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Mechanical stress-induced interleukin-1beta expression through adenosine triphosphate/P2X7 receptor activation in human periodontal ligament cells

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Background and Objective: Mechanical stress is an important factor in maintaining homeostasis of the periodontium. Interleukin-1beta (IL-1β) and adenosine triphosphate (ATP) are considered potent inflammatory mediators. In macrophages, ATP-activated P2X7 receptor is involved in IL-1β processing and release. Our previous works demonstrated mechanical stress-induced expression of osteopontin and RANKL through the ATP/P2Y1 receptor in human periodontal ligament (HPDL) cells. This study was designed to examine the effect of mechanical stress on IL-1β expression in HPDL cells, as well as the mechanism and involvement of ATP and the P2 purinergic receptor.

Material and Methods: Cultured HPDL cells were treated with continuous compressive loading. IL-1β expression was analyzed at both mRNA and protein levels, using RT-PCR and ELISA, respectively. Cell viability was examined using the MTT assay. ATP was also used to stimulate HPDL cells. Inhibitors, antagonists and the small interfering RNA (siRNA) technique were used to investigate the role of ATP and the specific P2 subtypes responsible for IL-1β induction along with the intracellular mechanism.

Results: Mechanical stress could up-regulate IL-1β expression through the release of ATP in HPDL cells. ATP alone was also capable of increasing IL-1β expression. The induction of IL-1β was markedly inhibited by inhibitors and by siRNA targeting the P2X7 receptor. ATP-stimulated IL-1β expression was also diminished by intracellular calcium inhibitors.

Conclusion: Our work clearly indicates the capability of HPDL cells to respond directly to mechanical stimulation. The results signified the important roles of ATP/P2 purinergic receptors, as well as intracellular calcium signaling, in mechanical stress-induced inflammation via up-regulation of the proinflammatory cytokine, IL-1β, in HPDL cells.

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Mechanical stress may affect the balance between tissue destruction and tissue regeneration. Under normal physiologic conditions, homeostasis of the periodontium is maintained by appropriate mechanical loading (1,2). Periodontal ligament (PDL) cells respond to mechanical stress by signaling the surrounding cells, thereby regulating bone-matrix resorption and formation (3–5). Improper loading can disturb the balance of PDL tissue remodeling (5). For instance, orthodontic tooth movement causes increased expression of cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha, RANKL and MMP-1 in PDL tissue (6–8). These cytokines may contribute to alveolar bone resorption during tooth movement.

Reports have shown increased numbers of inflammatory mediators, including proinflammatory cytokines (i.e. IL-1β, IL-6, IL-8 and tumor necrosis factor-alpha), adenosine triphosphate (ATP), prostaglandin E2 and nitric oxide, as well as the extracellular matrix degradation enzymes, MMPs, in various cell types upon mechanical activation (9–14). Among these, the release of IL-1β is considered significant in the initiation and regulation of inflammation (15–17).

ATP is an important extracellular messenger. Apart from its major physiological role as an energy source of the cell, it is now well established that ATP can be released under pathologic conditions (18–21). ATP functions as a signaling molecule released from mechanically stimulated cells, including human periodontal ligament (HPDL) cells (19,22–25). The released ATP consequently activates purinergic P2 receptors, which are broadly classified into two families, based on their modes of signal transduction, as the ligand-gated ion channels (P2X1-7) and the G-protein-coupled metabotropic receptors (P2Y1, 2, 4, 6, 11, 12, 13 and 14) (26–31). P2X7 receptor, a well-known calcium-permeable cation channel, is of interest because of its high responsiveness to ATP activation as well as the participation in IL-1β processing and release in macrophages (32,33). ATP causes formation of a reversible plasma membrane pore, which might be essential in P2X7 receptor-stimulated IL-1β release (34). The involvement of the P2X7 receptor in IL-1β release is also found in activated neutrophils, epithelial cell and microglial cells (35,36). P2X7 receptor is therefore considered vital in IL-1β processing and release (33). During orthodontic tooth movement, a role for the P2X7 receptor in mechanotransduction has also been suggested (37). We previously found mechanical stress-induced expression of osteopontin and RANKL through the release of ATP via the P2Y1 receptor in HPDL cells (23,24,38,39). Based on established evidence, it is hypothesized that ATP, as well as activation of its receptor, might contribute to the mechanical stress-induced expression of inflammatory cytokines in HPDL cells (23,38,39). However, the relationship among mechanical stress, IL-1β, ATP and the P2X7 receptor in HPDL cells has not been reported. This study was conducted in order to clarify the effect of mechanical stress on the expression of the inflammatory cytokine, IL-1β, in HPDL cells. The underlying mechanism and the involvement of ATP, as well as the P2X7 receptor, were also examined.

Material and methods

Cell culture

HPDL cells were obtained from extracted healthy third molars with patients' informed consent. The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Teeth were rinsed with sterile phosphate-buffered saline and periodontal tissues were removed from the middle third of root surfaces. Tissues were then grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA), 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 incubated at 37°C in a 5% CO_2 humidified air chamber. HPDL cells from the 3rd to the 5th passages were used. All experiments were performed in triplicate, using cells from three different donors.

Treatment of cells

HPDL cells were seeded in six-well plates at a density of 25,000 cells/cm^2 and were grown to 90% confluence. After 4 h of serum deprivation, cells were treated with or without 1–2.5 g/cm^2 of compressive force for 1–5 h or with 10–200 μM ATP (Sigma-Aldrich Chemical, St Louis, MO, USA) for up to 180 min. For stress application, plastic cylinders were placed in each well and metal coins were added to produce compressive force (23). IL-1β expression was measured at both mRNA and protein levels. Culture medium was collected for ELISA and mRNA was extracted for RT-PCR. Nontreated cells were used as controls.

MTT assay

Viability of HPDL cells was analyzed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (USB Corporation, Cleveland, OH, USA). Viable cells were capable of converting the yellow MTT solution into purple formazan crystals, which were then eluted in dimethylsulfoxide to form a colored solution. The final product was quantified using an absorbance microplate reader (BioTek ELx800; BioTek Instruments Inc., Winooski, VT, USA) at 570 nm. Data were expressed as percentage of viable cells compared with the control.

Measurement of IL-1β release by ELISA

IL-1β release into culture medium was measured using an IL-1β ELISA kit (Quantikine® Human IL-1β/IL-1F2 Immunoassay; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The optical density was determined at 450 nm, using an absorbance microplate reader (BioTek ELx800;

BioTek Instruments Inc.) Data were expressed as fold increase of IL-1β over control.

Measurement of ATP release using the bioluminescence assay

ATP release into culture medium was measured using a luciferin-luciferase base bioluminescence detection kit for ATP (ENLITEN® ATP Assay System; Promega, Madison, WI, USA), as previously described (24). The emitted light was measured at 560 nm, using a luminescence microplate reader (BioTek Synergy H1 Hybrid Reader; BioTek Instruments Inc.). Data were expressed as fold increase of ATP over control.

Extraction of RNA and semiquantitative PCR

Total RNA extraction, reverse transcription and PCR were performed as previously described (24,38). Primers were designed as reported in GenBank. The oligonucleotide sequences and PCR cycles were as follows: IL-1β (NM 000576.2, 40 cycles), sense 5′-GGA GCA ACA AGT GGT GTT CT-3′ and antisense 5′-AAA GTC CAG GCT ATA GCC GT-3'; P2X7 receptor (NM 002562.5, 35 cycles) sense 5′-TTTGCTCTGGTGAGTGA CAAGCTG3′ and antisense 5′CCT CTGGTTGTCCAGGAATCG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM 002046.3, 22 cycles) sense 5′-TGAAGGTCGGAG TCAACGGAT-3′ and antisense 5′-TC ACACCCATGACGAACATGG-3′

Western blot analysis for detection of protein

The protein extraction, separation and immunoblotting protocols used were as previously described (24,38). The primary antibodies used were raised against P2X7 receptor (rabbit polyclonal antibody to P2RX7, 1 : 200 dilution; Abcam, Cambridge, UK) and actin (mouse anti-actin monoclonal antibody, clone: C4, 1 : 1000 dilution; Chemicon International, Temecula, CA, USA). Secondary antibodies used were goat anti-rabbit IgG biotin-conjugated affinity-purified antibody (1 : 2000 dilution; Chemicon International) and goat anti-mouse IgG biotin-conjugated antibody (1 : 2000 dilution; Invitrogen, Eugene, OR, USA).

Inhibitors

After 4 h of serum deprivation of cultured HPDL cells, inhibitors were added 30 min before application of stress or ATP. The following inhibitors were used: 3.6 μM actinomycin D (a transcription blocker; Calbiochem, San Diego, CA, USA), 7.96 μM cyclohexamide (Sigma-Aldrich Chemical), 15 μM suramin (Calbiochem), 1 unit/mL of apyrase (Sigma-Aldrich Chemical), 10 μM KN-62 (Sigma-Aldrich Chemical), 10 μM MRS2179 (Sigma-Aldrich Chemical), 0.18 μM NF449 (Tocris Bioscience, Minneapolis, MN, USA), 300 nM NF023 (Tocris Bioscience), 5 μM 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; Enzo Life Sciences Inc., Farmingdale, NY, USA), 12.5 nM thapsigargin (Sigma-Aldrich Chemical) and 50 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB8; Biomol Research Laboratories Inc., Plymouth, PA, USA).

For transfection with small interfering RNA (siRNA), HPDL cells were cultured in antibiotic-free normal growth medium until reaching 70–80% confluence. Cells were treated with a mixed solution of siRNA oligonucleotide specific to P2X7 receptor (1 : 250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and transfection reagent (Lipofectamine™2000 Reagent; Invitrogen) for 24 h before application of stress or ATP. The ratio of siRNA to transfection reagent was 2 : 3. Untreated cells, cells treated with only control siRNA (1 : 250 dilution; Santa Cruz Biotechnology) or transfection reagents (MOCK, data not shown) were used as controls. Percentage knockdown of gene expression was determined from the relative band intensity of siRNA-transfected cells compared with control (untreated) cells, using SCION IMAGE software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Data were analyzed by one-way analysis of variance using statistical software (SPSS, Chicago, IL, USA) with Scheffé's test as post-hoc analysis ($p < 0.05$).

Results

Mechanical stress-induced IL-1β expression in HPDL cells

We first investigated whether mechanical stress could induce IL-1β expression and release in HPDL cells. After stress application, up-regulation of IL-1β expression was observed at both mRNA and protein levels (Fig. 1). The increase in IL-1β level correlated with the increased magnitude of compressive loading and with the duration of treatment (Fig. 1A, 1B, 1D and 1E). The MTT assay was used to evaluate the viability of HPDL cells after receiving mechanical stress (Fig. 1C and 1F). Maximal expression of IL-1β with the least harmful effect on HPDL cells was found when using 2 g/cm^2 stress stimulation for 3 h (Fig. 1C and 1F), which were used in all subsequent experiments.

ATP acts as a signaling molecule in mechanical stress-induced IL-1β expression

We explored the participation of ATP in stress-induced IL-1β expression by using ATP-degradading enzyme and P2 receptor antagonists. An ATP diphosphohydrolase, apyrase, as well as a nonspecific P2 receptor antagonist, suramin, nearly abolished the effect of mechanical stress on IL-1β expression at both mRNA and protein levels (Fig. 2A and 2B), indicating the involvement of ATP in the induction of IL-1β.

The role of ATP was investigated using ATP itself as a stimulator. After 4 h of serum deprivation, cultured HPDL cells were treated with or without 10–100 μM ATP for up to 3 h. ATP alone could stimulate IL-1β expression at both mRNA and protein levels, as shown by RT-PCR and ELISA (Fig. 2C–2F). The maximum

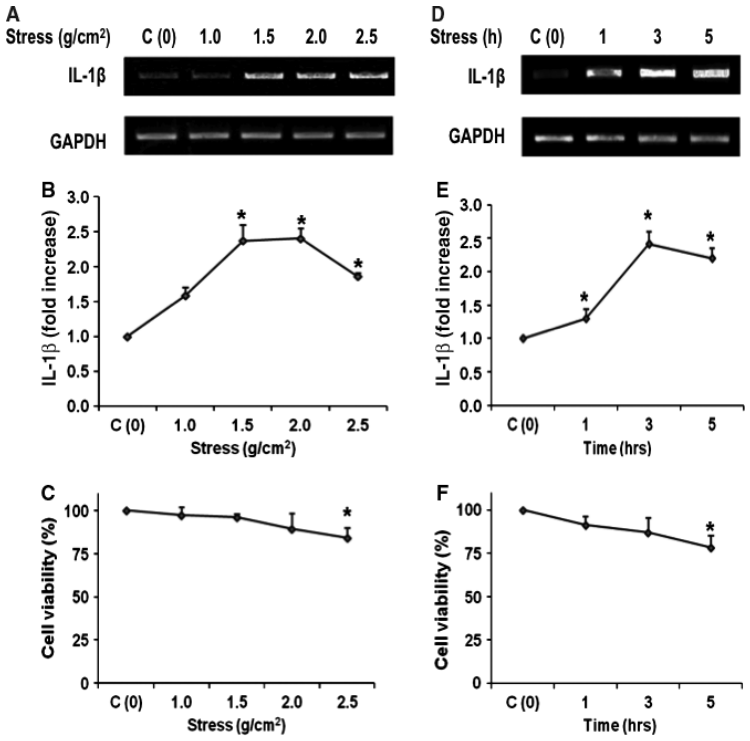

Fig 1. Up-regulation of interleukin (IL)-1β expression upon mechanical stress stimulation in human periodontal ligament (HPDL) cells. Cultured HPDL cells were treated with compressive loading, ranging from 0 to 2.5 g/cm^2, for 3 h. Expression of IL-1β was measured using RT-PCR (A) and ELISA (B). Cell viability was determined using MTT assays (C). To evaluate the time-course effect of stress, HPDL cells received 2 g/cm^2 of loading for up to 5 h. IL-1β expression was measured at both mRNA (D) and protein (E) levels. The MTT assay was also performed (F). Results are expressed as mean ± standard deviation from three different experiments. *Significant difference, $p < 0.05$. C, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

concentration of IL-1β was observed upon treatment with 40 μM ATP for 30 min, which was then used in subsequent experiments.

We examined the induction of IL-1β using a transcription blocker, actinomycin D, and a translation blocker, cyclohexamide. Both inhibitors markedly inhibited stress- as well as ATP-induced IL-1β expression at both mRNA and protein levels (Fig. 2G, 2H and 2J). However, the luciferin-luciferase assay revealed no significant effect of actinomycin D and cyclohexamide on stress-induced ATP release (Fig. 2I).

P2X7 receptor is a major P2 subtype responsible for IL-1β induction in HPDL cells

In order to determine the specific P2 receptor subtype involved in ATP-induced IL-1β release in stress-activated HPDL cells, we used several P2 receptor antagonists. A specific P2X7 receptor antagonist, KN-62, in the same way as suramin, significantly diminished ATP-induced IL-1β release, while no, or a less significant, effect was observed when using MRS2179 (a specific P2Y1 receptor antagonist), NF449 (P2X1, P2X3, P2Y1 and P2Y2 receptor antagonists) and NF023 (P2X1, P2X2, P2X3 and P2X4 antagonists) (Fig. 3A). Similar inhibitory effects of these antagonists on stress-induced IL-1β expression were also observed (data not shown).

The function of the P2X7 receptor was additionally confirmed using siRNA transfection. Treatment with siRNA targeting the P2X7 receptor for 24 h yielded approximately 75–80% knockdown of P2X7 receptor mRNA and protein expression in HPDL cells (calculated by SCION IMAGE Software, data not shown), as demonstrated by RT-PCR (Fig. 3B) and western blot analysis (Fig. 3C). After transfection with siRNA, mechanical stress or ATP was applied on cultured HPDL cells. IL-1β expression was detected using RT-PCR (Fig. 3D and 3E) and ELISA (Fig. 3F and 3G). Knockdown of P2X7 receptor resulted in dramatically decreased expression of IL-1β in stimulated HPDL cells, while an elevated level of IL-1β was still observed in all control groups.

As P2X7 receptor is well known as a cationic-channel that is highly permeable to calcium, the role of calcium was examined using calcium inhibitors. An intracellular calcium chelator, BAPTA, a calcium-ATPase inhibitor, thapsigargin, and an intracellular calcium antagonist, TMB8, were able to diminish IL-1β induction in HPDL cells (Fig. 4A and 4B), regardless of whether the induction occurred through stress or ATP application.

Discussion

It is well acknowledged that mechanical stress can induce tissue inflammation. Increased levels of pro-inflammatory cytokines, including IL-1β, IL-6 and IL-8, as well as tumor necrosis factor-alpha, have been reported in many cell types, for example chondrocytes (11,40), osteoblasts (9), temporomandibular joint (TMJ) synovial cells (10) and human dental pulp cells (11) receiving various types of stress. In our study, compressive stress induced inflammation in HPDL cells by increasing the expression of a major proinflammatory cytokine, IL-1β. When presented in the early phase of inflammation, IL-1β activates downstream inflammatory mediators, such as cyclooxygenase 2, prostaglandin E2 and MMPs (25,41–43), resulting in tissue damage.

We previously found that mechanical stress induced RANKL and osteopontin in HPDL cells through the release of a potent inflammatory mediator, ATP (38). By using ATPase as well as P2 receptor antagonists, we

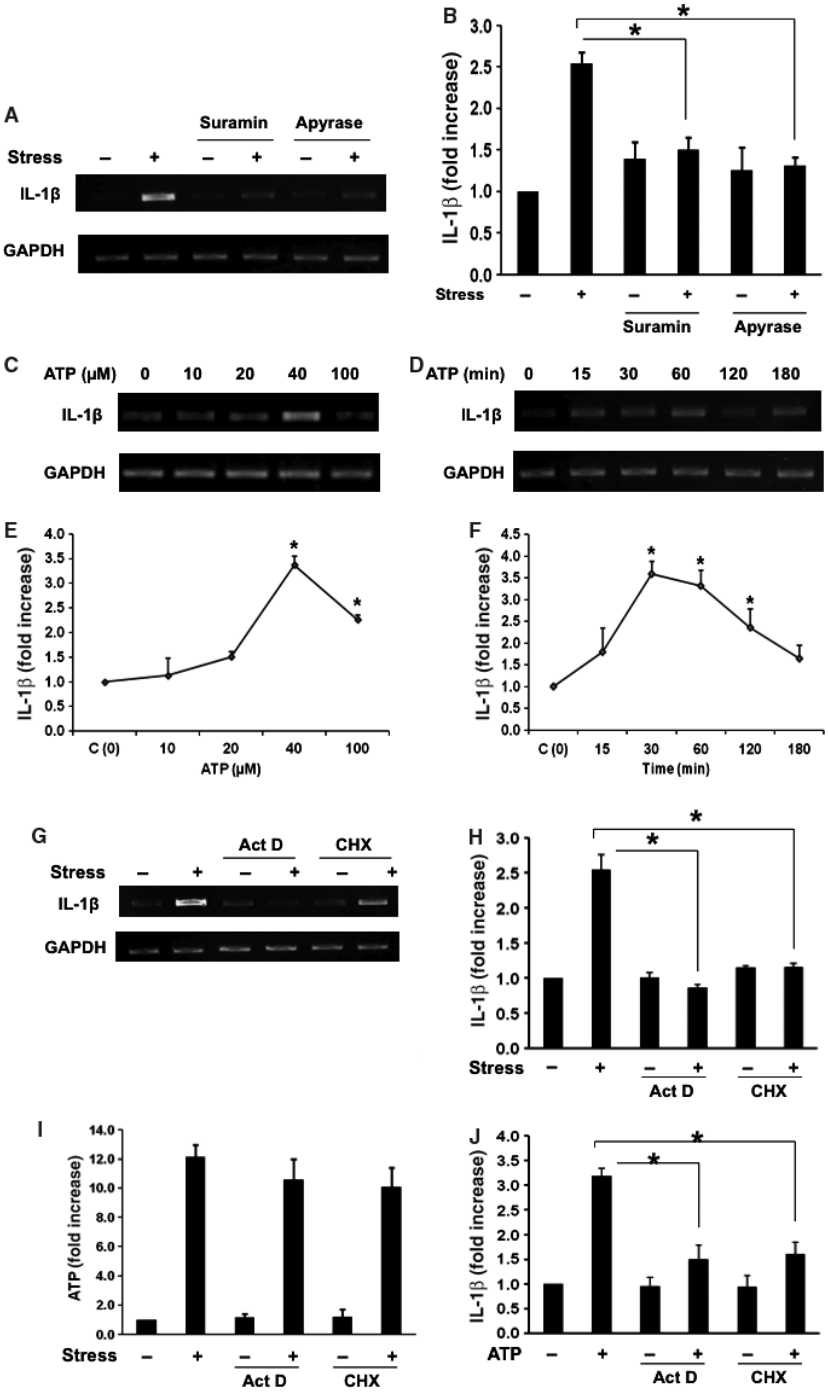

Fig 2. Adenosine triphosphate (ATP)-mediated stress-induced interleukin (IL)-1β expression. Mechanical stress (2 g/cm^2, 3 h) was applied on cultured human periodontal ligament (HPDL) cells in order to induce expression of IL-1β. RT-PCR (A) and ELISA (B) showed that suramin and apyrase strongly inhibited stress-induced IL-1β expression. ATP (10–100 μM) was then used to stimulate HPDL cells for 30 min. Expression of IL-1β was measured using RT-PCR (C) and ELISA (E). To evaluate the time-course effect of ATP, HPDL cells were treated with 40 μM ATP for up to 3 h. IL-1β was measured at both mRNA (D) and protein (F) levels. Actinomycin D (Act D) and cyclohexamide (CHX) strongly attenuated stress- or ATP-induced IL-1β expression at both mRNA and protein levels (G, H, J), while no significant effect was observed on stress-induced ATP release (I). Results are expressed as mean ± standard deviation from three different experiments. *Significant difference, $p < 0.05$. C, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

found that IL-1β induction upon mechanical stimulation also occurred through the release of ATP. In addition, ATP itself could act as an inducer of IL-1β, and the expression level of IL-1β was related to the applied force, to the ATP concentration as well as to the duration of treatments.

ATP can be released from various cell types receiving stress or trauma (18–24). Treatment of stress- or ATP-stimulated HPDL cells with a transcription blocker and a translation blocker markedly decreased stress-induced IL-1β expression, suggesting that IL-1β induction occurred at the mRNA level. However, the amount of ATP released from HPDL cells receiving mechanical stress was slightly affected by these two inhibitors, implying that ATP was probably released from an intracellular store, rather than by new protein synthesis. In our earlier study, an increase in the ATP level could be detected within 5 min after stress application in HPDL cells (24). Together, these results suggest the rather rapid response of HPDL cells and support the idea that ATP might play a role as an early inflammatory signaling molecule in response to mechanical stress, especially in HPDL cells.

The released ATP might subsequently activate P2 receptors. We previously reported that mechanical stress could up-regulate the expression of osteopontin and RANKL via ATP and activation of the P2Y1 receptor (38). In the present study, by using P2 receptor antagonists and siRNA silencing experiments, we found that P2X7 receptor is the main P2 subtype responsible for ATP-induced IL-1β expression in HPDL cells. ATP-induced IL-1β processing and release via activation of the P2X7 receptor has been illustrated in immune cells, including macrophages and monocytes (32,35), as well as in microglia (44,45). In genetically engineered human embryonic kidney cells (HEK-293) over-expressing the P2X7 receptor, activation of the P2X7 receptor resulted in rapid secretion of IL-1β (46) while P2X7 receptor-deficient macrophages in P2X7R$^{-/-}$ mice were not able to generate IL-1β in response

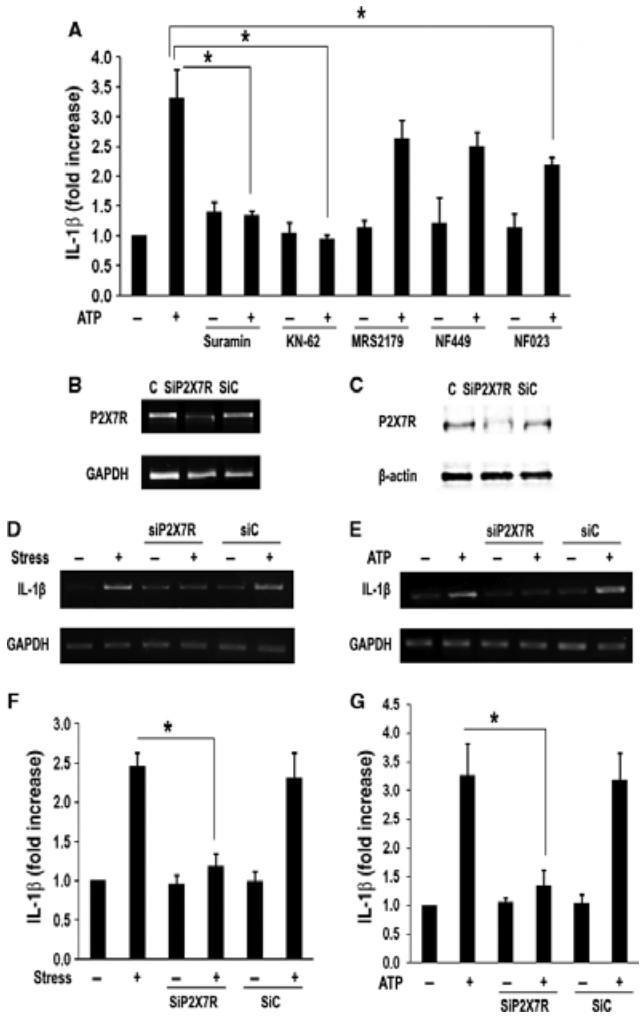

Fig 3. P2X7 receptor is a major P2 subtype responsible for induction of interleukin (IL)-1β. Human periodontal ligament (HPDL) cells were treated with P2 receptor antagonists before application of ATP. The ELISA results showed that suramin and KN-62 markedly inhibited ATP-induced IL-1β release, while MRS2179, NF449 and NF023 had no, or a less significant, effect (A). Transient knockdown of P2X7 receptor by small interfering (si) RNA resulted in a drastic decrease in the expression of P2X7 receptor, as shown in RT-PCR (B) and western blot analyses (C). RT-PCR and ELISA showed that siRNA targeting the P2X7 receptor dramatically decreased the release of IL-1β upon mechanical stress (D, F) or ATP (E, G) application, while no significant effects were observed on control groups. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference, $p < 0.05$. C, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siC, small interfering RNA control.

to ATP activation (35). Activation of the P2X7 receptor is therefore undoubtedly correlated with inflammation.

In orthodontic treatment, the induction of IL-1β mRNA was observed after application of orthodontic force in rats (6). Besides, experiments in P2X7R$^{-/-}$ mice revealed a significant role of the P2X7 receptor in mechanotransduction in orthodontic tooth movement (37). In knockout mice, decreased bone resorption along the bone–PDL interface was observed. Moreover, severe external root resorption in treated tooth was described as a consequence of an incompetent immune response to remove necrotic tissue around the stress area, as a result of the deficient levels of P2X7 and IL-1β. Our data clarified the role of the P2X7 receptor and ATP in stress-induced IL-1β expression in HPDL cells, which could explain the phenomena observed in the animal model. Despite that, blocking other P2 subtypes also resulted in a slightly decreased level of IL-1β upon activation of HPDL cells, suggesting an alternative signaling pathway aside from the P2X7 receptor.

ATP-induced IL-1β expression mechanisms have been proposed in macrophages and monocytes. Activation of the P2X7 receptor by ATP could activate caspase-1, an enzyme function in converting inactive precursor of IL-1β into its active mature form (17, 45). Caspase-1 activation by stimulation of the P2X7 receptor possibly occurs through the rapid decrease in the intracellular potassium (K^+) level, which activates caspase-1 from its proform to its active form (17). Although this pathway still remains unclear in HPDL cells, we demonstrated the involvement of intracellular calcium (Ca^{2+}). With high-affinity binding to Ca^{2+}, BAPTA acts as an intracellular calcium chelator, thapsigargin depletes intracellular calcium storage, while TMB8 acts as an intracellular Ca^{2+} antagonist, a calcium-channel blocker and an inhibitor of Ca^{2+} mobilization. Treatment of HPDL cells receiving mechanical stress or ATP with these inhibitors, in order to diminish intracellular calcium, resulted in a marked decrease in the release of IL-1β. Together with our previous study stating that mechanical stress-induced ATP release is calcium-dependent (24), these findings revealed a significant effect of intracellular calcium signaling in response to mechanical stimulation in HPDL cells.

The involvement of Ca^{2+} in P2X7 receptor-dependent IL-1β secretion was also found in macrophages and in monocytes (33,46). Moreover, considering that all P2X receptors could act as an ion channel, it is possible that they function in a similar way. This could also explain the slight inhibitory effect of NF449 and NF023 on IL-1β induction as a result of their specificity to P2X subtypes.

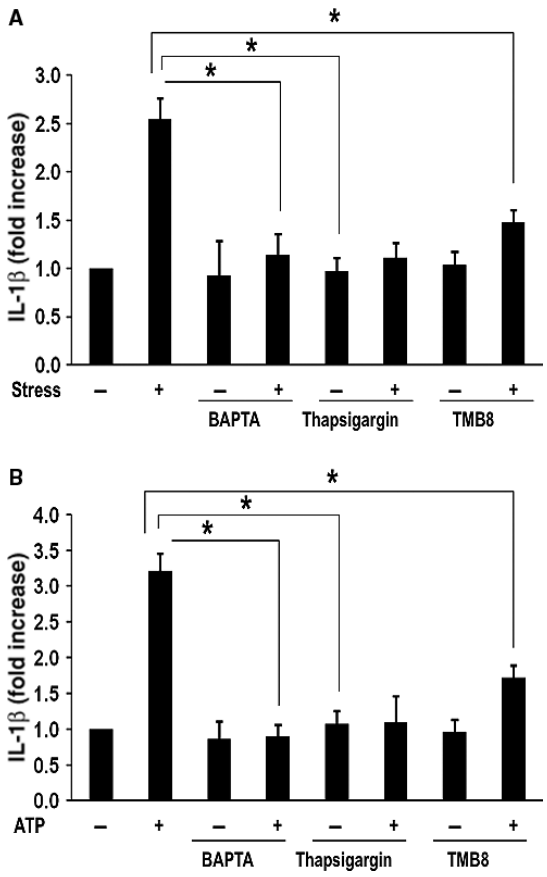

Fig 4. Calcium-dependent interleukin (IL)-1β release in human periodontal ligament (HPDL) cells. HPDL cells were treated with intracellular calcium antagonists before application of stress or ATP. The ELISA results showed that treatment of cells with 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), thapsigargin and 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB8) significantly diminished IL-1β release in stress-stimulated (A) or ATP-stimulated (B) HPDL cells. The results are expressed as mean ± standard deviation from three different experiments. *Significant difference, $p < 0.05$.

Furthermore, because there are several types of purinergic receptors, diverse ATP-induced cellular responses might be established. Combined with other evidence, we propose that ATP might serve as an early inflammatory signaling molecule responding to mechanical stress or injury.

In conclusion, we clearly demonstrated the effect of mechanical stress on the induction of IL-1β expression through the release of ATP in HPDL cells. The mechanism involved activation of the P2X7 receptor and intracellular calcium signaling. Our work clarified the role of ATP as an important, early-release inflammatory mediator in HPDL cells.

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Conflict of Interest Statement

The authors indicate no potential conflicts of interest.

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