropeptides 1988;11:23-32.
[44] Ryu PD, Murase K, Gerber G, Randic M. Actions of calcitonin gene-related peptide on rat sensory ganglion neurones. Physiol Bohemoslov 1988;37:259-65.
[45] sseur R, Sabatier JP, Potrel-Burgot C, Lecoq B, Creveuil C, Marcelli C. Sympathetic nervous system as transmitter of mechanical loading in bone. Joint Bone Spine 2003;70:515-9.
[46] Bataille C, Mauprivez C, Hay E, Baroukh B, Brun A, Chaussain C, et al. Different sympathetic pathways control the metabolism of distinct bone envelopes. Bone 2012;50:1162-72.
[47] Arai M, Nagasawa T, Koshihara Y, Yamamoto S, Togari A. Effects of beta-adrenergic agonists on bone-resorbing activity in human osteoclast-like cells. Biochim Biophys Acta 2003;1640:137-42.
[48] Zhang P, Tanaka SM, Jiang H, Su M, Yokota H. Diaphyseal bone formation in murine tibiae in response to knee loading. J Appl Physiol 2006;100:1452-9.
[49] de Souza RL, Pitsillides AA, Lanyon LE, Skerry TM, Chenu C. Sympathetic nervous system does not mediate the load-induced cortical new bone formation. J Bone Miner Res 2005;20:2159-68.
[50] Tran Van PT, Vignery A, Baron R. Cellular kinetics of the bone remodeling sequence in the rat. Anat Rec 1982;202:445-51.
[51] Dietrich JW, Mundy GR, Raisz LG. Inhibition of bone resorption in tissue culture by membrane-stabilizing drugs. Endocrinology 1979;104:1644-8.
[52] Serre CM, Farlay D, Delmas PD, Chenu C. Evidence for a dense and intimate innervation of the bone tissue, including glutamate-containing fibers. Bone 1999;25:623-9.
[53] Wada S, Kojo T, Wang YH, Ando H, Nakanishi E, Zhang M, et al. Effect of loading on the development of nerve fibers around oral implants in the dog mandible. Clin Oral Implants Res 2001;12:219-24.
[54] Bjurholm A. Neuroendocrine peptides in bone. Int Orthop 1991;15:325-9.
[55] Amano S, Arai M, Goto S, Togari A. Inhibitory effect of NPY on isoprenaline-induced osteoclastogenesis in mouse bone marrow cells. Biochim Biophys Acta 2007;1770:966-73.
[56] Ishizuka K, Hirukawa K, Nakamura H, Togari A. Inhibitory effect of CGRP on osteoclast formation by mouse bone marrow cells treated with isoproterenol. Neurosci Lett 2005;379:47-51.