ผลงานที่ได้อันเนื่องมาจากงานวิจัย

1. นำเสนอผลงานวิจัยแบบโปสเตอร์ในการประชุมวิชาการ IADR/SEA ณ ประเทศสิงคโปร์ ในระหว่างวันที่ 28-30 ตุลาคม 2554 เรื่อง *P. gingivalis*-LPS induces TNF-α in HPDLCs and HGFs via TLRs.
2. Thermal Stimulation of TRPV1 Up-regulates TNFα mRNA Expression in Human Periodontal Ligament Cells. (manuscript)

P. gingivalis-LPS induces TNF-α in HPDLCs and HGFs via TLRs

Neeracha Sanchavanakit, Jeeranan Manokawinchoke, Prasit Pavasant

Department of Anatomy, Research Unit of Mineralized Tissues, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

Objectives: *P. gingivalis* LPS has been implicated as a major etiology in periodontitis. Evidence shows that stimulation of toll-like receptor (TLR) 2 and 4 leads to the production of pro-inflammatory cytokines resulting in inflammatory response. The aim of the study was to investigate the roles of TLR2 and 4 in *P. gingivalis* LPS induced TNF-α expression in human periodontal ligament cells (HPDLCs) and human gingival fibroblasts (HGFs).

Methods: Cells were stimulated with *P. gingivalis* LPS at 10 μg/ml for 24 hours. TNF-α expression was analyzed by RT-PCR and ELISA. Activities of TLR2 or 4 on the cell membrane was inhibited by anti-human TLR2 or 4 neutralizing antibody, and confirmed by using TLR2 or TLR4 siRNA to inhibit mRNA expression.

Results: RT-PCR and ELISA demonstrated an increase of TNF-α in the presence of *P. gingivalis* LPS in HPDLCs and HGFs. Both kinds of cells constitutively express TLR2 and 4. Up-regulation of *P. gingivalis* LPS induced TNF-α in HPDLCs was inhibited by both anti-TLR2 and anti-TLR4 antibodies, whereas that in HGFs was inhibited only by anti-TLR4 antibody. The specificity of TLRs in response to *P. gingivalis* LPS was confirmed by the down-regulation of TLR2 or 4 using siRNA. Interestingly, *P. gingivalis* LPS increased expression of TLR2 in HPDLCs, while increased those of TLR2 and 4 in HGFs.

Conclusion: *P. gingivalis* LPS induced TNF-α in HPDLCs and HGFs via different TLRs. Up-regulation of TLR2 or 4 by *P. gingivalis* LPS may affect the recognition potential of those cells to bacterial antigens.

Key words: *P. gingivalis* LPS, TLRs, TNF-α, HPDLCs, HGFs

Thermal Stimulation of TRPV1 Up-regulates TNFα mRNA Expression in Human Periodontal Ligament Cells

Sireerat Sooampon[1, 2*], Waranyoo Phoolcharoen[3], Prasit Pavasant[2, 4]

[1]Department of Pharmacology, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330 Thailand
[2]Mineralized Tissue Research Unit, Faculty of Dentistry, Chulalongkorn Univesity, Bangkok 10330, Thailand
[3]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330 Thailand
[4]Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330 Thailand

[*]**CORRESPONDING AUTHOR:** Sireerat Sooampon, DDS, PhD

Department of Pharmacology, Faculty of Dentistry, Chulalongkorn University,

Henri-Dunant Road, Pathumwan, Bangkok, 10330, Thailand

Tel: +66-2-218-8882 Fax: +66-2-218-88882

E-mail: pl_sireerat@yahoo.com

RUNNING TITLE: Heat induced TNFα mRNA expression

KEY WORDS: TNFα, TRPV1, heat, human periodontal ligament cells

NUMBER OF TEXT FIGURE: 6 figures

Abstracts

Objectives: Transient receptor potential vanilloid 1 (TRPV1), a nociceptive ion channel receptor, was shown to express in human periodontal ligament (HPDL) cells. By using capsaicin, we previously demonstrated that TRPV1 activation led to the up-regulation of OPG/RANKL ratio. As oral cavity is often exposed to the change in temperature, this study further aims to investigate the effect of heat, another well-known TRPV1 activator, on the expression of OPG, RANKL, TNFα and IL-1β, the primarily genes involved in periodontitis.

Methods: HPDL cells were incubated at 45°C for thermal stimulation. The mRNA expression of OPG, RANKL, TNFα and IL-1β was determined by using RT-PCR. The OPG and TNFα secretions were detected by ELISA assay while RANKL protein expression was analyzed by Western blot analysis. The mechanisms of heat-induced TNFα expression were studied by using several inhibitors.

Results: Different from capsaicin, thermal stimulation had no effect on OPG and RANKL expression, suggested the agonist-specific action of TRPV1. In response to heat, the mRNA of TNFα expression, but not IL-1β, was clearly increased. However, the induction of TNFα was not detected at the protein level, suggesting that the negative post-transcriptional modification might occur. By using capsazepine and ruthenium red, the specific TRPV1 antagonists, we confirmed that the up-regulation of TNFα was mediated by TRPV1. While capsaicin was shown to up-regulate OPG expression via PLC, we found that PKC, but not PLC, was required for heat-induced TNFα expression. In addition, the use of Cytochalasin D, an inhibitor of actin polymerization, revealed that the cytoskeleton rearrangement might be an important mechanism for cellular sensing of thermal stimuli.

Conclusions: Our results indicated that TRPV1 played multi-functional role in HPDL cells depending on the stimuli. During thermal response, TRPV1 activation led to the induction of TNFα mRNA expression.

Introduction

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel that is predominantly expressed in sensory neuron. Recent evidence has shown that TRPV1 expression is not restricted to the neuronal tissues but can be detected in other tissues such as human epidermal keratinocyte, gastric epithelial cells, submandibular gland, and human hair follicles (1-4). In the previous study, we found that TRPV1 is also expressed in human periodontal ligament (HPDL) cells (5), a major cells type found in periodontal ligament (PDL). By using capsaicin, we demonstrated that the function of TRPV1, at least, involved in the up-regulation of osteoprotegerin (OPG). In response to capsaicin, TRPV1 presented in HPDL cells might implicate in the regulation of osteoclast formation.

In addition to capsaicin, TRPV1 is known to be activated by a wide range of noxious stimuli including proton and heat (6, 7). According to the temperature-related role, TRPV1 is also recognized as one of the thermo-sensitive cation channel which is activated when temperature is higher than 43°C (8). In sensory neuron, the exposure of heat to TRPV1 is thought to permit an influx of calcium and sodium ions, leading to membrane depolarization and thus pain sensitization (9). Disruption of *TRPV1* gene abrogates the development of inflammatory thermal hyperalgesia in TRPV1 null mice (10). On the other hand, the sensitization of TRPV1 by inflammatory protease leads to persistent thermal hyperalgesia (11). While the thermal function of TRPV1 in

neuronal tissues is widely studied, little is known about the thermal response of TRPV1 in non-neuronal tissues.

Oral cavity is a part of the body that often exposed to the change in temperature. The increase in intra-oral temperature can be occurred during some particular activities such as smoking, hot drinking or dental treatment (12, 13). However the effect of heat in oral tissue is largely unknown. Generally, heat is considered as one of the cellular stress. Cells respond to heat stress by altering a complex program of gene expression. The well-known genes whose expression is affected by heat shock are heat shock protein family (HSPs), which are thought to play a critical role in the development of thermotolerance and protection from stress-induced cellular damage (14). In addition to HSP, thermal stress also alters the expression of a wide variety of genes such as up-regulation of basic fibroblast growth factor (15) and down-regulation of c-fos (16).

As we previously found that TRPV1 activation by capsaicin involved in the regulation of OPG/RANKL axis, in this study, we investigated whether thermal stress, another TRPV1 activator, could induce the same response in HPDL cells. In addition, the implication of TRPV1 in the regulation of pro-inflammatory cytokine expression during thermal exposure will also be studied. Tumor necrosis factor α (TNFα) and Interleukin-1 β (IL-1β) are the primary regulators of inflammatory response with diverse biological effects including osteoclast activating function. Overproduction of TNFα and IL-1β are observed in several inflammatory diseases, including periodontitis (17, 18). However, the activation of these two cytokines upon disease progression is not fully understood. We hypothesize that heat, which is generally occurred during smoking and drinking, might be an important factor that contributes

to periodontal disease. Therefore, the aim of this study is to investigate the effect of heat on OPG, RANKL, TNFα and IL-1β expression in HPDL cells.

Material and Methods

Reagents

Goat anti-OPG immunoglobulin G and mouse anti-RANKL immunoglobulin G were from R&D Systems (Minneapolis, MN, USA). Mouse anti-β-actin immunoglobulin G1-kappa was purchased from Chemicon International (Temecula, CA, USA). Antigoat immunoglobulin G was purchased from Sigma (St Louis, MO, USA). Antimouse immunoglobulin G was from Invitrogen (Eugene, OR, USA). Capsazepine and ruthenium red were obtained from Sigma. U-73122 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Staurosporine was purchased from Millipore (Temecula, CA, USA). Cytochalacin D was from Calbiochem (La Jolla, CA, USA). Calcium-free Dulbecco's modified Eagle's medium was purchased from Invitrogen (Grand Island, NY, USA).

Cell culture and thermal stimulation

HPDL cells were retrieved from middle third of the root surface of healthy third molars extracted for orthodontic reasons. Human gingival fibroblasts (HGFs) were obtained from free gingival during the extraction of an impacted tooth. Primary human osteoblasts were obtained 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 12 patients (seven women and five men; 18–25 years of age). Briefly,

periodontal tissues, gingival tissues and torus mandibularis bone chips were rinsed with sterile phosphate-buffered saline (PBS) and were cut into small pieces. Bone chips were digested with 0.25% trypsin-EDTA. The explants were harvested on 60-mm culture dishes. HPDL cells and HGFs were maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA). Human osteoblasts were grown in Dulbecco's modified Eagle's medium containing 15% 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% CO2 at 37˚C. Cells from third through sixth passages were used. For the experiments, cells (1.5 x 10^5 cells/dish) were seeded into 35-mm culture dish and incubated for 48 h. Thermal stimulation was performed by incubating cells at 45˚C for indicated times. All experiments were done in triplicate using cells from three different donors.

Methylthiazol tetrazolium (MTT) Assay

After thermal stimulation, cells were washed with phosphate-buffered saline (PBS). Subsequently, 1 ml of MTT solution (Sigma Chemical Co, St Louis, MO, USA) was added and incubated for 10 minutes at 37°C. Cells were then washed 2 times with PBS. The purple formazan crystal was dissolved by adding 1 ml of 0.125% glycine buffer in dimethylsulfoxide and gentle shaking for 10 min at room temperature. The optical density was detected with a microplate reader (Biotek Instruments, Winooski, VT, USA)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) in accordance with the manufacturer's instructions. For cDNA synthesis, 1 μg of RNA was reverse-transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) for 1.5 hours at 42ºC. Subsequently, PCR was performed using Taq polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μl in the DNA thermal cycler (Biometra, Göttingen, Germany). The oligonucleotide sequences of the primers are as follows: OPG, forward, TCAAGCAGGAGTGCAATCG and reverse, AGAATGCCTCCTCACACAGG; RANKL, forward, CCAGCATCAAAATCCCAAGT, and reverse, CCCCTTCAGATGATCCTTC; TNFα, forward, AAGCCTGTAGCCCATGTTGT, and reverse, CAGATAGATGGGCTCATACC; IL-1β, forward, GGAGCAACAAGTGGTGTTCT, and reverse, AAAGTCCAGGCTATAGCCGT; HSP70, forward, ATCGACCTGGGCACCACCTA and reverse, CAGCACCATGGACGAGATCT; GAPDH, forward, TGAAGGTCGGAGTCAACGGAT, and reverse, TCACACCCATGACGAACATGG. The cycling condition for OPG was an initial 5-min denaturation step at 94ºC, followed by 24 cycles of denaturation at 94 ºC for 1 min, annealing at 60 ºC for 1 minute, and extension at 72 ºC for 2 minutes, followed by one extension cycle at 72 ºC for 10 minutes. The same condition was also used for RANKL (32 cycles), TNFα (37 cycles), IL-1β (37 cycles), HSP70 (25 cycles), and GAPDH (22 cycles). The PCR products were then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining.

Western blot analysis

Cells were washed twice in PBS and harvested 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. Thirty micrograms of cell lysates were resolved in SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated with primary antibody overnight at 4°C. Subsequently, the membranes were incubated for 1 h with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. Chemiluminescence was developed with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of OPG secretion in cell culture supernatants was determined using OPG ELISA kit (R&D system) in accordance with the manufacturer's protocol.

Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation. The two-tail Student's *t* test, assuming equal variances, was used to compare two groups of samples. One way analysis of variance and Tukey's HSD Post hoc test were used for comparisons among multiple groups. All statistical analysis was performed using Graph Pad Prism (La Jolla, CA, USA). Difference was considered significant when $p < 0.05$.

Results

The effect of heat on cell viability of HPDL cells

First, the effect of heat on cell viability was investigated. HPDL cells were incubated at 45 °C for 1 to 2 hours. By using MTT assay, we found that the exposure of heat for 1 hour did not significantly affect the viability of HPDL cells (Fig. 1). However, approximately 20% of cells were died after 2 hour of heat exposure. Therefore, the effect of heat on HPDL cells was then studied at 1 hour for the rest of the experiments.

Thermal stress did not alter OPG and RANKL expression

As we previously found that capsaicin treatment up-regulated OPG expression through TRPV1 in HPDL cells. We then studied whether the same effect would be occurred when cells were stimulated with heat, another TRPV1 activator. As shown in figure 2A, thermal stimulation for 1 hour did not affect mRNA expression of both OPG and RANKL. To confirm the heat-shock response, the mRNA expression of HSP70 was measured after heat treatment. As expected, the level of HSP70 was markedly increased after heat treatment (Fig. 2A). By using ELISA and Western Blot analysis, we demonstrated that the level of protein expression of OPG and RANKL were not changed by heat, which was corresponded to the mRNA levels (Fig. 2B and C).

Heat induced TNFα expression in a time-dependent manner

Next we studied whether thermal stress was able to stimulate pro-inflammatory cytokine production. HPDL cells were incubated at 45 °C for 10 to 120 minutes. RT-

PCR was performed to analyzed mRNA expression of TNFα and IL-1β, the major pro-inflammatory cytokines that involved in periodontal disease. As shown in Figure 3A, the up-regulation of TNFα mRNA expression could be observed since the first 10 minutes of heat exposure. The level of TNFα mRNA expression was gradually increased in a time-dependent manner. However, IL1-β mRNA expression was barely detectable and was not induced by heat, suggested that TNFα might be a thermal sensitive pro-inflammatory cytokine in HPDL cells. Moreover, the induction of TNFα expression was also found when cells were returned to the normal temperature (37°C) for 3 hours and 24 hours (Fig. 3B). In addition to HPDL cells, we found that the up-regulation of TNFα also observed in human gingival fibroblast and human osteoblast, the major cell components of periodontium (Fig. 3C). This data indicated the general effect of heat on cells in the oral cavity.

Blockade of TRPV1 inhibited TNFα up-regulation during thermal stress.

To confirm that thermal stress induced TNFα mRNA expression through TRPV1, HPDL cells were treated with capsazepine or ruthenium red, the TRPV1 antagonists. As shown in figure 4, the induction of TNFα mRNA expression after 1 hour exposure of heat was clearly abolished by both capsazepine and ruthenium red. The similar results were observed when cells were stimulated with heat for 1 hour and then returned to the normal temperature (37°C) for 24 hours. Interestingly, HSP70 mRNA expression was not affected by either capsazepine or ruthenium red, suggesting that the induction of HSP70 occurred through TRPV1-independent mechanisms. Taken together, our data indicated that TNFα up-regulation upon heat treatment was mediated by TRPV1.

Calcium and Protein Kinase C (PKC) were required for the up-regulation of TNFα by heat

Next the mechanisms how heat induced TNFα expression were investigated. As we previously found that calcium ion and Phospholipase C (PLC) were required for OPG up-regulation upon capsaicin treatment, we then tested whether the up-regulation of TNFα by heat was operated through the same pathway. As shown in figure 4, heat was unable to up-regulated TNFα expression when cells were cultured in the calcium-free medium, suggesting the important role of calcium in TNFα induction. However, treatment with U-73122, a PLC inhibitor, did not abolish the effect of heat on TNFα expression. Instead, we found that staurosporin, the non-specific PKC inhibitor, markedly attenuated TNFα up-regulation. Altogether, these data suggested that calcium and PKC are required for the induction of TNFα during heat treatment.

Actin polymerization was involved in heat-induced TNF expression

The mechanism how HPDL cells sensed thermal stress was then elucidated. We hypothesized that cytoskeleton rearrangement might occur during heat exposure. As expected, treatment with cytochalansin D, the inhibitor of actin polymerization, clearly abolished the effect of heat on TNFα induction. These data suggested the involvement of actin in the up-regulation of TNF during heat activation.

Discussion

In the previous study, we have demonstrated the novel function of TRPV1 in HPDL cells. Capsaicin, a well known TRPV1 agonist, was shown to up-regulate OPG expression. In this study, the role of TRPV1 in HPDL cells was studied by using heat, another TRPV1 activator. Here, we found that thermal stress did not affect the expression of OPG and RANKL, the key regulators of osteoclastogenesis. Instead, heat could induce mRNA expression of TNF, suggesting that TRPV1 played multi-functional role in HPDL cells depending on the stimuli.

The effect of heat on pro-inflammatory cytokine production was performed at 45 °C, which is the temperature that evokes noxious heat (19). At this temperature, TRPV1, which have the threshold at 43 °C, is activated and mediates membrane depolarization in neuron (9). Here, we reported for the first time that thermal stress could act as an inducer of TNFα mRNA expression, but not that of IL-1β, in HPDL cells. The increase in TNFα mRNA expression was not only observed in HPDL cells but also found in human gingival fibroblasts and human osteoblasts, the primary cell types of periodontium. However, the protein secretion of TNFα was barely detectable in these three cell types and could not be induced by heat (data not shown). The results implied that these three cells types were not the main sources of TNFα production. Our observation was corresponded to Keller's work whose could not detect the secretion of TNFα in human odontoblasts and dental pulp fibroblasts despite TNFα mRNA expression was strongly induced by lipoteichoic acid (20). The undetectable level of TNF protein might due to the negative post-transcriptional modification of TNFα in these cell types.

The crosstalk between TRPV1 and inflammation has been demonstrated. In addition to function as pain modulator, TRPV1 also plays a pivotal role as a molecular regulator of inflammatory response. In this study, the role of TRPV1 in the regulation of TNFα expression was investigated by using two TRPV1 antagonists; capsazepine and ruthenium red. Both antagonists clearly attenuated the effect of heat on TNFα expression. The involvement of TRPV1 was found in both the immediate and long term response to heat. Interestingly we found that HSP70, which is up-regulated by heat, was not altered by TRPV1 antagonists, suggesting that HSP70 might regulated by TRPV1-independent mechanism. It is of interest to study the mechanism of HSP70 induction in the future.

In this study, the mechanisms of heat-induced TNFα expression were also investigated. Though we previously found that calcium ion and PLC were required for OPG up-regulation upon capsaicin treatment (5), difference signal transduction was found in the case of thermal stress. In line with the characteristics of TRPV1 as a non-selective cation channel with high permeability to calcium (7), we found that calcium ion was essential for both capsaicin (5) and heat response. An influx of calcium ion upon TRPV1 activation might serve as secondary messenger that transduced the signal to up-regulate TNFα expression. In addition to calcium, a variety of signaling molecules have been shown to involve in signal transduction of TRPV1. For example, phospholipase C is shown to cleave phosphatidylinositol bisphosphate (PIP_2), resulting in the release of TRPV1 from its inactive state (21). Correspondingly, we previously found that PLC was required for capsaicin-induced OPG expression. However, this study showed that, instead of PLC, PKC is important to heat-induced

TNFα expression. As PKC has been shown to modulate TRPV1 function by phosphorylation at S502 and S800 (22), it is possible that PKC might directly gate TRPV1 ion channel. Also we could not exclude the possibility that PKC is important for the downstream signal to TRPV1 Further study is necessary to elucidate the pathway in details. Taken together, our study demonstrate for the first time that capsaicin and heat, both are known as the activators of TRPV1, might transduce signals through different cellular machinery, leading to agonist-specific response.

The agonist recognition sites of TRPV1 have been explored. For instance, capsaicin, a lipophilic molecule, is able to cross cell membrane and bind to intracellular domain of rat TRPV1 at Arg114 and Glu 761 residues (23, 24). In the case of heat, it has been demonstrated that C-terminal domain of TRPV1 is responsible for thermal sensitivity (25). Considering heat as a physical stimulus, it is of interest to study how it acts to gate TRPV1 channel. As thermal stress has been shown to induce a variety of cellular response, including actin polymerization (26, 27), we hypothesize that cytoskeleton alterations during heat exposure might be a crucial step in thermal sensitization. As expected, we found that the heat-induced TNFα expression was abolished when cells were treated with cytochalasin D, the inhibitor of actin polymerization. This finding suggests that cytoskeleton rearrangement might be an important mechanism for cellular sensing of thermal stimuli.

In conclusion, our results indicated that TRPV1 played multi-functional role in HPDL cells depending on the stimuli. During thermal response, TRPV1 activation led to the induction of TNFα mRNA expression. Moreover, calcium ion, PKC, and actin

polymerization were involved in the regulation of TNFα expression during thermal stimulation.

Acknowledgements

This work was supported by Thailand Research Fund (MRG5380146) and Government Research Budget, Chulalongkorn University 2010-2011

References

(1) Inoue K, Koizumi S, Fuziwara S, Denda S, Inoue K, Denda M. Functional vanilloid receptors in cultured normal human epidermal keratinocytes. *Biochemical and biophysical research communications* 2002; **291**: 124-129.
(2) Kato S, Aihara E, Nakamura A, et al. Expression of vanilloid receptors in rat gastric epithelial cells: role in cellular protection. *Biochem Pharmacol* 2003; **66**: 1115-1121.
(3) Zhang Y, Xiang B, Li YM, et al. Expression and characteristics of vanilloid receptor 1 in the rabbit submandibular gland. *Biochemical and biophysical research communications* 2006; **345**: 467-473.
(4) Bodo E, Biro T, Telek A, et al. A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *The American journal of pathology* 2005; **166**: 985-998.
(5) Sooampon S, Manokawinchoke J, Pavasant P. TRPV1 Regulates OPG/RANKL Homeostasis in Human Periodontal Ligament Cells. *J Periodontal Res* 2012 (In press).
(6) Tominaga M, Caterina MJ, Malmberg AB, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998; **21**: 531-543.
(7) Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997; **389**: 816-824.
(8) Jordt SE, McKemy DD, Julius D. Lessons from peppers and peppermint: the molecular logic of thermosensation. *Curr Opin Neurobiol* 2003; **13**: 487-492.
(9) Moran MM, Xu H, Clapham DE. TRP ion channels in the nervous system. *Curr Opin Neurobiol* 2004; **14**: 362-369.
(10) Davis JB, Gray J, Gunthorpe MJ, et al. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 2000; **405**: 183-187.
(11) Amadesi S, Nie J, Vergnolle N, et al. Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J Neurosci* 2004; **24**: 4300-4312.

(12) dos Santos RB, Katz J. Nicotinic stomatitis: positive correlation with heat in mate tea drinks and smoking. *Quintessence Int* 2009; **40**: 537-540.
(13) Nicoll BK, Peters RJ. Heat generation during ultrasonic instrumentation of dentin as affected by different irrigation methods. *Journal of periodontology* 1998; **69**: 884-888.
(14) Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 2002; **92**: 2177-2186.
(15) Erdos G, Lee YJ, Cho JM, Corry PM. Heat-induced bFGF gene expression in the absence of heat shock element correlates with enhanced AP-1 binding activity. *J Cell Physiol* 1995; **164**: 404-413.
(16) Chen C, Xie Y, Stevenson MA, Auron PE, Calderwood SK. Heat shock factor 1 represses Ras-induced transcriptional activation of the c-fos gene. *J Biol Chem* 1997; **272**: 26803-26806.
(17) Kurtis B, Tuter G, Serdar M, et al. Gingival crevicular fluid levels of monocyte chemoattractant protein-1 and tumor necrosis factor-alpha in patients with chronic and aggressive periodontitis. *Journal of periodontology* 2005; **76**: 1849-1855.
(18) Zhang F, Engebretson SP, Morton RS, Cavanaugh PF, Jr., Subbaramaiah K, Dannenberg AJ. The overexpression of cyclo-oxygenase-2 in chronic periodontitis. *J Am Dent Assoc* 2003; **134**: 861-867.
(19) Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature* 2001; **413**: 203-210.
(20) Keller JF, Carrouel F, Colomb E, et al. Toll-like receptor 2 activation by lipoteichoic acid induces differential production of pro-inflammatory cytokines in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. *Immunobiology* 2010; **215**: 53-59.
(21) Chuang HH, Prescott ED, Kong H, et al. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 2001; **411**: 957-962.
(22) Mandadi S, Tominaga T, Numazaki M, et al. Increased sensitivity of desensitized TRPV1 by PMA occurs through PKCepsilon-mediated phosphorylation at S800. *Pain* 2006; **123**: 106-116.
(23) Jung J, Hwang SW, Kwak J, et al. Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J Neurosci* 1999; **19**: 529-538.
(24) Jung J, Lee SY, Hwang SW, et al. Agonist recognition sites in the cytosolic tails of vanilloid receptor 1. *J Biol Chem* 2002; **277**: 44448-44454.
(25) Brauchi S, Orta G, Salazar M, Rosenmann E, Latorre R. A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *J Neurosci* 2006; **26**: 4835-4840.
(26) Han SI, Ha KS, Kang KI, Kim HD, Kang HS. Heat shock-induced actin polymerization, SAPK/JNK activation, and heat-shock protein expression are mediated by genistein-sensitive tyrosine kinase(s) in K562 cells. *Cell Biol Int* 2000; **24**: 447-457.
(27) Pawlik A, Nowak JM, Grzanka D, Gackowska L, Michalkiewicz J, Grzanka A. Hyperthermia induces cytoskeletal alterations and mitotic catastrophe in p53-deficient H1299 lung cancer cells. *Acta Histochem* 2012.

Figures

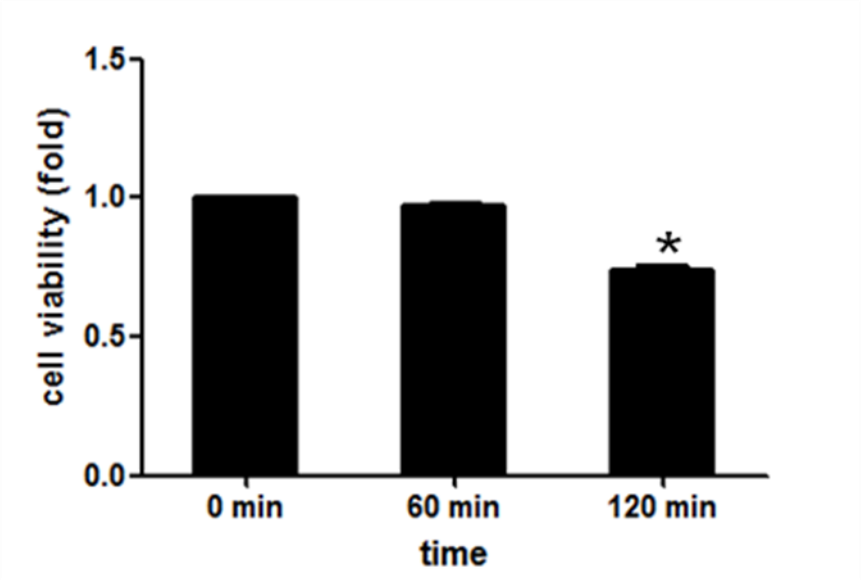

Figure 1. The effect of heat on cell viability. HPDL cells were incubated at 45 °C for indicated time. Cell viability was studied by MTT assay. Data were expressed as fold change under control (0 min) and represented as means ± SD of 3 independent experiments. * $p < 0.05$ *vs.* 0 min.

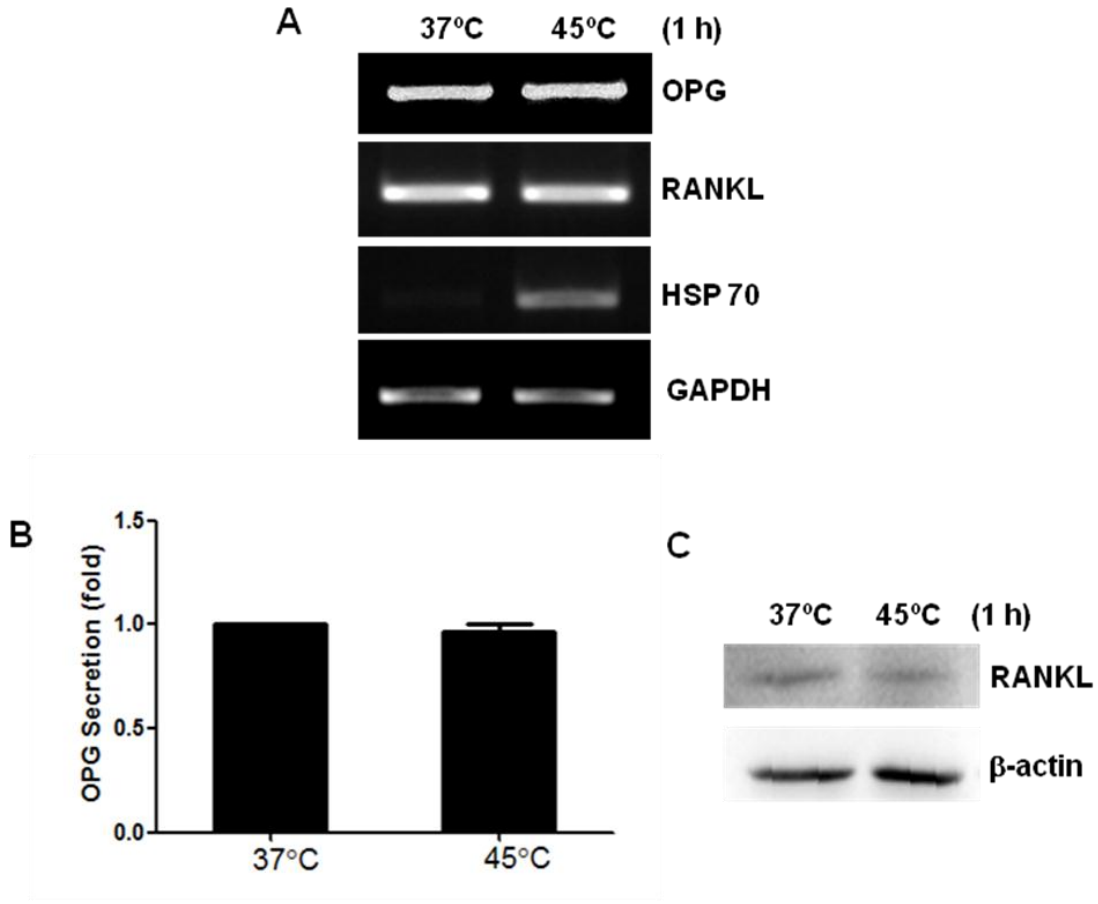

Figure 2. Thermal stress did not alter OPG and RANKL at both mRNA and protein level. HPDL cells were incubated at 45 °C for 1 hour. (A) The mRNA expression of OPG, RANKL and HSP70 was examined by RT-PCR. (B) Cell culture supernatants were tested for OPG secretion by ELISA assay. Data were expressed as fold change under control (0 min) and represented as means ± SD of 3 independent experiments. (C) Cell lysates were studied for RANKL protein expression by Western Blotting.

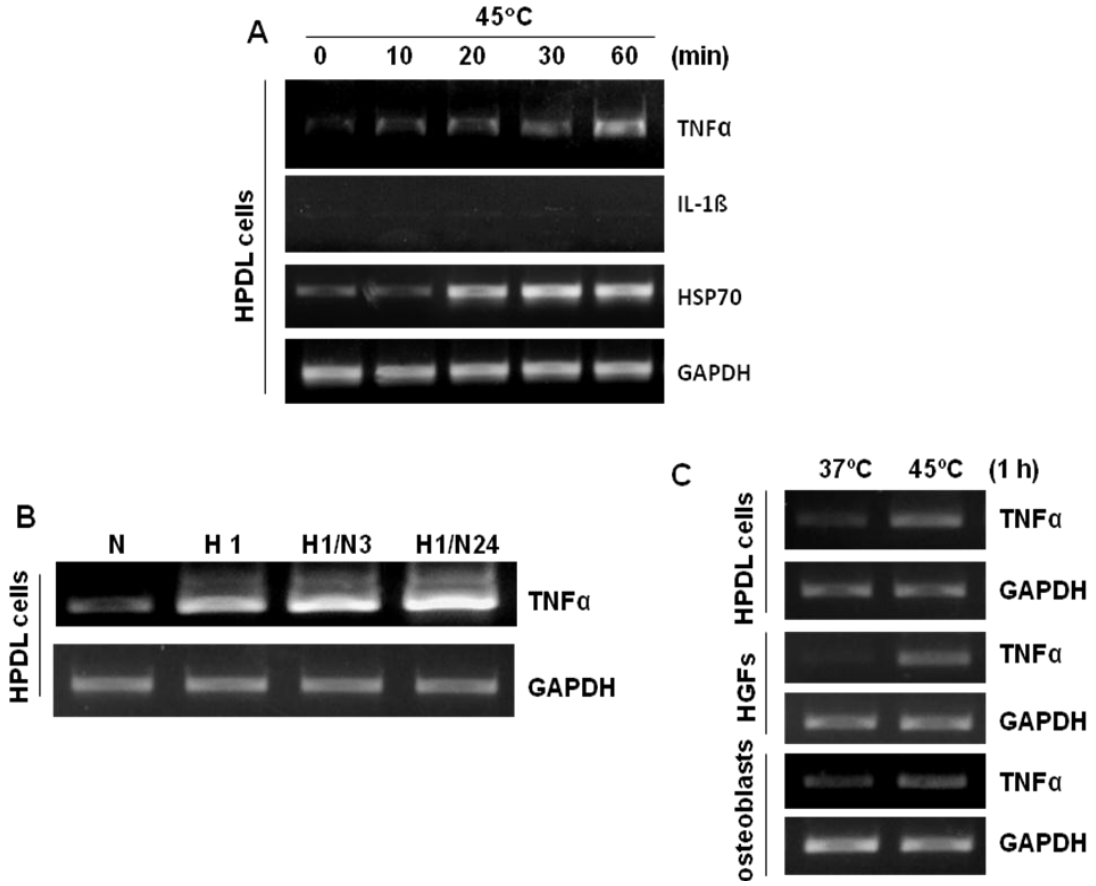

Figure 3. The induction of TNFα mRNA expression after heat exposure. (A, C) HPDL cells, HGFs and human osteoblasts were incubated at 45 °C for indicated time. The mRNA expression of TNFα, IL-1β and HSP70 was examined by RT-PCR. GAPDH mRNA expression was analyzed as loading controls. (B) HPDL cells were left untreated (N; 37°C) or incubated at 45 °C for 1 h (H1) and then return to 37°C for 3 h (H1/N3) or 24 h (H1/N24). The mRNA expression of TNFα was examined by RT-PCR. GAPDH mRNA expression was analyzed as loading controls.

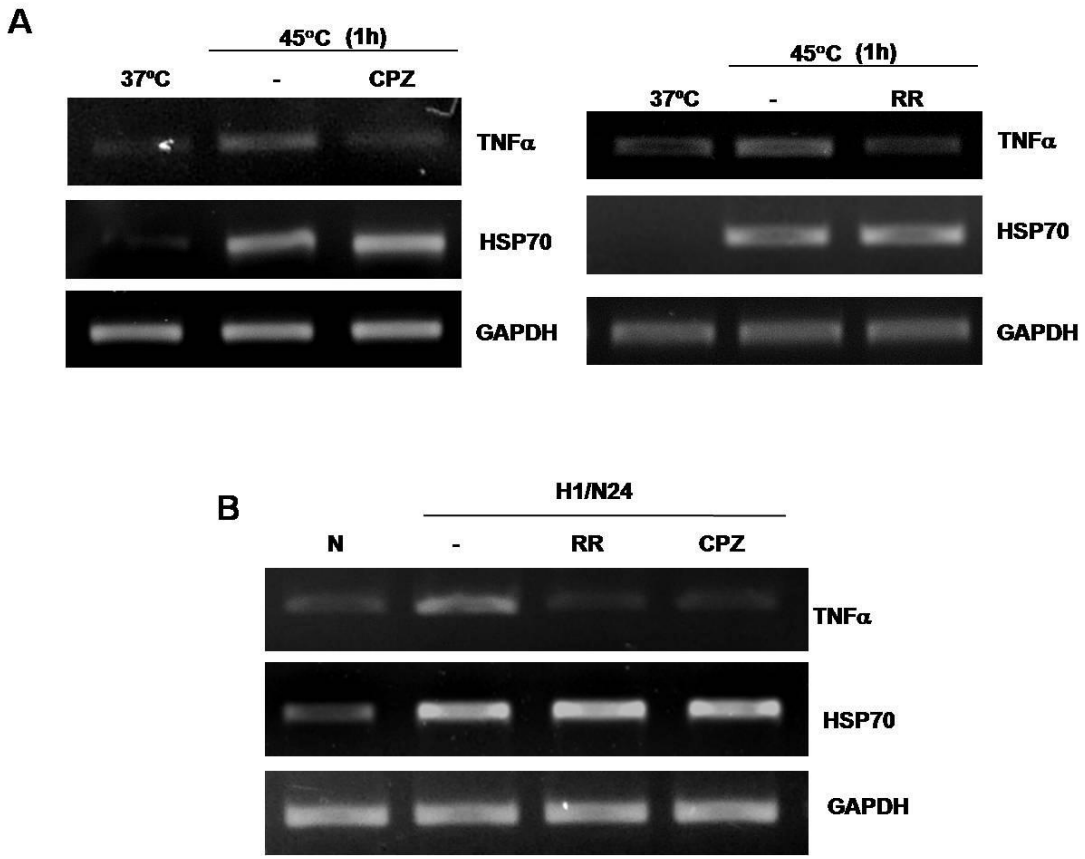

Figure 4. TRPV1 antagonists abolished the effect of heat on TNFα mRNA expression. HPDL cells were left untreated or pretreated with capsazepine (10 μM) or ruthenium red (10 μM) for 30 minutes. Then cells were incubated at 45 °C for 1 hour (A) or re-incubated at 37 °C for another 24 hours (B). The mRNA expression of TNFα and HSP70 was examined by RT-PCR. GAPDH mRNA expression was used as loading controls. CPZ, capsazepine; RR, ruthenium red

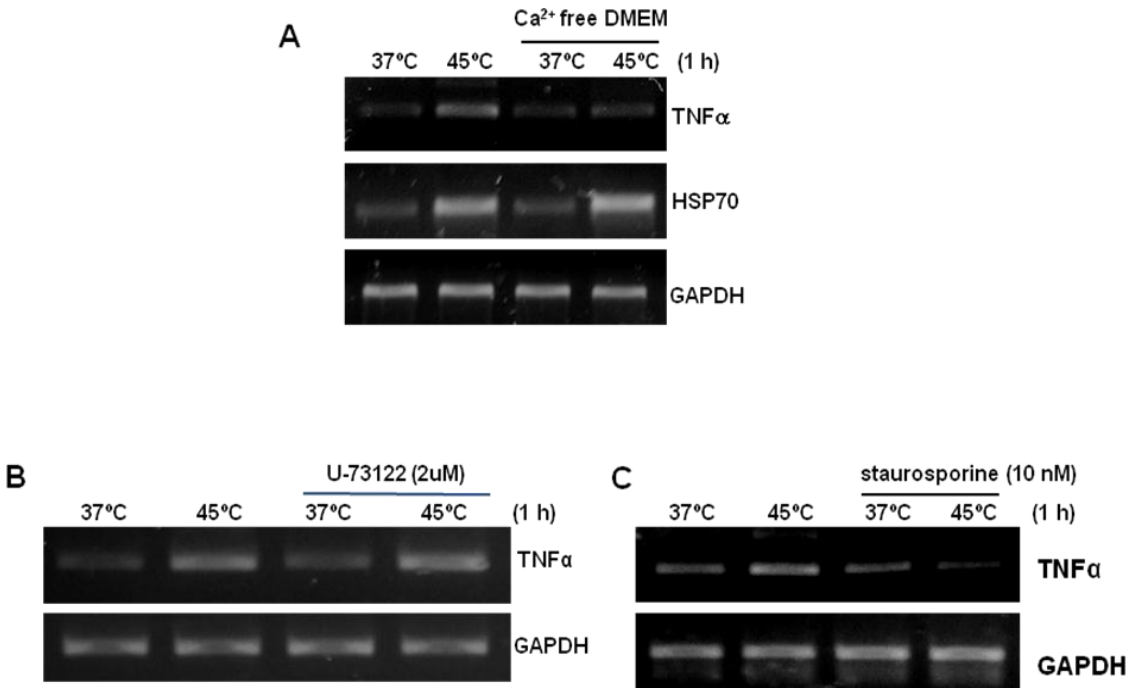

Figure 5. Calcium and PKC were required for heat-induced TNFα mRNA expression. HPDL cells were left untreated or pretreated with calcium-free medium (A), 2 μM of U-73122 (B) or 10 nM of staurosporin (C) for 30 minutes and then cells were incubated at 45 °C for 1 hour. The mRNA expression of TNFα was examined by RT-PCR. GAPDH mRNA expression was used as loading controls.

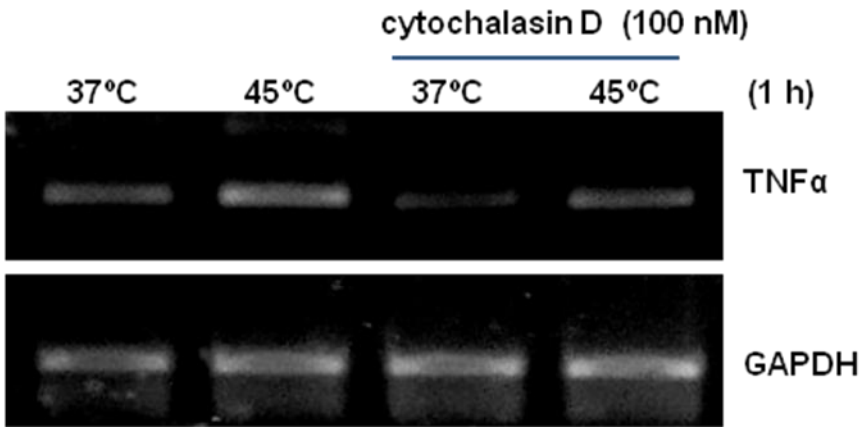

Figure 6. Actin polymerization was important for TNFα up-regulation upon heat treatment. HPDL cells were left untreated or pretreated with 100 nM of cytochalasin D for 30 minutes and then cells were incubated at 45 °C for 1 hour. The mRNA expression of TNFα was examined by RT-PCR. GAPDH mRNA expression was used as loading controls.