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Role of connexin43 hemichannels in mechanical stress-induced ATP release in human periodontal ligament cells

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Background and Objective: Our previous studies showed that mechanical stress could induce ATP release in human periodontal ligament (HPDL) cells. By signaling through P2 purinergic receptors, ATP increased the expression and the synthesis of osteopontin and RANKL. In this study, the mechanism of stress-induced ATP release was investigated.

Material and Methods: Continuous compressive forces were applied on cultured HPDL cells. The ATP released was measured using luciferin–luciferase bioluminescence. The expression of gap-junction proteins was examined using RT-PCR and western blot analysis. The opening of hemichannels was demonstrated by cellular uptake of a fluorescent dye, 5(6)-carboxyfluorescein, which is known to penetrate hemichannels. Intracellular signal transduction was investigated using inhibitors and antagonists.

Results: Mechanical stress induced the release of ATP into the culture medium, which was attenuated by carbenoxolone, a nonspecific gap-junction inhibitor. Addition of meclofenamic acid sodium salt, a connexin43 inhibitor, inhibited ATP release by mechanical stress. Knockdown of connexin43 expression by small interfering RNA reduced the amount of ATP released by mechanical stress, suggesting the role of connexin43 hemichannels. In addition, intracellular Ca^{2+} blockers could also inhibit mechanical stress-induced ATP release and the opening of the gap junction.

Conclusion: Our study demonstrated the involvement of gap-junction hemichannels, especially connexin43, in the stress-induced ATP-release mechanism. Furthermore, this mechanism may be regulated by the intracellular Ca^{2+} signaling pathway. These results suggest an important role of gap-junction hemichannels in the function and behavior of HPDL cells.

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ATP is an extracellular signaling molecule that regulates various biological processes, including cell proliferation, cell differentiation, muscle contraction and intercellular communication (1). Reports have indicated that ATP has a significant function in controlling cellular behavior. For example, extracellular ATP promotes collagen deposition in osteoblast culture (2) while inhibiting *in vitro* calcification (3). It is well known that, in periodontal tissue,

ATP is considered to be an inflammatory mediator that plays an important role in chronic periodontitis. ATP causes growth arrest and might influence periodontal tissue regeneration (4).

ATP can be released from cells in response to various types of stimulation, such as mechanical stress, hypotonic conditions and reduced extracellular Ca^{2+} level (5–9). The detailed mechanism of ATP release is still not fully understood. However, several mechanisms have been reported, including vesicular release, active transport via ABC transporters diffusion via stretch-activated channels, voltage-dependent anion channels and connexin hemichannels (10).

Gap junctions are specialized membrane structures which directly connect the cytoplasm of two neighboring cells. They function in cell–cell communication by allowing the passage of small molecules, such as amino acids, ions and second messengers. In addition, the hemichannel, or the half gap junction, on the cell surface has been reported to act as a channel connecting the cell with the extracellular space (11). In general, the hemichannel or connexon is composed of six connexin subunits joined together to form a nonselective channel that is permeable to molecules of < 1000 daltons. ATP, with a molecular weight of 507.21 daltons, could therefore be released via the opened hemichannels (5,12–14).

The involvement of hemichannels in ATP release has been directly demonstrated in several cell types. Studies in astrocytes, endothelial cells, cochlea and osteocytes showed that the release of ATP was inhibited by hemichannel blockers (5,9,13,15). In addition, certain types of connexins (Cxs) have been shown to be ATP-releasing channels. Among these, pannexin-1 and connexin43 (Cx43) were reported to be a conduit for ATP release after induction by various stimuli (16–20).

Cx43 is expressed in many tissues, including human periodontal ligament (HPDL) cells (21–23). The involvement of Cx43 in ATP release was demonstrated in retinal pigment epithelium (24), human microvascular endothelial cells (19), polymorphonuclear leukocytes (18), chondrocytes (25) and osteocytes (15).

Our previous studies showed that mechanical stress induced ATP release in HPDL cells. Exogenous ATP subsequently activated P2 receptors, resulting in increased expression and synthesis of osteopontin and RANKL. The addition of suramin, a P2 receptor antagonist, suppressed the induction of both osteopontin and RANKL but had no effect on the release of ATP. These results indicated that P2 receptors were involved in the induction of osteopontin and RANKL, but did not participate in the formation and release of ATP. The mechanism of ATP release in HPDL cells after application of mechanical stress is still unknown. In the present study, we aimed to investigate the mechanism and the intracellular signal transduction pathway involved in mechanical stress-induced ATP release in HPDL cells.

Material and methods

Cell culture

HPDL cells were obtained from healthy third molars extracted for orthodontic reasons and prepared as previously described (26). The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Informed consent was obtained from each patient (four women and two men; 18–22 years of age). Briefly, after rinsing with sterile phosphate-buffered saline, periodontal tissue was scraped from the middle third of the root surface and placed in culture vessels containing Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Hyclone), 2 mM L-glutamine (Gibco BRL, Carlsbad, CA, USA), 100 units/mL of penicillin (Gibco BRL), 100 μg/mL of streptomycin (Gibco BRL) and 5 μg/mL of amphotericin B (Gibco BRL), then incubated at 37°C in an atmosphere of air containing 5% CO_2. Each cell preparation was established from one donor. Cells from passages 3 to 5 were used. All experiments were performed in triplicate using cells prepared from three different preparations.

All the periodontal ligament cells obtained were characterized by the level of alkaline phosphatase activity and the ability to initiate an *in vitro* calcification after culture in osteogenic condition medium for 14–16 d.

Application of mechanical stress

Mechanical stress was applied as previously described (27). Briefly, cells were seeded in six-well plates, at a density of 25,000 cells/cm^2, for 16 h. A plastic cylinder containing metal coins was placed over the culture to generate compressive forces ranging from 0 to 2.5 g/cm^2 for 0 to 4 h.

Measurement of extracellular ATP (luciferin–luciferase bioluminescence assay)

Cells were seeded in six-well plates at a density of 25,000 cells/cm^2 and grown to approximately 90% confluence. After deprivation of serum for 6 h, mechanical stress was applied to the culture. For the inhibitory experiments, inhibitors were added 30 min before the application of stress. Culture medium was collected for extracellular ATP measurements using a highly sensitive luciferase-based technique (ENLITEN®ATP Assay System Bioluminescence Detection kit for ATP; Promega, Madison, WI, USA). The assay used recombinant luciferase to catalyze the following reaction: ATP + D-luciferin + O_2 → oxyluciferin + AMP + PPi + CO_2 + -light (560 nm). When ATP was the limiting component in the luciferase reaction, the intensity of the emitted light was proportional to the ATP concentration. Measurement of the light intensity using a luminometer (Victor Light Luminescence Counter; PerkinElmer Ltd., Salem, MA, USA) permitted direct quantification of ATP. A calibration curve was generated for each luciferase assay by serial dilution of an ATP standard.

Inhibitors

For inhibitory experiments, inhibitors or small interfering RNA (siRNA) were added 30 min or 24 h, respectively, before stress application. The inhibitors used included 5–10 μM carbenoxolone disodium salt (Sigma-Aldrich Chemical, St Louis, MO, USA), 20 μM meclofenamic acid sodium salt (Sigma-Aldrich Chemical), 100 μM spermine (Sigma-Aldrich Chemical), 12.5–25 nM thapsigargin (Sigma-Aldrich Chemical), 25–50 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) (Biomol Research Laboratories, Inc., Plymouth, PA, USA), 100–200 μg/mL of heparin sodium salt (Sigma-Aldrich Chemical), 25-50 μM 2-aminoethoxydiphenyl borate (2-APB; Sigma-Aldrich Chemical) and siRNA oligonucleotide specific to Cx43 and to the control oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Extraction of RNA and semiquantitative RT-PCR

Total cellular RNA was extracted using Tri-reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. One microgram of each RNA sample was converted to complementary DNA (cDNA) by reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega) for 1.5 h at 42°C. Subsequent to reverse transcription, PCR was performed. Primers were prepared using the reported sequences from GenBank. The oligonucleotide sequences are shown in Table 1. PCR was performed in DNA thermal cyclers (Biometra, Gottingen, Germany), using *Taq* polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μL. The amplification profile for RANKL (32 cycles) was one cycle at 94°C for 1 min, hybridization at 60°C for 1 min and extension at 72°C for 2 min, followed by one extension cycle at 72°C for 10 min. The same profile was also used for osteoprotegerin (24 cycles), pannexin1 (35 cycles), Cx26 (35 cycles), Cx32 (35 cycles), Cx37 (35 cycles), Cx40 (35 cycles), Cx43 (35 cycles), Cx45 (35 cycles), Cx50 (35 cycles) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 22 cycles). The amplified DNA was electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorostaining.

Western blot analysis

For protein detection, total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were quantified using a protein assay kit (BCA protein assay kit; Pierce Bio-technology, Rockford, IL, USA). Samples containing equal amounts of protein were subjected to electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane. To reduce nonspecific binding, membranes were incubated in 5% nonfat milk for 1 h before being incubated in primary antibody raised against RANKL (clone 70513; dilution 1:50; R&D Systems, Minneapolis, MN, USA), Cx43 (clone CX-1B1; dilution 1:250; Invitrogen Corporation, Camarillo, CA, USA) or actin (clone C4; dilution 1:1000; Chemicon International, Temecula, CA, USA). Membranes were then incubated in biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemiluminescence (SuperSignal® West Pico Stable Peroxide Solution and SuperSignal® West Pico Luminol/Enhancer Solution; Pierce Biotechnology).

Dye-uptake assay

Transmembrane flux of low-molecular-weight dyes is a commonly used method for assessing the presence and function of connexin hemichannels. For an independent verification that a gap junction-like channel is involved in ATP release, we evaluated the uptake of 5(6)-carboxyfluorescein, a fluorescent tracer molecule with a molecular weight of 376.32 daltons (Sigma-Aldrich Chemical), into HPDL cells after undergoing mechanical stress. Gap-junction inhibitors were added 30 min before mechanical stress application for 5 min. Cells were then incubated in 5 mM 5(6)-carboxyfluorescein for 10 min, washed with phosphate-buffered saline and Tyrode's solution, and observed under a fluorescence microscope. Background dye uptake was defined as fluorescence achieved in the absence of mechanical stress. Images were acquired using an Axiovert 40CFL microscope (Carl Zeiss, Jena, Germany).

Transfection of siRNA

HPDL cells were grown in six-well plates, in medium without antibiotics,

Table 1. Primers used in this study

Gene	Accession number	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)	Product size (bp)
RANKL	NM 033012.3	CCAGCATCAAAATCCCAAGT	CCCCTTCAGATGATCCTTC	602
OPG	NM 002546.3	TCAAGCAGGAGTGCAATCG	AGAATGCCTCCTCACACAGG	341
Px1	NM 015368.3	GGATCCTGAGAAACGACAGC	CTCTGTCGGGCATTCTTCTC	496
Cx26	NM 004004.5	GCAGAGACCCCAACGCCGAGAC	GCAGACAAAGTCGGCCTGCTCA	239
Cx32	NM 001097642.2	CTGCTCTACCCGGGCTATGC	CAGGCTGAGCATCGGTCGCTCT	330
Cx37	NM 002060.2	GGTGGGTAAGATCTGGCTGA	ATAGGTGCCCATCAGTGCTC	406
Cx40	NM 181703.2	GGGAGGCCATATTATTGCTG	GTGGCAGAGAAGGCAGAACT	486
Cx43	NM 000165.3	GGACATGCACTTGAAGCAGA	CAGCTTGTACCCAGGAGGAG	496
Cx45	NM 001080383.1	CACGGTGAAGCAGACAAGAA	GCAAAGGCCTGTAACACCAT	417
Cx50	NM 005267.3	TCATCCTGTTCATGTTGTCTGTGGC	AACCTCGGTCAAGGGGAAATAGT	238
GAPDH	NM 002046.3	TGAAGGTCGGAGTCAACGGAT	TCACACCCATGACGAACATGG	395

Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; Px, pannexin; RANKL, receptor activator of nuclear factor kB ligand.

to 70-80% confluence. Cells were treated with a mixed solution of siRNA oligonucleotides specific to Cx43, according to the manufacturer's instructions (Santa Cruz Biotechnology) and were transfected with siRNA for 24 h before treatment. As a control, cells were treated with control siRNA (Santa Cruz Biotechnology).

Statistical analysis

All data were analyzed by one-way analysis of variance using statistical software (SPSS, Chicago, IL, USA). Scheffé's test was used for *post hoc* analysis ($p < 0.05$).

Results

Carbenoxolone, a gap-junction inhibitor, attenuated stress-induced ATP release

To study the mechanism of extracellular ATP release in HPDL cells, we first measured the extracellular ATP within the culture media after stimulation of HPDL cells with mechanical stress. A force-dependent mechanism of ATP release was observed ($p < 0.05$) (Fig. 1A). A mechanical stress level of 2.5 g/cm^2 was selected for use in the time-course experiment of ATP release. An increase in ATP was noted within 1 min after stress application. The amount of ATP showed a decrease at 10 min but had increased again at 120 min (Fig. 1B).

Carbenoxolone, a nonspecific inhibitor of gap junctions, was added 30 min before stress application in order to investigate the influence of the gap junction in mechanical stress induced-ATP release in HPDL cells. Mechanical stress (2.5 g/cm^2) was applied on HPDL cells for 5 min in the presence or absence of carbenoxolone. Our results showed that carbenoxolone significantly suppressed mechanical stress-induced ATP release (Fig. 2A). Moreover, carbenoxolone inhibited the mechanical stress-induced upregulation of *RANKL* mRNA but not of *osteoprotegerin* mRNA (Fig. 2B), corresponding to previous results showing stress-induced expression of *RANKL* mRNA through the release of ATP.

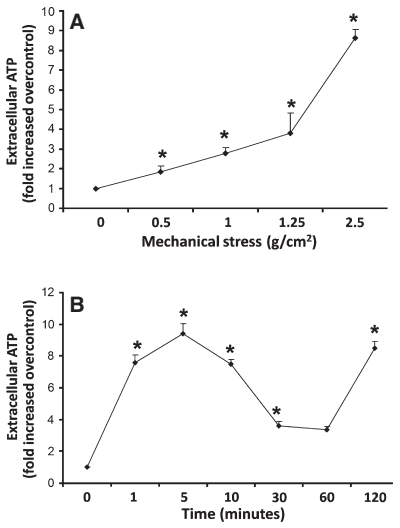

Fig. 1. Mechanical stress-induced ATP release in human periodontal ligament (HPDL) cells. The luciferin–luciferase assay was used to determine the amount of ATP released. HPDL cells were treated with different levels of mechanical stress, ranging from 0 to 2.5 g/cm^2, and the amount of ATP released was measured (A). The maximum extracellular ATP level was observed when a stress of 2.5 g/cm^2 was applied, and therefore this loading force was used in all subsequent experiments. (B) The amount of ATP released from HPDL cells was measured from 1 to 120 min after treatment with 2.5 g/cm^2 of stress. The results show that mechanical stress could induce ATP release in a dose- and time-dependent manner. The maximum extracellular ATP level was observed when a stress of 2.5 g/cm^2 was applied for 5 min. The results are expressed as mean ± standard deviation from three separate experiments. *Significant difference, $p < 0.05$.

The effect of carbenoxolone prompted us to examine the expression of gap junction proteins in HPDL cells. By using RT-PCR analysis, we observed that HPDL cells expressed at least seven gap-junction proteins, including Cx26, Cx32, Cx37, Cx40, Cx43, Cx45 and pannexin1 (Fig. 2C).

Cx43 is involved in stress-induced ATP release in HPDL cells

In order to clarify the type of gap junction involved in ATP release, specific gap-junction inhibitors were used. Specific inhibitors of Cx40 and Cx43 were selected based on previous reports (18–20,28). The results shown in Fig. 3A indicated that the Cx43 inhibitor (meclofenamic acid sodium salt), but not the Cx40 inhibitor (spermine), attenuated stress-induced ATP release. Application of the Cx43 inhibitor, but not of the Cx40 inhibitor, also suppressed the induction (by mechanical stress) of RANKL, but not of osteoprotegerin, at both mRNA (Fig. 3B) and protein (Fig. 3C) levels, supporting a role of Cx43 in mechanical stress-induced ATP release and RANKL upregulation.

The significance of Cx43 hemichannels in stress-induced ATP release was further investigated by transient transfection of HPDL cells with siRNA. Control siRNA and Cx43 siRNA were transfected into HPDL cells for 24 h before the application of mechanical stress. We observed that Cx43 siRNA effectively reduced the level of *Cx43* mRNA (Fig. 4A) and Cx43 protein (Fig. 4B), as shown in the far right lane of Fig. 4A. Downregulation of Cx43 expression by siRNA resulted in a strong inhibition of mechanical stress-induced ATP release (Fig. 4C). Mock-transfected cells (i.e. cells transfected with the transfection reagent alone) and cells transfected with control siRNA showed a normal pattern of ATP release in response to mechanical stress (Fig. 4C).

Mechanical stress-induced the opening of gap-junction hemichannels

Dye-uptake experiments were performed to examine the effect of mechanical stimulation on the opening of gap-junction hemichannels. The results showed increased carboxyfluorescein uptake by HPDL cells after mechanical stress application compared with the control (Fig. 5A,B). Furthermore, the inductive effect of mechanical stress on the opening of hemichannels was suppressed by carbenoxolone (data not shown) and by the Cx43 inhibitor (Fig. 5C,D) but not by the Cx40 inhibitor (Fig. 5E,F).

Intracellular calcium participated in stress-induced ATP release

It has been reported that an increase in intracellular calcium participates in the

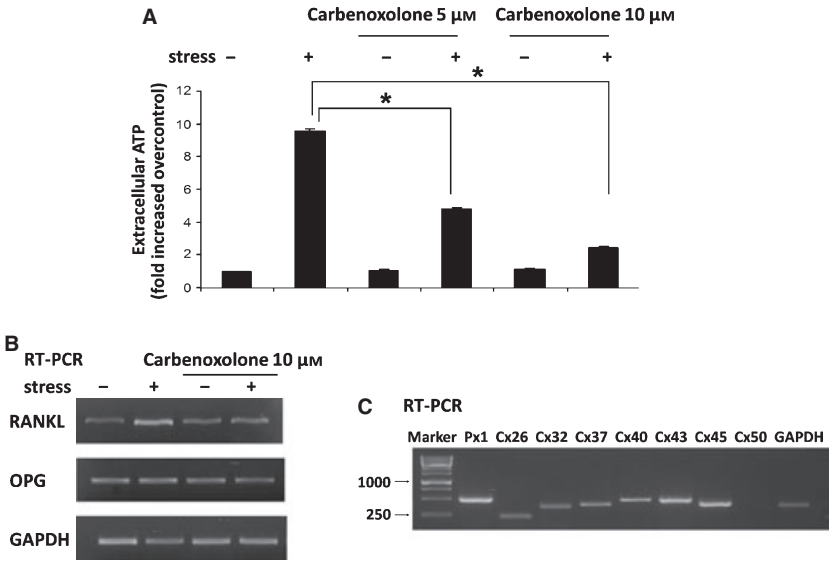

Fig. 2. The release of ATP was attenuated by a gap-junction inhibitor. Mechanical stress (2.5 g/cm^2) was applied on cultured human periodontal ligament (HPDL) cells for 5 min and 2 h to induce ATP release (A) and RANKL upregulation (B), respectively, in the presence or absence of the gap-junction inhibitor, carbenoxolone. The inhibitor was added 30 min before stress application. Carbenoxolone could attenuate the effect of mechanical stress on ATP release (A) and RANKL upregulation, as judged by RT-PCR (B). (C) Expression of gap-junction proteins, including connexin (Cx)26, Cx32, Cx37, Cx40, Cx43, Cx45 and pannexin-1 (Px1) in HPDL cells, as judged by RT-PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kB ligand. Results are expressed as mean ± standard deviation from three individual experiments. *Significant difference, $p < 0.05$.

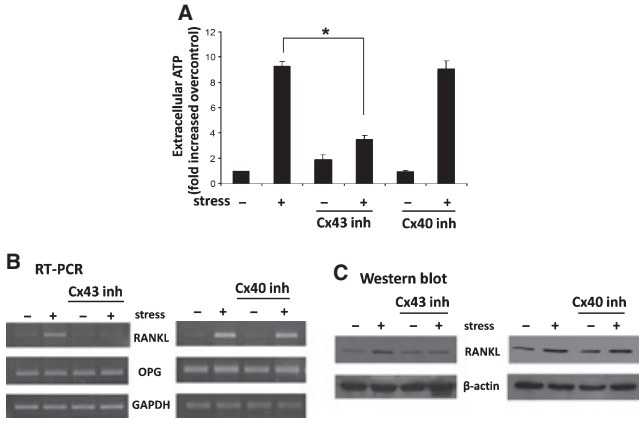

Fig. 3. Mechanical stress-induced ATP release and RANKL upregulation were strongly inhibited by a connexin43 inhibitor (Cx43 inh). Mechanical stress (2.5 g/cm^2) was applied on cultured human periodontal ligament (HPDL) cells in the presence or absence of 20 μM meclofenamic acid sodium salt (a Cx43 inhibitor) or 100 μM spermine [a connexin40 inhibitor (Cx40 inh)]. Inhibitors were added 30 min before stress application. Culture medium was collected after 5 min and ATP was measured using a luciferin–luciferase assay. RNA and protein were extracted after 2 and 4 h of stress application, respectively. The results showed that the Cx43 inhibitor, but not the Cx40 inhibitor, could inhibit mechanical stress-induced ATP release (A) and the upregulation of RANKL, but not OPG, at both mRNA (B) and protein (C) levels. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kB ligand. The results are expressed as mean ± standard deviation from three individual experiments. *Significant difference, $p < 0.05$.

opening of hemichannels (24,29). To test whether intracellular calcium is involved in stress-induced gap-junction opening, HPDL cells were incubated with TMB-8, which inhibits Ca^{2+} release from intracellular stores and Ca^{2+} influx, before treating the cells with mechanical stress. As shown in Fig. 6A, TMB-8 suppressed the amount of stress-induced ATP released in a dose-dependent manner. Moreover, the addition of thapsigargin (an inhibitor of calcium-ATPase), to deplete intracellular storage, significantly abolished the extracellular ATP released when compared with the control (Fig. 6B). These results support that intracellular Ca^{2+} storage is necessary for ATP release.

Next, two IP3 (inositol-1,4,5-trisphosphate) antagonists – heparin and 2-APB – were used to examine the role of the IP3 receptor. The results showed that both heparin (Fig. 6C) and 2-APB (Fig. 6D) significantly decreased mechanical stress-evoked ATP release, supporting the role of the IP3 receptor in stress-induced ATP release. Furthermore, carboxyfluorescein dye uptake was attenuated by thapsigargin and 2-APB (Fig. 7), suggesting that intracellular Ca^{2+} signaling might be the upstream signal to gap-junction opening in HPDL cells.

Discussion

Mechanical stress-induced ATP release has been reported in several cell types such as subepithelial fibroblasts (30), chondrocytes (31), airway epithelial cells (32), osteoblastic cells (33) and HPDL cells (6). In HPDL cells, increased extracellular ATP resulted in the upregulation of osteopontin and RANKL expression (6,26). In this study, we investigated the mechanism of mechanical stress-induced ATP release. Our results provide evidence that, in HPDL cells, ATP was released through Cx43 hemichannels. Moreover, the mechanism of ATP release was regulated by an intracellular Ca^{2+} signaling pathway.

The results of this study also indicate that HPDL cells express at least seven gap-junction proteins, including Cx26, Cx32, Cx37, Cx40, Cx43, Cx45 and pannexin1, similar to a study by

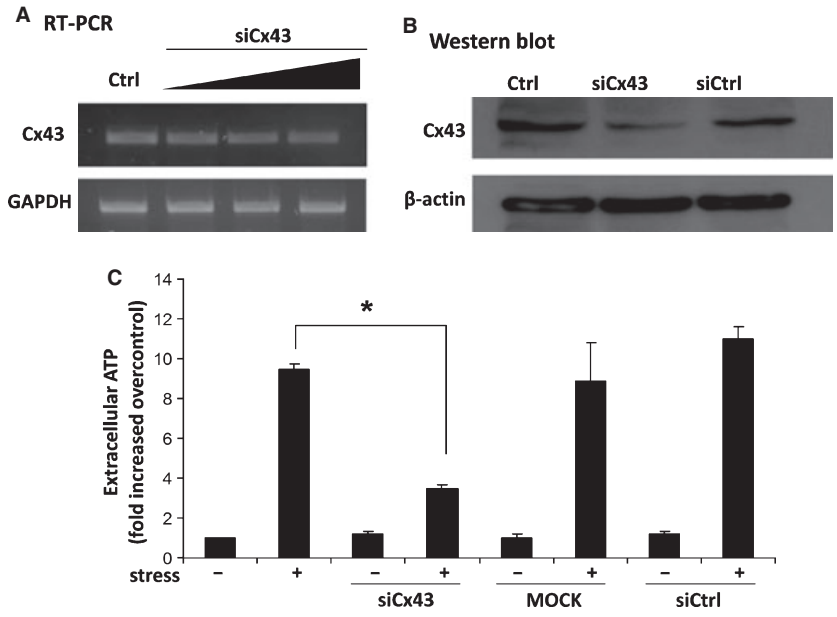

Fig. 4. Knockdown of connexin43 (Cx43) suppressed the inductive effect of mechanical stress on ATP release. The level of *Cx43* mRNA (A) and of Cx43 protein (B) in human periodontal ligament (HPDL) cells are shown after transfection with Cx43 small interfering RNA (siRNA). (C) Transfection with Cx43 siRNA (siCx43) inhibited the inductive effect of mechanical stress-induced ATP release, whereas the control siRNA (siCtrl) and the addition of transfection reagent alone (MOCK) had no significant effect. The results support a role for Cx43 in ATP release. The results are expressed as mean ± standard deviation from three individual experiments. *Significant difference, $p < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

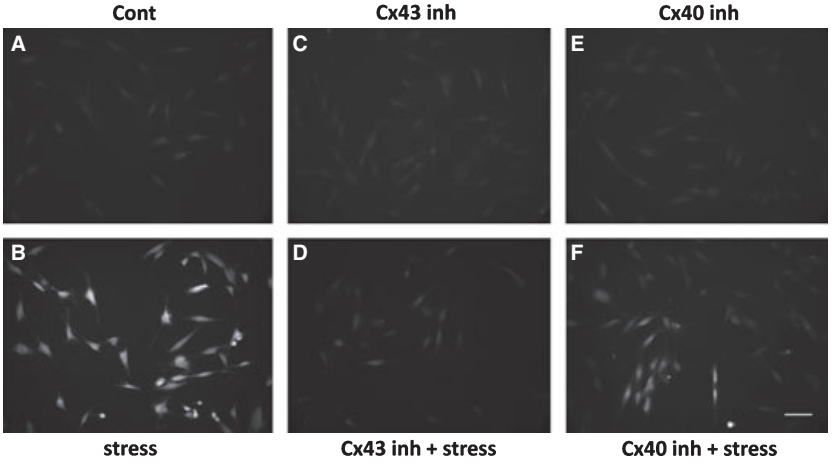

Fig. 5. Mechanical stress-induced opening of connexin43 (Cx43) hemichannels in human periodontal ligament (HPDL) cells. HPDL cells were incubated with 5 mM 5(6)-carboxyfluorescein before treatment with 2.5 g/cm^2 of mechanical stress for 5 min. Meclofenamic acid sodium salt [a Cx43 inhibitor (Cx43 inh)] or spermine [a connexin40 inhibitor (Cx40 inh)] were added 30 min before stress application. The cultures were washed with phosphate-buffered saline and Tyrode's solution, and observed under a fluorescent microscope. The presence of fluorescence signal indicated the influx of carboxyfluorescein into the cells after stress application (A, B). Addition of Cx43 (C, D) and Cx40 (E, F) inhibitors reduced the intensity of the fluorescence signal in HPDL cells receiving mechanical stress. By fluorescence analysis, the Cx43 inhibitor appeared to be more effective than the Cx40 inhibitor. Scale bar = 50 μm.

Yamaoka *et al.* (34), which found that HPDL cells expressed Cx32, Cx40, Cx43 and Cx45. The function of gap-junction hemichannels was demonstrated by using carbenoxolone disodium salt, a nonspecific gap-junction blocker. The inhibitory effect of carbenoxolone on mechanical stress-induced ATP release supports that gap-junction hemichannels are involved in the mechanism.

Our findings also correspond with those of Bennett *et al.* (35), which showed that ATP and other small molecules can be released via hemichannel opening. In our study, we focused on the roles of Cx43 and Cx40 because of evidence linking ATP efflux to these hemichannels in many cell types. For example, human osteoblast-like initial transfectant (HOBIT) cells expressed Cx43 and released ATP in response to mechanical stimulation (33). The efflux of ATP through Cx43 hemichannels in C6 cells was also identified in the membrane of hippocampal astrocytes (20). In addition, mechanical stimulation mediated ATP release through Cx40 hemichannels in an isolated glomerular endothelium cell (28). Based on the strong blocking effects of a Cx43 inhibitor and Cx43 siRNA, our results indicate that Cx43 is an ATP-releasing channel in HPDL cells.

The balance between RANKL and osteoprotegerin is important for osteoclast formation, activation and survival (36). Therefore, increased expression of RANKL, but not of osteoprotegerin, would increase the RANKL/osteoprotegerin ratio and promote osteoclastogenesis. Our studies revealed that mechanical stress, as well as ATP (26), could induce RANKL expression, but not osteoprotegerin expression, in HPDL cells. It has been reported that different signaling pathways regulate RANKL and osteoprotegerin. Kanzaki *et al.* (37) demonstrated that static mechanical compression promoted osteoclastogenesis via the upregulation of RANKL expression, while osteoprotegerin expression remained constant throughout the application of compressive forces in HPDL cells. In contrast, osteoprotegerin, but not

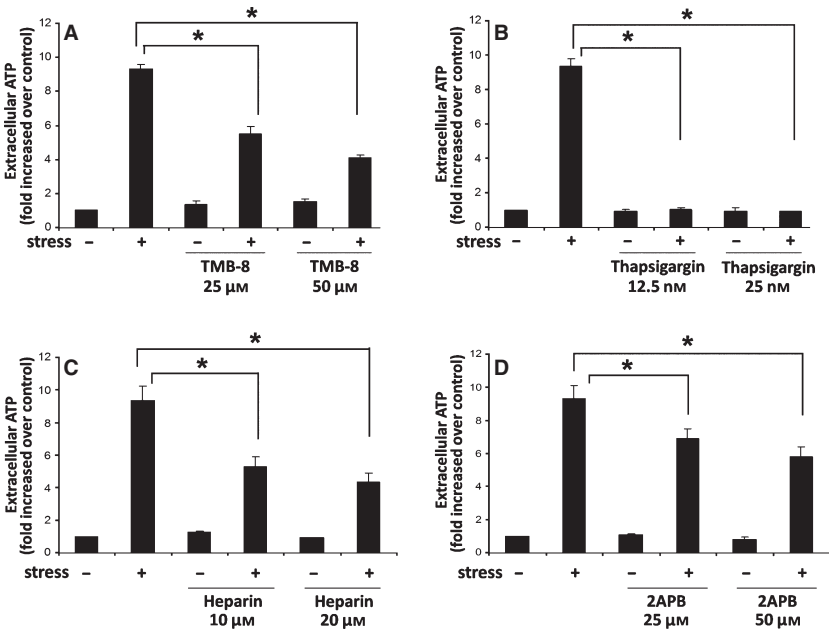

Fig. 6. The mechanism of ATP release is dependent on intracellular calcium signaling. Human periodontal ligament (HPDL) cells were pretreated with the intracellular calcium inhibitor TMB-8 (A) [3,4,5-trimethoxybenzoic acid 8-(Diethylamino)octyl ester], thapsigargin (B), and with the IP3 (inositol-1,4,5-trisphosphate) inhibitors heparin (C) and 2-aminoethoxydiphenyl borate (2-APB) (D) for 30 min before mechanical stress application. All inhibitors could significantly inhibit ATP release induced by mechanical stress. The results are expressed as mean ± standard deviation from three individual experiments. *Significant difference, $p < 0.05$.

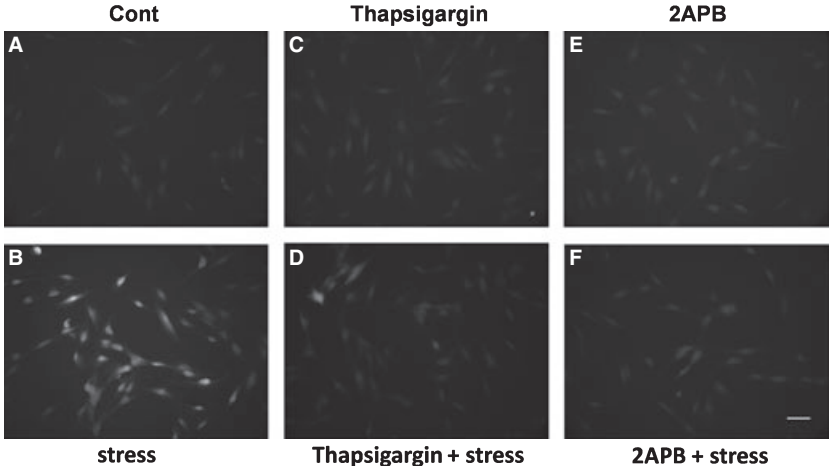

Fig. 7. Blockage of carboxyfluorescein influx by thapsigargin and 2-aminoethoxydiphenyl borate (2-APB). Human periodontal ligament (HPDL) cells were incubated with thapsigargin or 2-APB for 30 min before 5 min of mechanical stress application. The addition of carboxyfluorescein resulted in the influx of fluorescence into the cells after stress application (A, B). The presence of thapsigargin and 2-APB inhibited the uptake of carboxyfluorescein (C–F), indicating the role of an intracellular Ca^{2+} signaling pathway and IP3 (inositol-1,4,5-trisphosphate) receptor in stress-induced hemichannel opening. Scale bar = 50 μm.

RANKL, was upregulated in HPDL cells when cells were treated with strain or thrombin (38,39).

Transmembrane flux of the low-molecular-weight dye, 5(6)-carboxyfluorescein, is a commonly used method for assessing the presence and function of connexin hemichannels (40,41). Our study showed that mechanical stimulation of HPDL cells could evoke both dye uptake as well as ATP release. The fact that the Cx43 inhibitor suppressed dye uptake following mechanical stress stimulation suggested that Cx43 might be a major gap-junction protein which responds to mechanical stress. In addition, the present work demonstrated that mechanical stress-induced ATP release or dye uptake might require changes in intracellular Ca^{2+}. It is possible that the release of ATP by mechanical stress is not a direct effect of mechanical stress but is mediated by the Ca^{2+} signaling cascade.

Ca^{2+} signaling associated with ATP release has been reported previously (5,42). Increased intracellular Ca^{2+} has been implicated in ATP release from many cell types, including osteoblast-like cells (43,44). Furthermore, reports have suggested that the increase in the cytoplasmic concentration of free Ca^{2+} could trigger the opening of Cx hemichannels (24,29). In the present study, it is possible that mechanical stress altered the intracellular Ca^{2+} levels in the HPDL cells, leading to the opening of Cx43 hemichannels and allowing the release of ATP. In agreement with the role of increased intracellular Ca^{2+} levels in ATP release, application of a Ca^{2+} blocker could inhibit mechanical stress-induced ATP release, suggesting the importance of Ca^{2+} and Cx43 in regulating mechanical stress-induced ATP release in HPDL cells.

The question of whether stress induces ATP release or ATP synthesis remains unanswered. Generally, the amount of intracellular ATP is in the millimolar ratio (45). Our data indicated that an increased concentration of extracellular ATP could be detected within 1 min after stress application. Taken together, it is reasonable to postulate that mechanical stress induces the release of ATP from intracellular stores.

In conclusion, the present study showed that mechanical stress induced ATP release through Cx43 hemichannels. The mechanism of ATP release may depend on the intracellular Ca^{2+}

signaling pathway. Our results indicate an important role of Cx43 hemichannels in the function and behavior of HPDL cells. However, the identity of cell-surface receptor(s), if any, that respond to mechanical stress is still in question and needs further investigation.

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