กลไกระดับโมเลกุลในการเหนี่ยวนำการแสดงออกของ
รีเซบเตอร์แอกติเวเตอร์นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์
โดยแรงกดเชิงกลในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์

นางสาวพิมพ์พร รักพรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE MOLECULAR MECHANISM OF MECHANICAL STRESS-INDUCED RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS

Miss Pimporn Luckprom

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Oral Biology

Faculty of Dentistry

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พิมพ์พร รักพรม : กลไกระดับโมเลกุลในการเหนี่ยวนำการแสดงออกของรีเซบเตอร์แอกติเวเตอร์นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์โดยแรงกดเชิงกลในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์. (THE MOLECULAR MECHANISM OF MECHANICAL STRESS-INDUCED RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, 126 หน้า.

แรงกดเชิงกล เช่น แรงจากการจัดฟัน หรือแรงจากการบดเคี้ยวที่ผิดปกติ สามารถก่อให้เกิดการอักเสบและการทำลายของเนื้อเยื่อปริทันต์ รวมทั้งการละลายตัวของกระดูกเบ้าฟัน รีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ (receptor activator of nuclear factor-kappa B ligand; RANKL) และออสติโอโพรติจิลิน (osteoprotegerin; OPG) จัดเป็นโปรตีนที่มีบทบาทสำคัญในการควบคุมสมดุลของกระดูก การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของแรงกดเชิงกลต่อการควบคุมการแสดงออกของรีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ รวมถึงกลไกการถ่ายทอดสัญญาณที่เกิดขึ้นในเซลล์เอ็นยึดปริทันต์ของมนุษย์ แรงกดทับทำด้วยการให้น้ำหนักกดทับในจานเลี้ยงเซลล์เอ็นยึดปริทันต์ จากนั้นตรวจวัดระดับปริมาณ เอทีพี (Adenosine triphosphate; ATP) ด้วยหลักการลูซิเฟอรินลูซิเฟอเรส (Luciferin-luciferrase), ระดับเอ็มอาร์เอ็นเอของรีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ ด้วยเทคนิค รีเวอร์สทรานสคริบชัน-โพลีเมอร์เรสเชนรีแอคชัน และระดับโปรตีนของรีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ ด้วยเทคนิคเวสเทิร์น ส่วนกระบวนการถ่ายทอดสัญญาณที่เกิดขึ้น จะตรวจสอบโดยการใช้สารยับยั้งชนิดต่างๆ ผลการศึกษาในครั้งนี้ แสดงให้เห็นว่าแรงกดทับสามารถเหนี่ยวนำการหลั่งเอทีพีผ่านทางคอนเนคซินชนิด 43 (Connexin 43; Cx43) โดยกลไกที่เกิดขึ้นอาจถูกควบคุมจากระดับแคลเซียมภายในเซลล์ นอกจากนี้ยังพบว่าเอทีพีสามารถเพิ่มการแสดงออกของคือ รีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ โดยผ่านทางพีทูวายวันรีเซบเตอร์ (P2Y1 receptor) โดยสรุป การศึกษาในครั้งนี้แสดงให้เห็นว่าเซลล์เอ็นยึดปริทันต์ตอบสนองต่อแรงกดเชิงกลโดยเหนี่ยวนำให้เกิดการเพิ่มขึ้นของรีเซ็บเตอร์ แอคทิเวเตอร์ ออฟ นิวเคลียแฟคเตอร์แคปปาบี ไลแกนด์ ผ่านปริมาณของเอทีพีที่เพิ่มขึ้น ดังนั้นจึงกล่าวได้ว่าเอทีพีและพีทูวายวันรีเซบเตอร์มีบทบาทสำคัญในการควบคุมอัตราการทำลายกระดูก และสมดุลของกระดูก

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##5076453632: MAJOR ORAL BIOLOGY
KEYWORDS: ATP/ CONNEXIN43/ MECHANICAL STRESS/ PERIODONTAL LIGAMENT CELLS/ P2 RECEPTORS/ RANKL

PIMPORN LUCKPROM: THE MOLECULAR MECHANISM OF MECHANICAL STRESS-INDUCED RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS. ADVISOR: PROF. PRASIT PAVASANT, D.D.S.,Ph.D., 126 pp.

Mechanical stress such as orthodontic forces or abnormal mastication produce inflammation and damage of periodontium including alveolar bone resorption. The receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) are two key proteins important in bone homeostasis. This study aimed to examine the influence of mechanical stress on the expression and regulation of RANKL as well as the signaling pathway involved in human periodontal ligament (HPDL) cells. Continuous compressive force was applied on cultured HPDL cells. The released adenosine triphosphate (ATP) was measured using luciferin-luciferase bioluminescence. The level of mRNA and protein of RANKL expression in HPDL cells were examined by reverse transcription polymerase chain reaction (RT-PCR) and western analysis. Intracellular signal transduction was investigated using inhibitors and antagonists. The results of this study demonstrated that mechanical stress-induced ATP release through connexin 43 (Cx43) hemichannel. The mechanism of ATP release may depend on intracellular Ca^{2+} signaling pathway. In addition, extracellular ATP stimulates RANKL expression through P2Y1 receptor. In conclusion, this study showed that HPDL cells responded to mechanical stress by increasing the release of ATP, as well as the expression of RANKL. These finding suggests that ATP and P2Y1 receptor play an important role in regulating bone resorption and bone homeostasis.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADP	adenosine diphosphate
ADPβS	adenosine diphosphate analogue
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPγS	adenosine 5'-O-3-thiotriphosphate
Ca^{2+}	calcium ion
$[Ca^{2+}]_e$	extracellular calcium
$[Ca^{2+}]_i$	intracellular calcium
CD73	ecto-5'-nucleotidase
CFTR	cystic fibrosis transmembrane conductance regulator
COX-2	cyclooxygenase-2
Cx	connexin
Cx43	connexin43
EC_{50}	half maximal effective concentration
ECM	extracellular matrix
E-NPP	ectonucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase
GJIC	gap junction intercellular communication
HPDL	human periodontal ligament
HSCs	hematopoietic stem cells
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IP3	inositol (1,4,5)-trisphosphate

K^+	potassium ion
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MMP	matrix metalloproteases
2-meSADP	2-methylthio adenosine diphosphate
Na^+	sodium ion
NO	nitric oxide
NF-κB	Nuclear factor Kappa B ligand
OCP	osteoclast precursors
ODF	osteoclast differentiation factor
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand
P2	purinergic
PGE_2	prostaglandin E2
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
PTH	parathyroid hormone
RANK	receptor activator of Nuclear factor Kappa B
RANKL	receptor activator of Nuclear factor Kappa B ligand
RT-PCR	reverse transcription polymerase chain reaction
TRAFs	TNF receptor-associated factors
TRANCE	TNF-related activation induced cytokine
UDP	uridine diphosphate
UTP	uridine triphosphate
VRAC	volume-regulated anion channels

CHAPTER I

INTRODUCTION

The periodontal ligament is always exposed to mechanical loading during occlusion in healthy periodontal tissue. Under normal physiologic condition, the homeostasis of periodontal ligament tissues is maintained by appropriate mechanical loading (Tsuji et al., 2004). However, excessive mechanical force or tissue inflammation can induced periodontal tissue damage including alveolar bone resorption and tooth loss (Verna et al., 1999). When the mechanical stress loaded onto a tooth, the force is transduced to the periodontal ligament. The cells in the periodontal ligament respond to the mechanical stress to regulate the resorption and formation of bone matrix by signaling the surrounding cells (Lekic and McCulloch, 1996). In response to mechanical stress, adenosine triphosphate (ATP) were released into extracellular space (Wongkhantee et al., 2008).

ATP is one of the extracellular signaling molecules that regulates various biological processes including cell proliferation, cell differentiation, muscle contraction and intercellular communication (Burnstock, 1997). ATP can be released from cells in response to various types of stimulation such as mechanical stress, hypotonic condition and reduced extracellular calcium ($[Ca^{2+}]_e$) level (Stout et al., 2002; Wongkhantee et al., 2008; Wan et al., 2008; Boudreault and Grygorczyk, 2004; Zhao et al., 2005).

The detailed mechanism of ATP release is still not fully understood. However, several mechanisms have been reported including vesicular release, active transport via ATP-binding cassette (ABC) transporters, diffusion via stretch-activated

channels, voltage-dependent anion channels as well as connexin hemichannels (Lazarowski et al., 2003).

Gap junctions are specialized membrane structures that 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, function of hemichannel or half gap junction on cell surface has been reported as a gate connecting between cell and the extracellular space (Oviedo-Orta and Evans, 2002). In general, structure of hemichannel or connexon composes of six connexins subunits joined together to form a nonselective channel permeable to molecules of less than 1,000 daltons. ATP, with a molecular weight of 507.21 daltons, could therefore be released via the opened hemichannels (Stout et al., 2002; Ebihara, 2003; Gomes et al., 2005; Anselmi et al., 2008).

The involvement of hemichannels in ATP release has been directly demonstrated in several cell types. Studies in astrocytes, endothelial cells, cochlea as well as osteocytes showed that the released ATP was inhibited by the hemichannels blockers (Stout et al., 2002; Zhao et al., 2005; Gomes et al., 2005; Genetos et al., 2007). In addition, certain types of connexin (Cx) have been shown to be ATP releasing channel. Among these, connexin43 (Cx43) were reported to be a conduit for ATP-release after various stimulations (Eltzschig et al., 2006; Faigle et al., 2008; Kang et al., 2008).

Connexin43 is one of the widespread connexin types expressed in many tissues including human periodontal ligament (HPDL) cells (Ralphs et al., 1998; Yamaguchi et al., 1994; Yamaoka et al., 2000). The involvement of Cx43 in ATP release was demonstrated in retinal pigment epithelium (Pearson et al., 2005), human microvascular endothelial cells (Faigle et al., 2008), polymorphonuclear leukocytes

(Eltzschig et al., 2006), chondrocytes (Knight et al., 2009) and osteocytes (Genetos et al., 2007). However, the exact mechanism of mechanical stress-induced ATP release in HPDL cells has not been reported.

Previous study demonstrated the relationship of receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) expression stimulated by mechanical stress in periodontal ligament cells, that there was an up-regulation of RANKL expression while the OPG expression was not affected (Kanzaki et al., 2002). Moreover, ATP is also one of the regulators of bone homeostasis. It has been demonstrated that ATP can stimulate osteoclast activity via an up-regulation of the expression of RANKL in osteoblasts (Buckley et al., 2002). Extracellular ATP stimulated the resorptive activity of rat osteoclasts (Morrison et al., 1998). Pit formation in vitro was observed when ATP was added to cultures of human osteoclasts isolated from a giant cell tumor (Bowler et al., 1998). These lines of evidences indicate that mechanical stress and/or ATP participates in bone homeostasis through the regulation of RANKL.

RANKL is a molecule essential for osteoclastogenesis. It is expressed by osteoblasts as a membrane-associated factor. When RANKL binds to RANK, a receptor expressed on the cell surface of osteoclast precursors, it stimulates the differentiation of those precursors into mature osteoclasts. In contrast, an interaction of RANKL with RANK can be inhibited by OPG, a soluble factor secreted by osteoblasts. OPG acts as a decoy receptor for RANKL and thus prevents osteoclast differentiation. Therefore, RANKL and OPG are considered as major factors that regulate bone homeostasis.

It is well-documented that RANKL and OPG are the key proteins of proliferation, differentiation, and activation of osteoclasts. It is of interest that HPDL

cells also express RANKL and OPG and that their balance could be altered by mechanical stress (Kanzaki et al., 2002; Yamaguchi et al., 2006).

Previous study found that cells responded to mechanical stress by increasing the release of ATP, as well as the expression of RANKL and osteopontin, at both the mRNA and the protein level (Wongkhantee et al., 2007). However, the mechanism of stress-induced ATP release and the increased of RANKL expression by ATP has not been elucidated. On the basis of these findings, we hypothesized that the up-regulation of RANKL in HPDL cells was a consequence of release by ATP. In this study, we investigated the mechanism of mechanical stress-induced ATP release and the effect of ATP on RANKL expression in HPDL cells.

RESEARCH OBJECTIVES

1. To explore which type of gap junction involved in mechanical stress-induced ATP release in HPDL cells.
2. To investigate the mechanism involved in mechanical stress-induced ATP release in HPDL cells.
3. To elucidate the effect of ATP on the expression of RANKL in HPDL cells.
4. To examine the signaling pathway of RANKL expression in HPDL cells mediated by ATP.

RESEARCH HYPOTHESIS

1. Mechanical stress-induced ATP release via Cx43 in HPDL cells.
2. The mechanism of mechanical stress-induced ATP release in HPDL cells regulated by intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling pathway.
3. ATP induces RANKL expression at both mRNA and protein level in HPDL cells.
4. Up-regulation of RANKL in HPDL cells after stimulated with mechanical stress or ATP involves P2Y1 receptor signaling.

EXPECTED BENEFIT

The data from this study will provide the new scope for understanding the regulation of mechanical stress or ATP-induced RANKL expression in HPDL cells, which in turn will be useful to explain the behavior of HPDL cells after exposed to mechanical stress or ATP and may provide the new sights in orthodontic and periodontic therapeutics.

CHAPTER II

REVIEW OF RELATED LITERATURE

Mechanical stimulation

Mechanical loading is essential for the maintenance of skeletal architectural integrity (Kapur et al., 2005; Tan et al., 2008). Evidence of bone remodeling in response to loading have been reports. Consequently, numerous in vitro studies examining the application of mechanical loading including fluid flow, hydrostatic pressure, cyclic loading and mechanical strain on cells have been developed (Walboomers et al., 2005; Garcia nnd Knight, 2009; Cherian et al., 2005).

It is clear that bone cells are sensitive to their mechanical environment, and that the adaptive response can alter both mass and morphology of bone, what remains unclear is how the mechanical force is recognized by the bone cells, and transduced into a cellular signal that controls transcriptional activity to recruit osteoblasts or osteoclasts. Several studies demonstrated the activation of cell-surface receptors that respond for mechano-transmission of the biological signals have been demonstrated, including integrins, G protein-coupled receptors, cytoskeleton and ion channels (Rubin et al., 2006; Scott et al., 2008) as shown in figure 2.1.

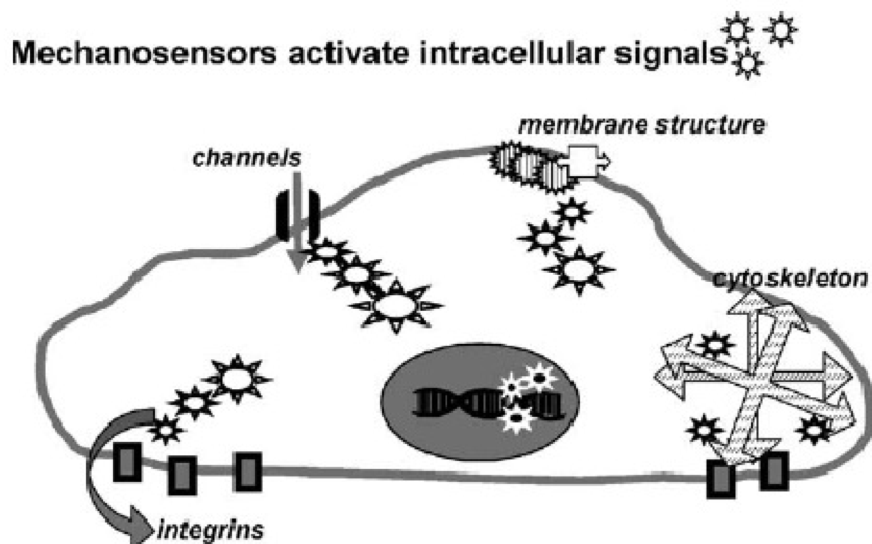

Figure 2.1 Mechanosensors activate intracellular signals. Multiple mechanosensors may be involved in receiving mechanical signals (Rubin et al., 2006).

The intracellular signaling systems after the mechanoreceptor is activated include G-proteins, calcium signaling, Mitogen-activated protein kinase (MAPK) signaling and nitric oxide (NO) signaling. Mechanical stimulation generates biochemical signals that transduce to the bone cells to exert biological effects (Cherian et al., 2003), and many factors including ATP, $[Ca^{2+}]_i$, NO, prostaglandin E2 (PGE_2), the expression of cyclooxygenase-2 (COX-2) (Bancroft et al., 2002; Romanello et al., 2001).

In summary, mechanical stimuli can regulate both gene expression and release of small signaling molecules. These mediators act as autocrine and paracrine signaling, resulting in cell responses such as cell proliferation, protein synthesis and alteration of cellular behaviors (Turner and Pavalko, 1998).

Adenosine triphosphate (ATP)

ATP is normally present in every living cell of the human body and is well-known for its role in intracellular energy metabolism. In addition to this intracellular role, ATP in the extracellular compartment is thought to contribute to the regulation of a variety of other biological processes, including cardiac function, neurotransmission, muscle contraction, vasodilatation, bone metabolism, liver glycogen metabolism and inflammation (Agteresch et al., 1999; Hoebertz et al., 2003; Burnstock and Knight, 2004).

One molecule of ATP contains three phosphate groups, and it is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Metabolic processes that use ATP as an energy source convert it back into its precursors. ATP is therefore continuously recycled in organisms, with the human body turning over its own weight in ATP each day.

The structure of this molecule consists of a purine base (adenine) attached to the 1' carbon atom of a pentose sugar (ribose). Three phosphate groups are attached at the 5' carbon atom of the pentose sugar as shown in figure 2.2. It is the addition and removal of these phosphate groups that inter-convert ATP, ADP and AMP.

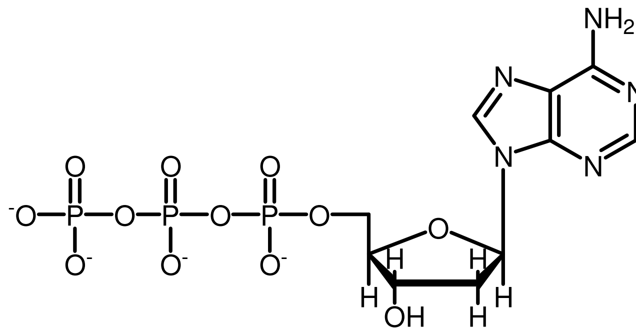

Figure 2.2 Molecular structure of ATP. ATP consists of an adenine base, a ribose sugar and a phosphate chain.

Concentrations of ATP and adenosine in the extracellular compartment are controlled by enzymes catalyzing their conversion as shown in Figure 2.3 (Bours et al., 2006). These so-called ecto-enzymes are located on cell surfaces or may be found in soluble form in the interstitial medium or in body fluids. The currently known ecto-enzymes, which are involved mainly in the breakdown of extracellular ATP, include four families that partially share tissue distribution and substrate specificity the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, alkaline phosphatases and ecto-5'-nucleotidase (CD73).

Levels of extracellular adenosine are also regulated by adenosine kinase, an intracellular enzyme catalyzing the rapid phosphorylation of adenosine to AMP (Kowaluk and Jarvis, 2000; McGaraughty et al., 2001).

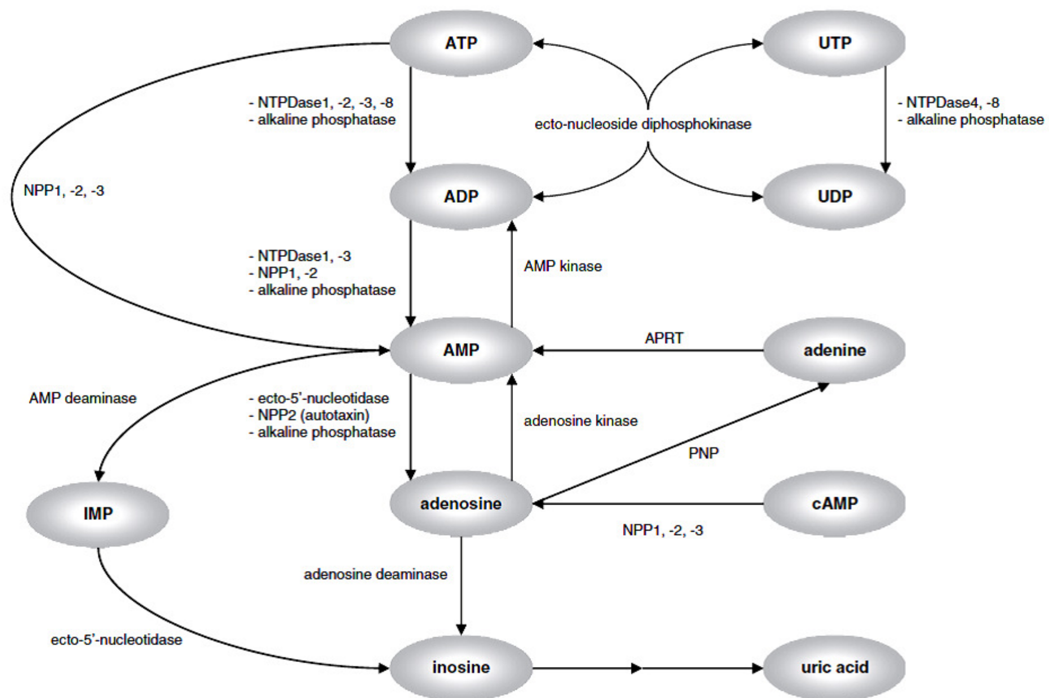

Figure 2.3 Overview of conversion pathways of ATP and adenosine (Bours et al., 2006).

Generally very high concentration of ATP is found intracellularly (3-10mM) compared to the concentration extracellularly. Physiological ATP concentrations in plasma are normally submicromolar (400-700 nM) (Coade and Pearson, 1989; Ryan et al., 1991; Ryan et al., 1996). However, extracellular concentrations of ATP can rise markedly under several conditions, including inflammation, hypoxia, ischemia and mechanical stress (Bodin and Burnstock, 1998; Bodin and Burnstock 2001; Latini and Pedata, 2001; Lazarowski et al., 2003).

In response to mechanical stimulation, ATP and other nucleotides including ADP and uridine triphosphate (UTP) may be released into the extracellular space (Ferrari et al., 1997; Pellegatti et al., 2005) and once released, they activate a family of plasma membrane receptors known as purinergic (P2) receptors.

Purinergic receptors

Growing evidence suggests the role of extracellular nucleotides in a wide range of biological processes, including smooth muscle contraction, inflammation, platelet aggregation and pain (Ralevic and Burnstock, 1998).Receptor of purines and pyrimidines have been classified into P1 and P2 receptors.

Adenosine can interact with at least four cell surface P1 receptors, designated as A1, A2a, A2b, and A3 receptors, and influence a variety of physiological functions. The endogenous ligand is adenosine (or AMP) (Gao et al., 2006). Receptor classification is based on receptor cloning, pharmacological studies, and mouse receptor knockout models. Adenosine receptors belong to the superfamily of seven transmembrane domain G protein-coupled receptors (Jacobson and Gao, 2006).

Currently, P2 purinergic receptors have been separated into two families; the P2X ligand-gated ion channels and P2Y G-protein-coupled receptors (Ralevic and Burnstock, 1998). Seven subtypes of P2X receptors (P2X1,2,3,4,5,6,7) and eight subtypes of P2Y receptors (P2Y1,2,4,6,11,12,13,14) have been cloned and characterized (Burnstock, 2006).

Physiology of purinergic signaling (Burnstock, 2009)

Previously, the role of purinergic signaling has been demonstrated, including cell proliferation, differentiation, motility and death during development, regeneration, wound healing, epithelial cell turnover, cancer and ageing (Abbracchio and Burnstock, 1998). For example, in blood vessels, there is a dual short-term control of vascular tone by ATP released as an excitatory cotransmitter from perivascular sympathetic nerves to act on P2X receptors in smooth muscle, while ATP released from endothelial cells during changes in blood flow (shear stress) and hypoxia acts on

P2X and P2Y receptors on endothelial cells leading to production of nitric oxide and relaxation (Burnstock, 2002). In addition, P2 receptors may regulate cell proliferation and differentiation, migration and death-involved neovascularization, restenosis following angioplasty and atherosclerosis (Erlinge and Burnstock, 2008).

Signal transduction pathways for P2X receptors and P2Y receptors

P2X receptors are cationic ligand-gated channels permeable to sodium ion (Na^+), potassium ion (K^+) and calcium ion (Ca^{2+}) and most are activated at low concentrations of ATP, with half maximal effective concentration (EC_{50})'s of 1-10 μM, and can be distinguished by their relative ion permeabilities, gating kinetics and sensitivity to ATP and a range of agonists and antagonists (Burnstock, 2007). P2X7 receptors are only activated at high concentrations of ATP, in the 0.1-1mM range, and are capable of pore formation, resulting in sustained influx of Ca^{2+} (Burnstock, 2007; Khakh and Burnstock, 2009; Abbracchio et al., 2009).

P2Y receptor have been cloned in mammals and they exhibit differential sensitivity to the adenine nucleotides ATP/ADP (P2Y1,11,12,13), the uracil nucleotides UTP/ uridine diphosphate UDP (P2Y4,6), both adenine and uracil nucleotides (P2Y2), or UDP-glucose (P2Y14). All P2Y receptors are G-protein-coupled and broadly indicating activate phospholipase C (PLC) /inositol triphosphate (IP3) and Ca^{2+} release from the smooth endoplasmic reticulum via G alpha (q/11) (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11), and stimulating or inhibiting adenylyl cyclase via G alpha(s) and G alpha(i/o) proteins, respectively (P2Y12, P2Y13, and P2Y14) (Burnstock, 2007).

Table 2.1 Characteristics of receptors for purines and pyrimidines (Burnstock, 2006).

Receptor	Main distribution	Agonists[c]	Antagonists[c]	Transduction mechanisms
P1 (adenosine)				
A_1	Brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA, CPA	DPCPX, N0840, MRS1754	G_{i1}, G_{i2} and G_{i3}, ↓cAMP
A_{2A}	Brain, heart, lungs, spleen	CGS21680	KF17337, SCH58261	G_S, ↑cAMP
A_{2B}	Large intestine, bladder	NECA (nonselective)	Enprofylline, MRE2029F20	G_S, ↑cAMP
A_3	Lung, liver, brain, testis, heart	IB-MECA, Cl-IB-MECA, DBXRM, VT160	MRS1220, L268605, MRS1191	G_{i2}, G_{i3} and $G_{q/11}$, ↓cAMP, ↑Ins(1,4,5)P_3
P2X				
$P2X_1$	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	α,β-meATP = ATP = 2-meSATP (rapid desensitization)	TNP-ATP, IP_5I, NF023, NF449	Intrinsic cation channel (Ca^{2+} and Na^+)
$P2X_2$	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP ≥ ATPγS ≥ 2-meSATP ≫ α,β-meATP (pH and zinc sensitive)	Suramin, iso-PPADS, RB2, NF770	Intrinsic ion channel (particularly Ca^{2+})
$P2X_3$	Sensory neurons, NTS, some sympathetic neurons	2-MeSATP ≥ ATP ≥ α,β-meATP ≥ Ap_4A (rapid desensitization)	TNP-ATP, PPADS A317491, NF110	Intrinsic cation channel
$P2X_4$	CNS, testis, colon	ATP ≫ α,β-meATP, CTP, ivermectin	TNP-ATP (weak), BBG (weak)	Intrinsic ion channel (particularly Ca^{2+})
$P2X_5$	Proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP ≫ α,β-meATP, ATPγS	Suramin, PPADS, BBG	Intrinsic ion channel
$P2X_6$	CNS, motor neurons in spinal cord	– (does not function as homomultimer)	–	Intrinsic ion channel
$P2X_7$	Apoptotic cells in, for example, immune cells, pancreas, skin	BzATP > ATP ≥ 2-meSATP ≫ α,β-meATP	KN62, KN04, MRS2427, Coomassie brilliant blue G	Intrinsic cation channel and a large pore with prolonged activation
P2Y				
$P2Y_1$	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	2-MeSADP > 2-meSATP = ADP > ATP, MRS2365	MRS2179, MRS2500	G_q/G_{11}; PLC-β activation
$P2Y_2$	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP = ATP, UTPγS, INS37217	Suramin > RB2, ARC126313	G_q/G_{11} and possibly G_i; PLC-β activation
$P2Y_4$	Endothelial cells	UTP ≥ ATP, UTPγS	RB2 > suramin	G_q/G_{11} and possibly G_i; PLC-β activation
$P2Y_6$	Some epithelial cells, placenta, T cells, thymus	UDP > UTP ≫ ATP, UDPβS	MRS2578	G_q/G_{11}; PLC-β activation
$P2Y_{11}$	Spleen, intestine, granulocytes	ARC67085 > BzATP ≥ ATPγS > ATP	Suramin > RB2, NF157	G_q/G_{11} and G_S; PLC-β activation
$P2Y_{12}$	Platelets, glial cells	2-MeSADP ≥ ADP ≫ ATP	CT50547, ARC69931MX, INS49266, AZD6140, PSB0413	G_i (G_o); inhibition of adenylyl cyclase
$P2Y_{13}$	Spleen, brain, lymph nodes, bone marrow	ADP = 2-meSADP ≫ ATP and 2-meSATP	MRS2211	G_i/G_o
$P2Y_{14}$	Placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP glucose = UDP-galactose	–	G_q/G_{11}

[a] Abbreviations: Ap_4A, diadenosine tetraphosphate; BBG, Brilliant blue green; BzATP, 2′- and 3′-*O*-(4-benzoyl-benzoyl)-ATP; CCPA, chlorocyclopentyl adenosine; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-N-methyl-5′-carbamoyladenosine; CPA, cyclopentyl adenosine; CTP, cytosine triphosphate; DBXRM, *N*-methyl-1,3-dibutylxanthine-7-β-D-ribofuronamide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; IB-MECA, N^6-(3-iodobenzyl)-N-methyl-5′carbamoyladenosine; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; Ip_5I, di-inosine pentaphosphate; α,β-meATP, α,β-methylene ATP; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5′-*N*-ethylcarboxamido adenosine; PLC, phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid; RB2, reactive blue 2; TNP-ATP, trinitrophenyl-substituted ATP.
[b] Table updated and reprinted from [94].
[c] See Chemical names.

Role of P2 receptors in bone biology

ATP, signaling through P2 receptors, is one of the most important extracellular regulatory molecules in the skeleton. Extracellular ATP and other nucleotides signal through P2 receptors, a diverse group of receptors that are widely expressed by bone cells. In order to activate P2 receptors, nucleotides must be released into the bone microenvironment. ATP is present in mmol concentrations in cells and can be released by cell lysis, cell trauma or physiological mechanisms. The capacity of extracellular nucleotides to provide a highly localized and transient signal coupled with the profound effects of P2 receptor activation on osteoblastic and osteoclastic cells and the synergistic interactions with systemic hormones, indicate that nucleotides have a strong influence over bone tissue growth and regeneration (Gallagher and Buckley, 2002).

Expression of P2 receptors in osteoblasts

P2X receptors

The expression of P2X receptors in osteoblasts is less well documented. Nakamura and coworkers demonstrated the presence of P2X4, P2X5, P2X6 and P2X7 receptors by reverse transcription polymerase chain reaction (RT-PCR) in human osteoblast-like MG-63 cells (Nakamura et al., 2000). Gartland and colleagues reported the expression of the P2X7 receptor in a sub-population of primary human osteoblasts by RT-PCR and immunohistochemistry (Gartland et al., 2001). Hoebertz et al. detected P2X2 and P2X5 protein in rat calvarial osteoblasts by imunohistochemistry and *in situ* hybridization (Hoebertz et al., 2000).

P2Y receptors

The first evidence for P2 receptors in bone was obtained from studies in which ATP was added to cultures of osteoblastic cells and shown to cause elevations in $[Ca^{2+}]_i$ (Kumagai et al., 1989; Kumagai et al., 1991; Schofl et al., 1992; Reimer and Dixon, 1992). Schofl et al. demonstrated responses to ATP in primary osteoblasts derived from human bone and in the SaOS-2 osteosarcoma cell line. They demonstrated that the cells expressed the P2U receptor (now is P2Y2) (Schofl et al., 1992). Subsequent investigation by Buckley et al. indicated that, in common with the human osteosarcoma line SaOS-2, the UMR-106 cell line predominantly expresses P2Y1 (Buckley et al., 2001).

Many studies were reporting that extracellular nucleotides elicit a range of proliferative and other responses in cultures of osteoblastic cells, and molecular studies have indicated that multiple subtypes of P2Y receptors are expressed in osteoblastic cells. P2Y1, P2Y2, P2Y4, and P2Y6 messenger RNA has been demonstrated to be present in human bone and osteoblastic cell lines by RT-PCR (Maier et al., 1997).

Expression of P2 receptors in osteoclasts

P2X receptors

Both functional and molecular evidence exists for the presence of P2X receptors on osteoclasts. Sequences encoding the P2X4 receptor have been identified in a rabbit osteoclast cDNA library, and by RT-PCR in purified preparations of rabbit osteoclasts (Naemsch et al., 1999). Application of ATP or ADP to these isolated rabbit osteoclasts activated an inward current, nonselective for cations, consistent with P2X receptor stimulation.

A study involving both patch clamping and fluorescent labeling, demonstrated that elevations in $[Ca^{2+}]_i$ in rat osteoclasts were consistent with the presence of both P2X and P2Y receptors (Weideman et al., 1997). P2X2 and P2X7 receptors have been detected by immunocytochemistry, in osteoclasts isolated from rat long bones, and mRNA encoding the P2X4 receptor was also detected in these cells (Hoebertz et al., 2000). P2X7 receptor in human osteoclasts has also been detected by RT-PCR, immunohistochemistry and functional assay in human osteoclasts generated from peripheral blood monocytes (Gartland et al., 1999).

P2Y receptors

ATP and other extracellular nucleotides have been shown to induce elevations in $[Ca^{2+}]_i$ in osteoclasts (Yu and Ferrier, 1993). Early studies revealed that these $[Ca^{2+}]_i$ elevations persisted in the absence of $[Ca^{2+}]_e$, and could be blocked by inhibiting G protein activation, implicating the involvement of P2Y receptors (Yu and Ferrier, 1994). P2Y2 receptor have been shown, by RT-PCR, to be expressed by osteoclasts isolated from human giant cell tumours (Bowler et al., 1995). Wiebe and co-worker study involving osteoclasts, isolated from long bone of neonatal rats, $[Ca^{2+}]_i$ elevations were observed in response to 10-100 μmol concentrations of ATP, consistent with the presence of functional P2 receptors (Wiebe et al., 1999).

Table 2.2 Evidence of expression of P2 receptors in bone (Gallagher and Buckley, 2002).

Type	Preparation Techniques and Transduction signal
$P2Y_1$	↑$[Ca^{2+}]_i$ in UMR-106 rat osteosarcoma cells, *Reimer and Dixon 1992*(9) ↑$[Ca^{2+}]_i$ in primary human osteoblasts, *Dixon et al. 1997*(15) RT–PCR of primary human osteoblasts, *Bowler et al. 1999*(29) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2Y_2$	↑$[Ca^{2+}]_i$ in primary human osteoblasts, *Schöfl 1992*(8) ↑$[Ca^{2+}]_i$ in UMR 106 cells, *Gallinaro et al. 1995*(11) RT-PCR and Northern analysis in primary human osteoblasts, whole bone, osteoclastoma, *Bowler et al. 1998*(21) *In situ* hybridization of human osteoclasts, *Bowler et al. 1998*(21) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2Y_4$	RT-PCR of human bone and bone cells, *Maier et al. 1997*(14) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(42)
$P2Y_6$	RT-PCR of human bone and bone cells, *Maier et al. 1997*(14) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2Y_{11}$	RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2Y_{12}$	Not detected RT-PCR of primary human osteoblasts and SaOS cells (Buckley, unpublished observations)
$P2X_1$	RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2X_2$	Immunohistochemistry and *in situ* hybridization of osteoblasts and osteoclasts, *Hoebertz et al. 2000*(18)
$P2X_3$	Not detected
$P2X_4$	RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41) Immunohistochemistry and in situ hybridisation osteoclasts, *Hoebertz et al. 2000*(18) RT-PCR and activation of cation current in rabbit osteoclasts, *Naemsch et al. 1999*(23) RT-PCR on MG63 cells, *Nakamura et al. 2000*(16)
$P2X_5$	Immunostaining in rat osteoblasts, *Hoebertz et al. 2000*(18) RT-PCR on MG63 cells, *Nakamura et al. 2000*(16) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2X_6$	RT-PCR on MG63 cells, *Nakamura et al. 2000*(16) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)
$P2X_7$	RT-PCR on MG63 cells, *Nakamura et al. 2000*(16) RT-PCR, immunohistochemistry, pore formation in human osteoblasts and human osteoclasts, *Gartland et al. 2001*(17) Immunohistochemistry of rat osteoclasts, *Hoebertz et al. 2000*(18) Pore formation in rat osteoclasts, *Modderman et al. 1994*(46) RT-PCR in RANKL derived human osteoclast cultures, *Buckley 2002 in press*(41)

Role of P2 receptors in osteoblasts and osteoclasts

Growing evidence suggests that extracellular nucleotides, signaling through P2 receptors, might play important roles in the regulation of bone and cartilage metabolism. ATP and other nucleotides can exert impressive stimulatory effects on the formation and activity of osteoclasts in addition to inhibiting bone formation by osteoblasts.

Role of P2 receptors in osteoblast biology

Several studies have shown that nucleotides act through P2 receptors to induce formation of IP3 and transiently elevate $[Ca^{2+}]_i$ in osteoblastic cells. Studies on rat osteoblast like cells demonstrated that extracellular nucleotides interact with at least two receptor subtypes; the pharmacological profiles were characteristic of P2Y1 and P2Y2 receptors (Yu and Ferrier, 1993; Sistare et al., 1994; Reimer and Dixon, 1992). Studies on single cells and populations of human osteoblasts revealed heterogeneity of receptor expression within one cell culture (Dixon et al.,1997). This might indicate that expression of P2 receptors changes during the osteoblast life cycle, depending on the differentiation state.

The expression of P2X5 receptor has been described in human osteoblasts. The P2X5 receptor has also been implicated in the stimulation of DNA synthesis by ATP (Nakamura et al., 2000). Earlier studies showed that P2X5 receptor immunoreactivity was indeed restricted to the metabolically active, differentiating cell layers in epithelial and hair follicles (Groeschel-Stewart et al., 1999). Thus, P2X5 receptors might participate in the regulation of osteoblastic proliferation and differentiation.

Activation of P2Y1 and P2Y2 receptors has been shown to potentiate subsequent parathyroid hormone (PTH) receptor-mediated $Ca2^{+}$ signalling (Buckley, et al.,2001; Bowler et al., 1999). For example, it has been suggested that PTH receptors are capable of activating adenylyl cyclase but might be unable to activate PLC until cells receive a signal as a consequence of P2 receptor activation (Bowler et al., 2001). These synergies suggest a mechanism through which systemic PTH could initiate bone remodelling at specific sites in the skeleton by co-operating with the localized release of nucleotides. Extracellular nucleotides present in the bone microenvironment might thus be capable of modulating bone cells and controlling the remodelling process by interacting with, and potentiating, both systemic hormones, such as PTH, and local growth factors (Bowler et al., 2001).

Furthermore, high concentrations of ATP (50-500 mM) have been shown to reduce the amount of bone formed by primary rat osteoblasts (Jones et al., 1997). While, ATP at concentrations as low as 1-10 mM, but not adenosine or ADP, caused strong inhibition of mineralized bone nodule formation by cultured rat osteoblasts (Hoebertz et al., 2002). The potent inhibitory actions of ATP and UTP point to the involvement of either P2Y2 or P2Y4 receptors. No evidence for the expression of the P2Y4 receptor in rat osteoblasts, which suggests that the P2Y2 receptor might mediate these inhibitory effects (Hoebertz et al., 2000). The earlier observation by Jones et al. that adenosine 5'-O-3-thiotriphosphate (ATPγS), a potent agonist at the P2Y2 receptor, also inhibited osteoblast formation (Jones et al., 1997). P2Y2 receptors have recently been shown to mediate oscillatory fluid flow-induced Ca^{2+}mobilization in murine osteoblasts (You et al., 2002). Mechanically stimulated human osteoblasts have also been shown to propagate fast intercellular Ca^{2+}waves via autocrine activation of P2Y2 receptors (Jorgensen et al., 1997). Intercellular signal propagation might represent a mechanism by which mechanically initiated signals, possibly from osteocytes, diffuse through the bone tissue to surface osteoblasts and

osteoclasts. However, signalling to osteoclasts was not mediated by P2Y receptors but appeared to require the P2X7 receptor (Jorgensen et al., 2002).

Role of P2 receptors in osteoclast biology

The first evidence that osteoclasts respond to nucleotides have been shown in cultured rabbit osteoclasts. ATP was shown to stimulate an increase in the concentration of $[Ca^{2+}]_i$ in these cells via influx of Ca^{2+} across the cell membrane and G protein-coupled release of Ca^{2+} from internal stores (Yu and Ferrier, 1993; Yu and Ferrier, 1994). Subsequent electrophysiological studies provided evidence for the coexistence of both P2X and P2Y receptors on osteoclasts (Weidema et al., 1997). More recent studies reported that the ADP analogue (ADPβS) elicited a Ca^{2+}-dependent K^+ current in rabbit osteoclasts, and raised $[Ca^{2+}]_i$ in rat osteoclasts, consistent with the presence of the P2Y1 receptor on osteoclasts (Naemsch et al., 1999; Weidema et al., 2001).

In addition, the role of P2 receptors in osteoclast biology was first proposed in 1995 when ATP was shown to stimulate bone resorption by cells derived from a human osteoclastoma (Bowler et al., 1995). At low concentrations of ATP was shown to stimulate not only the resorptive activity of osteoclasts but also the formation of rodent osteoclasts. The stimulatory effect of ATP on resorption was demonstrated in rat osteoclasts were co-activated by culture in acid medium (Morrison et al., 1998).

Recently, the first evidence to link a specific P2 receptor to the action of nucleotides on bone resorption was reported (Hoebertz et al., 2001). Extracellular ADP and 2-methylthioADP (2-meSADP), a potent ADP analog with selectivity for P2Y1 receptor, were shown to be potent stimulators of bone resorption at 20 nM to 2 μM concentrations (Hoebertz et al., 2001). The actions of ADP on resorption pit formation by mature rat osteoclasts were biphasic: no effects were evident at higher

concentrations (20-200 mM), which is in agreement with a bell-shaped response curve observed earlier for ADP at the P2Y1 receptor (Sak et al., 2000). AMP and adenosine had no significant effect on resorption, which suggests that ADP itself was the signalling agent. The ADP effect could be blocked in MRS2179, one of the most potent P2Y1 receptor antagonists (Boyer et al., 1998). The experiments indicated that extracellular ADP could stimulate resorption directly via the P2Y1 receptor expressed on mature osteoclasts or indirectly via receptors expressed on osteoblasts, which in turn release pro-resorptive local factors, or by both direct and indirect mechanisms. A recent study on human osteoclasts suggested that the effect of ATP on resorption is indirect through upregulation of RANKL in osteoblasts (Buckley et al., 2002). As mentioned above, ATP is also a potent stimulator of the activation and formation of rodent osteoclasts, an effect only evident at low pH 6.9, which suggests the involvement of the P2X2 receptor (Morrison et al., 1998).

Osteoclasts have been reported to undergo cell death when exposed to high concentrations of ATP (1-2 mM) (Morrison et al., 1998). However, high concentrations of ATP can also cause inactivating inward current that is permeable to small cations; this rules out pore formation and suggests the role for the P2X7 receptor in osteoclast biology (Naemsch et al., 2001).

Table 2.3 Evidence and possible functions for P2 receptors in osteoblasts and osteoclasts (Hoebertz et al., 2003).

Receptor subtype	Species	Evidence	Proposed function[c]
Osteoblasts			
$P2X_2$	Rat	Immunolabelling and *in situ* hybridization	–
$P2X_5$	Rat	Immunolabelling	Proliferation, differentiation
	Human	RT–PCR	
$P2X_6$	Human	RT–PCR	–
$P2X_7$	Human	RT–PCR	Active cell death at high ATP concentrations
	Human	Immunolabelling and RT–PCR	
P2Y	Rat	Ca^{2+} release from stores	
$P2Y_1$	Rat	*In situ* hybridization	Enhance PTH-induced Ca^{2+} signalling; release of pro-resorptive factors (e.g. prostaglandins and RANKL)
	Human	RT–PCR	
$P2Y_2$	Rat	*In situ* hybridization	Inhibition of bone formation; intercellular communication between osteoblasts
	Human	RT–PCR	
$P2Y_4$	Human	RT–PCR	–
$P2Y_6$	Human	RT–PCR	–
Osteoclasts			
P2X	Rat	Ca^{2+} influx	
$P2X_2$	Rat	Immunolabelling and *in situ* hybridization	Increased osteoclast activity
$P2X_4$	Rat	Immunolabelling and *in situ* hybridization	–
	Rat	Nonselective cation current	
	Rabbit	RT–PCR and cation current	
$P2X_7$	Rat	Immunolabelling	Intercellular communication between osteoblasts and osteoclasts; fusion of osteoclast progenitors; active cell death (at high ATP concentrations)
	Mouse	Permeabilization	
	Rabbit	Nonselective cation current	
	Human	RT–PCR	
P2Y	Rabbit	Ca^{2+} release from stores	
	Rat	Ca^{2+} release from stores	
$P2Y_1$	Rat	*In situ* hybridization	Increased osteoclast formation; increased resorptive activity
$P2Y_2$	Rat	*In situ* hybridization	–
	Human	RT–PCR	
	Human	*In situ* hybridization	

[a] Abbreviations: ATP, adenosine 5′-triphosphate; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor κB ligand; RT–PCR, reverse-transcriptase polymerase chain reaction.
[b] The references cited relate to the evidence for the presence of the receptor subtypes in osteoblasts and osteoclasts.
[c] The proposed functions relate to the specific receptor subtypes.

ATP release pathways and mechanisms

For many years, it is now known that many cell types release ATP physiologically in response to mechanical stimulation. ATP release is one of well-known mechanosensitive responses in endothelial cells. Released ATP induces Ca^{2+} responses and NO production in neighboring cells in an auto or paracrine manner. Mechanosensitive and agonist-induced ATP releases are also observed in many cell types, but the cellular mechanisms and pathways of ATP release are largely unknown. Reported candidates for ATP release pathways are ABC proteins including P-glycoprotein and cystic fibrosis transmembrane conductance regulator (CFTR), exocytosis of ATP-containing vesicles, and ATP-permeable anion channels. In vascular endothelium, vesicular exocytosis, volume-regulated anion channels (VRAC) and Cx hemichannels have been reported as candidates for ATP release pathways.

ATP concentrations need only reach middle to high nanomolar concentrations or low μM concentrations to stimulate ATP receptors. From all the recently developed assays to detect ATP release, the amount of ATP released from cells never exceeds 10 μmol/L. Thus, the cell is only releasing or sacrificing 0.1% or less of its total intracellular ATP pool (Schwiebert, 2001).

Table 2.4 Reported ATP release pathways and mechanisms (Oike et al., 2004).

	ATP release pathway	ATP releasing stimuli	cell types	properties, etc.
1	P-glycoprotein (*mdr-1* gene product)	spontaneous release	*mdr-1*-overexpressed CHO cell	conductance: 43.0pS (inward), 14.7pS (outward) (intra/extracellular: 100mM Tris-ATP)
2	CFTR	cAMP	CFTR-overexpressed adenocarcinoma cell line	conductance: 4.8pS (extracellular: 100mM MgATP, intracellular: 140mM NaCl)
3	connexin hemichannel	low Ca^{2+} solution, IP_3	Cx32-, Cx43-overexpressed C6 glioma and HeLa cells	No direct evidence for ATP permeation through the channel. Also reported in brain endothelium GP8.
4	vesicular release	shear stress	human umbilical cord vein endothelial cell	Release of ATP-containing intracellular vesicles. Suppressed by membrane transport inhibitors.
5	volume- and voltage-dependent ATP-conductive large conductance anion channel (VDACL)	hypotonic stress	C127i cell	conductance: ~400pS (100mM Na_2ATP) ATP release and currents were inhibited by Gd^{3+}.
6	volume regulated anion channel (VRAC)	hypotonic stress	bovine aortic endothelial cell	ATP suppressed VRAC current as a permeation-blocker. ATP release was inhibited by VRAC inhibitors.

Gap junctions (Jiang, et al., 2007)

Gap junctions are transmembrane channels, which connect the cytoplasm of adjacent cells. These channels permit molecules with molecular weights approximately less than 1,000 daltons such as small metabolites, ions, and intracellular signaling molecules (i.e. calcium, cAMP, IP3) to pass through. Gap junction channels have been demonstrated to be important in modulating cell signaling and tissue function in many organs, such as heart, liver, peripheral nerve, ovary, ear and lens of the eye (Bennett et al., 1978; Bergoffen et al., 1993; Britz-Cunningham et al., 1995; Reaume et al., 1995; Simon et al., 1997; Kelsell et al., 1997; Gong et al., 1997; Jiang et al., 1995).

Gap junctions are formed by members of a family of sequentially and structurally related proteins known as connexins. Approximately twenty Cx(s) have been identified and cloned from various tissues and cells (Eiberger et al., 2001; Willecke et al., 2002; Saez et al., 2003). Six monomers of Cx(s) are joined head-to-head across the extracellular "gap" between two adjacent cells to form intercellular channels. Cx(s) are membrane proteins, which consists of four conserved membrane spanning domains and two extracellular loop domains, sharing more than 95% homology. Sequences in the intracellular loop and especially, those in the carboxylterminus are divergent between Cx(s) (Goodenough et al., 1996).

Three types of Cx(s), Cx43, Cx45 and Cx46, are expressed in bone tissues. Cx43, a ubiquitously expressed connexin, is identified in virtually all types of bone cells, including cultured osteoblasts from newborn rat calvaria (Schirrmacher et al., 1992), human bone marrow stromal cells, trabecular bone osteoblasts (Civitelli et al., 1993), murine osteoblasts (Edelson, 1990), primary osteocytes in vivo (Mason et al., 1996), mandibular bone and periodontal ligament cells of rat teeth (Su et al., 1997), osteoblast-like MC3T3-E1 cells (Chiba et al., 1993; Yamaguchi et al., 1994),

osteocyte-like MLO-Y4 cells (Cheng et al., 2001; Thi et al., 2003) and chondrocytes from articular and growth plate cartilage (Donahue et al., 1995; Schwab et al., 1998; Chi et al., 2004). Cx45 and Cx46 are expressed in several established osteoblast cell lines. Both Cx43 and Cx46 are expressed in osteoblast-like ROS17/2.8 cells, however, the latter Cx fails to assemble into multimeric complexes and to be expressed on the cell surface to form gap junction channels (Koval et al., 1997). Cx45 is expressed in UMR-102 and hFOB 1.19 osteoblastic, and MLO-Y4 osteocytic cell lines, but to a much lesser degree than Cx43 (Thi et al., 2003; Steinberg et al., 1994; Donahue et al., 2000). However, we found that Cx45 protein is expressed in bone marrow, but not in osteoblasts, osteocytes and osteoclasts in the alveolar bone tissues of the tooth (Gluhak-Heinrich et al., 2006).

Functional gap junctions in osteoblasts were first demonstrated with electrical conductance and dye injection (Jeansonne et al., 1979). Voltage-sensitive gap junction currents were detected in osteoblastic cells derived from calvarias of new-born rats with a single gap junction channel conductance of approximate 100 pS (Schirrmacher et al., 1997). Fluorescent dye injected into rat calvarial subperiosteal osteoblasts spreads to neighboring osteoblastic cells via gap junction intercellular communication (GJIC). Using dye-transfer assays, it has also been observed that dye spreads rapidly between a numbers of odontoblasts (Ushiyama, 1989) and osteoblasts (Palumbo et al., 1990). Microinjection of anti-Cx43 antibody in MC3T3-E1 cells blocks cell coupling (Yamguchi et al., 1994). By using electron microscopy and histocytochemistry, the morphological proof of the existence of gap junction structures has also been obtained for periosteal fibroblasts, osteoblasts and osteocytes in vivo (Palumbo et al., 1990; Doty, 1981; Jones et al., 1993; Shapiro, 1997). Interestingly, in addition to linear gap junctions, stacked, oval and annual junction structures are found, especially within the osteocyte cytoplasm and in osteocyte cell processes within the canaliculi (Shapiro, 1997). However, the functional significance of these unusual gap

junction structures inside the cells is still unknown. Together, Cx(s) are highly expressed in bone cells and form functional gap junction channels.

Gap junction regulation by hormones, growth factors and other components (Jiang, et al., 2007)

The functions and expression of gap junctions and Cx43 are regulated by hormones, and other signaling and regulatory molecules. PTH stimulates the expression of Cx43 in osteoblasts and osteocytes (Civitelli et al., 1998; Massas and Benayahu, 1998; Schiller et al., 1992; Schiller et al., 1997). The stimulatory effect of PTH appears to depend upon the developmental state of the osteoblasts along the osteoblastic differentiation pathway (Schiller et al., 1997). Stable transfection antisense cDNA of Cx43 blocks the increase of cAMP elicited by PTH in ROS17/2.8 cells (Van der Molen et al., 1996). Additionally, blocking of GJIC attenuates the stimulatory effect of PTH on osteoblast mineralization (Schiller et al., 2001). Treatment with PGE_2, in most cases, increases GJIC and connexin expression in osteoblastic cell lines (Van der Molen et al., 1996; Civitelli et al., 1998; Massas et al., 1998; Schiller et al., 1997; Shen et al., 1986; Cheng et al., 2001; Cherian et al., 2003; Donahue et al., 1995). In contrast to PTH and PGE_2, transforming growth factor β, osteogenin and bone morphogenetic protein-2 inhibit GJIC in MC3T3-E1 cells (Rudkin et al., 1996).

pH plays a critical role in bone formation, by which alkaline pH supports mineral deposition while an acidic pH promotes mineral dissolution (Kreiger et al., 1992). GJIC is sensitive to pH changes in MC3T3-E1 cells and lower pH causes a reduction in cell coupling and the transcription rate of Cx43 (Yamaguchi et al., 1995; Yamaguchi and Mia, 2003).

Elevated intracellular cAMP that increases GJIC induces an increase in the expression of osteocalcin, a marker for osteoblast differentiation and a decrease in

alkaline phosphatase activity (Schiller et al., 1992; Romanello et al., 2001). ERK/PI3K signaling is suggested to functionally influence GJIC (Alford et al., 2003). Components of the extracellular matrix (ECM) have direct impact on osteoblast differentiation, GJIC and connexins. ECM components, such as, type 1 collagen, fibronectin, vitronectin, are present during specific stages of bone development and have been shown to have different roles in supporting adhesion of mechanically strained osteoblasts (Lacouture et al., 2002).

Modified extracellular matrix molecule, sulfated hyaluronan, enhances the expression of Cx43 and N-cadherin, further resulting in remarkable induction of the alkaline phosphatase activity in rat osteoblast cells (Nagahata et al., 2004). Hydroxy apatite (HA) microspheres, which enhance the differentiation of osteoblasts, increase GJIC in osteoblasts (Nakaoka et al., 2005).

Gap Junctions in Ca^{2+} signaling and propagation in bone cells (Jiang, et al., 2007)

Ca^{2+} signaling induced by hormones, growth factors and mechanical stimulation in bone cells is thought to be essential for bone formation and remodeling. Acting through the ERK signaling pathway in primary calvarial osteoblasts, extracellular Ca^{2+} induces COX-2 transcription and PGE_2 production (Choudhary et al., 2003). Gap junction-dependent and independent mechanisms have been proposed to modulate the Ca^{2+} wave propagation in bone cells. In Cx43-expressing ROS17/2.8 cells, calcium waves are propagated by GJIC and influx of extracellular calcium. However, Cx45 predominantly expressed in UMR106 cells, the propagation of Ca^{2+} is mediated through gap junction-independent pathway, by which calcium waves are transmitted by activation of P2 receptors, causing a release of intracellular calcium stores (McIntire et al., 1997). In cultured primary osteoblasts and in bone marrow stromal cells, elevated intracellular Ca^{2+} reduces intercellular coupling (Massas and

Benayahu, 1998; Schirrmacher et al., 1996). Extracellular Ca^{2+} influx through L-type Ca^{2+} channels triggers the surge of intracellular Ca^{2+}, which is transmitted via Cx43 gap junction channels (Jorgensen et al., 2003). GJIC is also demonstrated to mediate intercellular signaling in isolated chondrocytes (Donahue et al., 1995; Schwab et al., 1998; D'Andrea and Vittur, 1996) and paired chondrocytes in situ (Chi et al., 2004). Gap junctions mediate the propagation of intracellular Ca^{2+} waves in mechanically stimulated articular chondrocytes (D'Andrea et al., 2000) and between chondrocytes and synovial cells (D'Andrea et al., 1998; Grandolfo et al., 1998).

Recent evidence from long-term culture of human osteoblast-like cells shows that in less differentiated cells, P2Y-mediated intracellular calcium waves are primarily involved, but as cells differentiate in culture, gap junction-mediated intracellular Ca^{2+} become more prominent (Henriksen et al., 2006). Gap junctions provide a direct transmitting pathway for intracellular Ca^{2+} propagation, which results in bone formation and remodeling.

Gap junction hemichannels (Jiang, et al., 2007)

In addition to being the major components of gap junction channels, Cx(s) have recently been shown to exist and function in the form of un-apposed halves of gap junction channels called hemichannels (Goodenough and Paul, 2003; Bennett et al., 2003; Ebihara, 2003). These channels are localized at the cell surface, independent of physical contact with adjacent cells. Hemichannels, like gap junction channels, display relatively low substrate selectivity and permit molecules with molecular weights less than 1,000 daltons to pass through. However, the function of hemichannels is very different from gap junctions; the former mediate communication between cells and the extracellular matrix, while the latter is involved in the communication between adjacent cells. An atomic force microscopic study reveals the

structures of Cx43 hemichannels as randomly distributed individual particles and clusters, showing a lack of preferential orientation in a lipid membrane (Thimm et al., 2005). Extracellular domains of these undocked hemichannels are structurally different from connexons in docked gap junctional plaques.

Hemichannels appear to provide a mechanism for ATP and NAD^+ release, which raises $[Ca^{2+}]_i$ levels and promotes Ca^{2+} wave propagation in astrocytes, bone cells, epithelial cells and retinal cells (Stout et al., 2002; Vanoye et al., 1999; Gomes et al., 2005; Zhang and McMahon, 2001; Jorgensen et al., 2002; Guyot and Hanrahan, 2002; Romanello and D'Andrea, 2001; Bruzzone et al., 2000; Kamermans et al., 2001). Hemichannels in astrocytes are shown to be involved in the release of the neurotransmitter glutamate (Ye et al., 2003). Existence of functional hemichannels formed by Cx43 has been reported in many cell types such as neural progenitors, neurons (Hofer and Dermietzel, 1998; Boucher and Bennett, 2003), astrocytes (Contreras et al., 2003; Saez et al., 2003), and heart (John et al., 2003).

These hemichannels are regulated by voltage, protein kinase C (PKC), extracellular Ca^{2+}, and retinoic acid (Zhang and McMahon, 2001; Gomez-Hernandez et al., 2003; Jedamzik et al., 2000). Electrophysiological studies demonstrate the opening of hemichannels formed by Cx45 when the external Ca^{2+} concentration is reduced (Valiunas, 2002). The direct interaction of Ca^{2+} with Asp residues is responsible for preventing voltage-gated opening of Cx32 hemichannels. Disruption of the binding site, which is linked to a hereditary peripheral neuropathy, causes complete Ca^{2+} deregulation of hemichannels (Gomez-Hernandez et al., 2003). Hemichannels are reported to regulate cell volume in response to the change in extracellular physiological calcium (Quist et al., 2000).

Hemichannels are demonstrated to express in osteoblasts and osteocytes (Romanello and D'Andrea, 2001; Plotkin and Bellido, 2001; Plotkin et al., 2002). Previous reported that hemichannels formed by Cx43 serve as the pathway for the exit

of elevated intracellular PGE_2 in osteocytes induced by fluid flow shear stress (Jiang and Cherian, 2003; Cherian et al., 2005).

Gap Junctions and Hemichannels in Mechanical Signal Transduction of Bone (Jiang, et al., 2007)

Gap junctions through the propagation of intracellular signals contribute to mechanotransduction in bone and contribute to the regulation of bone cell differentiation (Donahue, 2000). Few studies have been reported the expression and regulation of Cx43 in response to mechanical stimulation using in vivo animal models. By using a rat experimental tooth movement model, Su et al. reported that Cx43 is increased in osteoclasts and periodontal ligament cells in compression zones, and in osteoblasts and osteocytes in tension zones of the periodontal ligament (Su et al., 1997). They also observed high expression of Cx43 mRNA in osteocytes. In studies of mouse tooth movement model show the similar stimulation of Cx43 mRNA expression by mechanical loading in osteoblasts and lining cells in bone formation sites and osteoclasts in resorption sites (Gluhak-Heinrich et al., 2006). However, there is no discernible increase of Cx43 mRNA in osteocytes, but in contrast, level of Cx43 protein in alveolar osteocytes is highly up-regulated in response to mechanical loading.

This discrepancy between their studies could be caused by the different mechanical models used, variation of tooth movement applied, and duration of loading regimes. These in vivo studies suggest that expression of Cx43 in bone is highly responsive to mechanical loading.

Several in vitro studies have been conducted to determine the relationship between mechanical stress and function and expression of gap junctions and connexins in bone cells. Mechanical loading through cyclical stretching enhances the phosphorylation of Cx43 and GJIC in osteoblastic cells, implying the formation of

functional gap junction channels (Ziambaras et al., 1998). Mechanical stress causes the release of PGE_2, which is shown to be dependent upon gap junctions (Saunders et al., 2001; Saunders et al., 2003). A dominant negative mutant of Cx43 diminishes fluid flow induced release of PGE_2, but not Ca^{2+} responses (Saunders et al., 2001).

Consistently, the fluid flow-induced PGE_2 response of ROS17/2.8 cells is gap junction-mediated and independent of intracellular Ca^{2+} (Saunders et al., 2003). Gap junction activities and accumulation of Cx43 protein in osteocytes are altered by mechanical loading (Cheng et al., 2001; Thi et al., 2003; Alford et al., 2003). Oscillating fluid flow has been shown to up-regulate GJIC in MLO-Y4 cells by an ERK1/2 MAP kinase-dependent mechanism (Alford et al., 2003). We have shown that fluid flow stimulates GJIC and increases Cx43 expression in osteocyte-like MLO-Y4 cells (Cheng et al., 2001). Cherian et al. found that in addition to gap junctions, Cx43 forming hemichannels mediate the biological responses elicited by fluid flow. Fluid flow increased surface expression of Cx43 and induced the rapid opening of hemichannels, which in turn mediated the release of PGE_2 in MLO-Y4 cells (Cherian et al., 2005). All this evidence suggests that signals generated by mechanical stimulation are likely to be transmitted between bone cells through gap junction channels, and between cells and the extracellular matrix through hemichannels. The role of hemichannel opening in response to mechanical stress is postulated in a model diagram as shown in figure 2.4 (Jiang et al., 2007).

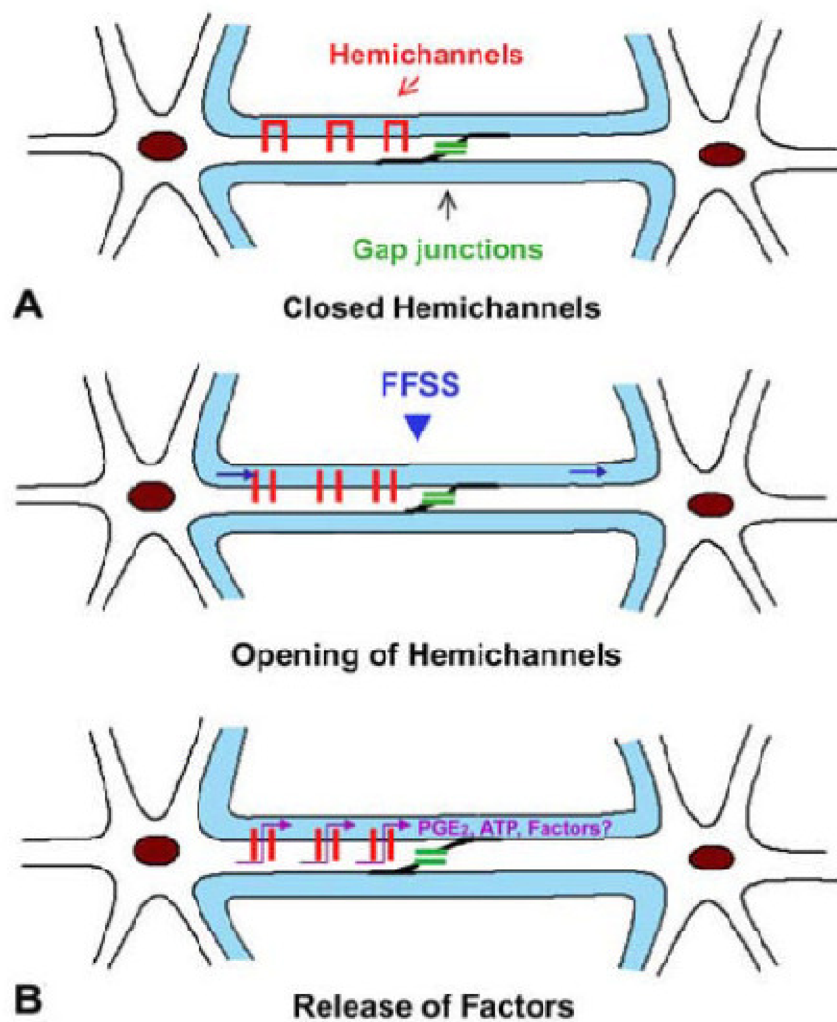

Figure 2.4 Model diagram for the role of hemichannels under fluid flow shear stress in osteocytes. Hemichannels are expressed on the plasma membrane away from cell-cell junction regions. (A) In the absence of mechanical stress, hemichannels remain closed, whereas gap junctions are kept open. (B) Fluid flow shear stress induces the opening of hemichannels (upper panel). PGE_2, possibly ATP and other responding physiological factors are released into canaliculi to mediate biological responses elicited by mechanical stress (lower panel) (Jiang et al., 2007).

Gap Junction Hemichannel Regulation (Jiang, et al., 2007)

The evidences for regulatory mechanisms of connexin forming hemichannels have been proposed. Dephosphorylation of Cx43 due to ATP depletion and activation of Ca^{2+}-dependent protein phosphatases are proposed to induce the opening of hemichannels (Contreras et al., 2004). The opening of hemichannels by ATP-depletion supports a role for hemichannels in causing injury in epithelial cells in general and in renal-tubule cells (Vergara et al., 2003; Vergara et al., 2003). Liposomes in which nonphosphorylated Cx43 is constituted show greater hemichannel permeability than those phosphorylated by MAPK (Kim et al., 1999).

Moreover, hemichannels formed by Cx43 (S368A), a PKC site mutant, remains preferentially open (Bao et al., 2004). Oxidation of Cx43 by enhanced generation of reactive oxygen-derived species also promotes the opening of hemichannels (Contreras et al., 2004). Conversely, a free-radical scavenger (Trolox) inhibits opening of Cx43 hemichannels in metabolically inhibited astrocytes (Contreras et al., 2002; Contreras et al., 2004), implying the involvement of redox potential in the opening of hemichannels. A recent study by Retamal et al. shows that metabolic inhibition increases the levels of Cx43 on the cell surface and induces dephosphorylation and nitrosylation of Cx43 (Retamal et al., 2006). Previous evidence suggests the involvement of integrins in regulation of GJIC and Cx43 expression (Lampe et al., 1998; Guo et al., 2001; Guo et al., 2003). Interaction of $\alpha 3\beta 1$ with laminin 5 promotes gap junction coupling (Lampe et al., 1998). Cx43 and Cx26 expression and distribution, formation of gap junction plaques and GJIC are regulated by matrix proteins (Guo et al., 2001). Previous studies have shown the co-localization of Cx43 with cell and substrate adhesion molecules during intramembranous bone formation by immunohistochemistry (Rundus et al., 1998). However, the role of integrin and adhesion molecules in regulation of hemichannels has not been elucidated.

It is likely that integrins and cell or substrate adhesion molecules serve as molecular tethers that sense mechanical stress and transducer the effects on gap junctions and hemichannels. Integrins are reported to be mechanical sensors on the cell surface (Wang and Ingber, 1993; Chen et al., 2004) and have been proposed to be the candidate mechanosensors in bone cells (Salter et al., 1997).

The involvement of hemichannels in ATP release

The involvement of hemichannels in ATP release has been directly demonstrated in several cell types. Studies in astrocytes, endothelial cells, cochlea as well as osteocytes showed that the released ATP was inhibited by the hemichannels blockers (Stout et al., 2002; Zhao et al., 2005; Gomes et al., 2005; Genetos et al., 2007).

In addition, certain types of Cx have been shown to be ATP releasing channel. Among these, Cx43 were reported to be a conduit for ATP-release after various stimulations (Huang et al., 2007; Ransford et al., 2009; Eltzschig et al., 2006; Faigle et al., 2008; Kang et al., 2008).

Connexin 43 is one of the widespread connexin types expressed in many tissues including HPDL cells (Ralphs et al., 1998; Yamaguchi et al., 1994; Yamaoka et al., 1998). The involvement of Cx43 in ATP release was demonstrated in retinal pigment epithelium (Pearson et al., 2005), human microvascular endothelial cells (Faigle et al., 2008), polymorphonuclear leukocytes (Eltzschig et al., 2006), chondrocytes (Knight et al., 2009) and osteocytes (Genetos et al., 2007).

Furthermore, Cx43 hemichannels in astrocytes and other cells may mediate the ATP release triggered by mechanical stimulation, thereby permitting the extracellular propagation of calcium waves (Cotrina et al., 1998; Cotrina et al., 2000; Stout et al., 2002; Arcuino et al., 2002). Recently, it was shown that photo release

intracellularly of caged IP3 induces opening of Cx43 hemichannels and release of ATP to the extracellular milieu (Braet et al., 2003). Increase intracellular Ca^{2+} has been implicated in ATP release from many cell types including osteoblast-like cells (Genetos et al., 2005; Liu et al., 2008). These results supported that the increase of intracellular calcium participated in the opening of hemichannels (Pearson et al., 2005; De Vuyst et al., 2006).

Regulation of Osteoclast Formation and Activation by RANKL, OPG and RANK (Boyce and Xing, 2008)

Osteoclasts are derived from mononuclear precursors in the myeloid lineage of hematopoietic cells that also give rise to macrophages. Understanding of the molecular mechanisms that regulate osteoclast formation and activation has since the discovery of the RANKL/RANK signaling system. Macrophage colony-stimulating factor (M-CSF) expression by osteoblastic stromal cells is required for progenitor cells to differentiate into osteoclasts, but M-CSF on its own is unable to complete this process. This requirement for M-CSF was discovered by the observation that *op/op* mice, which do not express functional M-CSF, have osteopetrosis because they lack osteoclasts (Yoshida et al., 1990). Completion of osteoclast precursors (OCP) differentiation requires expression of RANKL by osteoblastic stromal cells and of RANK by OCPs.

The requirement of osteoblastic stromal cell expression of a factor(s) that mediated OCP differentiation had been recognized for many years before the discovery of RANKL, its decoy receptor, OPG and its receptor, RANK.

Many groups had attempted to purify RANKL from bone cells. Researchers at Amgen unexpectedly discovered the naturally-occurring inhibitor of RANKL, a molecule they named osteoprotegerin (OPG) because it protected against bone loss. They were making transgenic mice over-expressing various TNF receptor-related cDNAs in attempts to find molecules that could interfere with TNF signaling. They observed that mice over-expressing a particular cDNA developed osteopetrosis due to a lack of osteoclasts (Simonet et al., 1997). Researchers at the Snow Brand Milk Products Co. in Japan discovered an identical molecule (Yasuda et al., 1998) by purifying a factor from ST-2 osteoblastic cells that inhibited osteoclast formation. Both

groups used expression cloning and OPG as a probe and quickly identified its ligand, which they called OPG ligand (ODGL) and osteoclast differentiation factor (ODF), respectively (Yasuda et al., 1998; Lacey et al., 1998). This protein turned out to be identical to a member of the TNF super-family, which had been identified the year before and called receptor activator of nuclear factor-κB ligand (RANKL) (Anderson et al., 1997) and TNF-related activation induced cytokine (TRANCE) (Wong et al., 1997). Researchers at Immunex had already discovered RANK while they were sequencing cDNAs from a human bone-marrow-derived myeloid dendritic cell cDNA library (Dougall et al., 1999) and it was soon identified as the receptor for OPGL/ODF. They had found that RANK was involved in the survival of dendritic cells and isolated RANKL by direct expression screening. They found that RANKL was expressed by T cells and that it increased proliferation and survival of dendritic cells. RANKL has come to be the generally accepted acronym for this cytokine following the recommendation of a nomenclature committee of the American Society for Bone and Mineral Research (The American Society for Bone and Mineral Research, 2000). RANKL/RANK/NF-κB signaling is also required for lymph node development and B cell maturation (Dougall et al., 1999; Franzoso et al., 1997). These discoveries that RANKL/RANK/NF-κB signaling is involved in osteoclast formation and immune responses and that T and B cells express RANKL have spawned the growing field of osteoimmunology.

OCP differentiation is regulated by a number of transcription factors and signaling pathways that are activated by RANKL/RANK interaction. The completion of OCP differentiation by RANKL requires the sequential expression of NF-κB, c-Fos and NFATc1 (Matsuo et al., 2004; Takayanagi et al., 2002; Yamashita et al., 2007). Interestingly, TNF, which like RANKL can induce osteoclast differentiation directly from wide type OCPs, also induces the sequential expression of

NF-κB, c-Fos and NFATc1 (Yamashita et al., 2007). Importantly, TNF can also induce osteoclast formation from OCPs from RANKL-/- and RANK -/- mice in vitro (Kim et al., 2005) and thus could augment the osteoclast formation induced indirectly by itself and other factors through induction of osteoblastic cell expression of RANKL. c-Fos or NFATc1 can substitute for NF-κB in OCPs in this sequential activation program by RANKL and TNF (Yamashita et al., 2007), and when either of these transcription factors is over-expressed in OCPs. IL-1 can induce osteoclast formation directly from them also (Yao et al., 2008). RANKL and TNF induce c-Fos expression in OCPs (Yamashita et al., 2007; Yao et al., 2008). This may be an important role for these cytokines at sites of inflammation in bone where IL-1 concentrations are increased because this could facilitate direct induction of osteoclast formation by IL-1 and thus augment the effects of these cytokines on osteoclastogenesis.

To resorb bone effectively, osteoclasts must attach themselves firmly to the bone surface using specialized actin-rich podosomes. By means of these podosomes, they form tight seals with the underlying bone matrix in roughly circular extensions of their cytoplasm and within these sealed zones they form ruffled border membranes. This ruffling of the cytoplasmic membrane increases the area of the cell surface for secretion of the proteolytic enzyme, cathepsin K, and hydrochloric acid onto the bone surface (Teitelbaum and Ross, 2003). By this sealing and secretory mechanism, they simultaneously degrade the matrix and dissolve the mineral of bone, while protecting neighboring cells from the harmful effects of hydro chloric acid. RANKL and beta integrin-mediated signaling from bone matrix activate osteoclasts (Teitelbaum, 2006). OCPs fuse with one another and become multinucleated under the influence of RANKL. This fusion requires expression by OCPs of DC-STAMP (Yagi et al., 2005) and of Atp6v0d2, a subunit of v-ATPase, a component of the V-type H^+ ATP6i proton pump complex that secretes H^+ from osteoclasts (Lee et al., 2006).

RANKL also induces expression of tartrate-resistant acid phosphatase and cathepsin K through NFATc1 (Matsuo et al., 2004).

RANKL (Boyce and Xing, 2008)

RANKL exists as a homotrimeric protein and is typically membrane-bound on osteoblastic and activated T cells or is secreted by some cells, such as activated T cells (Kearns et al., 2007; Wada et al., 2006; Takayanagi, 2007). The secreted protein is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing (Ikeda et al., 2001). The proteolytic cleavage of RANKL is carried out by matrix metalloproteases (MMP-3 or 7) (Lynch et al., 2005) or disintegrin and metalloprotease domain (Hikita et al., 2006). Most of the factors known to stimulate osteoclast formation and activity induce RANKL expression by osteoblastic stromal cells. However, RANKL is also highly expressed in lymph nodes, thymus, mammary glands and lung and at low levels in a variety of other tissues, including spleen and bone marrow (Kearns et al., 2008). It is expressed by synovial cells and activated T cells in joints of patients with inflammatory arthritis to contribute at least in part to the joint destruction seen in patients with rheumatoid arthritis.

This joint destruction in rheumatoid arthritis is also mediated by TNF by a number of mechanisms: it increases the proliferation of OCPs in the bone marrow and the number of them circulating systemically by promoting their egression from the bone marrow (Li et al., 2004); it also promotes the egression of OCPs from the blood to inflamed joints where along with RANKL and IL-1 it promotes fusion of these cells into osteoclasts (Li et al., 2004). RANKL also stimulates the release of OCPs into the circulation; and recent studies using PTP -knockout mice suggest that osteoclasts themselves regulate the egression of hematopoietic stem cells (HSCs) from niches within the marrow under the control of RANKL. Osteoclasts from PTP -knockout

mice have defective adhesion to bone and impaired resorption and RANKL did not induce HSC mobilization in these mice (Kollet et al., 2006). Thus, RANKL-induced osteoclast activation appears to regulate HSC mobilization as part of homeostasis and host defense mechanisms, linking bone remodeling with the regulation of hematopoiesis.

RANKL is also expressed in epithelial cells in mammary gland lobules during pregnancy and is required for hyperplasia of these cell during lactation and thus milk production in mice (Fata et al., 2000). It is expressed by some malignant tumor cells which also express RANK, and thus RANKL signaling may regulate tumor cell proliferation (Kim et al., 2006) by either an autocrine or a paracrine mechanism when it is produced by accessory cells. More recent studies have identified a role for RANKL signaling in tumor cell migration and bone metastasis (Jones et al., 2006). T cell production of RANKL also induces expression of interferon-beta (INF-β) by activated osteoclasts through c-Fos signaling to negatively regulate their formation (Takayanagi et al., 2002), a mechanism that can be enhanced by T cell produced interferon-gamma (INF-γ) which degrades TRAF6, an essential adapter protein recruited to RANK to mediate RANK signaling (Takayanagi et al., 2000). IFN-γ is a cytokine secreted primarily by activated T cells and NK cells and was originally characterized as a powerful macrophage activator that upregulated nitric oxide production and MHC class II expression in macrophages (Chen et al., 2007). It has been reported to be a strong suppressor of osteoclastogenesis in vitro through inhibition of RANKL signaling (Takayanagi et al., 2000; Takayanagi et al., 2002). However, its effects on osteoclast formation are controversial. IFN-γ also enhances osteoclast generation in cultures of peripheral blood from osteopetrotic patients, in part by normalizing superoxide production (Madyastha et al., 2000) and has been shown to be efficacious in the treatment of osteoporosis in humans (Key et al., 1995). A recent

study analyzed the in vivo effects of IFN-γ in 3 mouse models of bone loss, including ovariectomy, LPS injection, and inflammation induced by silencing TGF- signaling in T cells. It demonstrated that the net effect of IFN-γ in these conditions was bone loss due to increased osteoclast formation through the following mechanism: IFN-γ increased the antigen presenting function of bone marrow cells by up-regulating their expression of MHC class II molecules and thus stimulating T lymphocytes to produce RANKL, TNF and IFN-γ (Gao et al., 2007). It is likely that the effects of IFN-γ on osteoclast formation are complex and will depend on specific conditions in the bone microenvironment and relative concentrations of other cytokines that can affect the differentiation of OCPs. c-Fos/NFATc1-induced OCP differentiation can also be inhibited by reverse signaling through the OCP-expressed ligand, ephrin B2 (Zhao et al., 2006). Interaction between ephrin B2 and its receptor, Eph 4 on osteoblast precursors prevents c-Fos activation of NFATc1 to inhibit OCP differentiation. Intriguingly, forward signaling through Eph 4 in osteoblast precursors promotes their differentiation (Zhao et al., 2006), providing further evidence that osteoclastic cells have functions in bone other than bone resorption.

RANK (Boyce and Xing, 2008)

RANK is a homotrimeric transmembrane protein member of the TNF receptor superfamily. It appears to be expressed in fewer tissues than RANKL at the protein level, but in addition to OCPs, mature osteoclasts and dendritic cells, it is expressed in mammary glands (Fata et al., 2000) and some cancer cells, including breast and prostate cancers (Kim et al., 2006; Chen et al., 2006), two tumors with high bone metastatic potential. No humans with osteopetrosis have been identified to date with mutations in *rank*. However, a deletion mutation that occurred spontaneously in

rank was reported in transgenic mice. These mice had all of the features of mice with targeted deletion of RANK, confirming the importance of RANK for osteoclastogenesis (Kapur et al., 2004). In contrast, activating mutations in exon 1 of *rank* have been reported in humans to account for the increased osteoclast formation, activity and osteolysis seen in some patients with familial Paget's disease, confirming the importance of this system in humans (Hughes et al., 2000). A potential role for RANK in tumor cell proliferation (Kim et al., 2006) is being investigated and if proven could be a future target for anti-tumor therapy.

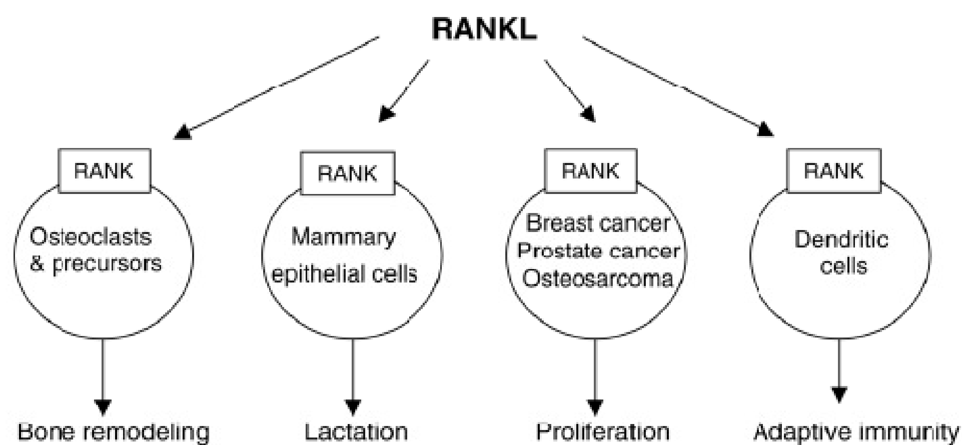

Figure 2.5 The role of the RANKL/RANK system in bone and other tissues. RANKL is produced by a variety of cell types and its expression is regulated by many physiologic and pathologic factors. Preclinical studies in mice and studies of human tissues have revealed functions for RANKL/RANK signaling in normal and pathologic states. OPG can bind to RANKL and prevent its interaction with RANK to inhibit osteoclast formation, but its effects on other cellular functions of RANKL have yet to be determined (Boyce and Xing, 2007).

OPG (Boyce and Xing, 2008)

OPG is secreted by many cell types in addition to osteoblasts, including those in the heart, kidney, liver, and spleen. A recent study reports that B cells may be responsible for 64% of total bone marrow OPG production and B cell-deficient mice are consistently osteoporotic, consistent with B cells being a major source of OPG in the bone marrow of normal mice (Li et al., 2007). Most of the factors that induce RANKL expression by osteoblasts also regulate OPG expression (Hofbauer and Schoppet, 2004). Although there some are contradictory data, in general when RANKL expression is up-regulated, OPG expression is down-regulated or not induced to the same degree as RANKL, such that the RANKL/OPG ratio changes in favor of osteoclastogenesis (Kearns et al., 2007; Theoleyre et al., 2004). Osteoclast numbers and activity can increase if there is a change in the RANKL/OPG ratio due to either an increase in the former or a decrease in the latter or a change in both that leads to a change in the ratio in favor of RANKL.

OPG's osteoprotective role in humans has been supported by homozygous partial deletions of *opg* in patients with juvenile Paget's disease, an autosomal recessive disorder in which affected individuals have increased bone remodeling, osteopenia, and fractures (Whyte et al., 2002); in addition, some patients with idiopathic hyperphosphatasia, an autosomal recessive bone disease in which affected children have increased bone turnover associated with deformities of long bones, kyphosis and acetabular protrusion have an inactivating deletion in exon 3 of *OPG* (Cundy et l., 2002).

OPG expression is regulated in osteoblasts not only by a variety of cytokines, hormones and growth factors (Theoleyre et al., 2004), but also by Wnt/β-catenin (Glass et al., 2005; Kieslinger et al., 2005). The Wnt/β-catenin pathway also regulates osteoblastic bone formation and the commitment of mesenchymal cells to the

osteoblast lineage (Hill et al., 2005). Jagged1/Notch1 signaling negatively regulates osteoclast formation both directly in osteoclast precursors and indirectly by affecting the OPG/RANKL expression ratio in stromal cells (Bai et al., 2007). Thus, bone mass is determined by many influences on osteoblasts and osteoclasts and is regulated by osteoblasts through three major signaling pathways: RANKL/RANK, Wnt/β-catenin and Jagged1/Notch1.

Transcription factor activation by RANKL/RANK in osteoclasts and OCPs
(Boyce and Xing, 2008)

A key preliminary step in downstream signaling after RANKL ligation to RANK is the binding of TNF receptor-associated factors (TRAFs) to specific sites in the cytoplasmic domain of RANK (Wong et al., 1997; Kim et al., 1999). RANK is a transmembrane protein, which like other TNF family receptors has no intrinsic protein kinase activating activity to mediate signaling. TRAFs 2, 5 and 6 all bind to RANK (Kim et al., 1999), but only TRAF6 appears to have essential functions in OCPs and osteoclasts, since deletion of only TRAF6 and no other TRAFs results in osteopetrosis (Lomaga et al., 1999; Naito et al., 1999). TRAF6-deficient mice produced by 2 independent groups of investigators developed osteopetrosis (Lomaga et al., 1999; Naito et al., 1999). Surprisingly, however, one set of mice has normal numbers of osteoclasts, but they are inactive (Lomaga et al., 1999), while the other has no osteoclasts (Naito et al., 1999). How inactivation of TRAF6 resulted in 2 different osteoclast phenotypes remains unexplained. Several signaling pathways are activated by RANK/TRAF-mediated protein kinase signaling; 4 directly mediate osteoclasts formation (inhibitor of NF-κB kinase (IKK)/NF-κB, c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1), c-myc, and calcineurin/NFATc1) and 3 mediate

osteoclast activation (Src and MKK6/p38/MITF) and survival (Src and extracellular signal-regulated kinase) (Boyle et al., 2003).

Several adapter molecules bind to the intracytoplasmic domain of RANK along with TRAFs to mediate signaling. These include Grb-2-associated binder (Gab) protein 2, a member of a family of proteins that are phosphorylated at tyrosine residues and recruit signaling molecules that contain Src homology-2 domains. Gab2-deficient mice have reduced RANKL-induced osteoclast differentiation, decreased bone resorption and mild osteopetrosis (Wada et al., 2005). The development of mild, rather than marked osteopetrosis in these mice suggests that Gab2 plays a significant, but not essential role in RANKL-induced osteoclast formation.

Essential roles for NF-κB, AP-1 and NFATc1 signaling in osteoclast formation was discovered after the generation of mice with targeted deletion of the genes encoding the precursor molecules of both p50 and p52 sub-units of NF-κB (Franzoso et al., 1997; Iotsova et al., 1997), c-Fos (Wang et al., 1992), and NFATc1 (Takayanagi et al., 2002). Over-expression of c-Fos rescues the defect in osteoclast formation in M-CSF-treated NF-κB p50/p52 double knockout osteoclast precursors in the absence of RANKL (Yamashita et al., 2007), and expression of NFATc1 in Fos-/- OCPs rescues their defect in differentiation (Matsuo et al., 2004). Activation of c-Fos by RANKL signaling requires expression of NF-κB p50 and p52, indicating that c-Fos and NFATc1 (Yamashita et al., 2007) are downstream from NF-kB (Figure6). On the basis of all these studies, NFATc1 has been described as the master regulator of osteoclast formation (Takayanagi et al., 2002). It is activated transiently in OCPs within 60 minutes of treatment with RANKL when it interacts with NF-κB p65 (Asagiri et al., 2005) and again during the fusion phase around 50–60 hours later by calcium-dependent calcineurin dephosphorylation. Several additional factors that are activated by RANKL and also participate in NFATc1 up-regulation include c-Fos and

RNA polymerase II (Fretz et al., 2008). Cyclosporine A, a calcineurin inhibitor, inhibits NFATc1 activation; yet treatment of patients with this immunosuppressant is associated with bone loss (Thiebaud et al., 1996). NFATc1 also positively regulates expression of osterix, a transcription factor that regulates osteoblast differentiation and function (Nakashima et al., 2002). Thus the likely reason why cyclosporine A induces bone loss in vivo is that it has a greater inhibitory effect on osteoblasts than on osteoclasts (Koga et al., 2005).

Recently, signaling molecules other than the transcription factors mentioned above have been implicated in mediating the effect of RANKL/RANK in osteoclasts. Differential screening of a human osteoclastoma cDNA library demonstrated that the regulator of G-protein signaling 10 (RGS10) is specifically expressed in osteoclasts (Yang et al., 2007). The expression of RGS10 is induced by RANKL in OCPs, and ectopic expression of RGS10 dramatically increases the sensitivity of osteoclast differentiation to RANKL signaling. RGS10-/- mice exhibit severe osteopetrosis and impaired osteoclast differentiation. Deficiency of RGS10 resulted in the absence of $[Ca2+]_i$ oscillations and loss of NFATc1, which can be rescued by over-expression of NFATc1. RGS10 competitively interacts with Ca2+/calmodulin and PIP3 in a $[Ca2+]_i$-dependent manner to mediate PLCγ activation and $[Ca2+]_i$ oscillations in osteoclasts (Yang and Li, 2007). These studies indicate that RGS10 specifically regulates RANKL-evoked RGS10/calmodulin–$[Ca2+]_i$ oscillation–calcineurin–NFATc1 signaling in osteoclast differentiation and may be a potential therapeutic target for the treatment of bone diseases in which bone resorption is increased.

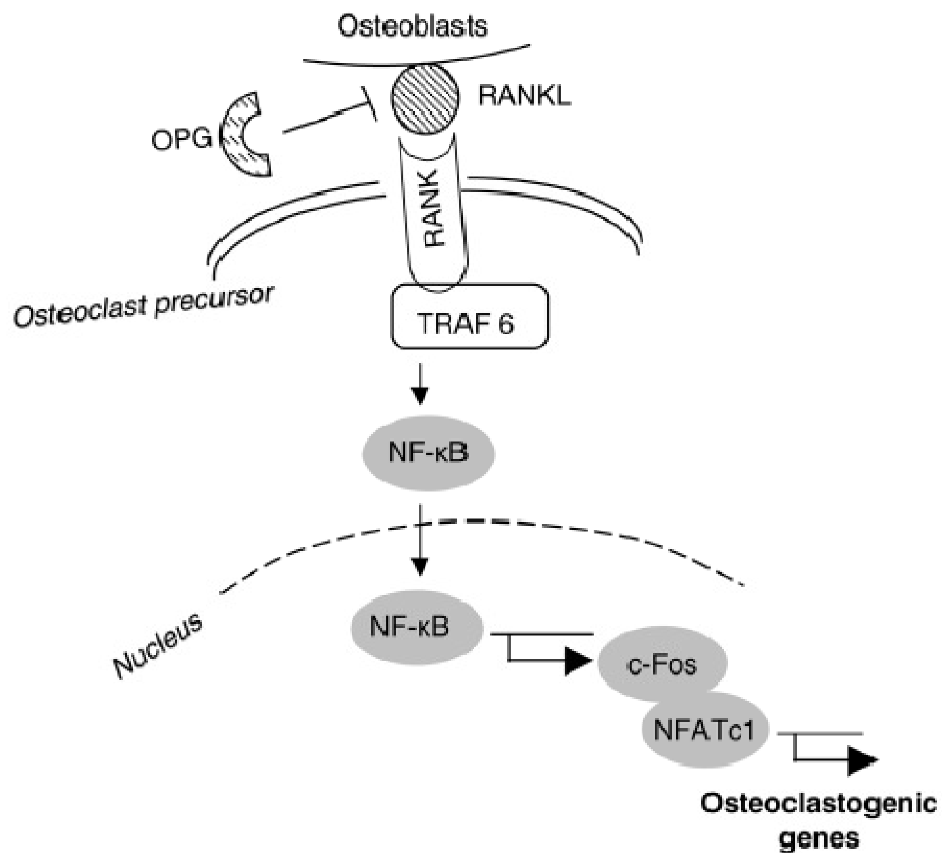

Figure 2.6 The essential signaling pathway for normal osteoclastogenesis. Under physiologic conditions, RANKL produced by osteoblasts binds to RANK on the surface of osteoclast precursors and recruits the adaptor protein TRAF6, leading to NF-κB activation and translocation to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes. OPG inhibits the initiation of the process by binding to RANKL (Boyce and Xing, 2007).

CHAPTER III

RESEARCH METHODOLOGY

1. Cell cultures

HPDL cells were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars, or extracted for orthodontic reasons at Faculty of Dentistry, Chulalongkorn University. The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. All patients gave informed consent. Briefly, teeth were rinsed with sterile phosphate buffered saline (PBS), 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 penicillin (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 5 μg/mL amphotericin B (Gibco BRL), then incubated in an atmosphere of air containing 5% CO_2 at 37°C. Each cell preparation was established from one donor. Cells from the third to the fifth passages were used. All experiments were performed in triplicate using cells prepared from three different preparations.

2. Application of mechanical stress

Mechanical stress was applied as previously described (Wongkhantee et al., 2007). Briefly, cells were seeded in six-well plates at a density of 25,000 cells cm^{-2} for 16 hours. 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 hours.

3. Luciferin-luciferase bioluminescence assay

HPDL 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 hours, mechanical stress was applied to the culture. For the inhibitory experiments, inhibitors were added 30 min prior to stress application. Culture medium was collected for extracellular ATP measurement 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 ATP concentration. Measurement of the light intensity using a luminometer (Victor Light Luminescence Counter, PerkinElmer Ltd., Salem, MA, USA) permitted direct quantitation of ATP. A calibration curve was generated for each luciferase assay by serial dilution of an ATP standard.

4. Treatment of cells

HPDL 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 hours, cells were stimulated with ATP (Sigma-Aldrich Chemical, St Louis, MO, USA).

To examine the effect of ATP on RANKL, cyclo-oxygenase (COX) mRNA expression and PGE_2 secretion, cells were treated with 0, 10, 20 or 40 μM ATP in serum-free medium. The RNA was extracted for RT-PCR analysis after 16 hours of treatment, and the medium was collected for ELISA at 2 hours after exposure to ATP.

Cell protein extracts and culture medium were collected from a parallel set of cultures after 48 hours of stimulation for western analysis.

The effective dose was selected and used for the rest of the experiments. To confirm the activity of adenylylcyclase on RANKL expression and PGE_2 release, forskolin (Sigma-Aldrich Chemical) at 0.1 and 1 mg/mL was used.

5. Measurement of PGE_2 by ELISA

The amount of PGE_2 was determined by ELISA methods according to the manuals of ELISA kits (Parameter PGE_2 Immunoassay R&D Systems). In brief, a conditioned medium was added to plates pre-coated with mouse monoclonal antibodies for 16 hours and becomes bound to the goat anti mouse antibody. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity for 20 min. The color development is stopped, and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of PGE_2 in the sample.

6. Inhibitors

The inhibitors used were 10 μM indomethacin (Sigma-Aldrich Chemical), 20 μM pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich Chemical), 2 μM phospholipase C inhibitor (U73122; Sigma-Aldrich Chemical), 5 nM cAMP-dependent protein kinase inhibitor (H89 dihydrochloride hydrate; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 5 μM P2Y1 receptor antagonist (MRS2179; Sigma-Aldrich Chemical), 5-10 μM carbenoxolone disodium salt (Sigma-Aldrich Chemical), 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 heparin sodium salt (Sigma-Aldrich Chemical), 25-50 μM 2-Aminoethoxydiphenyl borate; 2-APB (Sigma-Aldrich Chemical) and small interfering RNA or control oligonucleotide (Santa Cruz Biotechnology). Cells were treated with each inhibitor for 30 min (24 h for small interfering RNA or control oligonucleotide) prior to the addition of 40 μM ATP or mechanical stress.

7. RNA Extraction and Semiquantitative RT-PCR

Total cellular RNA was extracted with Tri-reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1.5 hours at 42°C. Subsequent to reverse transcription, PCR was performed. Primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences were shown in Table 3.1.

The 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 minute, hybridizationat 60°C for minute and extension at 72°C for 2 minutes, followed by one extension cycle at 72°C for 10 minutes. The same profile was also used for OPG (24 cycles), COX-1 (30 cycles), COX-2 (27 cycles), P2Y1 (35 cycles) Px1 (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 PCR was performed in the

DNA thermal cycles (Biometra, Gottingen , Germany). The amplified DNA was electrophoresed on a 1% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the gel bands were measured by imaging software analysis (Scion Image, Scion, Frederick, MD).

Table 3.1 Details of primers in this research.

Gene	Accession number	Primer Sequence	Product size (bp)
RANKL	NM 033012.3	forward,5'-CCAGCATCAAAATCCCAAGT-3' reverse,5'-CCCCTTCAGATGATCCTTC-3'	602
OPG	NM 002546.3	forward,5'-TCAAGCAGGAGTGCAATCG-3' reverse,5'- AGAATGCCTCCTCACACAGG-3'	341
COX-1	NM 080591.1	forward,5'-GCAGCTGAGTGGCTATTTCC-3' reverse,5'- ATCTCCCGAGACTCCCTGAT-3'	213
COX-2	NM 000963.2	forward,5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' reverse,5'-AGATCATCTCTGCCTGAGTATCTT-3'	305
P2Y1	NM 002563.2	forward,5'-CGGTCCGGGTTCGTCC-3' reverse,5'-CGGACCCCGGTACCT-3'	527
Px1	NM 015368.3	forward,5'-GGATCCTGAGAAACGACAGC-3' reverse,5'-CTCTGTCGGGCATTCTTCTC-3'	496
Cx26	NM 004004.5	forward,5'- GCAGAGACCCCAACGCCGAGAC-3' reverse,5'- GCAGACAAAGTCGGCCTGCTCA-3'	239
Cx32	NM 001097642.2	forward,5'- CTGCTCTACCCGGGCTATGC-3' reverse,5'- CAGGCTGAGCATCGGTCGCTCT-3'	330
Cx37	NM 002060.2	forward,5'- GGTGGGTAAGATCTGGCTGA-3' reverse,5'- ATAGGTGCCCATCAGTGCTC-3'	406
Cx40	NM 181703.2	forward,5'- GGGAGGCCATATTATTGCTG-3' reverse,5'- GTGGCAGAGAAGGCAGAACT-3'	486
Cx43	NM 000165.3	forward,5'- GGACATGCACTTGAAGCAGA-3' reverse,5'- CAGCTTGTACCCAGGAGGAG-3'	496
Cx45	NM 001080383.1	forward,5'- CACGGTGAAGCAGACAAGAA-3' reverse,5'- GCAAAGGCCTGTAACACCAT-3'	417
Cx50	NM 005267.3	forward,5'- TCATCCTGTTCATGTTGTCTGTGGC-3' reverse,5'- AACCTCGGTCAAGGGGAAATAGT-3'	238
GAPDH	NM 002046.3	forward,5'- TGAAGGTCGGAGTCAACGGAT-3' reverse,5'- TCACACCCATGACGAACATGG-3'	395

8. Protein Extraction and Western Analysis

The amount of OPG was prepared from the culture medium. The medium was collected and lyophilized to concentrate. The lyophilized medium was dissolved in sample buffer, boiled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to detect OPG.

For protein detection, total protein was extracted with radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were quantified using a protein assay kit (BCA protein assay kit; Pierce Bio-technology, Rockford, IL, USA). Equal amounts of protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane. To reduce non-specific binding, membranes were incubated in 5% non-fat milk for 1 hour before being incubated in primary antibody raised against RANKL (dilution 1:50; R&D Systems, Minneapolis, MN, USA), OPG (dilution 1:250; R&D Systems), P2Y1 (dilution 1:300; Abcam, Cambridge, MA, USA), Cx43 (dilution 1:250; Invitrogen Corporation), or actin (dilution 1:1000; Chemicon International). 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 Bio-technology, Rockford, IL, USA).

The relative intensities of bands were measured by imaging software analysis (Scion Image, Scion, Frederick, MD).

9. Transfection of small interfering RNA

HPDL cells (2 x 10^5 cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for 18-24 hours. For the generation of transfected with siRNA duplexes (40-80 pmol; P2Y1 siRNA (h): sc-42577, 60 pmol; Cx43 siRNA (h): sc-29276 and control siRNA-A: sc-37007, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in a 10-cm dish, using Lipofectamine 2000 (Invitrogen). Cells were transfected with siRNA 6 hours, after that medium was supplemented with 20% fetal bovine serum. Cells were analyzed after 24 hours for mRNA expression, and analyzed 48 hours for protein expression.

10. 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 prior to 5 min mechanical stress application. Cells were then incubated in 5 mM 5(6)-carboxyfluorescein for 10 min, washed with PBS 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, Germany).

11. Detection of nuclear factor kappa B (NF-κB) localization by immunofluorescence

Cells were seeded in a chamber slide (Lab-TEK® II Chamber slideTM System; Nalge Nunc International Corp., Naperville, IL, USA) at a density of 50,000 cells per chamber and grown to subconfluence. After being starved in serum-free conditions for 6 hours, cells were treated with ATP in the presence or absence of inhibitors. At the indicated time, cells were washed in phosphate-buffered saline (for 5 min, twice) and fixed with cold methanol (Merck KGaA, Darmstadt, Germany) for 10 min. Cells were incubated overnight at 4°C with primary antibody against NF-κB p50 (NLS; dilution 1:200 in 10% fetal bovine serum, sc-114; Santa Cruz Biotechnology), followed by biotinylated secondary antibody (dilution 1:1000; Zymed, S. San Francisco, CA, USA) and streptavidin-fluorescein isothiocyanate (dilution 1:1000; Sigma Aldrich Chemical) for 40 min. The chamber slide was then washed with phosphate-buffered saline and mounted. Cells were observed using a scanning photographic system (MIRAX MIDI, Carl Zeiss MicroImaging Inc., Germany).

12. Data analysis

All experiments were repeated at minimum of three times. All data are expressed as mean ± SD. All data were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS, Chicago, IL, USA). Scheffe’s test was used for post-hoc analysis (P value of less than 0.05 was considered statistically significance.

CHAPTER IV

RESULTS

PART I : Role of Cx43-hemichannel in mechanical stress-induced ATP release in HPDL cells

Mechanical stress-induced ATP release in HPDL cells.

To study the mechanism of extracellular ATP release in HPDL cells, we first measured the extracellular ATP within the supernatant after stimulated with mechanical stress. A force dependent mechanism of stress-induced ATP release was observed ($p<0.05$) (Figure 4.1 A). The 2.5 g/cm^2 of mechanical stress was selected to examine in the time course experiment for ATP release. The increase of ATP was noted within 1 min after stress application. The amount of ATP decreased after 10 min but increased again at 2 hours (Figure 4.1 B).

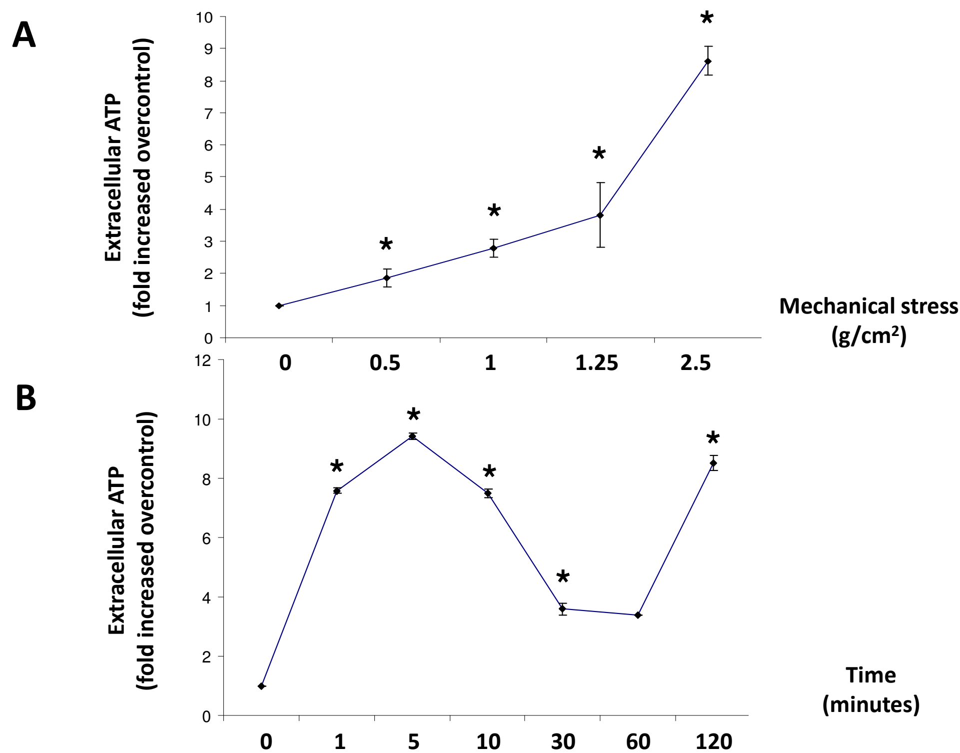

Figure 4.1 Luciferin-luciferase assay was used to determine the amount of ATP release. HPDL cells were treated with different levels of mechanical stress ranging from 0 to 2.5 g/cm^2 and the amount of ATP released was measured (Figure 4.1 A). Maximum extracellular ATP level was observed when applied 2.5 g/cm^2 stress, therefore, this loading force was used in all latter experiments. (Figure 4.1 B) The amount of ATP released from HPDL cells was measured from 1 min to 2 hours after treated with 2.5 g/cm^2 stress. Results showed that mechanical stress could induce ATP release in a dose dependent and time dependent manner. Maximum extracellular ATP level was observed when applied 2.5 g/cm^2 stress for 5 min. Results were expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

The release of ATP was attenuated by gap junction inhibitor.

Carbenoxolone, a non-specific inhibitor of gap junction, was added 30 min prior to stress application in order to investigate the influence of gap junction in mechanical stress induced-ATP release in HPDL cells. The 2.5 g/cm^2 of mechanical stress was applied on HPDL cells for 5 min in the presence or absence of carbenoxolone. Results showed that carbenoxolone significantly suppressed mechanical stress-induced ATP release (Figure 4.2 A). Moreover, carbenoxolone inhibited the upregulation of RANKL but not OPG mRNA expression induced by mechanical stress (Figure 4.2 B). corresponded to the previous results showing that stress-induced RANKL mRNA expression through the release of ATP (Wongkhantee et al., 2008).

The effect of carbenoxolone prompted us to examine the expression of gap junction protein in HPDL cells. By using RT-PCR analysis, results revealed that HPDL cells expressed at least 7 gap junction proteins including Cx26, 32, 37, 40, 43, 45 and pannexin1 (Figure 4.2 C).

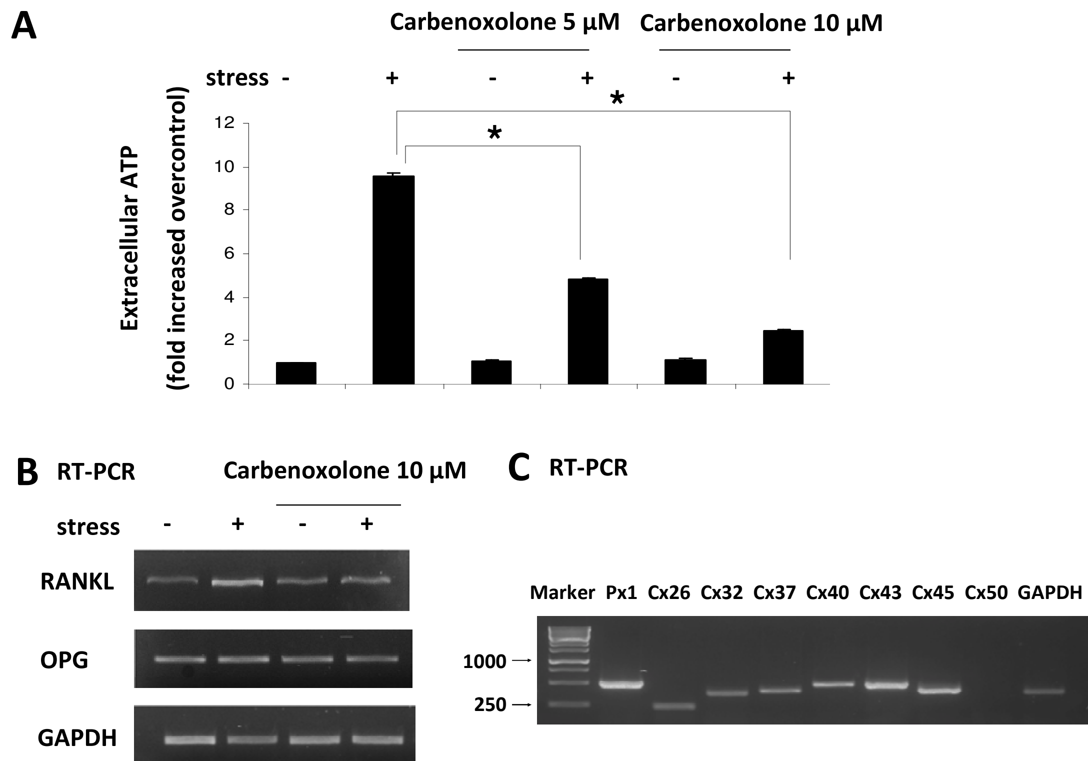

Figure 4.2 Mechanical stress (2.5 g/cm^2) was applied on cultured HPDL cells for 5 min and 2 hours in order to induce ATP release (Figure 4.2 A) and RANKL upregulation (Figure 4.2 B) respectively, in the presence or absence of a gap junction inhibitor carbenoxolone. The inhibitor was added 30 min prior to stress application. Carbenoxolone could attenuate the effect of mechanical stress on ATP release (Figure 4.2 A) and RANKL upregulation as judged by reverse transcription polymerase chain reaction (RT-PCR) (Figure 4.2 B).

Figure 4.2 C revealed the expression of gap junction proteins including connexin 26, 32, 37, 40, 43, 45 and pannexin-1 in HPDL cells using RT-PCR approach. Results were expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

Cx43 involved in stress-induced ATP release in HPDL cells

In order to clarify the type of gap junction involved in ATP release, the specific gap junction inhibitors were used. Specific inhibitors to Cx40 and Cx43 were selected based on the previous reports (Eltzschig et al., 2006; Faigle et al., 2008; Kang et al., 2008; Toma et al., 2008). Results from Figure 4.3 A indicated that Cx43 inhibitor (meclofenamic acid sodium salt), but not Cx40 inhibitor (spermine) attenuated the stress-induced ATP release. Application of Cx43 inhibitor, but not Cx40 inhibitor, also suppressed the mechanical stress-induced RANKL but not OPG at both mRNA (Figure 4.3 B) and protein levels (Figure 4.3 C), supporting the 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 hrs before mechanical stress application. Results indicated that Cx43 siRNA effectively reduced the level of Cx43 mRNA (Figure 4.4 A) and protein expression (Figure 4.4 B) as shown in lane 4 of Figure 4.4 A. Downregulation of Cx43 expression by siRNA resulted in a strong inhibition of mechanical stress-induced ATP release (Figure 4.4 C). MOCK transfected cells (transfection reagent alone) and cells transfected with control siRNA showed a normal pattern of ATP release in responses to mechanical stress (Figure 4.4 C).

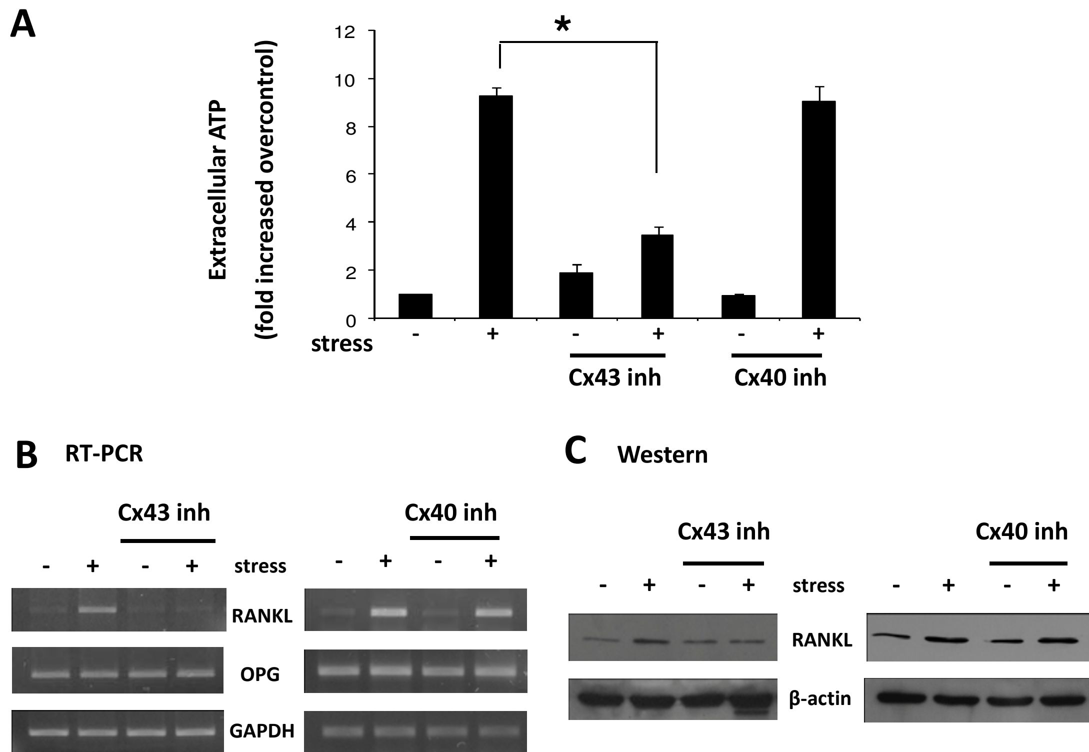

Figure 4.3 Mechanical stress (2.5 g/cm^2) was applied on cultured HPDL cells in the presence or absence of 20 μM meclofenamic acid sodium salt (Cx43 inhibitor) or 100 μM spermine (Cx40 inhibitor). Inhibitors were added 30 min prior to stress application. Culture medium was collected after 5 min and ATP was measured using Luciferin-luciferase assay. RNA and protein were extracted after 2 hours and 4 hours stress application respectively. Results showed Cx43 inhibitor, but not Cx40 inhibitor, could inhibit mechanical stress-induced ATP release (Figure 4.3 A) and upregulation of RANKL, but not OPG, at both mRNA (Figure 4.3 B) and protein level (Figure 4.3 C). Results were expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

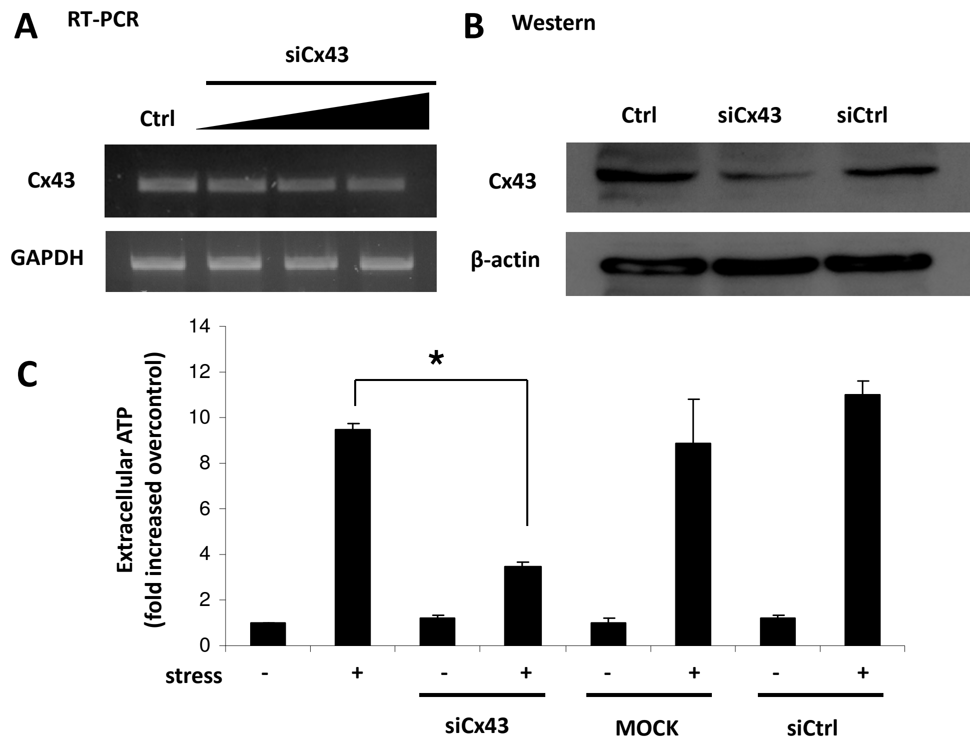

Figure 4.4 Figure 4.4 A and 4.4 B showed the mRNA and protein level of Cx43 in HPDL cells respectively, after transfected with Cx43 small interfering (si) RNA. Graph in Figure 4.4 C indicated that Cx43 siRNA transfection inhibited the inductive effect of mechanical stress-induced ATP release while the control siRNA (siCtrl) and the addition of transfection reagent alone (MOCK) had no effect. The results supported the function of Cx43 in ATP release. Results were expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

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. Results showed increase carboxyfluorescein uptake by HPDL cells after mechanical stress application, compared to the control (Figure 4.5 A - B). Furthermore, the inductive effect of mechanical stress on the opening of hemichannels was suppressed by Cx43 inhibitor (Figure 4.5 C - D) but not Cx40 inhibitor (Figure 4.5 E-F).

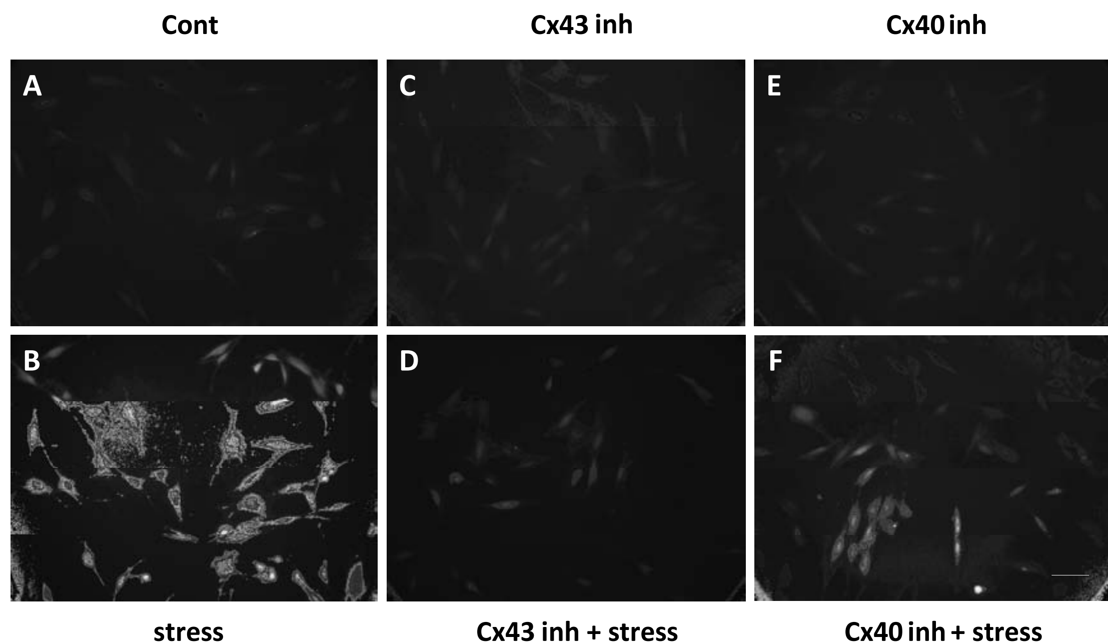

Figure 4.5 HPDL cells were incubated with 5 mM 5(6)-carboxyfluorescein before treated with 2.5 g/cm^2 of mechanical stress for 5 min. Meclofenamic acid sodium salt (Cx43 inhibitor) or spermine (Cx40 inhibitor) was

added 30 min prior to stress application. Cultures were washed with PBS and Tyrode's solution, and observed under fluorescent microscope. The presence of fluorescence signal indicated the influx of carboxyfluorescein into the cells after stress application (Figure 4.5 A-B). Addition of Cx43 (Figure 4.5 C-D) and Cx40 (Figure 4.5 E-F) inhibitors could reduce the fluorescence signal in HPDL cells receiving mechanical stress. Cx43 inhibitor seemed to be more effective than Cx40 inhibitor. Scale bar = 50 μm.

Intracellular calcium participated in stress-induced ATP release

It has been reported that the increase of intracellular calcium participated in the opening of hemichannels (Pearson et al., 2005; De Vuyst et al., 2006). To test whether intracellular calcium is involved in stress-induced gap junction opening, HPDL cells were incubated with TMB-8, an inhibitor that inhibits Ca^{2+} release from intracellular stores and Ca^{2+} influx, before treated with mechanical stress. As shown in Figure 4.6 A, TMB-8 suppressed the amount of stress-induced ATP release in a dose dependent manner. Moreover, addition of thapsigargin, an inhibitor of calcium-ATPase to deplete intracellular stores, could significantly abolish the extracellular ATP released when compared to control (Figure 4.6 B). These results supported that intracellular Ca^{2+} store is necessary for ATP release.

Next, two IP3 antagonists, heparin and 2-APB were used to examine the role of IP3 receptor. Results showed that both heparin (Figure 4.6 C) and 2-APB (Figure 4.6 D) significantly decreased mechanical stress-evoked ATP release,

supporting the role of IP3 receptor in stress-induced ATP release. Furthermore, carboxyfluorescein dye uptake was attenuated by thapsigargin and 2APB (Figure 4.7), suggested that intracellular Ca^{2+} signaling might be the upstream signal to gap junction opening in HPDL cells.

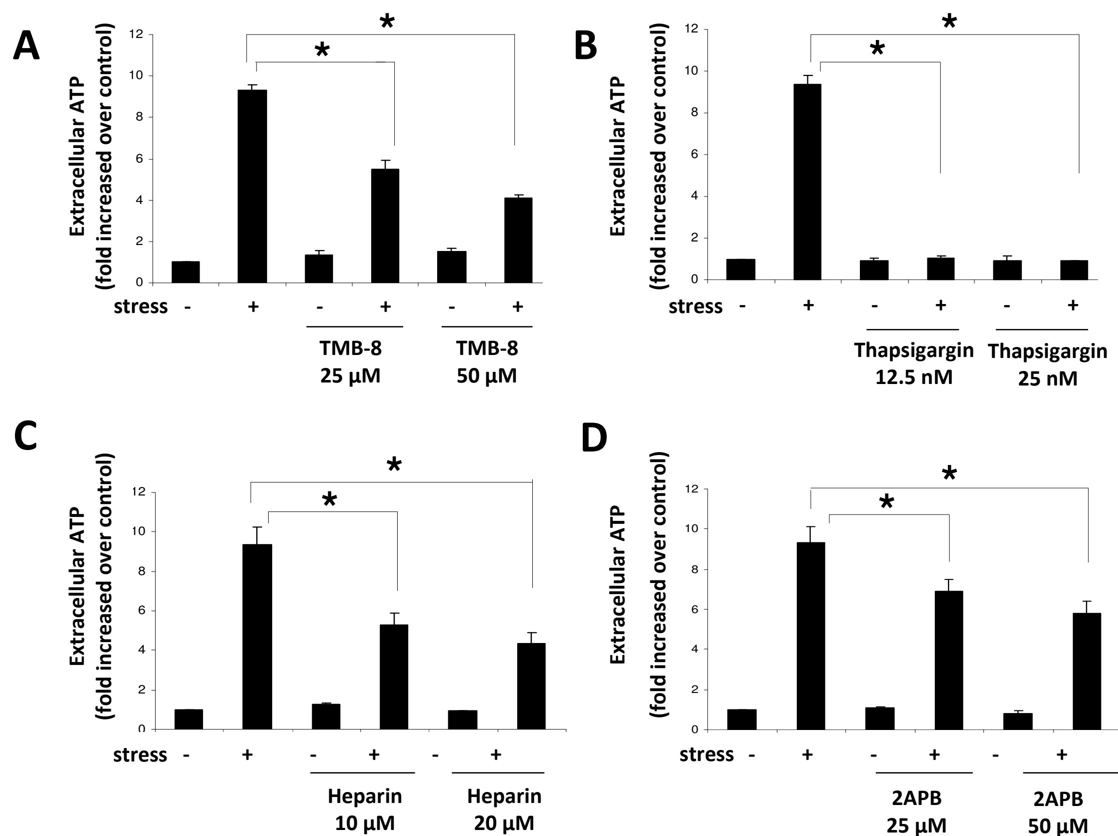

Figure 4.6 HPDL cells were pre-treated with intracellular calcium inhibitor TMB-8 (Figure 4.6 A), thapsigargin (Figure 4.6 B), IP3 inhibitors heparin (Figure 4.6 C) and 2-APB (Figure 4.6 D) for 30 min prior to mechanical stress application. All inhibitors could significantly inhibit ATP release induced by mechanical stress. Results were expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

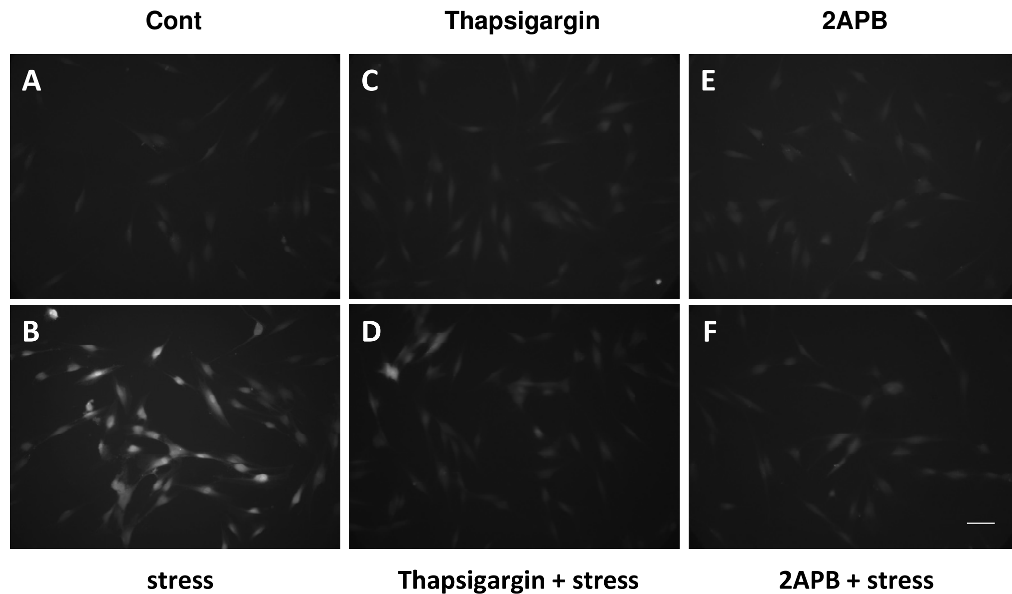

Figure 4.7 HPDL cells were incubated with thapsigargin or 2APB for 30 min prior to 5 min mechanical stress application. Addition of carboxyfluorescein showed the influx of fluorescence into the cells after stress application (Figure 4.7 A-B). The presence of thapsigargin and 2APB inhibited the uptake of carboxyfluorescein (Figure 4.7 C-F), indicating the role of intracellular Ca^{2+} signaling pathway and IP3 receptor in stress-induced hemichannel opening. Scale bar = 50 µm.

PART II : ATP stimulates RANKL expression through P2Y1 receptor–COX dependent pathway in HPDL cells

Extracellular ATP induced RANKL mRNA and protein expression.

HPDL cells were activated with 0, 10, 20 or 40 μM ATP, and the expressions of RANKL mRNA and protein were analyzed at 16 and 48 hours, respectively. The mRNA expression of RANKL was normalized to the expression level of GAPDH, while the amount of RANKL protein was normalized to the amount of actin. The results showed that ATP increased the expression of RANKL in a concentration-dependent manner at both transcriptional and translational levels (Figure 4.8 A, C). However, changes alteration neither in mRNA nor protein expression of OPG was observed (Figure 4.8 A, C). The relative band densities of RANKL/OPG from each experiment are depicted as histograms (Figure 4.8 B, D). The effective concentration (40 μM) of ATP was used for the rest of the experiments.

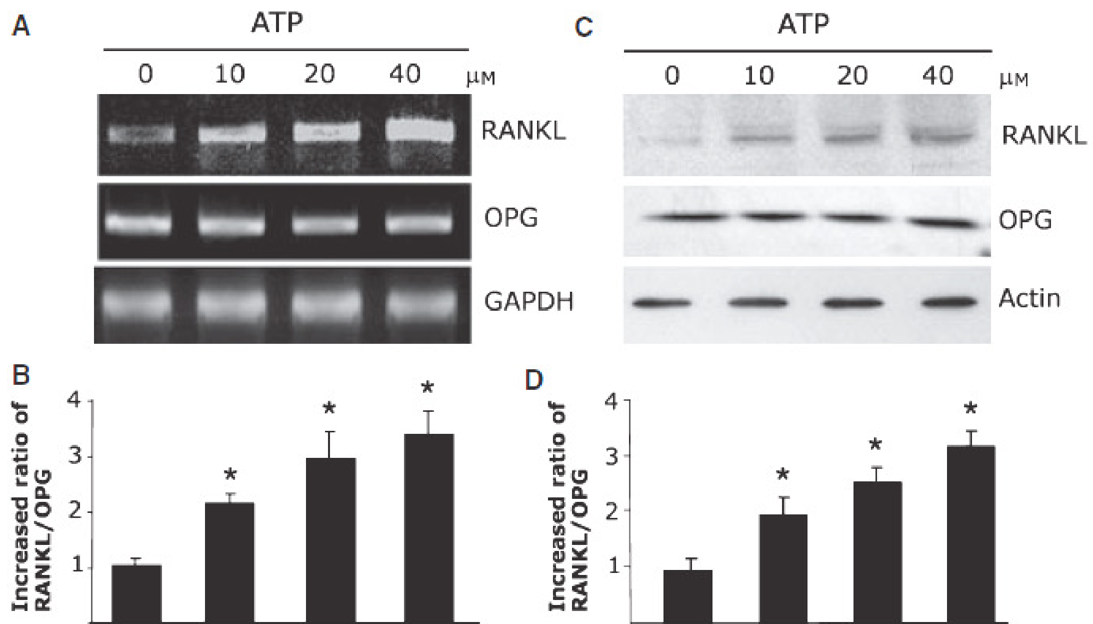

Figure 4.8 HPDL cells were treated with various concentrations of ATP ranging from 0 to 40 μM for 16 hours for RT-PCR and 48 hours for western analysis in serum-free conditions. The results from both RT-PCR (Figure 4.8 A) and western (Figure 4.8 C) showed that ATP increased the expression of RANKL, but not that of OPG, in a dose-dependent manner. The graphs represent the ratio of RANKL to OPG from RT-PCR (Figure 4.8 B) and western analysis (Figure 4.8 D). The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

Extracellular ATP stimulates RANKL production through COX-dependent pathway

To determine the intracellular pathway involved in the regulation of RANKL by ATP, indomethacin was used as an inhibitor. The results demonstrated that the elevation of RANKL induced by ATP was suppressed by indomethacin, a non-specific inhibitor of COX activity (Figure 4.9 A, B). To confirm that ATP mediated its signal via the COX pathway, the expressions of COX-1 and COX-2 and the production of PGE_2 were examined after stimulating the cells with 0–40 μM ATP. The result showed that ATP increased the expression of COX-2 but not COX-1 (Figure 4.9 C). Production of PGE_2 increased remarkably at concentrations of 20 and 40 μM (Figure 4.9 D). We also investigated which molecules are involved in the ATP-induced RANKL expression. Inhibitors were used to explore the signals involved. Pretreatment with NF-κB inhibitor PDTC or H89 dihydrochloride hydrate, a potent inhibitor of cAMP-dependent protein kinase (PKA), inhibited the elevation of both RANKL (Figure 4.10 A) and PGE_2 (Figure 4.10 B), suggesting that cAMP and NF-κB are the upstream signals of the PGE_2–RANKL axis in response to ATP in HPDL cells. To confirm the involvement of cAMP, an adenylyl cyclase activator, forskolin, was used. It appeared that forskolin increased the synthesis of RANKL and increased the release of PGE_2 from HPDL cells (Figure 4.10 C, D). Immunofluorescent staining was also used to explore the NF-κB nuclear translocation. The results showed that ATP induced NF-κB translocation and the effect was attenuated by H89 as well as by PDTC (Figure 4.11).

To investigate whether P2Y1 is the receptor through which ATP mediates its signal to up-regulate COX-2 and RANKL expression, a specific P2Y1

antagonist, MRS2179, was applied to the cultures prior to addition of ATP. It appeared that MRS2179 inhibited the stimulatory effect of ATP on COX-2 expression (Figure 4.12 A) and PGE_2 production (Figure 4.12 B). In addition, the expression of RANKL mRNA and protein were attenuated (Figure 4.12 C, D). The results suggested that ATP could act through the P2Y1 receptor.

Small interfering RNA was used as another approach to confirm the results obtained using P2Y1 receptor antagonist. Control small interfering RNA and P2Y1 small interfering RNA (120, 140 and 180 pmol) were transfected into HPDL cells for 24 hours before application of ATP. The results confirmed that P2Y1 small interfering RNA reduced P2Y1 mRNA and protein expression effectively at 120 pmol (Figure 4.13 A) and that it exerted an inhibitory effect on both PGE_2 release (Figure 4.13 B) and RANKL expression (Figure 4.13 C, D) similar to that exerted by P2Y1 receptor antagonist.

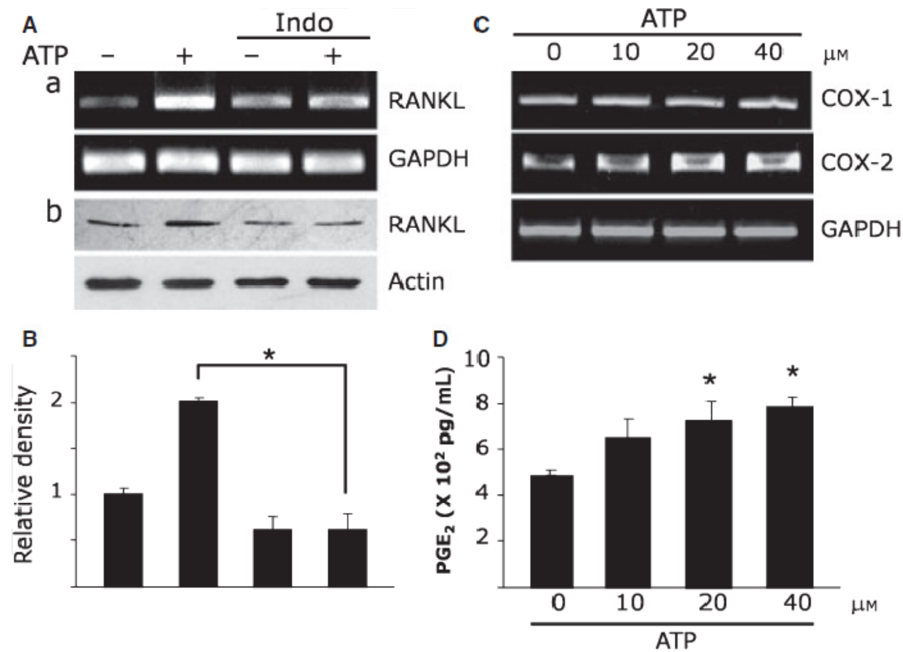

Figure 4.9 (Figure 4.9 A) HPDL cells were treated with 40 μM ATP in the presence or absence of indomethacin, a non-specific COX inhibitor, to determine the role of COX in the mechanism. The inhibitor was added 30 min before the treatment. The RNA was extracted at 16 hours, and the cell protein extract was collected at 48 hours after the treatment. The results showed that application of indomethacin could inhibit the up-regulation of RANKL expression induced by ATP at both the mRNA (Figure 4.9 Aa) and the protein levels (Figure 4.9 Ab). The graph (Figure 4.9 B) represents the band density from western analysis. The results are expressed as means ± SD from three different experiments. (Figure 4.9 C) ATP induced COX-2 mRNA after treatment with various concentrations of ATP. The results revealed that the expression of COX-2 increased in a concentration-dependent manner. (Figure 4.9 D) The amount of PGE_2 in the culture medium was also measured by ELISA after 2 hours of exposure to ATP. We found that ATP increased PGE_2 production significantly in a dose-dependent manner. The graph shows the mean ± SD of band density from three separate experiments. *Significant difference, $p < 0.05$.

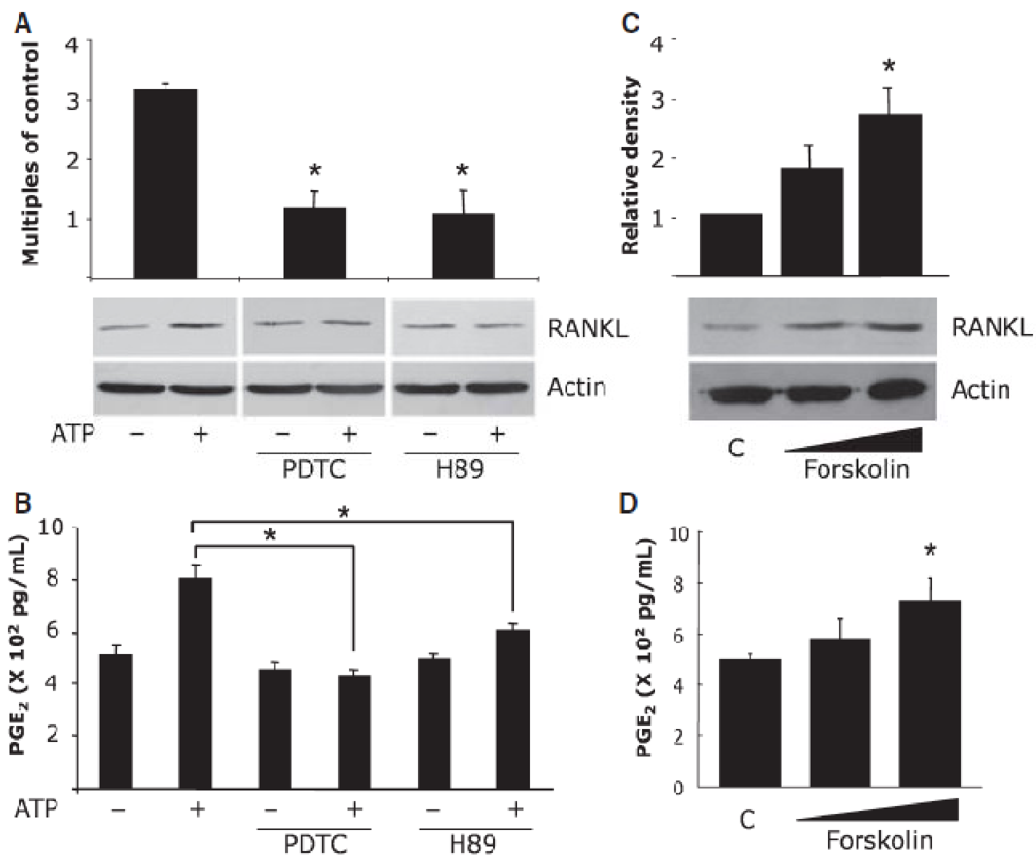

Figure 4.10 The stimulatory effect of ATP was abolished by PDTC and H89. Cells were treated with PDTC, an NF-κB inhibitor, and H89, a cAMP-dependent protein kinase inhibitor, 30 min before the addition of ATP. The protein extract was collected at 48 hours for western analysis and the culture medium was collected at 2 hours for ELISA after the treatment. The results showed that ATP-induced RANKL (Figure 4.10 A) and PGE_2 production (Figure 4.10 B) were inhibited by both PDTC and H89. The effects of forskolin, an activator of adenylylcyclase, on RANKL expression and PGE_2 release are shown in (Figure 4.10 C) and (Figure 4.10 D), respectively. *Significant difference, $p < 0.05$.

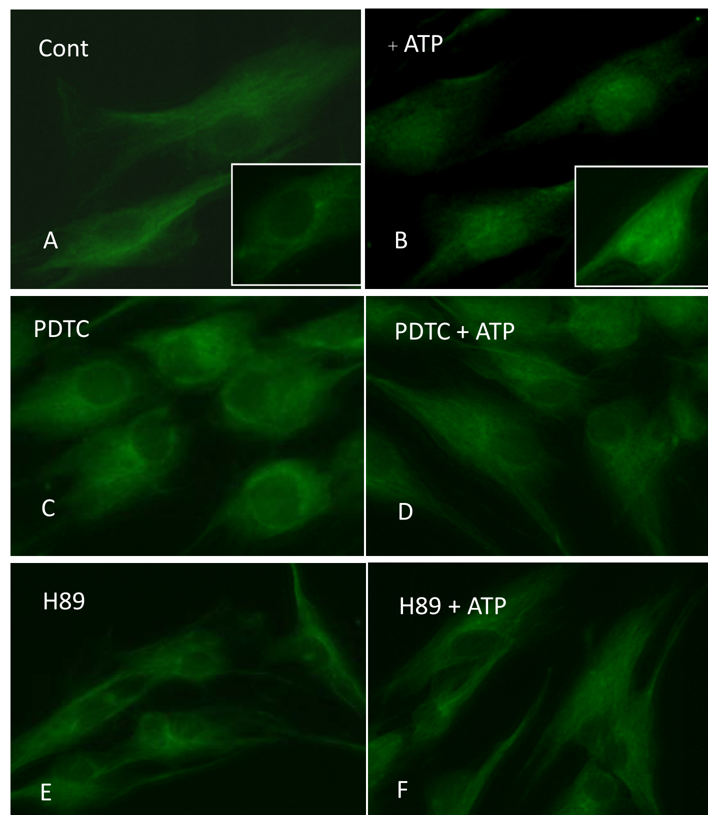

Figure 4.11 HPDL cells were incubated with or without inhibitor, PDTC or H89, for 30 min before being treated with ATP for 2 hours. Immunofluorescent staining showed that ATP enhanced nuclear localization of NF-κB (Figure 4.11 B) when compared with the control treatment (Figure 4.11 A). The effect of ATP was abolished by PDTC (Figure 4.11 D) and H89 (Figure 4.11 F) when compared with their corresponding control cultures (Figure 4.11 C, Figure 4.11 E).

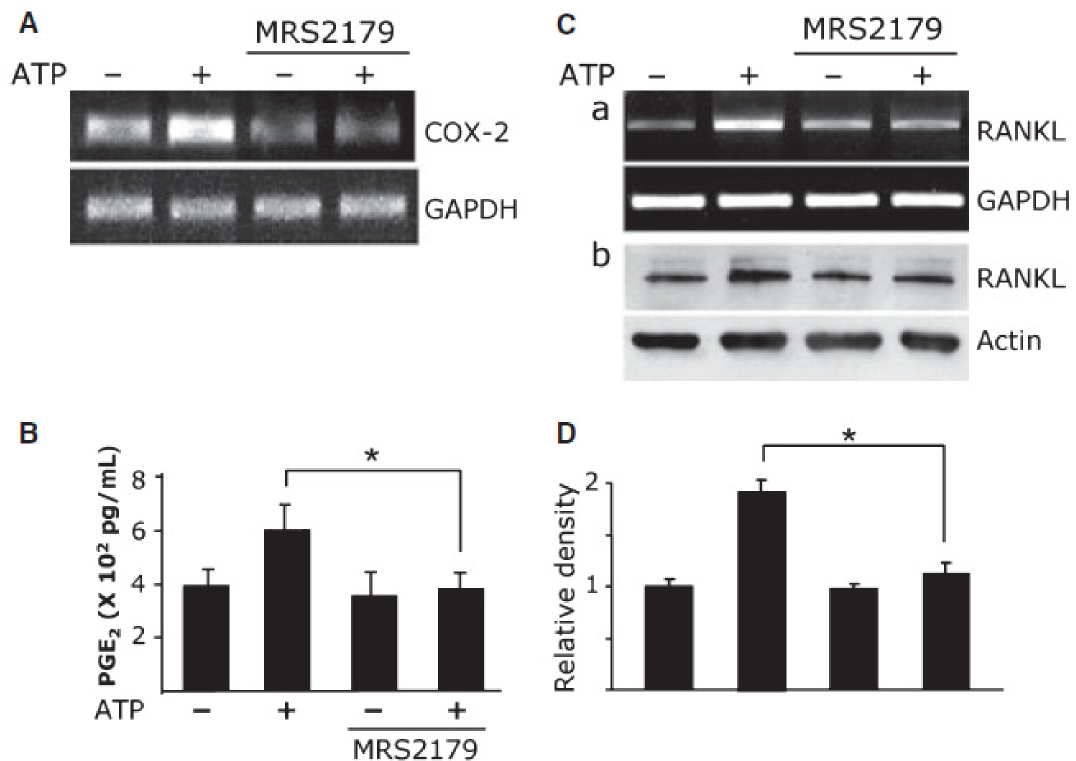

Figure 4.12 HPDL cells were pre-incubated with MRS2179, a specific P2Y1 receptor antagonist, for 30 min prior to addition of ATP. After 16 and 48 hours of exposure to ATP, the RNA and protein extract were collected for RT-PCR and western analysis, respectively. The culture medium from a parallel set of experiments was collected after 2 hours of treatment for ELISA. The results showed that MRS2179 exerted an inhibitory effect on the ATP-induced COX-2 expression (Figure 4.12 A) and PGE_2 production (Figure 4.12 B). MRS2179 attenuated the ATP-induced RANKL expression at both the mRNA (Figure 4.12 Ca) and the protein level (Figure 4.12 Cb). The graph (Figure 4.12 D) represents the band density from western analysis. The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

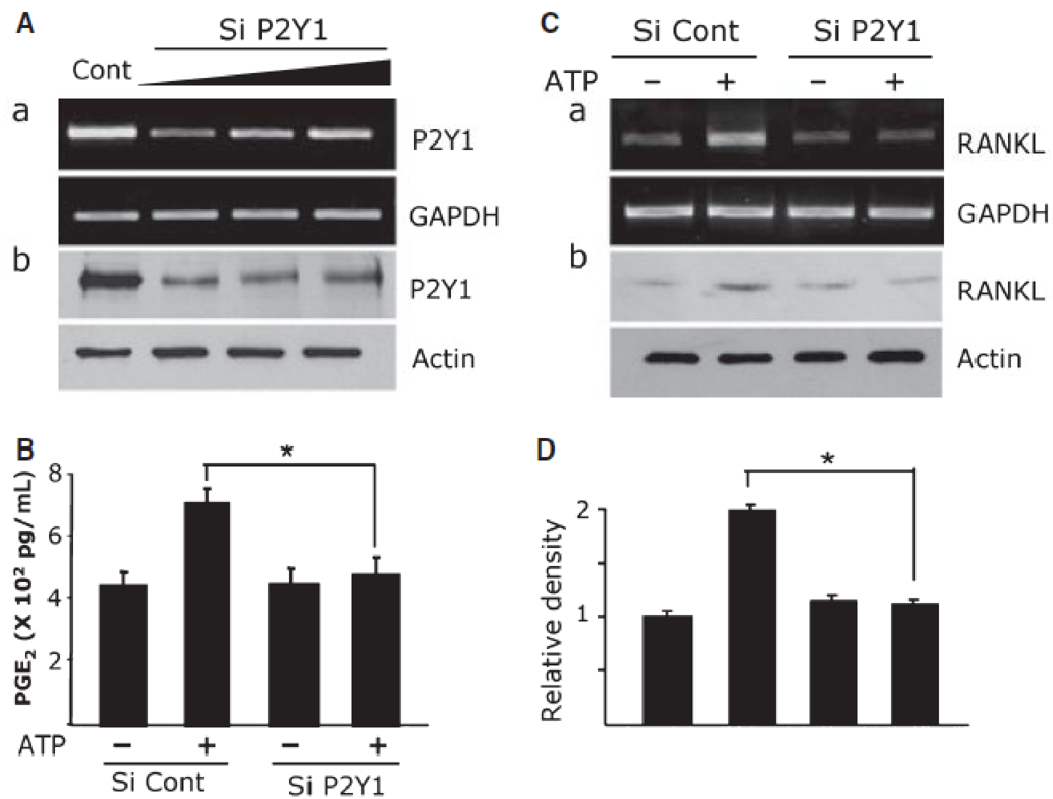

Figure 4.13 HPDL cells were transfected with P2Y1 small interfering (Si) RNA. (Figure 4.13 A) The P2Y1 small interfering RNA could interfere with the expression of the P2Y1 receptor at both the mRNA (Figure 4.13 Aa) and the protein level (Figure 4.13 Ab). The P2Y1 small interfering RNA inhibited the release of PGE_2 (Figure 4.13 B) as well as the expression of RANKL (Figure 4.13 C). The graph represents the band density from western analysis (Figure 4.13 D). The results are expressed as means ± SD from three different experiments. *Significant difference, $p < 0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

HPDL cells are responsible for the mechanical stress transduced from the tooth, which is significant during periodontal tissue remodeling and repair (Lekic and McCulloch, 1996). Moderate mechanical stress is the key factor in maintaining bone mass (Deschner et al., 2003). Excessive mechanical stress can induce bone loss through imbalance of the RANKL and OPG (Kanzaki et al., 2002; Yamaguchi et al., 2006). This study provided the first evidence that nucleotide could act as a signaling molecule in HPDL cells.

From the results in this study, the model of the molecular mechanism of mechanical stress-induced RANKL expression in HPDL cells could be propose as shown in figure 5.1.

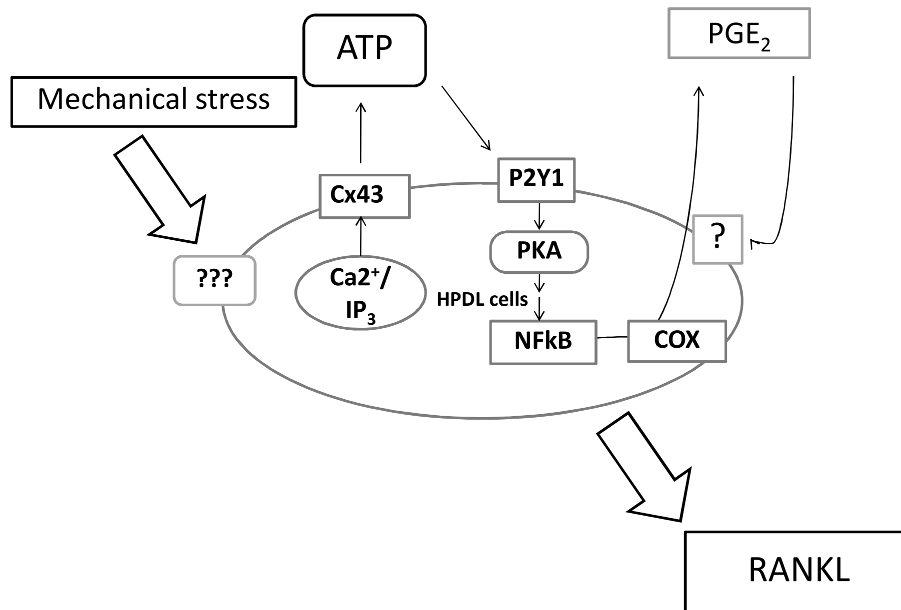

Figure 5.1 The mechanism of RANKL expression induced by mechanical stress in HPDL cells.

As shown in the figure, mechanical stress-induced ATP release through Cx43 hemichannel. The mechanism of ATP release may depend on intracellular Ca^{2+} signaling pathway. Our results also indicated that extracellular ATP stimulates RANKL production through the COX-dependent pathway. In addition, the stimulatory effect is mediated through the P2Y1 receptor in HPDL cells, which mediates its signal through PKA and NF-κB. Activation of NF-κB causes the release of PGE_2 via a COX-dependent pathway. The activation of RANKL expression is a consequence of the released PGE_2, after which the cascade of signaling molecules involved requires further clarification.

A number of studies have demonstrated that mechanical stress or biological activation could induce ATP release via lytic or non-lytic mechanisms involving vesicle-mediated secretion, carrier-mediated transport or plasma membrane channels (Burrell et al., 2005; Fitz., 2007). The mechanical stress-induced ATP release has been reported in several cell types such as subepithelial fibroblasts (Furuya et al., 2005), chondrocytes (Graff et al., 2000), airway epithelial cells (Homolya et al., 2000), osteoblastic cells (Romanello et al., 2001) and HPDL cells (Wongkhantee et al., 2008). However, the detailed mechanism of ATP release is still not fully understood.

In the present study, we demonstrated that mechanical stress induced ATP release in HPDL cells. The amount of ATP released from HPDL cells could be detected as fast as 5 minutes after stress application. The 5 min period was selected in all experiment.

The mechanism of mechanical stress-induced ATP release was study in details. Results provided evidences that, in HPDL cells, ATP was released through Cx43 hemichannels. Moreover, the mechanism of ATP release was regulated by intracellular Ca^{2+} signaling pathway.

Our results indicated that HPDL cells expressed at least 7 gap junction proteins including Cx26, 32, 37, 40, 43, 45 and pannexin1, similar to the study of Yamaoka et al. whose report the expression of Cx32, Cx40, Cx43, and Cx45 in PDL cells (Yamaoka et al., 2002). The function of other Cx in HPDL cells is still unknown.

Our findings were corresponded with Bennett et al. report, which showed that ATP and other small molecules can be released via hemichannel opening (Bennett et al., 2003). In this study, we focused on the role of Cx43 and Cx40 since evidences were 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 (Romanello et al., 2001). The

efflux of ATP through Cx43 hemichannels in C6 glioma cells was also identified in the membrane of hippocampal astrocytes in acutely prepared slices (Kang et al., 2008). In addition, mechanical stimulation mediated ATP release through Cx40 hemichannel in a single glomerular endothelium cells (Toma et al., 2008). Based on the strong blocking effects of Cx43 inhibitor and Cx43 siRNA, the results indicated that Cx43 was one of the ATP releasing channels in HPDL cells.

The balance of RANKL and OPG is important for osteoclast formation, activation and survival (Hofbauer and Heufelder, 2001). Therefore, increase expression of RANKL, but not OPG, would increase the RANKL/OPG ratio and promote the osteoclastogenesis. In this study showed Cx43 inhibitor, but not Cx40 inhibitor, could inhibit upregulation of RANKL, but not OPG, by mechanical stress. This result is consistent with previously reported that mechanical stress evoked RANKL expression, but not OPG, could be a consequence of ATP release (Wongkhantee et al., 2008).

Moreover, the function of gap junction hemichannels was demonstrated by using carbenoxolone disodium salt, a nonspecific gap junction blockers. Inhibitory effect of carbenoxolone on mechanical stress-induced ATP release supported that gap junction hemichannels are involved in the mechanism.

Transmembrane flux of low molecular weight dye, 5(6)-carboxyfluorescein, is a commonly used method for assessing the presence and function of connexin hemichannels (Li et al., 1996; Vergara et al., 2003). Our study showed that mechanical stimulation of HPDL cells could evoke both dye uptake as well as ATP release. The fact that Cx43 inhibitor suppressed dye uptake by mechanical stress stimulation suggested Cx43 might be a major gap junction protein that response to mechanical stress. In addition, the present work demonstrated that

mechanical stress-induced ATP release or dye uptake might require the 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 Ca^{2+} signaling cascade.

The Ca^{2+} signaling associated with ATP release has been reported (Stout et al., 2002; Cotrina et al., 1998). Increase intracellular Ca^{2+} has been implicated in ATP release from many cell types including osteoblast-like cells (Genetos et al., 2005; Liu et al., 2008). The question remains whether the increased of intracellular Ca^{2+} came from the extracellular Ca^{2+} influx. Pretreatment with EGTA, a calcium chelator, in the medium or using Ca^{2+} free medium in the experiments did not alter mechanical stress induced ATP released, suggesting that the extracellular calcium is not involved in the induction Application of TMB-8 and thapsigargin to abolish the intracellular calcium could significantly abolish the mechanical stress induced ATP released. These data suggest mechanical stress induced ATP release depends on the intracellular Ca^{2+} release from Ca^{2+} storage.

Furthermore, reports suggested that the increase in cytoplasmic free Ca^{2+} concentration could trigger Cx hemichannels opening (Pearson et al., 2005; De Vuyst et al., 2006). It is possible that mechanical stress altered intracellular Ca^{2+} level, leading to the opening of Cx43 hemichannel and causing the release of ATP in HPDL cells. In agreement with the role of increase intracellular Ca^{2+} levels in ATP release, application of Ca^{2+} blocker could inhibit mechanical stress-induced ATP release suggested the importance of Ca^{2+} and Cx43 in regulating mechanical stress induced ATP release in HPDL cells.

ATP is one of the extracellular signaling molecules that regulates various biological processes including cell proliferation, cell differentiation, muscle contraction and intercellular communication (Burnstock, 1997). The significant

function of ATP in controlling cellular behavior has been suggested. For example, extracellular ATP could promote collagen deposition in osteoblast culture (Nakano et al., 2007) while inhibiting in vitro calcification (Orriss et al., 2007). The role of ATP on the up-regulation of RANKL has been reported mostly in osteoblasts. Buckley et al. demonstrated that ATP stimulated human osteoclast activity via the upregulation of osteoblast expressed RANKL (Buckley et al., 2002). Jin-Man Kim and co-workers also demonstrated that blocking ATP generation significantly decreases RANKL-stimulated osteoclast differentiation (Kim et al., 2007). However, the increase in the level of RANKL generated by ATP in HPDL cells has not been elucidated.

The involvement of COX/PGE_2 in the regulation of RANKL expression has been reported by Kanzaki and co-workers (Kanzaki et al., 2002). They demonstrated that compressive force stimulated osteoclastogenesis in periodontal ligament cells by increasing the expression of RANKL and PGE_2 production. We considered the possibility that PGE_2 might be an intermediate mediator of the ATP-induced RANKL expression.

The expression of COX-1 and COX-2 and the production of PGE_2 were examined, and the results showed that COX-2 and PGE_2 responded to ATP stimulation. The involvement of PGE2 was supported by the fact that of induction of ATP released was abrogated by indomethacin, an inhibitor of prostanoid synthesis. In addition, these results were consistent with previous reports demonstrating the effect of ATP on the release of PGE_2 in many cell types, such as endothelial cells (Hashimoto et al., 1995), astrocytes (Xu et al., 2003) and epithelial cells (Ruan et al., 2008). We conclude that, in HPDL cells, the up-regulation of RANKL by ATP is via a COX-dependent pathway.

The blocking effect exerted by NF-κB inhibitor and PKA inhibitor on the release of PGE_2 and the expression of RANKL also suggests that NF-κB and

cAMP are the upstream regulators of the PGE_2 release. Our study indicated that ATP induced the translocation of NF-κB and the translocation was suppressed by the inhibitor of cAMP-dependent protein kinase. The results suggest that cAMP could be the up-stream signal of NF-κB. It is tempting to speculate that ATP induces the formation of cAMP and causes activation of NF-κB. Subsequently, activation of NF-κB will activate cyclo-oxygenase, resulting in the release of PGE_2. The involvement of cAMP in ATP-induced RANKL expression was further confirmed by the use of forskolin. Addition of forskolin without ATP could increase the expression of RANKL at both the mRNA and the protein level. The finding that NF-κB is one of the downstream targets of the P2 receptor agreed with the report by Korcok et al., which demonstrated that nucleotides acted through P2Y6 receptors to initiate NF-κB signaling in osteoclasts (Korcok et al., 2005). In addition, the role of NF-κB in controlling the release of PGE_2 had been reported (Jung et al., 2003). However, our data suggest that the increase of PGE_2 and RANKL stimulated by ATP are not a consequence of PLC activity. This finding is different from those found in other cell types including epithelial cells (Ruan et al., 2008; Bucheimer and Linden, 2004), which proposed that ATP-induced release of PGE_2 was dependent on intracellular Ca^{2+} and phospholipase C activity.

In regard to the ATP receptor, ATP is able to act through almost all subtypes of P2 receptors to exert various effects (Hoebertz et al., 2003). It has been shown to initiate NF-κB signaling and enhance survival through P2Y6 (Korcok et al., 2005) and P2X7 receptors (Korcok et al., 2004) in osteoclasts, to activate DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells (Nakamura et al., 2000) and to generate resorption pits on dentin disks by elevating RANKL expression in osteoblast-like UMR-106 cells, where the P2Y1 receptor is predominantly expressed (Buckley et al., 2002). These results indicate that locally

acting ATP may play a pivotal role in osteoclast activation at bone-resorbing sites by inducing elevated expression of RANKL in bone cells. For HPDL cells, we found in the previous study that perturbing the function of the P2Y1 receptor using MRS2179, a specific inhibitor of the P2Y1 receptor, attenuated the inductive effect of ATP on osteopontin expression (Wongkhantee et al., 2008). In the present study, a similar blocking effect on ATP-induced RANKL expression was seen using MRS2179 as well as small interfering RNA. In addition, it is notable that interference with the function of the P2Y1 receptor exerted an almost complete inhibition on the stimulatory effect of ATP, indicating that the P2Y1 receptor could be the main receptor involved in the regulation of both RANKL and osteopontin expression by ATP in HPDL cells.

Since mechanical stress induces expression of PGE_2 (Kanzaki et al., 2002) as well as proinflammatory cytokines such as interleukin-1beta (Nakao et al., 2007), which is able to stimulate the release of PGE_2, it is possible that the release of PGE_2 is a result of the action of those proinflammatory cytokines. We demonstrated in the previous report that mechanical stress increased the level of ATP (Wongkhantee et al., 2008) and revealed in the present study that ATP could cause a rise in PGE_2 production within 2 hours. Our results advance the understanding that, besides proinflammatory cytokines, ATP is one of the upstream signaling molecules for PGE_2 release in response to mechanical stress in HPDL cells and plays a role in periodontal tissue homeostasis through the P2Y1 receptor.

In conclusion, the present study showed that mechanical stress-induced ATP release through Cx43 hemichannel. The mechanism of ATP release may depend on intracellular Ca^{2+} signaling pathway. After release, ATP can activate the P2Y1 receptor, which signal through PKA and NF-κB signaling cascade, resulting in PGE_2 release via a COX-dependent pathway. The activation of RANKL expression is a consequence of the released PGE_2, after which the cascade of signaling molecules involved requires further clarification.

Future Studies

The interesting topics for future investigation include:

1. To find the cell surface receptors that respond to mechanical stress.
2. To determine the downstream target of PGE_2 signaling cascade in ATP-induced RANKL synthesis.
3. To investigate the function of other type of P2 receptors in HPDL cells.
4. To investigate the function of other type of Cx hemichannels in HPDL cells.

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