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Transient receptor potential vanilloid-1 regulates osteoprotegerin/RANKL homeostasis in human periodontal ligament cells

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Background and Objective: Increasing evidence has shown the presence of transient receptor potential vanilloid-1 (TRPV1) in a variety of nonneuronal tissues; however, the function of TRPV1 in these cells is not well understood. In this study, we aimed to investigate the expression and function of TRPV1 in human periodontal ligament (HPDL) cells. As HPDL cells are known to play an important role in the bone-remodeling process, we hypothesized that TRPV1 might be implicated in the regulation of osteoprotegerin (OPG) and RANKL expression.

Material and Methods: TRPV1 expression was examined by western blot analysis. The function of TRPV1 was studied using capsaicin, a well-known TRPV1 agonist. RT-PCR was performed to study the expression of *OPG* and *RANKL* mRNAs. The expression of OPG and RANKL proteins was analyzed by ELISA and western blotting, respectively. The mechanisms of capsaicin-induced OPG expression in HPDL cells were studied using inhibitors.

Results: In this study we found that TRPV1 was present in HPDL cells. Treatment with capsaicin induced OPG expression in a dose-dependent manner but did not affect the expression of RANKL. The increase of the OPG/RANKL ratio was also found in human osteoblasts, but not in MC3T3-E1 cells, a mouse osteoblastic cell line, suggesting species specificity. Capsazepine, the competitive TRPV1 antagonist, significantly abolished the effect of capsaicin on OPG expression in HPDL cells. In addition, studies investigating the effects of a calcium chelator and a phospholipase C inhibitor indicated that calcium ions and phospholipase C were required for the induction. Interestingly, capsaicin was able to increase the OPG/RANKL ratio, even in the presence of prostaglandin E2, a potent inducer of RANKL.

Conclusion: Our study demonstrates that activation of TRPV1 leads to an increase of the OPG/RANKL ratio in HPDL cells. These findings suggest the novel function of TRPV1 in periodontal tissues, at least, as the regulator of the OPG/RANKL axis.

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The transient receptor potential vanilloid 1 (TRPV1, formerly known as vanilloid receptor 1) is a nonselective cation channel that can be activated by a wide range of stimuli such as noxious heat (> 43°C), protons and capsaicin, a pungent ingredient of hot chilli peppers (1,2). An *in vivo* study showed that mice lacking TRPV1 have an impaired response to noxious stimuli (3). In addition, elevated expression of TRPV1 has been observed in an animal models of inflammatory hyperalgesia and in a mouse model of diabetic neuropathy (4,5). Pharmacological blockade of TRPV1 by its antagonist attenuates mechanical stimuli-induced hyperalgesia and bone pain in animal models of cancer (6–8). Therefore, TRPV1 is a promising target for pain relief. Although TRPV1 is predominantly expressed in primary sensory neurons, increasing evidence has shown the presence of TRPV1 in a variety of nonneuronal tissues, such as epidermal keratinocytes of human skin, gastric epithelial cells, human dental pulp cells and submandibular gland (9–12). However, the function of TRPV1 in these cells is not clearly understood. Investigating the role of TRPV1 in nonneuronal tissues may provide a better understanding of the cellular response to physical and pathological stimuli.

Periodontal ligament (PDL) is the soft connective tissue that attaches teeth to the alveolar bone socket. PDL cells, a major cell type found in periodontal ligament, have multifunctional features. They are crucial for producing new PDL during periodontal regeneration (13). PDL also contain progenitor cells that can differentiate into osteoblasts and cementoblasts (14,15). Moreover, it has been demonstrated that PDL cells can act as the local regulators of osteoclastogenesis. Direct co-culture of human PDL (HPDL) cells with human peripheral blood mononuclear cells (PBMCs) increases the area of resorption pits on dentin slices (16). In contrast, an inhibitory action of HPDL cells on osteoclast differentiation is observed when PBMCs are indirectly co-cultured with HPDL cells (16).

Osteoprotegerin (OPG) and RANKL are known to play an important role in the regulation of osteoclastogenesis. The balance between OPG expression and RANKL expression is one of the key factors that determine the role of HPDL cells in osteoclast differentiation. RANKL is a membrane-bound protein that can induce the differentiation of osteoclast precursors into mature osteoclasts by binding with its specific receptor, RANK (17). On the other hand, OPG is a secreted protein that functions as a decoy receptor for RANKL and thereby inhibits osteoclast differentiation (18). HPDL cells are shown to constitutively express both OPG and RANKL (19). The deregulation of the OPG/RANKL system is observed under various microenvironments. Proinflammatory mediators (such as interleukin-1α) and mechanical stress (a common occurrence during orthodontic treatment) have been shown to up-regulate RANKL expression but to down-regulate OPG expression in HPDL cells (20,21). Moreover, clinical studies have shown a correlation between an increased RANKL/OPG ratio and the progression of periodontal disease (22). Although OPG and RANKL are recognized as the key molecules involved in pathological bone loss, the new regulators of this axis are still being uncovered.

In this study, we investigated whether TRPV1 is expressed in HPDL cells. The function of TRPV1 was studied using capsaicin, a well-known TRPV1 agonist. As HPDL cells have been shown to play an important role in osteoclastogenesis, we hypothesized that TRPV1 might be implicated in the regulation of bone resorption by modulating the OPG/RANKL ratio.

Material and Methods

Reagents

Goat anti-OPG immunoglobulin G and mouse anti-RANKL immunoglobulin G were purchased from R&D Systems (Minneapolis, MN, USA). Goat anti-TRPV1 immunoglobulin G was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-β-actin immunoglobulin G1-kappa was from Chemicon International (Temecula, CA, USA). Anti-goat immunoglobulin G was from Sigma (St Louis, MO, USA). Anti-mouse immunoglobulin G was purchased from Invitrogen (Eugene, OR, USA). Capsaicin and capsazepine were obtained from Sigma. BAPTA-AM (a calcium chelator) was from Enzo Life Sciences (Plymouth, PA, USA). U-73122 [a phospholipase C (PLC) inhibitor] was from Santa Cruz Biotechnology. Prostaglandin E2 (PGE_2) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture and treatment

HPDL cells were retrieved from healthy third molars extracted for orthodontic reasons. Primary human osteoblasts were retrieved from torus mandibularis removed for prosthodontic reasons. Cells were prepared according to the protocol approved by the Ethics Committee (Faculty of Dentistry, Chulalongkorn University). Informed consent was obtained from 10 patients (six women and four men; 18–23 years of age). Briefly, third molars and torus mandibularis bone chips were rinsed with sterile phosphate-buffered saline. HPDL tissues were removed from the middle third of the root surface. Bone chips were cut into small pieces and digested with 0.25% trypsin-EDTA. The explants were harvested on 60-mm culture dishes. HPDL cells and human osteoblasts were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA) and 15% fetal bovine serum, respectively. MC3T3-E1 cells, a cell line derived from mouse calvaria, were cultured in minimum essential medium (Hyclone) containing 10% fetal bovine serum. All culture media were supplemented with 2 mM L-glutamine (Gibco BRL), 100 units/mL of penicillin (Gibco BRL), 100 μg/mL of streptomycin (Gibco BRL) and 5 μg/mL of amphotericin B (Gibco BRL), and the cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For the experiments, cells were seeded into six-well plates, at 1.5×10^5

cells per well, and incubated for 2 d. To exclude the effect of other factors in the serum, the cells were starved with serum-free medium for 24 h before treatment with capsaicin. The inhibitors were added 1 h before stimulation with capsaicin. All experiments were performed in triplicate. MC3T3-E1 cells from passages 19 to 22 were used in the experiments. HPDL cells and human osteoblasts from passages 3 to 6 were used. The cells from three donors were studied in each experiment.

RT-PCR amplification

RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. Aliquots of 1 μg of total RNA were converted to complementary DNA by RT using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1.5 h at 42°C. Subsequently, PCR was performed using Taq polymerase (Qiagen, Hilden, Germany) in a total reaction volume of 25 μL. The oligonucleotide sequences of the primers were as follows. Human OPG: forward, TCAAGCAGGAGTGCAA TCG; and reverse, AGAATGCCTCC TCACACAGG. Mouse OPG: forward, CCACTCTTATACGGACA GCT; and reverse, TCTCGGCATTC ACTTTGGTC. Human RANKL: forward, CCAGCATCAAAATCCCA AGT; and reverse, CCCCTTCAGAT GATCCTTC. Mouse RANKL: forward, AGCACGAAAAACTGGTCG GGC; and reverse, TGCAGGTCCC AG CGCAATGTA. Human TRPV1: forward, GGCTGTCTTCATCATCC TGCTGCT; and reverse, GTTCTTGC TCTCCTGTGCGATCTTGT. Mouse TRPV1: forward, ACCACGGCTGCT TACTATCG; and reverse, CAC TGTAGCTGTCCACAAAC. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, TGAA GGTCGGAGTCAACGGAT; and reverse, TCACACCCATGACGAAC ATGG. Mouse GAPDH, forward: ACTTTGTCAAGCTCATTTCC; and reverse, TGCAGCGAACTTTATTG ATG. The number of PCR cycles for amplification of OPG RNA from human and mouse cells was 24 and 30, respectively. Cycling conditions were 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, followed by one extension cycle at 72°C for 10 min. The same cycling conditions were used for RANKL TRPV1 and GAPDH, but the number of PCR cycles differed. The number of PCR cycles for amplification of RANKL RNA from human and mouse cells was 33 and 37, respectively, and the number of PCR cycles for amplification of TRPV1 and GAPDH RNAs from both human and mouse cells was 33 and 22, respectively. PCR was performed in a DNA Thermal Cycler (Biometra, Göttingen, Germany). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate and protease inhibitor cocktail. Protein samples were electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibody, biotinylated secondary antibody and then with peroxidase-labeled streptavidin. Chemiluminescence was detected by adding SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

ELISA

The amount of OPG secreted into the culture supernatant was measured using the OPG ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was analyzed by one-way analysis of variance using Graph Pad Prism (La Jolla, CA, USA). The Turkey test was used for post-hoc analysis. The difference was considered significant when $p < 0.05$.

Results

Capsaicin dose-dependently increased the OPG/RANKL ratio

First, the expression of TRPV1 in HPDL cells was examined. Four lines of HPDL cells, collected from different patients, were analyzed by western blot analysis. As shown in Fig. 1A, TRPV1 protein could be found in all lines of HPDL cells. To study whether TRPV1 was functionally active in HPDL cells, the cells were stimulated with capsaicin, a TRPV1 agonist, for 24 h. RT-PCR amplification indicated that capsaicin was able to induce the expression of *OPG* mRNA in a dose-dependent manner (Fig. 1B). The expression of *OPG* mRNA began to increase when cells were exposed to 10 μM capsaicin, and expression reached a plateau at 50 μM capsaicin (Fig. 1B). However, expression of *RANKL* mRNA was unchanged during the treatment (Fig. 1B). Next, we checked whether the mRNA level observed corresponded to the level of protein expressed in the cells. The ELISA results showed that the baseline levels of OPG secreted in HPDL cells from different patients varied in the range of 1000–5000 pg/mL. However, the relative ratio of OPG induction was quite constant among individuals. Therefore, the effect of capsaicin on OPG secretion was presented as fold OPG induction compared with no induction. As shown in Fig. 1C, secretion of OPG protein was increased in a dose-dependent manner after exposure to capsaicin. However, western blot analyses showed that expression of RANKL protein was not changed by capsaicin treatment (Fig. 1D). To analyze the relative expression of OPG and RANKL, Scion Image was used to quantify the ratio of OPG/RANKL expression. As the expression of both OPG and RANKL varied from patient to patient, data were presented as fold change of OPG/RANKL expression compared with the control (0 μM capsaicin). As shown in Fig. 1E, capsaicin treatment induced a dose-dependent increase in the OPG/RANKL ratio at both mRNA and protein levels.

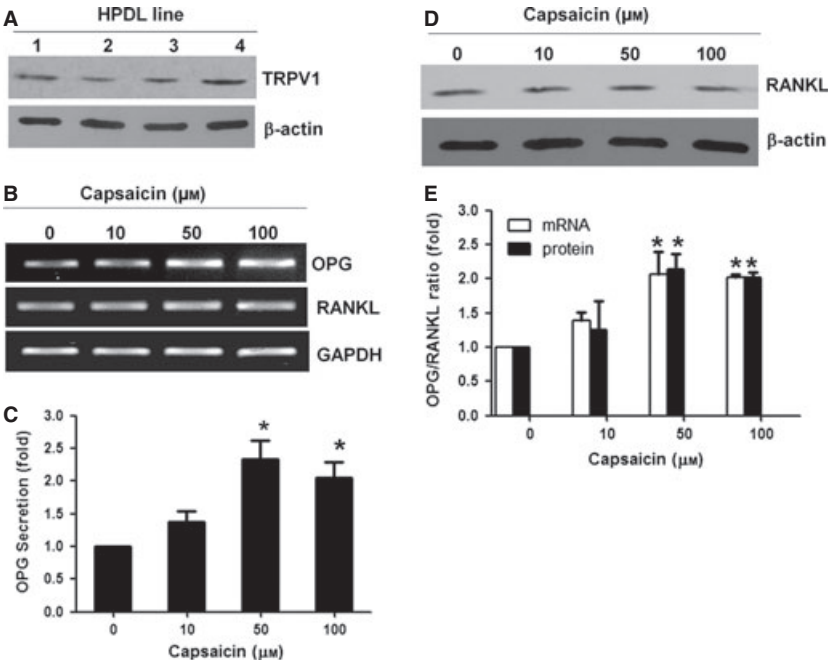

Fig. 1. Effect of capsaicin on the expression of osteoprotegerin (OPG) and RANKL in human periodontal ligament (HPDL) cells. (A) Whole-cell lysates of four lines of HPDL cells were analyzed for expression of transient receptor potential vanilloid-1 (TRPV1) protein by western blotting. (B–D) HPDL cells were treated with 0–100 μM capsaicin for 24 h. The expression of *OPG* and *RANKL* mRNAs was analyzed by RT-PCR (B). OPG secretion in cell-culture supernatants was analyzed by ELISA (C). Whole-cell lysates were analyzed for RANKL protein expression by western blotting (D). Bar chart showing quantitative analysis of the OPG/RANKL ratio analyzed using Scion Image (E). Data are expressed as fold change relative to the control (0 μM capsaicin) and represent the mean ± SD of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. $*p < 0.05$ vs. 0 μM capsaicin.

TRPV1 inhibition abolished the effect of capsaicin on OPG expression

To confirm that the observed response was mediated by TRPV1, HPDL cells were treated with capsazepine, a competitive TRPV1 antagonist. The ELISA results clearly indicated that capsazepine completely abolished the effect of capsaicin on OPG expression (Fig. 2A). However, RANKL expression was not changed during the treatment (Fig. 2B). These results indicated that capsaicin up-regulated OPG expression through a TRPV1-dependent mechanism.

Capsaicin increased the OPG/RANKL ratio in human osteoblasts but not in mouse MC3T3E-1 cells

To study whether the observed function of TRPV1 was specific to HPDL cells, the effect of capsaicin on the OPG/RANKL axis was explored in osteoblasts, the major cells involved in the regulation of osteoclastogenesis. MC3T3E-1 cells and three lines of primary human osteoblasts, collected from different patients, were analyzed for TRPV1 expression. The results of RT-PCR amplification showed that TRPV1 was expressed in all lines of primary human osteoblasts as well as in MC3T3E-1 cells (Fig. 3A). The expression of *OPG* mRNA was increased when primary human osteoblasts were treated with 10–50 μM capsaicin (Fig. 3B). In contrast, the expression of *RANKL* mRNA was markedly decreased when cells were exposed to 50 μM capsaicin (Fig. 3B). Surprisingly, the opposite findings were observed in MC3T3E-1 cells. The expression of *OPG* mRNA was found to decrease when cells were stimulated with 10–50 μM capsaicin, whereas the expression of *RANKL* mRNA was increased in a dose-dependent manner (Fig. 3C). Collectively these data indicate that TRPV1 shows a similar response to capsaicin in both HPDL cells and human osteoblasts by increasing the OPG/RANKL ratio. As we found that capsaicin decreased the OPG/RANKL ratio in MC3T3E-1 cells, it is possible that TRPV1 functions differently in human and mouse osteoblasts.

Calcium ions and PLC are required for capsaicin-induced OPG expression

Next, the mechanism of how TRPV1 transduced the signal to up-regulate OPG expression in HPDL cells was investigated. As TRPV1 is a nonselective cation channel with high permeability to calcium ions (1), the involvement of calcium ions in this pathway was studied. As expected, treatment with BAPTA-AM, a calcium chelator, totally abolished the effect of capsaicin to stimulate OPG expression (Fig. 4A). Moreover, PLC was also implicated in capsaicin-induced OPG expression. Treatment with U-73122, a PLC inhibitor, partially inhibited the effect of capsaicin on OPG production (Fig. 4C). Similarly to capsazepine, neither BAPTA-AM nor U-73122 had any effect on RANKL expression (Fig. 4B and 4D).

Capsaicin attenuated the effect of PGE_2 on OPG and RANKL expression

As bone resorption commonly occurs during the inflammatory process, it would be interesting to study whether the observed effect of capsaicin could be found during this condition. HPDL cells were treated with 1 μM PGE_2 to mimic inflammatory conditions. As shown in Fig. 5, PGE_2 treatment decreased OPG secretion but increased the expression of RANKL protein. Interestingly, co-treatment with capsaicin was able to reverse the effect of PGE_2 on the expression of OPG and RANKL. In the presence of PGE_2, capsaicin was still able to increase OPG secretion. Moreover, capsaicin could induce the expression of RANKL

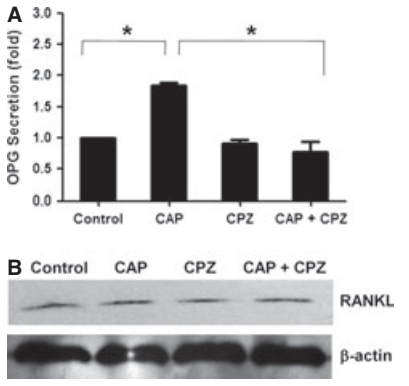

Fig. 2. Capsaicin-induced osteoprotegerin (OPG) expression is mediated by transient receptor potential vanilloid-1 (TRPV1). Human periodontal ligament (HPDL) cells were left untreated or were pretreated with capsazepine (10 μM) for 30 min and then cells were exposed to capsaicin (50 μM) for 24 h. (A) OPG secretion in cell-culture supernatants was analyzed by ELISA. Data are expressed as fold change relative to the control and represent the mean ± SD of three independent experiments. (B) Whole-cell lysates were analyzed for RANKL protein expression by western blotting. *$p < 0.05$ vs. control. CAP, capsaicin; CPZ, capsazepine.

protein when cells were co-treated with PGE_2. These results indicate that the effect of capsaicin on the OPG/RANKL ratio was stronger than that of PGE_2.

Discussion

TRPV1 has emerged as an essential molecule responsible for the sensitization of painful stimuli. In fact, TRPV1 agonists and antagonists have been used in clinical trials for many painful conditions such as postherpatic neuralgia and osteoarthritis (23–25). Besides its role in the neuronal tissues, the function of TRPV1 in nonneuronal tissues is not widely understood. In this study, the novel function of TRPV1 in HPDL cells was demonstrated. The results of this study showed that HPDL cells expressed TRPV1. In line with the role of HPDL cells in the regulation of bone homeostasis, we found that TRPV1 is involved in the regulation of the OPG/RANKL ratio. TRPV1 activation by capsaicin led to the up-regulation of OPG without affecting RANKL expression.

To our knowledge, the inflammatory-related phenotypes of TRPV1 knockout mice have not been reported. In fact, TRPV1 knockout mice showed only a benign phenotype, such as altered bladder function (26). Correspondingly, we found that pharmacological blockade of TRPV1 by capsazepine did not affect the basal secretion of OPG (Fig. 2A). This suggested that TRPV1 might not be functionally active in physiological conditions. However, upon external stimulation with capsaicin, TRPV1 became active and mediated the up-regulation of OPG. To understand, in greater detail, the possible role of TRPV1 in the onset and/or progression of periodontal disease, further studies to investigate the response of TRPV1 to other stimuli, such as noxious heat and low pH, are needed. In addition, identification of its endogenous ligand would help to understand the function of TRPV1 in HPDL cells.

In this study, capsaicin was used to demonstrate the role of TRPV1 in HPDL cells. Although the basal levels of OPG and RANKL varied among patients, our results showed that capsaicin could increase the OPG/RANKL ratio in all experiments. As the main function of OPG is to act as a decoy receptor for RANK, resulting in the inhibition of osteoclast differentiation, our observation on the ability of capsaicin to increase the OPG/RANKL ratio in HPDL cells suggested that capsaicin might have a protective effect on bone architecture. In contrast, it has been reported that TRPV1 agonists, such as resinferatoxin and low-dose capsaicin (1–10 μM), induced osteoclast formation when human and mouse PBMCs were exogenously stimulated with macrophage colony-stimulating factor and RANKL (27,28). This discrepancy might be a result of differences in the types and concentrations of agonists used in the experiments. Moreover, the differing response between cell types is of concern. It is plausible that TRPV1 might function differently in osteoclasts compared with PDL cells. In fact, it has been demonstrated that the same cell type may behave differently when located in different tissue. For example, osteoclasts derived from jaw and long bone have a different shape and respond differently to osteoclasts derived from bone and dentin (29). To prove whether capsaicin can reduce bone destruction in periodontal

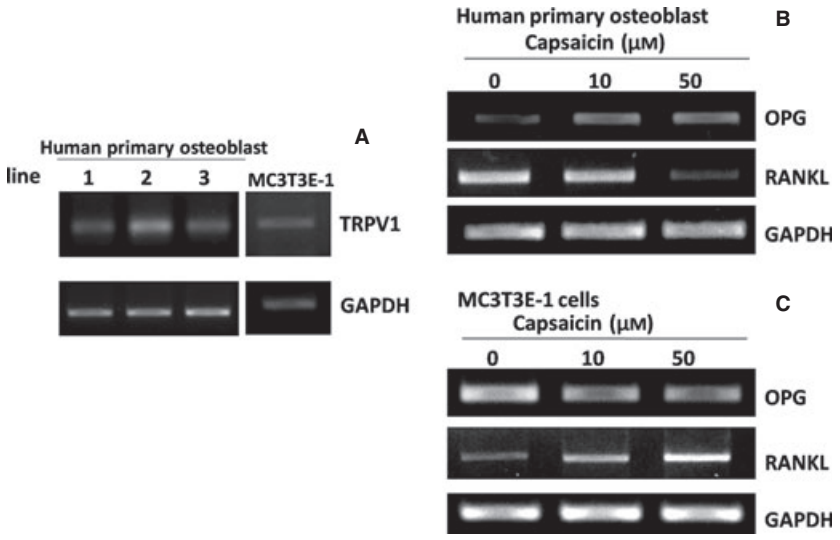

Fig. 3. Effect of capsaicin on osteoprotegerin (OPG) and RANKL expression in osteoblasts. (A) Primary human osteoblasts and MC3T3E-1 cells were analyzed for expression of transient receptor potential vanilloid-1 (TRPV1) mRNA by RT-PCR. Primary human osteoblasts (B) and MC3T3E-1 cells (C) were treated with 0–50 μM capsaicin for 24 h. The expression of *OPG* and *RANKL* mRNAs was analyzed using RT-PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

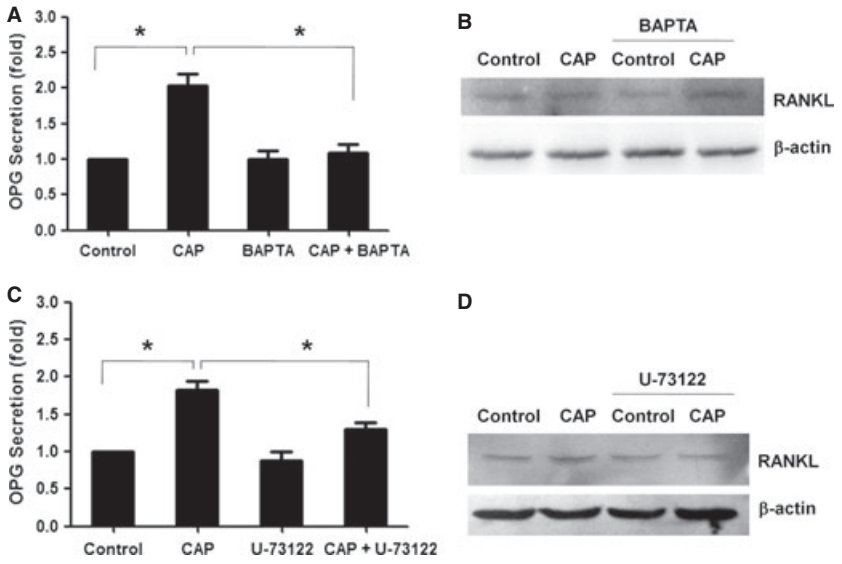

Fig. 4. Involvement of calcium and phospholipase C (PLC) in capsaicin-induced osteoprotegerin (OPG) expression. Human periodontal ligament (HPDL) cells were left untreated or were pretreated with BAPTA-AM (5 μM) or U-73122 (2 μM) for 30 min. For capsaicin stimulation, cells were exposed to 50 μM capsaicin for 24 h. (A and C) OPG secretion in cell culture supernatants was analyzed by ELISA. Data are expressed as fold change relative to the control and represent the mean ± SD of three independent experiments. (B and D) Whole-cell lysates were analyzed for expression of RANKL protein by western blotting. $*p < 0.05$. CAP, capsaicin.

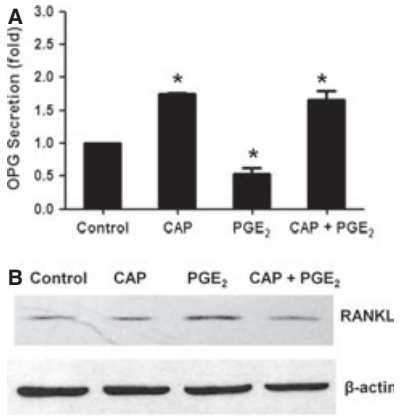

Fig. 5. Capsaicin reversed the effect of prostaglandin E2 (PGE_2) on osteoprotegerin (OPG) and RANKL expression. Human periodontal ligament (HPDL) cells were treated with capsaicin (50 μM) and/or PGE_2 (1 μM) for 24 h. (A) OPG secretion in cell culture supernatants was analyzed by ELISA. Data are expressed as fold change relative to the control and represent the mean ± SD of three independent experiments. (B) Whole-cell lysates were analyzed for expression of RANKL protein by western blotting. $*p < 0.05$ vs. control. CAP, capsaicin.

disease, further studies using osteoblast/osteoclast co-culture are needed.

To date, the role of TRPV1 in the regulation of bone resorption has been confirmed only in mouse. Although capsazepine was shown to inhibit osteoclast formation in mouse osteoblast–bone marrow co-cultures, as well as to inhibit bone resorption in an ovariectomy mouse model (27), the same effect might not be found in humans. In this study, we found that mouse and human ostoblasts showed a different response to capsaicin. While capsaicin increased the OPG/RANKL ratio in human osteoblasts, the opposite results were found in mouse osteoblasts. Therefore, the use of TRPV1 antagonist to protect bone resorption in humans might be reconsidered and requires further investigations. In fact, it has been demonstrated that TRPV1 pharmacology is different between human and rat. For example, the cloned human TRPV1 expressed in Chinese hamster ovary cells is found to have a lower threshold for proton activation than the cloned rat TRPV1 (30). Moreover, capsazepine is more effective in blocking the response of human TRPV1 than the response of rat TRPV1 to noxious heat, capsaicin and low pH (30). The species-dependent inhibitory effect of capsazepine is also found for rat, mouse and guinea-pig. For example, capsazepine effectively reduced mechanical and thermal hyperalgesia in guinea-pig models of inflammatory and neuropathic pain but had no effect in rat or mouse (7). Until now, the mechanisms of how TRPV1 responds in different species is not yet known. It is possible that the protein machinery which participates in the signal transduction is different between species. Further studies are needed to investigate the pathway in detail. Regarding the pharmacological difference of TRPV1 among species, further studies using human cells are necessary to clarify the role of TRPV1 in the regulation of bone resorption.

The proposed mechanism of capsaicin-induced OPG expression in HPDL cells is presented in Fig. 6. Using capsazepine, we confirmed that the effect of capsaicin was mediated by TRPV1. As TRPV1 is a nonselective cation channel that has high permeability to calcium ions (1), the involvement of calcium ions in this pathway is demonstrated using BAPTA-AM, the calcium chelator. Our data suggest that calcium is required for capsaicin-induced OPG expression. The increased concentration of intracellular calcium might function as a secondary messenger that transduces the signal to up-regulate OPG. Although protein kinase A and protein kinase C have been shown to phosphorylate TRPV1 and thereby modulate its function (31,32), the results from this study revealed that these two kinases were not responsible for capsaicin-induced OPG expression (data not shown). Instead, the mechanism of capsaicin-induced OPG expression involved signalling through the PLC pathway. However, the mechanism of PLC activation during capsaicin treatment is not yet known. As the PLCδ isoform is shown to be activated by the increased concentration of cytoplasmic calcium (33), we suspect that calcium entry after

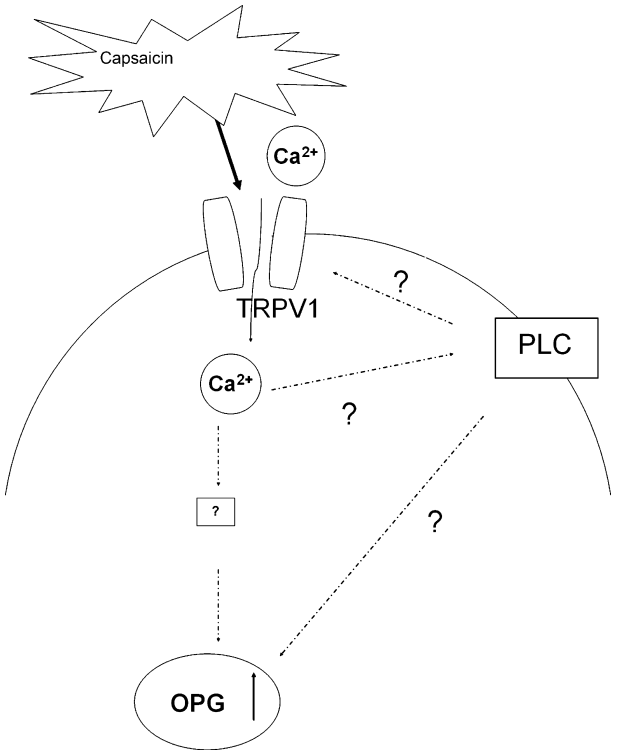

Fig. 6. Schematic diagram representing the pathway of capsaicin-induced osteoprotegerin (OPG) expression in human periodontal ligament (HPDL) cells. Capsaicin treatment leads to the opening of the transient receptor potential vanilloid-1 (TRPV1) ion channel and thus to calcium influx. Calcium ions act as a secondary messenger that transduces the signal to up-regulate OPG expression. The mechanism of how phospholipase C (PLC) is involved in this pathway is not yet known. Further studies are needed to elucidate this pathway in detail.

capsaicin treatment leads to the activation of PLC. According to the work of Chuang *et al*., PLC might increase TRPV1 channel activity by releasing TRPV1 from the endogenous inhibitor, phosphatidylinositol bisphosphate (34). However, the possibility that PLC might regulate OPG expression through other mechanisms could not be excluded. As PLC inhibition partially abolished OPG expression, it is possible that some other signals are implicated in the up-regulation of OPG expression. Further studies are needed to elucidate this pathway in detail.

The association of inflammation and pathological bone loss is well documented. During the process of inflammation, the release of inflammatory mediators stimulates osteoclast function, resulting in active bone destruction. In this study, the inflammatory condition of HPDL cells was mimicked using PGE_2, a major proinflammatory mediator, which is known to play an important role in osteoclast formation. PGE_2 stimulates osteoclastogenesis and cementoclastogenesis by inhibiting OPG secretion and by up-regulating RANKL expression in osteoblasts and cementoblasts (35,36). Correspondingly, we found that in HPDL cells, PGE_2 stimulation led to the up-regulation of RANKL and to the down-regulation of OPG. In addition, we showed that capsaicin co-treatment could abolish the effect of PGE_2 on the expression of RANKL and OPG. Although treatment with capsaicin alone had no effect on RANKL expression, co-treatment with PGE_2 led to the down-regulation of RANKL. This finding indicated that capsaicin might not be involved in the basal regulation of RANKL expression but that it could interfere with the pathway of PGE_2-induced RANKL expression. As capsaicin was able to increase the OPG/RANKL ratio, even in inflammatory conditions, further studies are needed to prove whether capsaicin could be used to promote alveolar bone regeneration in periodontal disease.

In conclusion, we report, for the first time, that TRPV1 is expressed in HPDL cells. In this cell type, the function of TRPV1, at least, involves the regulation of OPG expression. Moreover, we found that calcium ions and PLC are involved in the up-regulation of OPG upon capsaicin treatment. According to this novel function of TRPV1, our findings suggest the possible application of capsaicin in periodontal treatment.

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