บทบาทของอะดีโนซีนไตรฟอสเฟต (เอทีพี) และแกปจังชันเฮมิแชนแนล ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ที่ถูกกระตุ้นด้วยแรงกดเชิงกล

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Roles of adenosine triphosphate (ATP) and gap junction hemichannels in mechanically-stimulated human periodontal ligament cells

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กวิตา กาญจนเมฆานันต์ : บทบาทของอะดีโนซีนไตรฟอสเฟต (เอทีพี) และแกปจังชันเฮมิแชนแนลในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ที่ถูกกระตุ้นด้วยแรงกดเชิงกล. (ROLES OF ADENOSINE TRIPHOSPHATE (ATP) AND GAP JUNCTION HEMICHANNELS IN MECHANICALLY-STIMULATED HUMAN PERIODONTAL LIGAMENT CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: Prof. Vincent Everts, 111หน้า.

แรงกดเชิงกลจัดเป็นปัจจัยสำคัญในการคงสภาวะสมดุลของเนื้อเยื่อปริทันต์ การศึกษาก่อนหน้าพบว่า แรงกดเชิงกลสามารถกระตุ้นการหลั่งของเอทีพี (ATP) ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ ในการศึกษาครั้งนี้ พบว่า เอทีพีมีบทบาทในการกระตุ้นการแสดงออกในระดับเอ็มอาร์เอ็นเอ (mRNA) และการหลั่งของสารเหนี่ยวนำการอักเสบ อินเทอร์ลิวคินวันเบต้า ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ที่ได้รับแรงกดเชิงกล เอทีพีที่หลั่งออกมานี้สามารถส่งสัญญาณเข้าสู่เซลล์ผ่านทางพีทูรีเซปเตอร์ (P2 receptor) การทดลองโดยใช้สารยับยั้งการทำงานและการแสดงออกของพีทูรีเซปเตอร์แสดงให้เห็นว่า พีทูเอ็กซ์เซเว่นรีเซปเตอร์ (P2X7 receptor) ทำหน้าที่เป็นรีเซปเตอร์หลักที่เกี่ยวข้องกับการกระตุ้นการแสดงออกของอินเทอร์ลิวคินวันเบต้าโดยเอทีพี ในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ที่ได้รับแรงกดเชิงกล รายงานในเซลล์แมคโครเฟจ (macrophages) นำเสนอหลักฐานว่า แพนเนกซินวันเฮมิแชนแนล (Pannexin-1 hemichannel) จะรวมตัวกับพีทูเอกซ์เซเว่นรีเซปเตอร์ กลายเป็นสารเชิงซ้อนที่มีความสามารถในการรับรู้แรงกดเชิงกลและมีส่วนในการควบคุมการแสดงออกของอินเทอร์ลิวคินวันเบต้า ในการศึกษาครั้งนี้ ผู้วิจัยพบว่าในภาวการณ์ขาดแพนเนกซินวัน การเหนี่ยวนำการแสดงออกของอินเทอร์ลิวคินวันเบต้าทั้งจากการกระตุ้นด้วยแรงกดเชิงกลและเอทีพีจะลดลงอย่างมาก นอกจากนี้ การศึกษาด้วยเทคนิคอิมมูโนไซโตเคมิสตรี (Immunocyto-chemistry) และโค-อิมมูโนเพรซิพิเทชั่น (Co-immunoprecipitation) เปิดเผยถึงการแสดงออกในบริเวณเดียวกัน (co-localization) ของพีทูเอกซ์เซเว่นรีเซปเตอร์และแพนเนกซินวันเฮมิแชนแนลในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ ซึ่งพบว่ามีการเพิ่มขึ้นหลังจากได้รับแรงกดเชิงกล ผลการศึกษายังพบว่าแพนเนกซินวันเฮมิแชนแนลอาจทำหน้าที่เป็นช่องทางหนึ่งในการหลั่งของเอทีพีออกจากเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ การศึกษากระบวนการหลั่งของอินเทอร์ลิวคินวันเบต้าโดยการใช้สารยับยั้งการเคลื่อนของเวซิเคิลภายในเซลล์ พบว่าสารดังกล่าวสามารถลดการหลั่งของอินเทอร์ลิวคินวันเบต้าในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ที่ได้รับแรงกดเชิงกลโดยไม่มีผลกับการแสดงออกในระดับโมเลกุล นอกจากนี้ยังพบว่า สแนปทเวนที้ไฟว์ (SNAP-25) ซึ่งเป็นโปรตีนหลักในการเกิดสารเชิงซ้อนขณะมีการเชื่อมระหว่างเวซิเคิลและเมมเบรน มีการแสดงออกที่บริเวณเดียวกันกับพีทูเอกซ์เซเว่นรีเซปเตอร์และแพนเนกซินวัน ข้อมูลดังกล่าวบ่งชี้ว่าการหลั่งอินเทอร์ลิวคินวันเบต้าในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์อาจเกิดผ่านการปลดปล่อยเวซิเคิล กล่าวโดยสรุป แรงกดเชิงกลสามารถควบคุมการแสดงออกและการหลั่งอินเทอร์ลิวคินวันเบต้าในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ผ่านกระบวนการที่เกี่ยวข้องกับเอทีพีและพีทูเอกซ์เซเว่นรีเซปเตอร์/แพนเนกซินวัน โดยแพนเนกซินวันและพีทูเอกซ์เซเว่นรีเซปเตอร์อาจมีหน้าที่สำคัญในการกระตุ้นการแสดงออกของอินเทอร์ลิวคินวันเบต้า รวมถึงอาจมีบทบาทในการเป็นบริเวณที่เหนี่ยวนำให้เกิดการหลั่งอินเทอร์ลิวคินวันเบต้าผ่านทางเวซิเคิล

สาขาวิชา .....ชีววิทยาช่องปาก..... ลายมือชื่อนิสิต .....

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5176451432 : MAJOR ORAL BIOLOGY
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KAVITA KANJANAMEKANANT: ROLES OF ADENOSINE TRIPHOSPHATE (ATP) AND GAP JUNCTION HEMICHANNELS IN MECHANICALLY-STIMULATED HUMAN PERIODONTAL LIGAMENT CELLS. ADVISOR: PROF. PRASIT PAVASANT, D.D.S., Ph.D., CO-ADVISOR: PROF. VINCENT EVERTS, Ph.D., 111 pp.

Mechanical stress is an important factor in maintaining periodontium homeostasis. We previously reported that mechanical stress could activate ATP release in human periodontal ligament (HPDL) cells. In this study, we found that ATP mediated mechanical stress-induced mRNA expression and release of the pro-inflammatory cytokine IL-1β. The released ATP later activates P2 receptor. By using inhibitors and siRNA experiments, we found that P2X7 receptor was the main P2 subtype responsible for ATP-induced upregulation of IL-1β in HPDL cells. In macrophages, Pannexin-1 (Panx1) hemichannel has been proposed to combine with P2X7 receptor, forming a mechanosensitive complex involving in the regulation of IL-1β. In this work, we found that, in the absence of Panx1, upregulation of IL-1β upon both mechanical and ATP stimulation was drastically diminished. Additionally, immunocytochemistry and co-immunoprecipitation revealed the co-localization of P2X7 receptor and Panx1 hemichannel in HPDL cells, which found to be increased after stress application. Results also showed that Panx1 hemichannel might serve as one of the ATP release pathway in HPDL cells. The IL-1β releasing mechanism was also investigated. Pretreatment with vesicular trafficking inhibitors significantly reduced IL-1β release from HPDL cells, while mRNA induction remained unaffected. Membrane co-localization of SNAP-25, a core protein of vesicular-membrane fusion SNARE complex, with P2X7 receptor/Panx1 was observed. Collectively, these data indicated vesicular release of IL-1β. In conclusion, mechanical stress could directly regulate IL-1β expression and vesicular release in HPDL cells through ATP-gated P2X7 receptor/Panx1-dependent pathway. Function of Panx1/P2X7 receptor might be required in the IL-1β induction mechanism, and their possible novel function as docking sites for IL-1β vesicular release was also indicated.

Field of Study : Oral Biology Student's Signature .....

Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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CONTENTS

Page

Abstract (Thai) iv

Abstract (English) v

Acknowledgements vi

Contents vii

Lists of Tables x

Lists of Figures xi

Lists of Abbreviations xiii

CHAPTER I INTRODUCTION 1

CHAPTER II REVIEW OF RELATED LITERATURE 8

Mechanical stress 8

Adenosine Triphosphate (ATP) 13

Purinergic receptors 17

Gap junctions 28

Inflammatory cytokines 37

Interleukin-1 (IL-1) 37

CHAPTER III RESEARCH METHODOLOGY 45

Cell culture..... 45

HPDL cells stimulation..... 45

MTT assay..... 47

Measurement of interleukin1-beta (IL-1β) by enzyme-linked immunosorbent assay (ELISA) 47

Page

Measurement of ATP release by bioluminescence assay..... 47
Inhibitors and peptides 48
Transfection of small interfering RNA (siRNA) 49
RNA Extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) 49
Western blot analysis protein detection..... 52
Co-immunoprecipitation (Co-IP)..... 52
Immunocytochemistry..... 53
Dye-uptake assay..... 54
Statistical analysis..... 55

CHAPTER IV RESULTS 56
Mechanical stress induced IL-1β expression and nucleotide release in in HPDL cells 56
ATP acts as a signaling molecule in mechanical stress-induced IL-1β expression 59
Mechanical stress induced gap junction hemichannels opening in HPDL cells 62
Panx1 and Cx43 hemichannels participated in mechanical stress-induced IL-1β expression 64
P2X7 receptor is a major P2 subtype responsible for IL-1β induction in HPDL cells 69
The IL-1β induction is calcium-dependent 72
Co-expression of Panx1 and P2X7 receptor in HPDL cells 74
IL-1β release mechanism in HPDL cells 76

Page

CHAPTER V DISCUSSION AND CONCLUSION 79

REFERENCES 89

VITA 111

LIST OF TABLES

		Page
Table 2.1	Characteristics of purine-mediated receptors ……	20
Table 2.2	Principal P2X receptors expressed by excitable tissues and nonneuronal cells ……..	22
Table 2.3	Evidences and possible functions for P2 receptors in bone cells ……..	24
Table 2.4	Properties of P2Y receptors ……	27
Table 2.5	IL-1 family members ……..	39
Table 2.6	The IL-1 receptor family ……..	42
Table 3.1	Primers details ……	51

LIST OF FIGURES

		Page
Figure 2.1	Mediators of cellular mechanotransduction	11
Figure 2.2	Molecular structure of ATP and ADP	14
Figure 2.3	Proposed model for ATP release mechanism from non-excitable cells	17
Figure 2.4	The purinergic receptors	19
Figure 2.5	Schematic drawing of transmembrane topology of P2X receptors	21
Figure 2.6	Schematic drawing of the metabotropic P2Y receptors	27
Figure 2.7	Schematic drawing of gap junctions	30
Figure 2.8	Innexins, pannexins and connexins	32
Figure 2.9	Connexins and pannexins	32
Figure 2.10	The participation of plasma membrane hemichannels in ATP release and activation of purinergic signaling	34
Figure 2.11	Proposed steps in the synthesis and secretion of IL-1β in macrophage	41
Figure 2.12	IL-1 receptor family	42
Figure 3.1	Compressive stress model	46
Figure 4.1	Upregulation of IL-1β expression upon mechanical stimulation in HPDL cells	57
Figure 4.2	Mechanical stress-induced ATP release in HPDL cells	58
Figure 4.3	ATP mediated stress-induced IL-1β expression in HPDL cells	60
Figure 4.4	IL-1β mRNA induction in mechanically-stimulated HPDL cells	61
Figure 4.5	Gap junctions opening upon mechanical stimulation	63

Page

Figure 4.6 Role of gap junction hemichannels in mechanical stress-induced IL-1β expression …… 65

Figure 4.7 Function of Panx1 and Cx43 hemichannels is important in stress-Induced IL-1β release in HPDL cells …… 67

Figure 4.8 Roles of Panx1 and Cx43 hemichannels in stress-induced ATP release and ATP-induced IL-1β secretion in HPDL cells …… 68

Figure 4.9 ATP-induced IL-1β release involved P2 purinergic signaling …… 70

Figure 4.10 P2X7 receptor is a major P2 subtype responsible for IL-1β induction …… 71

Figure 4.11 Calcium-dependent IL-1β release in HPDL cells …….. 73

Figure 4.12 Co-localization of Panx1 and P2X7 receptor in HPDL cells …….. 75

Figure 4.13 IL-1β release occurred through vesicles …….. 77

Figure 4.14 Panx1 / P2X7 receptor might act as docking site for IL-1β release in HPDL cells …… 78

Figure 5.1 Roles of ATP and gap junction hemichannels in mechanically-stimulated HPDL cells ……..... 88

LIST OF ABBREVIATIONS

Act D	actinomycin D
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BFA	brefeldin A
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CBX	carbenoxolone
COX-2	cyclooxygenase type 2
Cx	connexin
Cx43	connexin 43
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GJ	gap junction
HPDL	human periodontal ligament
IL-1β	interleukin 1 beta
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IP	immunoprecipitate
K^{+}	potassium ion
LPS	lipopolysaccharide
MA	meclofenamic acid
MMP	matrix metalloproteinase

MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
Na^+	sodium ion
NEM	N-ethylmaleimide
NO	nitric oxide
P2	purinergic
P2X7R	P2X7 receptor
$P2X7R^{-/-}$	genetic knockdown of P2X7 receptor
Panx	pannexin
Panx1	pannexin 1
PB	probenecid
PDL	periodontal ligament
PGE2	prostaglandin E2
PLA2	phospholipase A2
QN	quinine
RANKL	receptor activator of nuclear factor-kappa b ligand
RT-PCR	reverse transcription-polymerase chain reaction
siRNA	small interference RNA
Sp	spermine
TMJ	temporomandibular joint
TNF-α	tumor necrosis factor-alpha

CHAPTER I

INTRODUCTION

Periodontium consists of four components including gingiva, cementum, alveolar bone, and periodontal ligament. The periodontal ligament (PDL) is a specialized connective tissue fiber connecting tooth and its alveolar bone socket, providing support and helping tooth withstanding mechanical loading during mastication. PDL also exhibits propioceptive and sensory functions. Under normal physiologic condition, homeostasis of PDL tissues is maintained by appropriate mechanical loading (Tsuji et al., 2004; Wang et al., 2007). PDL cells response to mechanical stress by signaling surrounding cells thereby regulating bone matrix resorption and formation (Henneman, Von den Hoff and Maltha, 2008; Ingber, 2003; Lekic and McCulloch, 1996). Improper loading from orthodontic force can disturb the balance of PDL tissue remodeling (Henneman et al., 2008; Verna, Zaffe and Siciliani, 1999). Orthodontic tooth movement causes increased expression of cytokines such as interleukin1-beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), receptor activator of nuclear factor-kappa B ligand (RANKL), and matrix metalloproteinase-1 (MMP-1) from PDL tissue (Alhashimi et al., 2001; Garlet et al., 2007; Yamaguchi, 2009). These cytokines may contribute to alveolar bone resorption during tooth movement.

Reports showed increased inflammatory mediators including proinflammatory cytokines (i.e. IL-1β, IL-6, IL-8, and TNF-α), adenosine triphosphate (ATP), prostaglandin E2 (PGE2), nitric oxide (NO) as well as extracellular matrix degradation enzymes matrix metalloproteinases (MMPs) in various cell types upon mechanical activation. For example, expression of IL-1β, IL-6, IL-8, IL-11 and TNF-α were increased in response to compressive forces in osteoblast cell line Saos-2 (Koyama et al., 2008). Cyclic compression loading of temporomandibular joint (TMJ) synovial cells induced expression of IL-8, MMP-2 and MMP-3 (Muroi, Kakudo and Nakata, 2007). Exposure of human dental pulp cells to mechanical strain induced IL-1β, TNF-α, and IL-6 expression (Lee et al., 2008). Fluid flow shear stress induced ATP secretion, PGE2 and nitric oxide release in osteoblasts (Bakker et al., 2001; Genetos et al., 2005; Johnson, McAllister and Frangos, 1996; Klein-Nulend et al., 1995). Among these, the release of IL-1β is considered crucial in the initiation and regulation of inflammation (Dinarello, 1996; Dinarello, 2002; Dinarello, 2009). The induction of cyclooxygenase type 2 (COX-2) expression and synthesis by IL-1β consequently resulted in PGE2, phospholipase A2 and inducible nitric oxide synthase (iNOS) production, all of which are key regulators of inflammation (Auron, 1998; Dinarello, 1996; Dinarello, 2009).

ATP is an important extracellular messenger (Grygorczyk and Hanrahan, 1997; Hazama et al., 1999; Knight and Burnstock, 2004; Praetorius and Leipziger, 2009; Sabirov, Dutta and Okada, 2001). Apart from its major physiologic role as energy source of the cell, it is well established now that ATP can be released under

pathologic conditions including inflammation and trauma (Skaper, Debetto and Giusti, 2010). ATP functions as a signaling molecule released from mechanical stimulated cells such as epithelial cells and human periodontal ligament (HPDL) cells (Grygorczyk and Hanrahan, 1997; Hazama et al., 1999; Knight and Burnstock, 2004; Maroto and Hamill, 2001; Praetorius and Leipziger, 2009; Sabirov et al., 2001; Wongkhantee, Yongchaitrakul and Pavasant, 2008). The released ATP consequently activated purinergic P2 receptors, which are broadly classified into two families based on their mode of signal transduction as the ligand-gated ion channels (P2X1-7) and the G-protein-coupled metabotropic receptors (P2Y1, 2, 4, 6, 11, 12, 13, 14) (Burnstock, 1976; Burnstock, 2006; Burnstock, 2007b; Fredholm et al., 1997; Ralevic and Burnstock, 1998). Among these P2 receptor subtypes, P2X7 receptor is of interest due to its high responsiveness to ATP activation as well as its participation in IL-1β processing and release in macrophages (Ferrari et al., 1997a; Ferrari et al., 2006). ATP causes formation of a reversible plasma membrane pore which might be essential in P2X7 receptor stimulated IL-1β release (Sluyter, Shemon and Wiley, 2004). Studies in $P2X7R^{-/-}$ mice also identified P2X7 receptor as a receptor responsible for ATP-dependent IL-1β release from macrophages (Ferrari et al., 1997a; Solle et al., 2001). The involvement of P2X7 receptor in IL-1β release is also found in activated neutrophils, epithelial and microglia cells (Elssner et al., 2004; Solle et al., 2001). P2X7 receptor is therefore considered vital in IL-1β processing and release (Ferrari et al., 2006). During orthodontic tooth movement, the role of

P2X7 receptor in mechanotransduction has also been suggested (Viecilli et al., 2009).

Increasing evidence suggests roles of gap junctions in response to mechanical stimulation (Bao, Sachs and Dahl, 2004b; Barbe, Monyer and Bruzzone, 2006; Goodenough and Paul, 2009; Praetorius and Leipziger, 2009). Gap junctions composed of specialized membrane proteins, forming channels connecting cytoplasm of two adjacent cells. By allowing passages for molecules smaller than 1 kDa such as small metabolites, ions and intracellular signaling molecules (i.e. calcium, cAMP, inositol triphosphate), gap junctions are considered major cell components functioning in cell-to-cell communication (Dubyak, 2009; Goodenough and Paul, 2009). There are two major types of gap junction proteins, connexins (Cx) and pannexins (Panx). Some of them, for example Panx1 and Cx43, have been reported to form non-junctional hemichannels, allowing cell-to-extracellular matrix communication and serving as releasing channels for small molecules such as ATP (Cotrina et al., 1998; Cotrina et al., 2000; Goodenough and Paul, 2009; Hofer and Dermietzel, 1998; Praetorius and Leipziger, 2009; Stout and Charles, 2003). Panx1 and Cx43 hemichannels are found to be mechanosensitive, permeable to ATP and could serve as ATP releasing channel (Bao, Locovei and Dahl, 2004a; Bruzzone et al., 2003; Dubyak, 2009; Eltzschig et al., 2006; Kang et al., 2008; Pelegrin and Surprenant, 2006). Panx1 is proposed to be part of the pore-forming unit of P2X7 receptor, and direct interaction between Panx1 and P2X7 receptor has been stated (Iglesias et al., 2008; Locovei et al., 2007; Pelegrin and Surprenant, 2006; Pelegrin

and Surprenant, 2009). In macrophages, ATP-induced P2X7 receptor activation leads to IL-1β secretion, which is a Panx1-dependent pathway (Dubyak, 2009; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009). Several release mechanisms of IL-1β have been proposed, including an exocytosis via lysosome, protein carrier or vesicular release (Dinarello, 2009; Le Feuvre, Brough and Rothwell, 2002). However, with the size 17 kDa of IL-1β, it is hypothesized that IL-1β probably be released through vesicles in HPDL cells.

Previous studies demonstrated that mechanical stress induced expression of osteopontin and nuclear factor kappa-B ligand (RANKL) through the release of ATP via P2Y1 receptor in HPDL cells (Luckprom et al., 2010; Wongkhantee, Yongchaitrakul and Pavasant, 2007; Wongkhantee et al., 2008). These data indicated the capability of HPDL cells in directly response to mechanical stimulation. However, the relationship among mechanical stress, IL-1β, ATP, P2X7 receptor and Panx1 hemichannel in HPDL cells has not been reported.

This work was conducted in order to examine the influence of mechanical stress on the inflammation in HPDL cells, focusing on the role of ATP in the induction of IL-1β. The mechanism and the participation of P2X7 receptor and Panx1 hemichannel were also investigated.

RESEARCH OBJECTIVES

1. To examine the effect of mechanical stress on the inflammatory cytokine IL-1β expression in HPDL cells
2. To investigate the involvement of ATP and its P2 receptors activation in mechanical stress-induced IL-1β expression in HPDL cells
3. To determine the role of gap junction hemichannels in mechanical stress-induced ATP release and IL-1β expression in HPDL cells
4. To explore the IL-1β release pathway in HPDL cells

RESEARCH HYPOTHESES

1. Mechanical stress induced IL-1β expression in HPDL cells
2. Upregulation of IL-1β upon stress activation occurred through the release of ATP and activation of P2X7 receptor in HPDL cells
3. Gap junction hemichannels, particularly Panx1 and Cx43, might be required in stress-induced ATP release, as well as IL-1β expression in HPDL cells
4. IL-1β release occurred through vesicles in HPDL cells

EXPECTED BENEFITS

Results from this study would emphasize the role of mechanical stress in regulating periodontal tissue inflammation. Understanding the prominent functions

of ATP and Panx1/P2X7 receptor in stress-induced IL-1β upregulation would provide an insight into how mechanical stress regulates inflammation. The results might suggest possible new therapeutic targets for clinical treatment in the future.

CHAPTER II

Review of related literatures

Mechanical stress

Mechanical loading plays an important role in the maintenance of tissue homeostasis and can be found throughout the body. Basically, three major types of mechanical loads are tension, compression and shear stress. Living cells sense and response to mechanical stress in different ways depending on their primary functions (Bao and Suresh, 2003). Various cell types including endothelial cells, smooth muscle cells, osteoblasts, chondrocytes, and fibroblasts are found to be responsive to mechanical loading (Wang et al., 2007).

Mechanical stresses alter structural and functional properties of cells at the cellular, molecular, and genetic levels. This processed is called mechanotransduction. Cellular responses to direct mechanical stresses involve interaction between cytoskeletal and biochemical second messengers (Davies, 2009; Davies and Tripathi, 1993; Papadaki and Eskin, 1997; Wang et al., 2007). The signal transduction mechanisms by which mechanical stresses can activate cellular response is becoming in focus (Ingber, 2003; Ingber, 2006). Cell surface proteins and extracellular matrix activate ion channels and enzymes by mechanical deformation. Stretch-activated ion channels, integrins, cadherins, cytoskeletal filaments, nuclei, extracellular matrix, and numerous other structures and signaling molecules are contributed to the

mechanotransduction response (Davies and Tripathi, 1993; Ingber, 2003; Ingber, 2006). A change in the extracellular concentration of cell surface bioactive ligands as a result of fluid movement has also been identified as an indirect mechanism of mechanotransduction (Davies and Tripathi, 1993).

The microfilament network confers tension to the cell. Stress transmission pathway seemed to involve association between cytoskeleton network and mechanotransducers which could be located at different location in the cells (Burger, Klein-Nulend and Veldhuijzen, 1992; Davies and Tripathi, 1993; Wang et al., 2007; Watson, 1991). The existence of mechanically activated ion channels emerges as a new candidate for rapid stress transduction to an electrophysiological response (Davies, 1995; Davies and Tripathi, 1993; Guharay and Sachs, 1984). An effect of stress on integral membrane enzymes and ion channels may generate second messenger activity by similar mechanisms (Watson, 1991). However, the mechanism of how these elements co-operatively produce signals for cellular response is remained unclear and becoming a new challenge for future research.

Mechanical stress could induce tissue inflammation. Increased level of inflammatory cytokines including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), as well as tumor necrosis factor-alpha (TNF-α) in various cell types after undergoing mechanical stress have been reported. In cultured chondrocytes, cyclic tension force could induce extracellular matrix degradation via matrix metalloproteinases (MMPs) and interleukin-1 upregulation (Fujisawa et al., 1999). The expression of IL-1β, IL-6, IL-8, IL-11 and TNF-α were increased in response

to compressive forces in osteoblast cell line Saos-2 (Koyama et al., 2008). Exposure of human dental pulp cells to mechanical strain induced IL-1β, TNF-α, and IL-6 (Lee et al., 2008). Increased TNF-α and IL-6 expression in rat myocardium were found in response to graded mechanical stretch, suggesting potential implications with regard to compensatory hypertrophy and heart failure (Palmieri et al., 2002). In arthritis, mechanical stress and IL-1β are considered critical factors in disease onset and progression. Cyclic compression loading of TMJ synovial cells induce expression of IL-8, MMP-2 and MMP-3 (Muroi et al., 2007). Mechanical compression of articular cartilage could increase the production of nitric oxide (NO), COX-2 and PGE2, which is partly due to IL-1β induction, resulting in tissue inflammation (Fermor et al., 2002). IL-1β is also accounted for a significant upregulation of matrix degrading enzymes, MMPs, in the presence of mechanical loading (Archambault et al., 2002a; Tsuzaki et al., 2003a; Tsuzaki et al., 2003b). IL-1β could induce expression of MMP-1, MMP-3 and MMP-13 in tendon cells receiving fluid flow shear stress (Archambault et al., 2002b; Tsuzaki et al., 2003b). These data indicated a significant role of IL-1β in response to mechanical loading (Wang et al., 2007).

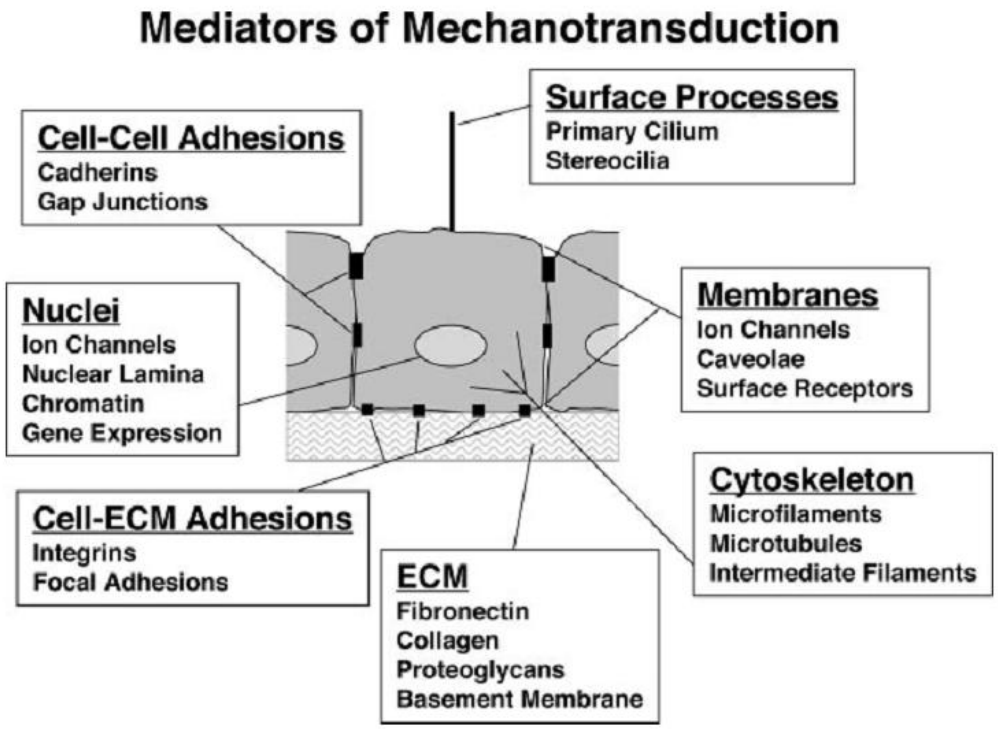

Figure 2.1 Mediators of cellular mechanotransduction (Ingber, 2006). A number of molecules and cellular components, as well as extracellular structures contribute to mechanotransduction. The mechanism of how these elements co-operatively produce signals for cellular response is remained unclear and becoming a new challenge for future research.

Mechanical stimulation may cause cellular damages by activating the release of cellular content, especially nucleotide release from the compromised cell. (Cotrina et al., 1998; Grygorczyk and Hanrahan, 1997; Hazama et al., 1999; Lazarowski et al., 1997; Maroto and Hamill, 2001; Praetorius and Leipziger, 2009). Gentle rotating forces (Harden, Lazarowski and Boucher, 1997), flow over an epithelial/endothelial sheet, or gentle mechanical stimuli are prominent triggers for nucleotide release

(Jensen et al., 2007; Tarran et al., 2005). In non-excitable cells, mechanical stimulation lead to increase in Ca^{2+} waves spreading in neighboring cells, as a result of the released nucleotides spreading (Arcuino et al., 2002; Cotrina et al., 1998).

Reports found ATP release via gap junction hemichannels and the purinergic P2X7 receptor (Anselmi et al., 2008; Bao et al., 2004a; Bao et al., 2004b; MacVicar and Thompson, 2010; Pelegrin and Surprenant, 2006; Praetorius and Leipziger, 2009). Activation of P2X7 receptor with high ATP concentrations (>100–300 μM) could trigger cell membrane permeabilisation to medium size molecular markers (<900 Da) (North, 2002). It was therefore speculated that this receptor-mediated permeabilisation may also allow ATP to exit cells. Evidence for this was generated in cells which expressed membrane-bound luciferase and the P2X7 receptor (Pellegatti et al., 2005).

Mechanical stress plays an important role in maintaining bone homeostasis (Henneman et al., 2008). Mechanical unloading by prolonged bed rest, immobilization, or microgravity in space causes marked bone loss due to a serious imbalance between bone formation and resorption, and the impairment of bone formation constitutes an important mechanism for the unloading-induced bone loss (Burger et al., 1992). Bone cells also respond to fluid shear stress with increases in intracellular calcium concentration as well as the release of signaling molecules ATP, which accounts for the prostaglandin release (Bakker et al., 2001; Genetos et al., 2005; Johnson et al., 1996; Klein-Nulend et al., 1998; Klein-Nulend et al., 1995). Reduce in fluid flow could lead to apoptosis of osteocytes, which subsequently

attracts osteoclasts to the site (Tan et al., 2006). This event might occur in unloading side of bone in which a reduction of fluid flow leads to increased osteocytes apoptosis followed by bone resorption (Aguirre et al., 2006; Henneman et al., 2008). In periodontium, after receiving excessive external force, the balance in synthesis and degradation of periodontal structures is disturbed. Increase in PDL and bone remodeling could lead to tooth movement (Henneman et al., 2008). Excessive forces could induce bone destruction. In patients receiving excessive occlusal forces such as traumatic occlusion or periodontitis as well as orthodontic tooth movement forces, alveolar bone loss was observed.

Adenosine-5'-triphosphate (**ATP**)

Adenosine-5'-triphosphate (ATP) is an important biomolecule functioning as the main energy source of the cells. Chemically, ATP comprises of a purine base (adenosine) attached to the 1’carbon atom and three phosphate groups attached to the 5’carbon atom of the pentose sugar (ribose). It can be produced from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP) by ATP synthase through cellular respiration or photosynthesis. Cells obtain energy via hydrolysis of high energy phosphate bond and convert ATP back to its precursors. ATP is therefore continuously recycled in organisms.

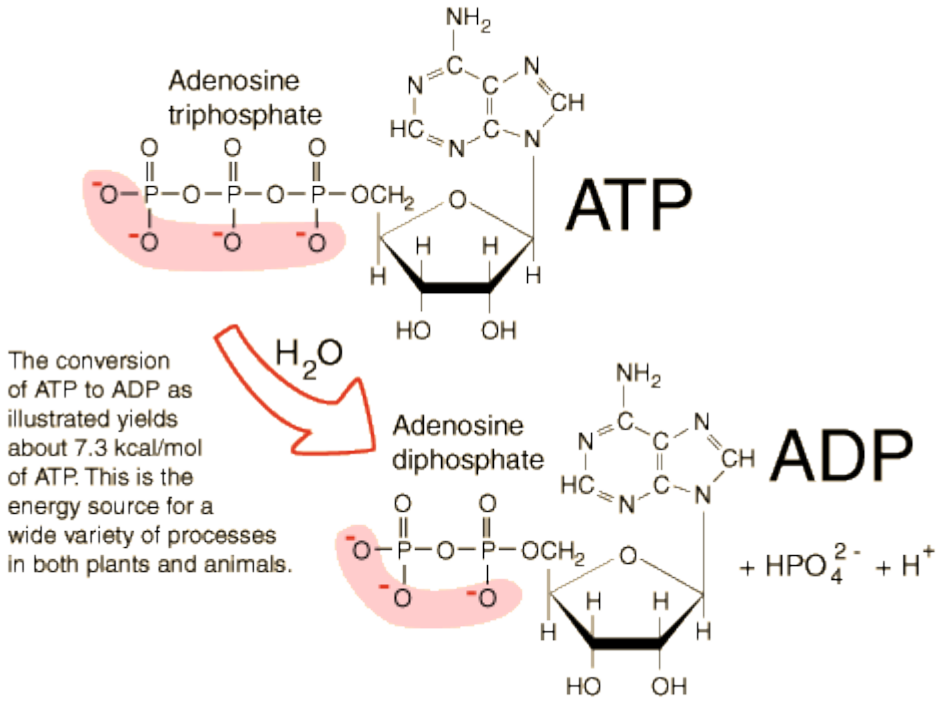

Figure 2.2 Molecular structures of ATP and ADP (http://hyperphysics.phy-astr.gsu.edu/hbase/biology/atp.html). ATP consists of an adenine base, ribose sugar and three phosphate groups. The two outermost phosphates in ATP are held to the rest of the molecule by high-energy phosphoanhydride bonds. The hydrolysis of ATP converts ATP into ATP and yields approximately 7.3 kcal/mol.

ATP is an important extracellular signaling molecule. ATP, ADP and adenosine are recognized by purinergic receptors. In humans, this signaling role is important in both the central and peripheral nervous system. Activity-dependent

release of ATP from synapses, axons and glia activates purinergic membrane receptors known as P2 (Fields and Burnstock, 2006).

ATP plays critical role in intracellular signal transduction processes. It is used as a substrate in signal transduction pathways by kinases that phosphorylate proteins and lipids (Cotrina et al., 2000; Kamenetsky et al., 2006). It is also used by adenylate cyclase and is transformed to the second messenger molecule, cyclic AMP (cAMP), which is involved in triggering calcium signals by the release of calcium from intracellular stores (Kamenetsky et al., 2006). This form of signal transduction is particularly important in brain function.

ATP is also considered a signaling molecule triggered by mechanical stress or other physical stimulation (Arcuino et al., 2002; Jensen et al., 2007). The role of ATP as a danger signal released from damaged cells has been indicated (Di Virgilio et al., 2003). Mechanical stimulation is a common trigger for ATP release (Cotrina et al., 1998; Grygorczyk and Hanrahan, 1997; Hazama et al., 1999; Lazarowski et al., 1997; Maroto and Hamill, 2001; Praetorius and Leipziger, 2009). ATP could activate the the inflammation by inducing inflammatory mediators such as IL-1β, COX-2 and PGE2 expression, as well as matrix metalloproteinases (MMPs), resulting in extracellular matrix degradation and tissue destruction (Tsuzaki et al., 2003a; Wang et al., 2007). (Di Virgilio et al., 2003). ATP released from injured lung cells could act as an inducer of IL-1β maturation and lung fibrosis (Riteau et al., 2010). ATP deriving from dying brain cells or astrocytes could stimulate microvesicle shedding and IL-1β release in microglia (Bianco et al., 2005). In central nervous system, the release of ATP may

contribute to systemic inflammation (Gourine et al., 2007), and the roles of ATP in neurodegeneration has been indicated (Le Feuvre et al., 2002). An increase in ATP release has been shown in endothelial cells, during acute inflammation induced by LPS or shear stress (Bodin and Burnstock, 1998). ATP is a powerful stimulus for IL-1β induction in LPS-primed macrophages and monocytes (Ferrari et al., 1997a; Laliberte et al., 1997).

In general, the concentrations of intracellular or cytosolic ATP, which is the source for extracellular ATP, are in millimolar range, approximately 3-10 mM. The physiological plasma ATP concentrations are only in submicromolar, around 400-700 nM (Coade and Pearson, 1989; Gribble et al., 2000; Ryan et al., 1996). In pathologic conditions such as inflammation, ischemia, hypoxia or under stress, extreme rising in extracellular ATP concentration is normally observed (Bodin and Burnstock, 1998; Latini and Pedata, 2001; Lazarowski, Boucher and Harden, 2003; Lazarowski et al., 1997). Although detailed mechanism of ATP release is not fully understood, several ATP release pathways have been proposed, including transport via a specific carrier, exocytosis, release upon cellular lysis, necrosis or apoptosis, as well as transport via conductive pore, voltage-dependent anion channels or hemichannels (Lazarowski et al., 2003; Praetorius and Leipziger, 2009).

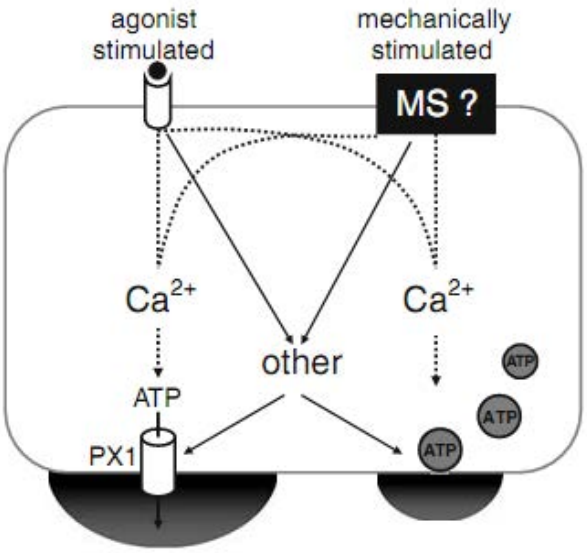

Figure 2.3 Proposed model for ATP release mechanism from non-excitable cells (Praetorius and Leipziger, 2009). Two major stimuli to trigger ATP release are agonist and mechanical stimulation. However, the mechano-sensor (MS, black box) remains unknown. Two major ATP release pathways are conductive pore, which might be Panx1, and exocytosis. Intracellular Ca^{2+} signaling is crucial in both pathways.

Purinergic receptors

Purinergic receptors or purinoceptors were first defined by Burnstock et al. in 1976 (Burnstock, 1976). These receptors can be distinguished into two types, identified as P1 and P2. P1 receptors are selective for adenosine, a breakdown product of ATP, produced after degradation by ectonucleotidases. Four different P1 receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, have been cloned and characterized. Adenosine receptors are members of the rhodopsin-like family of G protein-coupled

receptors that differentially regulate the production of cAMP. The A2A and A2B types could activate adenylate cyclase and increase the levels of cAMP, while A1 and A3 receptors inhibit adenylate cyclase (Baroja-Mazo, Barbera-Cremades and Pelegrin, 2012; Burnstock, 1976; Burnstock, 2006; Burnstock, 2007b; Burnstock and Knight, 2004).

P2 receptors are activated by purines, some subtypes also by pyrimidines. P2 recognized primarily extracellular ATP, ADP, UTP and UDP (Ralevic and Burnstock, 1998). Two P2 subtypes have been proposed, the ionotropic P2X receptors with seven subtypes (P2X1-7) and the metabotropic G-protein-coupled P2Y receptors with eight subtypes (P2Y1, 2, 4, 6, 11-14) (Baroja-Mazo et al., 2012; Burnstock, 1976; Burnstock, 2006; Burnstock, 2007b; Burnstock and Knight, 2004).

P2X receptors are membrane ion channels widely expressed in animal tissues. On presynaptic and postsynaptic nerve terminals throughout the central, peripheral and autonomic nervous systems, P2X receptors have been shown to modulate synaptic transmission. Furthermore, P2X receptors are capable of initiating contraction in heart muscle, skeletal muscle, and various smooth muscle cells (Baroja-Mazo et al., 2012; Burnstock, 1976; Burnstock, 2006; Burnstock, 2007b; Burnstock and Kennedy, 2011; Khakh and North, 2006).

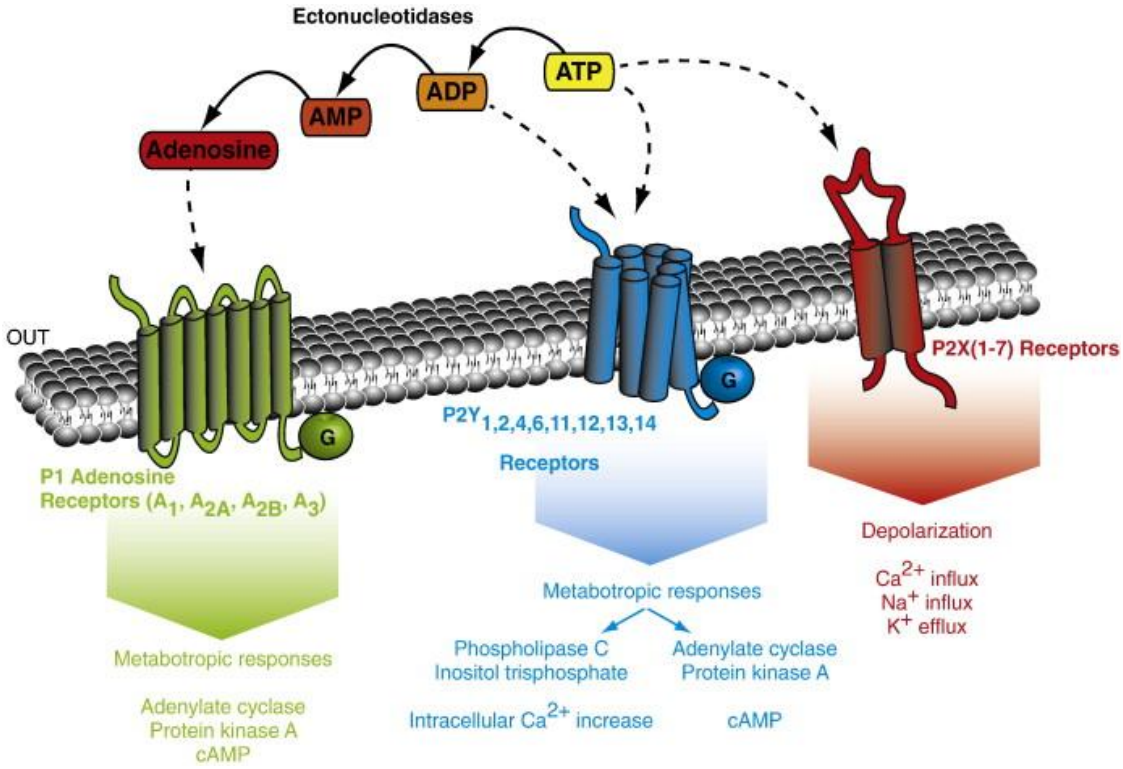

Figure 2.4 **The purinergic receptors** (Baroja-Mazo et al., 2012). Two types of purinergic receptors are P1 and P2 receptors. Extracelluar ATP is the agonist of both P2X and P2Y receptors. Ectonucleotidases degrade ATP into adenosine, which activates P1 or adenosine receptors. The G protein-coupled P1 receptors regulate the production of cAMP via activating and inhibiting adenylate cyclase. Activation of ionotropic P2X receptors open cationic channels, allowing and extracellular Na^+ and Ca^{2+} influx and K^+ efflux, resulting in cellular hyperpolarization. The metabotropic P2Y receptors stimulation leads to the activation of phospholipase C, production of inositol trisphosphate, increase in cytosolic Ca^{2+} due to the release from intracellular storages, as well as the activation of adenylate cyclase.

Table 2.1 Characteristics of purine-mediated receptors (Burnstock, 2007a)

Receptor		Main distribution	Agonists*	Antagonists	Transduction mechanisms
P1 (adenosine)	A_1	brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA, CPA, S-ENBA, CVT-510	DPCPX, N-0840, MRS1754, N-0840, WRC-0571	$G_{i/o}$ ↓cAMP
	A_{2A}	brain, heart, lungs, spleen	CGS 21680, HE-NECA, CVT-3146	KF17837, SCH58261, ZM241385, KW 6002	G_S ↑cAMP
	A_{2B}	large intestine, bladder	NECA (non-selective)	enprofylline, MRE2029-F20, MRS17541, MRS 1706	G_S ↑cAMP
	A_3	lung, liver, brain, testis, heart	IB-MECA, 2-Cl-IB-MECA, DBXRM, VT160	MRS1220, L-268605, MRS1191, MRS1523, VUF8504	$G_{i/o}$ $G_{q/11}$ ↓cAMP ↑IP_3
P2X	$P2X_1$	smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	α,β-meATP = ATP = 2-MeSATP, L-β,γ-meATP (rapid desensitisation),	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 + zinc-sensitive)	suramin, isoPPADS, RB2, NF770, NF279	intrinsic ion channel (particularly Ca^{2+})
	$P2X_3$	sensory neurones, NTS, some sympathetic neurons	2-MeSATP≥ATP≥α,β-meATP≥Ap_4A (rapid desensitisation)	TNP-ATP, PPADS, A317491, NF110, Ip_5I, phenol red	intrinsic cation channel
	$P2X_4$	CNS, testis, colon	ATP>>α,β-meATP, CTP, Ivermectin potentiation	TNP-ATP (weak), BBG (weak), phenolphthalein	intrinsic ion channel (especially 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, O-ATP 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= ADPβS>2-MeSATP = ADP>ATP, MRS2365	MRS2179, MRS2500, MRS2279, PIT	G_q/G_{11}; PLC-β activation
	$P2Y_2$	immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP=ATP, UTPγS, INS 37217, INS 365	suramin > RB2, AR-C126313	G_q/G_{11} and possibly G_i; PLC-β activation
	$P2Y_4$	endothelial cells	UTP≥ATP, UTPγS, INS 37217	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, IDP	MRS2578	G_q/G_{11}; PLC-β activation
	$P2Y_{11}$	spleen, intestine, granulocytes	AR-C67085MX>BzATP≥ATPγS>ATP	suramin > RB2, NF157, 5'-AMPS	G_q/G_{11} and G_S; PLC-β activation
	$P2Y_{12}$	platelets, glial cells	2-MeSADP≥ADP>>ATP	CT50547, AR-C69931MX, INS49266, AZD6140, PSB0413, ARL66096, 2-MeSAMP	$G_{i/o}$; inhibition of adenylate cyclase
	$P2Y_{13}$	spleen, brain, lymph nodes, bone marrow	ADP=2-MeSADP>>ATP=2-MeSATP	MRS2211, 2-MeSAMP	$G_{i/o}$
	$P2Y_{14}$	placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP glucose = UDP-galactose	–	G_q/G_{11}

*Abbreviations: BBG, Brilliant Blue Green; BzATP, 2'-&3'-*O*-(4-benzoyl-benzoyl)-ATP; cAMP, cyclic AMP; CCPA, chlorocyclopentyl adenosine; CPA, cyclopentyl adenosine; CTP, cytosine triphosphate; IP_3, inosine triphosphate; Ip_5I, di-inosine pentaphosphate; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5'-*N*-ethylcarboxamido adenosine; PLC, phospholipase C; RB2, reactive blue 2

The ionotropic P2X receptors consist of two transmembrane-spanning regions, intra-cellular N- and C-termini, a large extracellular loop and a hydrophobic region. P2X receptors can be opened in response to binding of their principal ligand, extracellular ATP. All P2X receptors are permeable to small monovalent cations, some have significant Na^+, K^+, Ca^{2+} or anion permeability and, on activation, cause cell depolarization (Zambon et al., 2001). Seven subunits of the ligand-gated ion channels P2X receptors have been identified, which form both homomultimers and heteromultimers. The P2X1-7 receptors show 30-50% sequence identity at the peptide level. Among these, P2X7 receptor is of interest for its important role as an ATP receptor as well as in interleukin-1 processing and release (Ferrari et al., 2006).

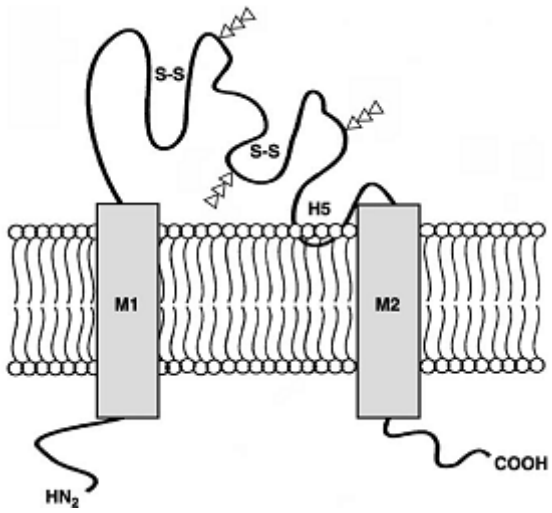

Figure 2.5 Schematic drawing of transmembrane topology of P2X receptors (Burnstock and Kennedy, 2011)**.** Diagram shows two putative transmembrane domains (TM1 and TM2) of P2X receptors, with both N-terminus and C-terminus in the cytoplasm. The extracellular domain contains two disulfide-bonded loops (S–S) and three N-linked glycosyl chains (triangles).

Table 2.2 Principal P2X receptors expressed by excitable tissues and nonneuronal cells (Burnstock and Kennedy, 2011)

Neuronal	
Sympathetic neurons	P2X1–7
Parasympathetic neurons	P2X2, P2X3, P2X4, P2X5
Sensory neurons	P2X1–7, predominantly P2X3, and P2X2/3
Enteric neurons	P2X2, P2X3, P2X4, P2X7
Central nervous system	P2X2, P2X4, and P2X6 (including heteromultimers) and P2X7
Retinal neurons	P2X2, P2X3, P2X4, P2X5, P2X7
Muscle cells	
Smooth muscle	P2X1–7, predominantly P2X1
Skeletal muscle	
Developing	P2X2, P2X5, P2X6
Adult	P2X1–7
Cardiac muscle	P2X1, P2X3, P2X4, P2X5, P2X6
Nonneuronal	
Osteoblasts	P2X1, P2X2, P2X5, P2X7
Osteoclasts	P2X1, P2X2, P2X4, P2X7
Cartilage	P2X2
Keratinocytes	P2X2, P2X3, P2X5, P2X7
Fibroblasts	P2X7
Adipocytes	P2X1
Epithelial cells (lung, kidney, trachea, uterus, cornea)	P2X4, P2X5, P2X6, P2X7
Astrocytes	P2X1–7
Oligodendrocytes	P2X1
Microglia	P2X4, P2X7
Müller cells	P2X3, P2X4, P2X5, P2X7
Enteric glial cells	P2X7
Sperm	P2X2, P2X7
Endothelial cells	P2X1, P2X2, P2X3, predominately P2X4
Erythrocytes	P2X2, P2X4, P2X7
Platelets	P2X1
Immune cells (thymocytes, macrophages, neutrophils, eosinophils, lymphocytes, mast cells, dendritic cells)	P2X4 and predominately P2X7, but some P2X1, P2X2, P2X5
Exocrine secretary cells	P2X1, P2X4, P2X7
Endocrine secretory cells (pituitary, pancreas, adrenal, thyroid, testis)	P2X1–7, predominately P2X2/6
Cholangiocytes	P2X2, P2X3, P2X4, P2X6
Interstitial cells of Cajal	P2X2, P2X5
Kupffer cells	P2X1, P2X4, P2X7
Special senses	
Inner ear	P2X1, P2X2, P2X3, P2X7
Eye	P2X2, P2X7
Tongue	P2X2, P2X3
Olfactory organ	P2X2, P2X4
Cochlea hair cells	P2X1, P2X2, P2X7

P2X7 receptor has been characterized as an important player in IL-1β release from macrophages and microglia primed with endotoxin such as lipopolysaccharide (LPS) (Ferrari et al., 1997a; Ferrari et al., 1997b). ATP is the only known physiological activator of P2X7 receptor (Chakfe et al., 2002; Ferrari et al., 1997b; Skaper et al., 2010). Under normal conditions, extracellular ATP is present in low concentration. However, activated cells including immune cells, macrophages, microglia, or dying cells may release high concentration of ATP into extracellular space (Skaper et al., 2010). Moreover, proinflammatory cytokines and bacterial products can upregulate P2X7 receptor expression and increase its sensitivity to extracellular ATP (Humphreys and Dubyak, 1998; Narcisse et al., 2005; Skaper et al., 2010).

A role of P2X7 in the regulation of bone remodeling has been stated (Grol et al., 2009). Remodeling provides a mechanism for preventive maintenance of the skeleton and the targeted replacement of fatigued or damaged bone. P2X7 receptor activation in osteoblasts enhances differentiation and bone formation (Panupinthu et al., 2008), whereas its activation in osteoclasts results in apoptosis (Korcok et al., 2004; Ohlendorff et al., 2007). Studies in $P2X7R^{-/-}$ mice demonstrated the effects of mechanical stimulation on periosteal bone formation are dependent on the P2X7 receptor.

Table 2.3 Evidences and possible functions for P2 receptors in bone cells (Hoebertz, Arnett and Burnstock, 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.

Activation of P2X7 receptor, when exposed to high concentration of ATP (1–2 mM), could initiate active cell death in osteoclasts and may involve in the formation of giant cells by mediating the fusion of murine macrophage-like cells (Chiozzi et al., 1997). Fusion of osteoclast precursors is also initiated by P2X7-receptor-mediated pore formation in the membranes of adjacent cells, leading to the development of cytoplasmic bridges. Jorgensen et al. (Jorgensen et al., 2002) suggested P2X7 receptors in osteoclasts play a role in mechanically induced intercellular signaling between osteoblasts and osteoclasts, and among osteoclasts. It was proposed that,

in osteoclasts, mechanical stimulation caused ATP release, leading to an elevation of intracellular calcium concentration in neighboring cells, and that this response is mediated by P2X7 receptors.

In addition to the usual rapid opening of the cation-selective ion channel, studies revealed P2X7 sustained activation by high concentration extracellular ATP caused the formation of the reversible plasma membrane pore permeable to hydrophilic molecules with molecular mass up to 900 Da (Di Virgilio, 1995; Surprenant et al., 1996), as well as large dye molecules e.g. ethidium and YO-PRO-1. This pore formation is found essential for P2X7 stimulated IL-1β release (Sluyter et al., 2004). In genetic engineered human embryonic kidney cells HEK-293, overexpressing P2X7 receptor resulted in rapid secretion of IL-1β, while non-expressed P2X7R wild-type cells failed to do so. Similar events also occurred in human monocytes and murine macrophage (Gudipaty et al., 2003). It is also proposed that this process is dependent on extracellular Ca^{2+} influx and a sustained rise in cytosolic Ca^{2+} (Gudipaty et al., 2003). In $P2X7R^{-/-}$ mice, P2X7R deficient macrophage failed to generate IL-1β in response to ATP activation (Solle et al., 2001). IL-1β release via P2X7 receptor is also found in activated neutrophils and epithelial cells (Elssner et al., 2004). Thus, P2X7 receptor is considered a key player in IL-1β processing and release (Di Virgilio, 1995; Elssner et al., 2004; Ferrari et al., 2006; Gudipaty et al., 2003; Sluyter et al., 2004; Solle et al., 2001)

There are five main types of P2X7 blockers: ions (calcium, magnesium, zinc, copper and protons), the suramin analog NF279, oxidized ATP, Brilliant Blue G, which

is most effective at rat P2X7 receptors, and KN-62, which is selective for the human P2X7 receptor. BzATP is a potent agonist at the P2X7 receptor(Burnstock, 2007b).

P2Y receptors are metabotropic G-protein-coupled receptors stimulated by nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose. They are presented in almost all human tissues where they exert various biological functions based on their G-protein coupling. P2Y receptors can be broadly subdivided into five Gq-coupled subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) and three Gi-coupled subtypes (P2Y12, P2Y13, P2Y14). Differences in their agonist preference are used experimentally to identify their subtypes (Burnstock, 2007a). In general, each P2Y receptor binds to a single heterotrimeric G protein, although P2Y11 can couple to both Gq and Gs (Zambon et al., 2001). Whereas the Gq-coupled receptors activate phospholipase C and increase cytosolic concentrations of Ca^{2+}, the Gi-coupled subtypes inhibit adenylyl cyclase and lower cAMP levels (Baroja-Mazo et al., 2012; Burnstock, 2006; Burnstock, 2007b; Burnstock and Knight, 2004).

Table 2.4 Properties of P2Y receptors [modified from Boeynaemas et al., 2012 (Boeynaems, Communi and Robaye, 2012)]

Group	Receptor	Agonist (human)	G protein
A	$P2Y_1$	ADP	G_q
	$P2Y_2$	ATP = UTP	G_q (+G_i)
	$P2Y_4$	UTP	G_q (+G_i)
	$P2Y_6$	UDP	G_q
	$P2Y_{11}$	ATP	$G_q + G_s$
B	$P2Y_{12}$	ADP	G_i
	$P2Y_{13}$	ADP	G_i
	$P2Y_{14}$	UDP-glucose	G_i
		UDP	

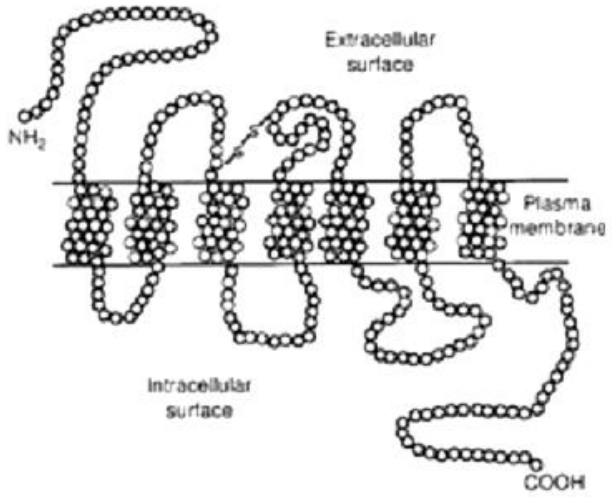

Figure 2.6 Schematic drawing of the metabotropic P2Y receptors (Burnstock, 2007b). Diagram shows characteristic subunit topology of an extracellular N-terminus and an intracellular C-terminus. The intracellular loops and C-terminus posses structural diversity among the eight P2Y subtypes**.**

Gap junctions

Gap junctions, firstly discovered by J.P. Revel & M.J. Karnovsky in 1967 (Revel and Karnovsky, 1967), composed of membrane proteins that form channels connecting cytoplasm of adjacent cells. These channels allow molecules less than 1kDa molecular weights such as small metabolites, ions and intracellular signaling molecules (i.e. calcium, cAMP, inositol triphosphate) to pass freely between cells. They are considered to be universal feature of all multicellular animals. Gap junction channels have been demonstrated to be important in modulating cell signaling and tissue function in many organs, including heart, liver, peripheral nerve, ovary, ear and ocular lens (Goodenough, Goliger and Paul, 1996; Goodenough and Paul, 2009; Goodenough and Revel, 1970; Rackauskas, Neverauskas and Skeberdis, 2010; Shestopalov and Panchin, 2008).

Gap junctions are formed by proteins known as connexins. One gap junction is composed of two connexons (or hemichannels) connecting across the intercellular space. A connexon is formed by 6 connexins, which joined head-to-head across the extracellular gap between two adjacent cells to form intercellular channels. Connexins (Cx) are membrane proteins, consist of four conserved membrane spanning domains with both C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extracellular loop domains (EL-1 and EL-2), sharing more than 95% homology. Sequences in the intracellular loop and especially, those in the carboxyl terminus are divergent between connexins (Goodenough et al., 1996; Rackauskas et al., 2010). Various types of connexins have been identified and cloned,

approximately twenty-one in human and twenty in rodents (Rackauskas et al., 2010; Willecke et al., 2002). The protein weighs between 26 and 60 kDa. At gap junctions, the intercellular space is 2-4 nm (Goodenough and Paul, 2009; Maeda et al., 2009; Rackauskas et al., 2010) and connexons in the membrane of each cell are lined up with one another (Goodenough and Paul, 2009; Perkins, Goodenough and Sosinsky, 1998). Different functional properties of gap junctions include pore conductance, size selectivity, charge selectivity, voltage gating and chemical gating (Goodenough and Paul, 2009; Shestopalov and Panchin, 2008).

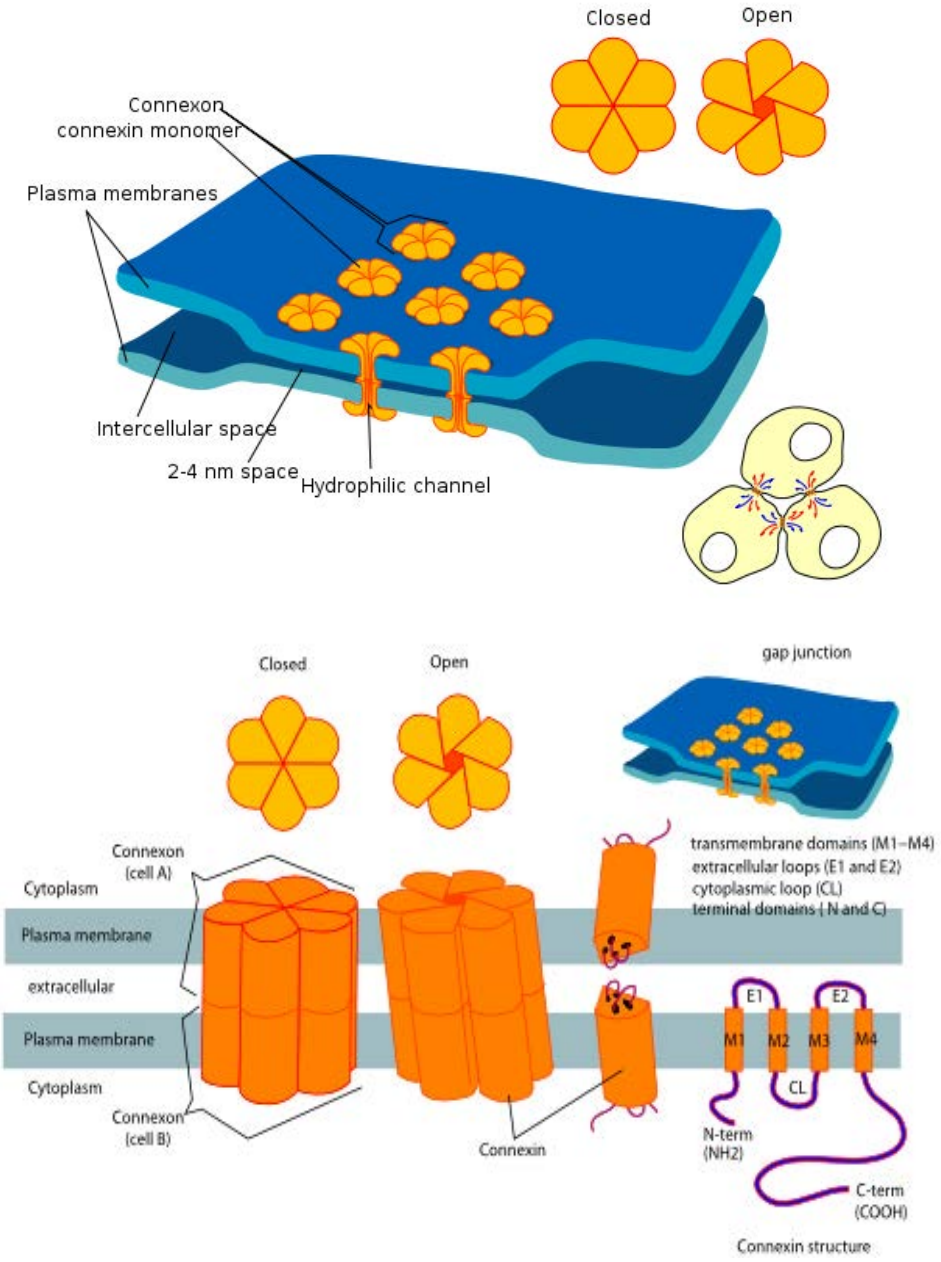

Figure 2.7 Schematic drawing of gap junctions

(http://php.med.unsw.edu.au). One gap junction is composed of two connexons (or hemichannels) which connect across the 2-4 nm intercellular space.

Pannexins (Panx) are newly discovered gap junction proteins, representing a second family of gap junction proteins in vertebrates with no sequence homology to connexins. Instead, they are related to innexins, the invertebrates gap junction proteins (Panchin et al., 2000; Phelan, 2005). Three vertebrate pannexins have been found. Panx1 is the most widely distributed, particularly in immune cells, endothelia and epithelia. Panx2 appears to be more restricted to the central nervous system, whereas Panx3 shows fairly localized expression to joints, synovial fibroblasts, cartilage and skin (Baranova et al., 2004; Barbe et al., 2006). Pannexins are proposed to formed gap junction as well as non-junctional hemichannels, which function in communication between cells and extracellular matrix. Similar to gap junction formed by connexins, the three pannexins subtype were thought to formed pannexon hemichannels (Bruzzone et al., 2003; MacVicar and Thompson, 2010; Shestopalov and Panchin, 2008).

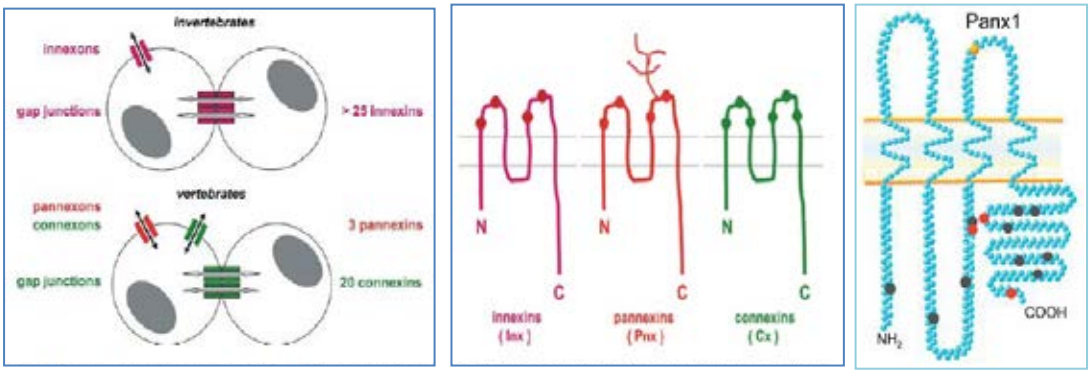

Figure 2.8 Innexins, pannexins and connexins. *Left:* Innexins, pannexins and connexins form different types of gap junction; *Middle:* Membrane topography of the proteins forming gap junctions and hemichannels (Scemes, Spray and Meda, 2009); *Right:* Proposed model of Panx1 based on sequence analysis (Penuela et al., 2007). Panx1 are predicted to be polytopic and contain several predicted N-glycosylation (red and orange residues) and phosphorylation (black) sites.

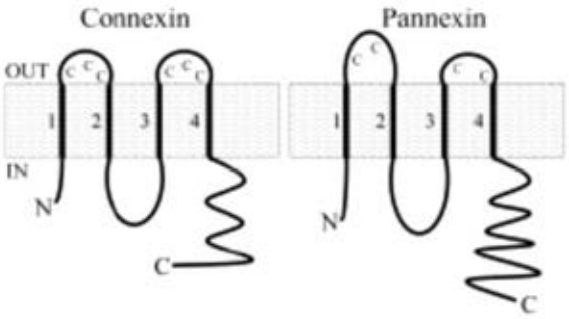

Figure 2.9 Connexins and pannexins (Boassa et al., 2007). Another predicted transmembrane topology for pannexins and connexins.

Bruzzone et al. study in 2003 (Bruzzone et al., 2003) firstly revealed the formation of intercellular channels and functional role of pannexins as gap junction proteins in Xenopus oocytes. Functional Panx1 intercellular channels were also implicated in the formation of Ca^{2+}-permeable gap junction channels (Vanden Abeele et al., 2006). Evidences support a role of Panx1 in forming functional single-membrane channels, commonly referred to as hemichannels (Barbe et al., 2006; Bruzzone et al., 2005). Panx1 also forms functional hemichannels in erythrocytes and may be responsible for ATP release in response to mechanical or osmotic stimulation (Locovei, Bao and Dahl, 2006). This hemichannel opening during stroke has been proposed to be involved in ischemic neuronal cell death (Thompson, Zhou and MacVicar, 2006). Additionally, functional role of gap junction hemichannel as ATP conduit has been reported in various cells including macrophages, erythrocytes, neutrophils, astrocytes and endothelial cells (Baroja-Mazo, Barbera-Cremades and Pelegrin, 2013; Eltzschig et al., 2006; Faigle et al., 2008; Iglesias et al., 2009; Locovei et al., 2006; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009).

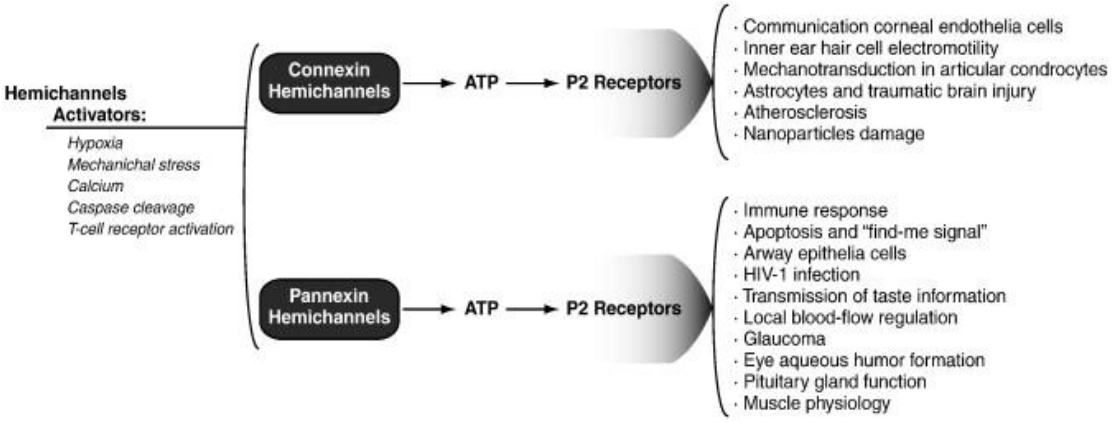

Figure 2.10 The participation of plasma membrane hemichannels in ATP release and activation of purinergic signaling (Baroja-Mazo et al., 2013)

Pannexin 1 has been found to co-immunoprecipitate with the P2X7R protein and is proposed to be part of the pore-forming unit of P2X7 receptor (Locovei et al., 2007; Pelegrin and Surprenant, 2006). Selective inhibition of Panx1 by small interference RNA (siRNA) and Panx1-mimetic inhibitory peptide could inhibit P2X7R-mediated dye uptake (Pelegrin and Surprenant, 2006). Panx1 is also known to combine with P2X7 receptor to form channel that allow ATP, as well as IL-1β, release from cells (Iglesias et al., 2009; Locovei et al., 2007; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009; Vessey, Li and Kelley, 2010). Panx1/P2X7 channel is found to mediate the release of cardioprotectants, including ATP, induced by ischemic pre- and post-conditioning in rat (Vessey et al., 2010). Interestingly, this Panx1/P2X7 receptor complex has been shown to be mechanosensitive (Bao et al., 2004a; Bruzzone et al., 2003; Pelegrin and Surprenant, 2006) and playing important role in the release of inflammatory cytokine IL-1β (Bao et al., 2004a).

Studies showed expression of gap junction proteins in bone and dental-related cells. In osteoblasts, functional gap junctions were first demonstrated by using electrical conductance and dye injection (Jeansonne et al., 1979). Voltage-sensitive gap junction currents were detected in osteoblasts derived from new-born rats calvarias with a single gap junction channel conductance of approximate 100 pS (Schirrmacher et al., 1997). Using dye-transfer assays, rapid dye spreading between a numbers of odontoblasts (Ushiyama, 1989) and osteoblasts (Palumbo, Palazzini and Marotti, 1990) were observed, which indicated cells communication, namely via gap junction. Fluorescent dye injected into rat calvarial subperiosteal osteoblasts spreads to neighboring osteoblastic cells via gap junctional intercellular communication (GJIC). Microinjection of anti-Cx43 antibody in MC3T3-E1 cells blocks cells coupling (Yamaguchi et al., 1994). Electron microscopy and histocytochemistry revealed morphological proof of the existence of gap junction structures in periosteal fibroblasts, osteoblasts, and osteocytes in vivo (Palumbo et al., 1990; Shapiro, 1997). In addition to linear gap junctions, stacked, oval, and annual junction structures were also found, especially within the osteocyte cytoplasm and in osteocyte cell processes within the canaliculi (Shapiro, 1997).

Functional gap junctions including Cx43 are also expressed in the bone resorbing cell osteoclasts. Gap junctions are suggested to play important functional roles in osteoclast precursor fusion to form multinucleated mature osteoclasts, cells communication in the bone multicellular unit, as well as bone remodeling and osteoclast formation (Ilvesaro and Tuukkanen, 2003; Ilvesaro, Vaananen and

Tuukkanen, 2000; Ransjo, Sahli and Lie, 2003). Mechanical loading could stimulate expression of Cx 43 in alveolar bone cells in the tooth movement model (Gluhak-Heinrich et al., 2006). Gap junction inhibitors, 18 α-glycyrrhetinic acid and oleamide, inhibited parathyroid hormone and 1,25-$(OH)_2D_3$ (an active form of vitamin D_3)-stimulated osteoclastic pit formation, an assay for detection of osteoclast formation (Ransjo et al., 2003). The connexin mimetic peptide GAP 27, which inhibits communication among gap junctions, decreased the numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts and increased the numbers of apoptotic osteoclasts (Ilvesaro, Tavi and Tuukkanen, 2001). Gap junction intercellular communication is crucial for osteoclast formation and survival. Carbenoxolone (CBX), a nonspecific gap junction inhibitor, could significantly inhibit osteoclastogenesis stimulated by PTH, PGE2 and 1,25$(OH)_2D_3$ in mouse bone marrow cultures (Matemba, Lie and Ransjo, 2006), which further implicating the role of gap junction in conveying the stimulating signals of these hormones on osteoclast formation and bone remodeling.

In periodontal ligaments, studies revealed diversity of gap junction expression. Yamaoka Y et al. (Yamaoka et al., 2002; Yamaoka et al., 2000) confirmed the expression of Cx32, Cx40, Cx43 and Cx50 in PDL fibroblasts. Report found Cx43 played a role in the coordination of events during experimentally induced alveolar bone remodeling (Su et al., 1997). However, functional role of gap junction in human periodontal ligament cells has not been well investigated.

Inflammatory cytokines

Cytokines are small proteins secreted by specific cells that carry signals locally between cells. They are signaling molecules extensively used in cellular communication. The action of cytokines may be autocrine or paracrine in chemotaxis and endocrine as a pyrogen. Cytokines could function as immune modulating agents. Inflammatory cytokines are immunoregulatory cytokines that promotes inflammation. The major pro-inflammatory cytokines that are responsible for early responses are IL-1α, IL-1β, IL-6, and TNF-α. Other pro-inflammatory mediators include IFN-γ, TGF-β, IL-11, IL-12, IL-17, IL-18 and a variety of other chemokines that chemoattract inflammatory cells. These cytokines either act as endogenous pyrogens (IL-1, IL-6, TNF-α), upregulate the synthesis of secondary mediators and pro-inflammatory cytokines by both macrophages and mesenchymal cells (including fibroblasts, epithelial and endothelial cells), stimulate the production of acute phase proteins, or attract inflammatory cells.

Interleukin-1 (IL-1)

The interleukin-1 family is a group of major inflammatory cytokines playing crucial role in initiating and regulating inflammation. More than any other cytokine family, IL-1 is closely linked to the innate immune response. There are 11 members of the IL-1 family. Among these, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18 have been widely studied in vitro, in animal models of disease, and in humans

(Dinarello, 2009). IL-1 and IL-18 are considered significant mediators of inflammatory process.

Unlike other cytokine families, the IL-1 family exerts control over inflammation at both receptor and nuclear levels. Members of the IL-1 family receptors contain activators and suppressors of inflammation. For example, the IL-1 type II receptor (IL-1RII) functions as a decoy receptor, the single Ig IL-1-related receptor (SIGIRR) suppresses inflammation (Garlanda et al., 2004; Wald et al., 2003), and the IL-18-binding protein (IL-18BP) is the high-affinity endogenous neutralizer of IL-18 activity (Novick et al., 1999).

IL-1β is the most studied member of the IL-1 family because of its role in mediating inflammation, as well as in auto-inflammatory diseases, which are highly responsive to IL-1β blockade. IL-1β is considered an important initiator of the inflammation. IL-1 family members have an indirect effect on immune function. IL-1β is capable of inducing COX-2 expression and synthesis, which consequently stimulated the production of PGE2, phospholipase A2 and inducible nitric oxide synthase (iNOS). IL-6 production is particularly sensitive to IL-1β, and blocking IL-1β in systemic disease resulting in decreased IL-6 levels (Dinarello, 1996; Dinarello, 2007).

Table 2.5 IL-1 family members (Dinarello, 2009)

New Name	Other Name	Property
IL-1F1	IL-1α	Agonist
IL-1F2	IL-1β	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18; IFN-γ-inducing factor	Agonist
IL-1F5	FIL1δ	Anti-inflammatory
IL-1F6	FIL-1ε	Agonist
IL-1F7	IL-1H4, IL-1ζ	Anti-inflammatory
IL-1F8	IL-1H2	Agonist
IL-1F9	IL-1ε	Agonist
IL-1F10	IL-1Hy2	Receptor antagonist (?)
IL-1F11	IL-33	Agonist

IL-1β could increase expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on mesenchymal cells and vascular cell adhesion molecule−1 (VCAM-1) on endothelial cells, along with chemokines induction which further promote the infiltration of inflammatory and immunocomponent cells from circulation into extravascular space and then into tissues. IL-1β is an angiogenic factor and plays a role in tumor metastasis and blood vessel formation (Voronov et al., 2003). It also functions as a costimulator of T cell functions, as well as growth factor for B cell proliferation (Dinarello, 2009).

The primary sources of IL-1β are blood monocytes, tissue macrophages, and dendritic cells. B lymphocytes and NK cells also produce IL-1β. However, fibroblasts and epithelial cells generally do not produce the cytokine. Circulating blood monocytes or bone marrow aspirates from healthy humans do not constitutively

express mRNA for IL-1β. In contrast, several malignant tumors express IL-1β as part of their neoplastic nature, particularly acute myelogenous leukemia, melanoma, multiple myeloma, and juvenile myelogenous leukemia (Dinarello, 2002; Dinarello, 2009).

IL-1β synthesis and secretion mechanism has been proposed in macrophage or monocyte. Activation of P2X7 receptor by ATP could stimulate IL-1β synthesis. Upon P2X7 receptor activation, rapid efflux of potassium causes falling in intracellular potassium level, which later triggers the assembly of the NALP3 inflammasome. The assembled components of the inflammasome initiate the processing of procaspase-1, resulting in the formation of the active caspase-1, which processes the IL-1β precursor. The calcium influx with an increase in intracellular calcium level somehow drives the release of mature IL-1β from cells (Dinarello, 2009). The secretion of IL-1β includes the classical pathway, which normally occurred in macrophage and monocytes, and the non-classical pathway. In classical pathway, IL-1β accumulates as a biologically inactive 33-kDa pro-cytokine (pro IL-1) in cytoplasm, which is conversed to biologically active 17-kDa mature form by caspase-1, as mentioned above. Other IL-1β release mechanisms in non-classical pathway include exocytosis of secretory lysosomes, shedding of plasma membrane microvesicles, and direct efflux through plasma membrane transporters (Qu et al., 2007).

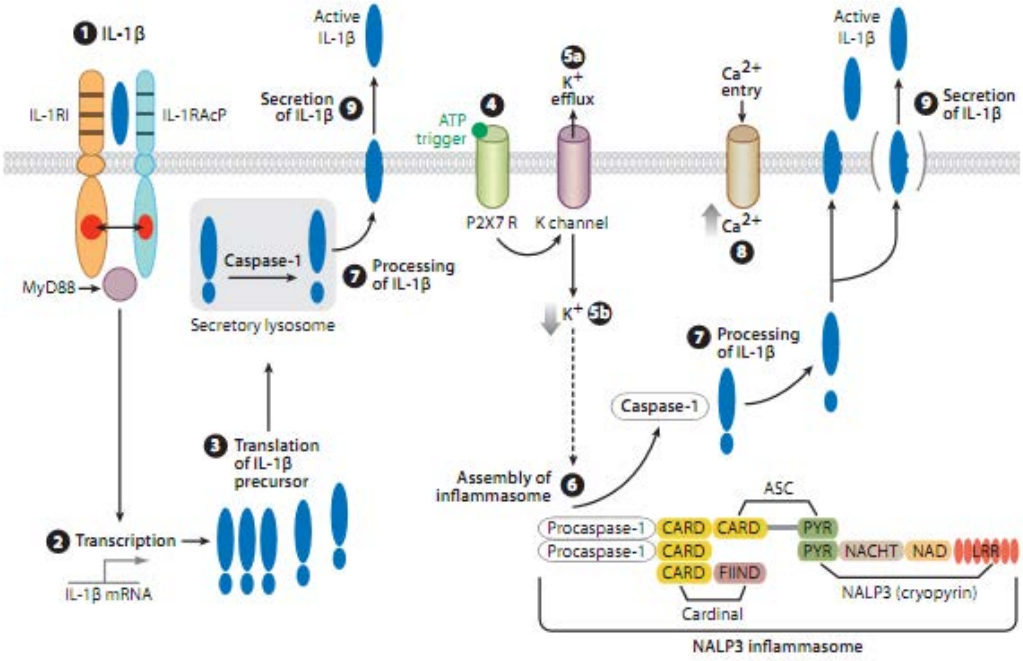

Figure 2.11 Proposed steps in the synthesis and secretion of IL-1β in macrophage (Dinarello, 2009).

The IL-1 receptor family encodes ten members, mostly consists of three IgG-like segments for extracellular domain of the receptor. IL-1 signaling system is critical for innate immunity especially in responding to infectious agents. IL-1β and IL-1α binds to IL-1 Receptor type I (IL-1RI), IL-1 Receptor type II (IL-1RII) and IL-1 Receptor type III (IL-1RAcP). In crystallization studies, IL-1RI undergoes a conformational change when binding to IL-1β, which then allows the IL-1RAcP to form the heterodimer. The cytoplasmic domain of IL-1RI is unique in that it contains homology to the Drosophila Toll protein, TIR domain. The TIR domain is also found in the cytoplasmic domains of each TLR (Toll-like receptors). The TIR domains of IL-1RI and also of the co-receptor IL-1RAcP are necessary for signal transduction. Fundamental

inflammatory responses such as the induction of COX-2, increased expression of adhesion molecules, or synthesis of nitric oxide are indistinguishable responses of both IL-1 and TLR ligands (Dinarello, 1998; Dinarello, 2009).

Table 2.4 **The IL-1 receptor family** (Dinarello, 2009)

Name	Designation[1]	Ligands	Coreceptor
IL-1RI	IL-1R1	IL-1α, IL-1β, IL-1Ra	IL-1RAcP (IL-1R3)
IL-1RII	IL-1R2	IL-1β, IL-1β precursor	IL-1RAcP (IL-1R3)
ST2/Fit-1	IL-1 R4 (IL-33Rα)	IL-33	IL-1RAcP (IL-1R3)
IL-18Rα	IL-1R5	IL-18, IL-1F7	IL-18Rβ (IL-1R7)
IL-1Rrp-2	IL-1R6	IL-1F6, IL-1F8, IL-1F9	IL-1RAcP (IL-1R3)
TIGIRR-2/IL-1RAPL	IL-1R8	unknown	unknown
TIGIRR-1	IL-1R9	unknown	unknown
SIGIRR	TIR8	unknown	unknown

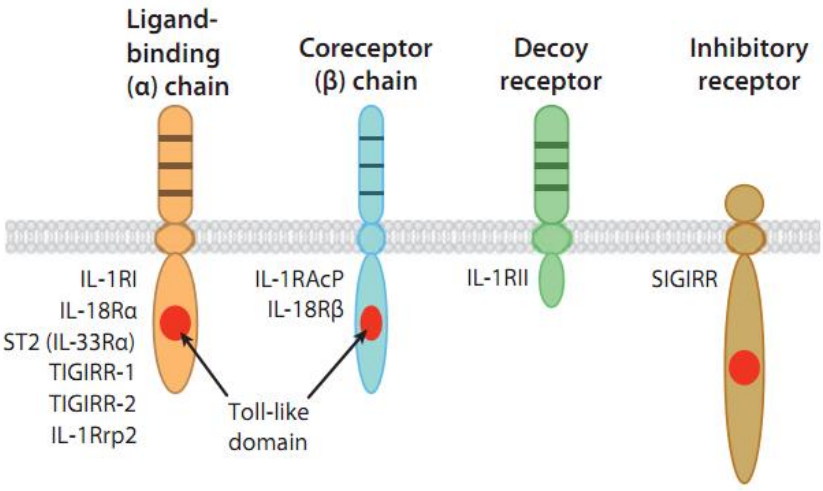

Figure 2.11 **IL-1 receptor family** (Dinarello, 2009)

In primary blood monocytes or tissue macrophages, IL-1β mRNA rise rapidly following LPS or IL-1β itself stimulation. Raising intracellular cAMP levels also enhances IL-1-induced IL-1 gene expression and protein synthesis. The IL-1β precursor accumulates in the cytosol, and processing by caspase-1, which is triggered by the activation of P2X7 receptor by ATP.

IL-1, particularly IL-1β, is one of the major cytokines produced at inflamed sites and involved in the initiation and progression of connective tissue destruction. As described above, monocytes are the cell type responsible for the majority of IL-1β production, although other cells may contribute in disease states. Microbial products, such as lipopolysaccharide (LPS) or endotoxin, potently upregulate IL-1β synthesis (Auron, 1998). IL-1β stimulates a variety of cell types to produce connective tissue catabolic and bone-resorptive mediators, including IL-1β, IL-6, TNF-α, PGE2 and MMPS (Dinarello, 1996). These factors lead to degradation of connective tissue such as collagen, along with the recruitment and activation of osteoclasts. Increase in IL-1β level has been found involved in many inflammatory diseases, for example rheumatoid arthritis (Dinarello, 1996).

Studies reported a strong relationship between severity of adult periodontitis and increased IL-1β in gingival crevicular fluid. In chronic periodontitis, microbial components, especially LPS, have the capacity to activate macrophages to synthesize and secrete a wide array of molecules including the IL-1β, TNF-α, PGE2 and hydrolytic enzymes, resulting in the destruction of alveolar bone and periodontal

connective tissue (Boch, Wara-aswapati and Auron, 2001; Dinarello, 1996; Kanzaki et al., 2002).

CHAPTER III

RESEARCH METHODOLOGY

1. Cell culture

Human periodontal ligament (HPDL) cells were obtained from extracted healthy third molars with patients' informed consent. The protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Teeth were rinsed with sterile phosphate buffer saline (PBS) and periodontal tissues were removed from the middle third of root surfaces. Tissues were then grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 2 mM L-glutamine (Gibco BRL), 100 units/mL penicillin (Gibco BRL), 100 µg/mL streptomycin (Gibco BRL) and 5 µg/mL amphotericin B (Gibco BRL), and incubated at 37°C in 5% CO_2 humidified air chamber. HPDL cells from the 3^{rd} to 5^{th} passages were used. All experiments were performed in triplicate, using cells from three different donors.

2. HPDL cells stimulation

HPDL cells were seeded in six-well plates at a density of 25,000 cells/cm^2 and grown to 90% confluence. After 4 hours of serum deprivation, cells were treated

with or without 1-2.5 g/cm^2 compressive force for up to 5 hours, or 10-100 µM ATP (Sigma-Aldrich Chemical, MO, USA) for up to 180 minutes. For stress application, plastic cylinders were placed in each well and metal coins were added to generate compressive force (Wongkhantee et al., 2008). IL-1β expression was measured at both mRNA and protein levels. Culture medium was collected for enzyme-linked immunosorbent assay (ELISA) and mRNA was extracted for reverse transcription-polymerase chain reaction (RT-PCR). Non-treated cells were used as controls.

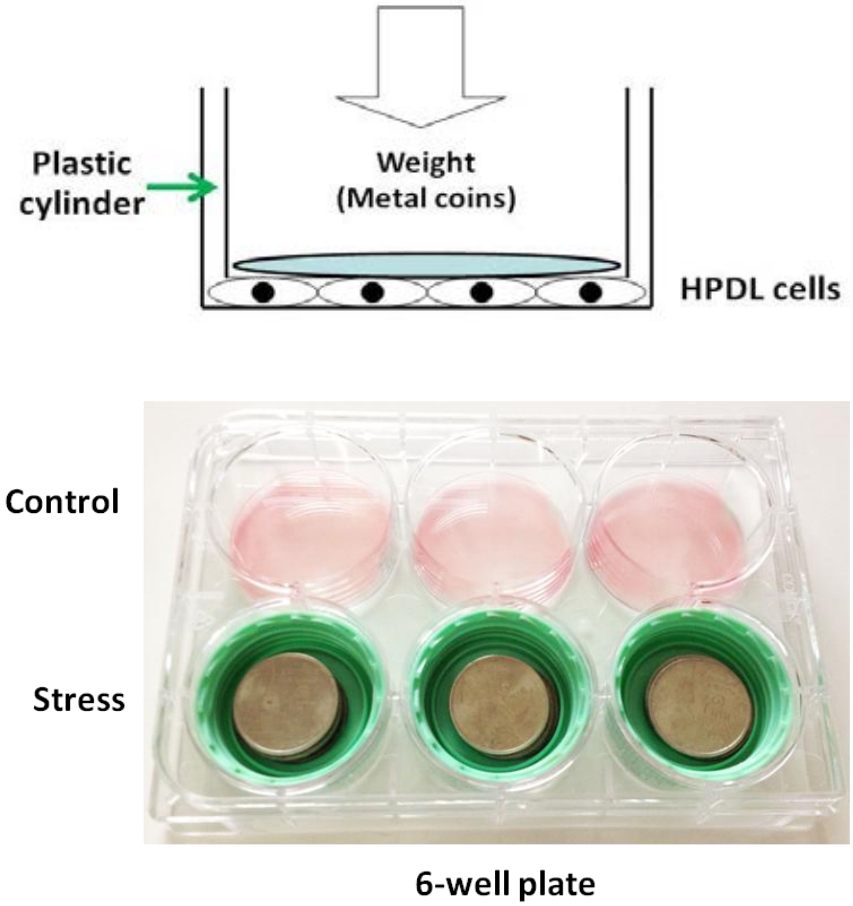

Figure 3.1 **Compressive stress model** [modified from *Kanzaki et al., 2002* (Kanzaki et al., 2002; Wongkhantee et al., 2007)]

3. MTT assay

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

4. Measurement of IL-1β release by enzyme-linked immunosorbent assay (ELISA)

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

5. Measurement of ATP release by bioluminescence assay

ATP release into culture medium was measured by using luciferin-luciferase base bioluminescence detection kit for ATP (ENLITEN® ATP Assay System; Promega, Madison, WI, USA) according to the manufacturer's instruction. The emitted light

was measured at 560 nm, using luminescence microplate reader (BioTek Synergy H1 Hybrid Reader, BioTek Instruments Inc.). Data were expressed as fold increase of ATP over control.

6. Inhibitors and peptides

After 4 hours of serum deprivation of cultured HPDL cells, inhibitors or peptides were added 30 minutes prior to stress or ATP application. Inhibitors used were as follows; 3.6 μM actinomycin D (ActD, transcription blocker; Calbiochem, San Diego, CA, USA), 7.96 μM cyclohexamide (CHX; Sigma-Aldrich Chemical), 15 μM suramin (Calbiochem), 1 unit/mL apyrase (Sigma-Aldrich Chemical), 10 μM KN-62 (Sigma-Aldrich Chemical), 10 μM MRS2179 (Sigma-Aldrich Chemical), 0.18 μM NF449 (Tocris Bioscience, Minneapolis, MN, USA), 300 nM NF023 (Tocris Bioscience), 5 μM 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA, Enzo Life Sciences Inc., Farmingdale, NY, USA), 12.5 nM thapsigargin (Sigma-Aldrich Chemical), 50 μM 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB8, Biomol Research Laboratories Inc., Plymouth, PA, USA), 10μM carbenoxolone disodium salt (Sigma-Aldrich Chemical), 10 μM probenecid (Sigma-Aldrich Chemical), 20 μM meclofenamic acid sodium salt (Sigma-Aldrich Chemical), 10 μM spermine (Sigma-Aldrich Chemical), 10 μM quinine (Sigma-Aldrich Chemical), 100 μM monensin (Sigma-Aldrich Chemical), 400 μM N-ethylmaleimide (NEM, Sigma-Aldrich Chemical), and 36 μM brefeldin A (BFA, Sigma-Aldrich Chemical). Peptides used were 100 μM

10Panx (Panx1 mimetic inhibitory peptide, Tocris Bioscience) and 100 μM Scrambled 10Panx (scrambled version of 10Panx, Tocris Bioscience).

7. Transfection of small interfering RNA (siRNA)

HPDL cells were cultured in antibiotic-free normal growth medium until 70-80% confluence. Cells were treated with a mixed solution of siRNA oligonucleotide specific to P2X7 receptor (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Panx1 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Cx43 (1:250, Santa Cruz Biotechnology) and transfection reagent (LipofectamineTM2000 Reagent, Invitrogen) for 24 hours prior to stress or ATP application. The ratios of siRNA to transfection reagent used were 2:3 for P2X7 receptor, and 1:1 for Panx1 and Cx43. Non-treated cells, cells treated with only control siRNA (SiC, 1:250, Santa Cruz Biotechnology) or transfection reagents (MOCK, data not shown) were used as controls. Percent knockdown of gene expression was determined from relative band intensity of siRNA-transfected cells, compared to control (non-treated cells), using Scion Image software (Scion Corporation, Frederick, MD, USA).

8. RNA Extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Cultured HPDL cells were collected and total cellular RNA was extracted using Tri-reagent (Molecular Research Center, Cincinnati, OH, USA) according to the

manufacturer's instructions. Avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) was used to convert RNA (1 µg of each sample) to cDNA. Polymerase chain reaction (PCR) was subsequently performed. The cDNA was amplified using Taq polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 µl in DNA thermal cyclers (Biometra, Gottingen, Germany). Amplification profile was cycles of 1 minute denaturation at 94°C, 1 minute hybridization at 60°C and 2 minutes extension at 72°C, followed by a final extension at 72°C for 10 minutes. The amplified DNA was then electrophoresed on 1% agarose gel and visualized by ethidium bromide fluoro-staining. Primers were designed as reported in GenBank database. The oligonucleotide sequences and PCR cycles were shown in Table 3.1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for internal control.

Table 3.1 Primers details

Gene	Accession number	Oligonucleotide sequence	Product size (bp)	PCR cycles
IL-1β	NM 000576.2	forward 5'GGA GCA ACA AGT GGT GTT CT3' reverse 5'AAA GTC CAG GCT ATA GCC GT3'	459	40
P2X7	NM 002562.5	forward 5'TTT GCT CTG GTG AGT GAC AAG CTG3' reverse 5'CCT CTG GTT GTC CAG GAA TCG3'	1246	40
Panx1	NM 015368.3	forward 5'GGA TCC TGA GAA ACG ACA GC3' reverse 5'CTC TGT CGG GCA TTC TTC TC3'	496	35
Cx26	NM 004004.5	forward 5'- GCAGAGACCCCAACGCCGAGAC-3' reverse 5'- GCAGACAAAGTCGGCCTGCTCA-3'	239	35
Cx32	NM 001097642.2	forward 5'- CTGCTCTACCCGGGCTATGC-3' reverse 5'- CAGGCTGAGCATCGGTCGCTCT-3'	330	35
Cx37	NM 002060.2	forward 5'- GGTGGGTAAGATCTGGCTGA-3' reverse 5'- ATAGGTGCCCATCAGTGCTC-3'	406	35
Cx40	NM 181703.2	forward 5'- GGGAGGCCATATTATTGCTG-3' reverse 5'- GTGGCAGAGAAGGCAGAACT-3'	486	35
Cx43	NM 000165.3	forward 5'GGA CAT GCA CTT GAA GCA GA3' reverse 5'CAG CTT GTA CCC AGG AGG AG3'	496	35
Cx45	NM 001080383.1	forward 5'- CACGGTGAAGCAGACAAGAA-3' reverse 5'- GCAAAGGCCTGTAACACCAT-3'	417	35
Cx50	NM 005267.3	forward 5'- TCATCCTGTTCATGTTGTCTGTGGC-3' reverse 5'- AACCTCGGTCAAGGGGAAATAGT-3'	238	35
GAPDH	NM 002046.3	forward 5'TGA AGG TCG GAG TCA ACG GAT3', reverse 5'TCA CAC CCA TGA CGA ACA TGG3'	395	22

9. Western blot analysis protein detection

Total proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer. Proteins were then separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred onto nitrocellulose membrane, which was incubated in 5% non-fat milk for 1 hour prior to an overnight incubation in primary antibody to reduce non-specific binding. Primary antibodies used were raised against P2X7 receptor (rabbit polyclonal antibody to P2RX7, 1:200, Abcam, Cambridge, UK), Panx1 (rabbit polyclonal antibody to Pannexin1, 1:500, Abcam, Cambridge, UK), Cx43 (mouse anti-connexin 43 monoclonal antibody, 1:250, Invitrogen, Camarillo, CA, USA) and actin (mouse anti-actin monoclonal antibody, clone: C4, 1:1000, Chemicon International, Temecula, CA, USA). Membrane was then incubated in biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. Secondary antibodies used were goat anti-rabbit IgG biotin-conjugated affinity purified antibody (1:2000, Chemicon International) and goat anti-mouse IgG biotin conjugated antibody (1:2000, Invitrogen). Signal was captured by chemiluminescence (SuperSignal® West Pico Stable Peroxide Solution and SuperSignal® West Pico Luminol/Enhancer Solution, Pierce Bio-technology, Rockford, IL, USA).

10. Co-immunoprecipitation (Co-IP)

Co-IP of Panx1 and P2X7 receptor in HPDL cells was performed using Co-IP Kit (Pierce® Co-Immunoprecipitation (Co-IP) Kit, Pierce Biotechnology) according to the

manufacturer's instruction. Cultured HPDL cells were washed with PBS and lysed with IP Lysis/Wash buffer. Cell lysates were centrifuged at 13,000 x g and supernatants were used for immunoprecipitation. P2X7 receptor antibody (rabbit polyclonal antibody to P2RX7, 20 µl, Abcam) or Panx1 antibody (rabbit polyclonal antibody to Pannexin1, 10 µg, Abcam) was immobilized into AminoLink Plus Coupling resin by incubating on a rotator at room temperature for 90 minutes. Lysate pre-clearing was performed using control agarose resin prior to Co-IP steps. The supernatants were then incubated in P2X7 receptor- or Panx1-tagged coupling resin overnight at 4^{o}C, washed three times with IP Lysis/Wash buffer and centrifuged after each wash. Immunoprecipitates (IP) were eluted with elution buffer, and separated using western blot analysis. Whole cell lysates without undergoing Co-IP steps and lysates precipitated from control resin with no antibody immobilization were used as controls.

11. Immunocytochemistry

Cultured HPDL cells were washed with PBS and fixed in cold methanol for 10 minutes, washed with PBS and incubated overnight in primary antibodies diluted in 10% FBS in PBS. Primary antibodies used were for P2X7 receptor (rabbit polyclonal antibody to P2RX7, 1:200, Abcam), Panx1 (rabbit polyclonal antibody to Pannexin1, 1:100, Abcam) and SNAP-25 (goat anti-SNAP-25 affinity purified polyclonal antibody, 1:100, Millipore, Temecula, CA, USA). Cells were washed with PBS and incubated in secondary antibodies for 40 minutes. Secondary antibodies used were goat anti-

rabbit IgG biotin-conjugated affinity purified antibody (1:2000, Chemicon International) and rabbit anti-goat IgG biotin-conjugated specific antibody (1:2000, Sigma-Aldrich Chemical). Cells were then washed with PBS, incubated in 0.1 mg/ml DAPI for 15 minutes in the dark, washed again with PBS and incubated in rhodamine or FITC for 40 minutes in the dark, washed with PBS and mounted on slide using ProLong® Gold antifade reagent (Invitrogen). Digital images were taken using Zeiss Axio Observer Z1 (Carl Zeiss MicroImaging GmbH, Germany) fluorescent microscope and Axiovision 4.8 image processing software (Carl Zeiss).

12. Dye-uptake assay

To investigate the involvement of gap junction upon mechanical stimulation, dye-uptake assay was used. Carboxyfluorescein is a well-known low molecular weight (376.32 daltons) fluorescent dye that has been used to examine the opening of gap junction. After stress application, cells were washed with PBS and incubated in Tyrode's solution containing 5 mM 5,6-carboxyfluorescein (Sigma-Aldrich Chemical) for 10 minutes and visualized by fluorescence microscope (Axiovert 40CFL, Carl Zeiss, Germany). Background dye uptake is defined as fluorescence achieved in the absence of mechanical stimulation.

13. Statistical analysis

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 ($p < 0.05$).

CHAPTER IV

RESULTS

Mechanical stress induced IL-1β expression and nucleotide release in HPDL cells

We first investigated whether mechanical stress could induce IL-1β expression and release in HPDL cells. After stress application, upregulation of IL-1β expression was observed at both mRNA and protein levels (Fig. 4.1). IL-1β level increased in correlation with the magnitude of compressive loading and duration of treatments (Fig. 4.1 A, 4.1 B, 4.1 D, 4.1 E). MTT assay was used to evaluate HPDL cells viability after receiving mechanical stress (Fig. 4.1 C, 4.1 F). A maximum IL-1β expression with the least harmful effect to HPDL cells was found when using 2 g/cm^2 stress stimulation for 3 hours (Fig. 4.1 C, 4.1 F), which was then used in all latter experiments.

To examine whether mechanical stress could also induce ATP release from HPDL cells, luciferin-luciferase assay was used. HPDL cells were treated with compressive force for up to 120 minutes, and extracellular ATP level was measured. Mechanical stress could stimulate ATP release in HPDL cells, in a dose- and time-dependent manner (Fig. 4.2 A, 4.2 B). Interestingly, an extreme rise in extracellular ATP level could be detected within 5 minutes after mechanical stimulation.

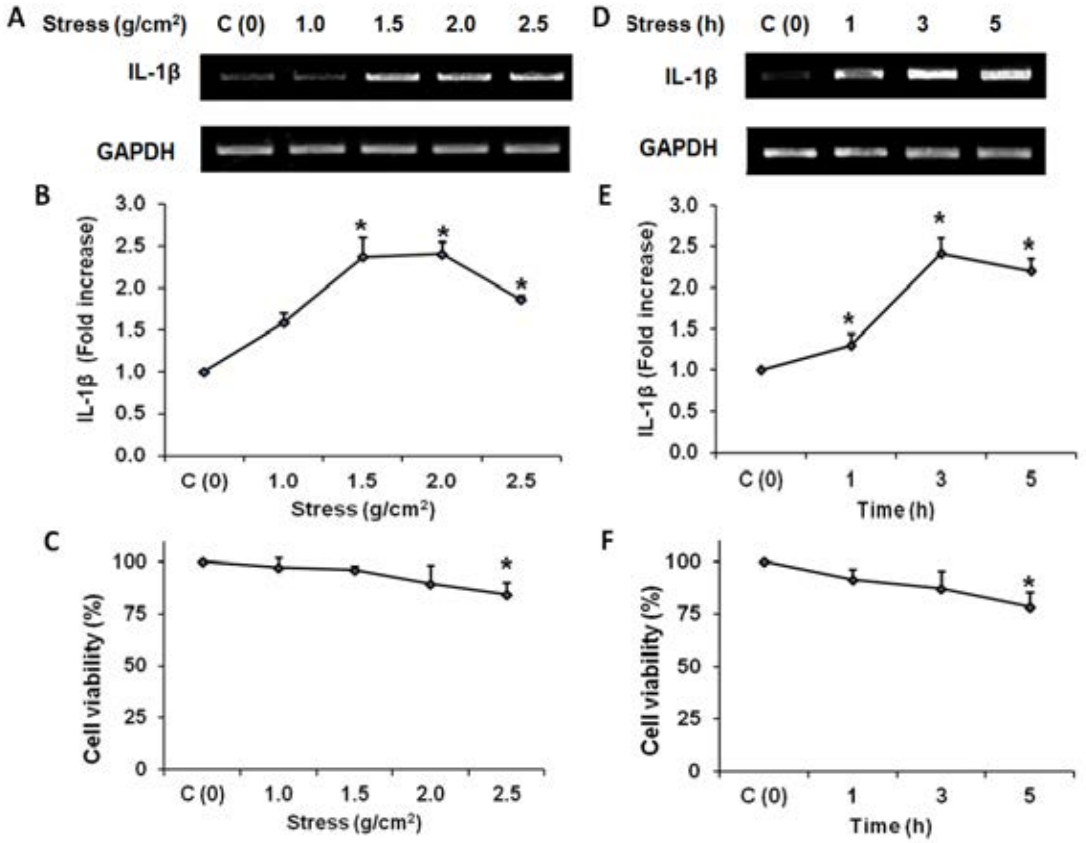

Figure 4.1 Upregulation of IL-1β expression upon mechanical stimulation in HPDL cells. Cultured HPDL cells were treated with compressive loading ranging from 0 to 2.5 g/cm^2 for 3 hours. Expression of IL-1β was measured using RT-PCR (A) and ELISA (B). Cell viability was observed using MTT assays (C). To evaluate the time-course effect of stress, HPDL cells were received 2 g/cm^2 loading for up to 5 hours. IL-1β expression was measured at both mRNA (D) and protein (E) levels. MTT was also performed (F). Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

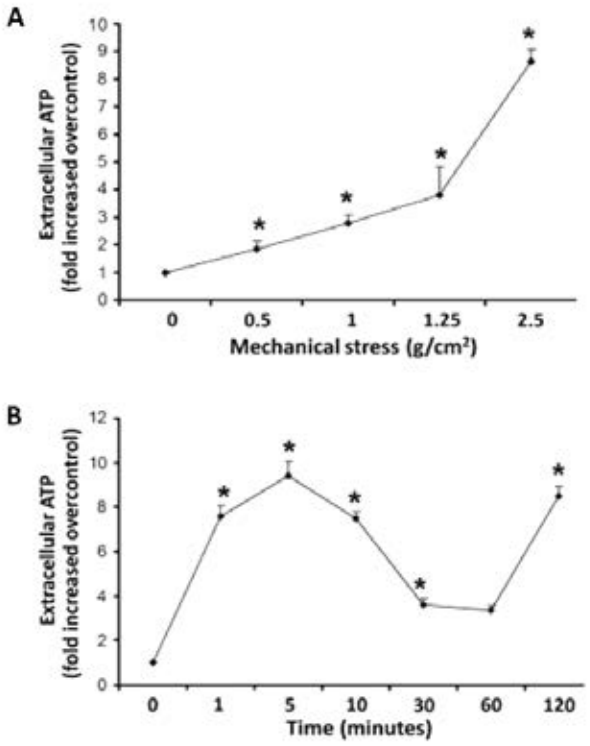

Figure 4.2 Mechanical stress-induced ATP release in HPDL cells. Cultured HPDL cells were treated with compressive loading ranging from 0 to 2.5 g/cm^2 for up to 120 minutes. The amount of ATP released into culture medium was determined using luciferin-luciferase assay. A force-dependent increase in extracellular ATP level was observed with the highest extracellular ATP level when treated cells with 2.5 g/cm^2 loading, which therefore used to determine the time-course effect. The maximum extracellular ATP level could be detected within 5 minutes after stress application (B). Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

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

The participation of ATP in stress-induced IL-1β expression was explored by using ATP degradating enzyme and P2 receptor antagonists. An ATP diphosphohydrolase, apyrase, as well as a nonspecific P2 receptor antagonist, suramin, nearly abolished the effect of mechanical stress on IL-1β expression at both mRNA and protein levels (Fig. 4.3 A, 4.3 B), indicating the involvement of ATP in IL-1β induction.

The role of ATP was investigated by using ATP itself as a stimulator. After 4 hours of serum deprivation, cultured HPDL cells were treated with or without 10-100 μM ATP for up to 3 hours. ATP alone could stimulate IL-1β expression at both mRNA and protein level, as shown by RT-PCR and ELISA (Fig. 4.3 C, 4.3 F). The maximum concentration of IL-1β was observed when using a treatment of 40 μM ATP for 30 minutes, which was then used in latter experiments.

The IL-1β induction was further examined by using a transcription blocker, actinomycin D (Act D), and a translation blocker, cyclohexamide (CHX). Both inhibitors markedly inhibited stress- as well as ATP-induced IL-1β expression at both mRNA and protein levels (Fig. 4.4 A, 4.4 B, 4.4 D). However, luciferin-luciferase assay revealed no significant effect of Act D and CHX on stress-induced ATP release (Fig. 4.4 C).

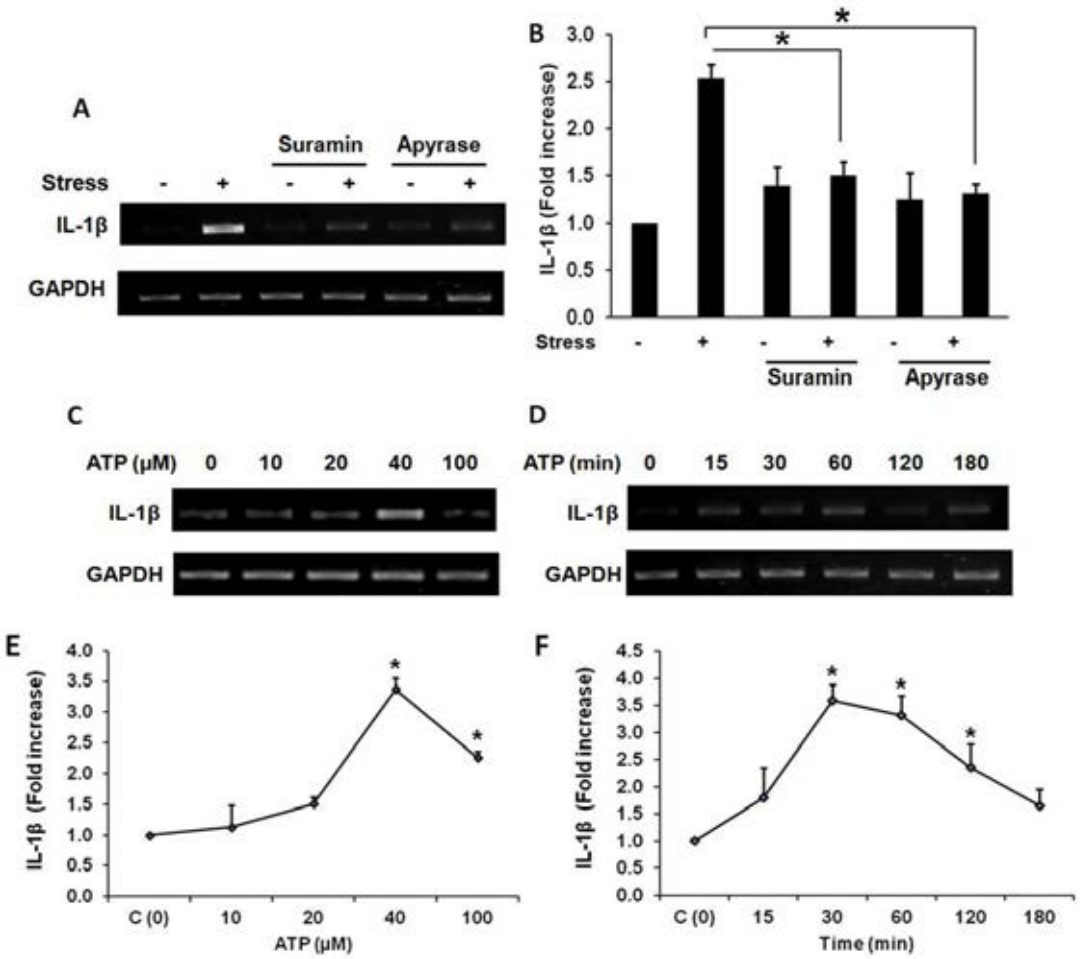

Figure 4.3 ATP mediated stress-induced IL-1β expression in HPDL cells.

Mechanical stress (2 g/cm^2, 3 hours) was applied on cultured HPDL cells in order to induce IL-1β expression. RT-PCR (A) and ELISA (B) showed suramin and apyrase strongly inhibited stress-induced IL-1β expression. ATP (10-100 μM) was then used to stimulate HPDL cells for 30 minutes. Expression of IL-1β was measured using RT-PCR (C) and ELISA (E). To evaluate the time-course effect of ATP, HPDL cells were treated with 40 μM ATP for up to 3 hours. IL-1β was measured at both mRNA (D) and protein (F) levels. Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

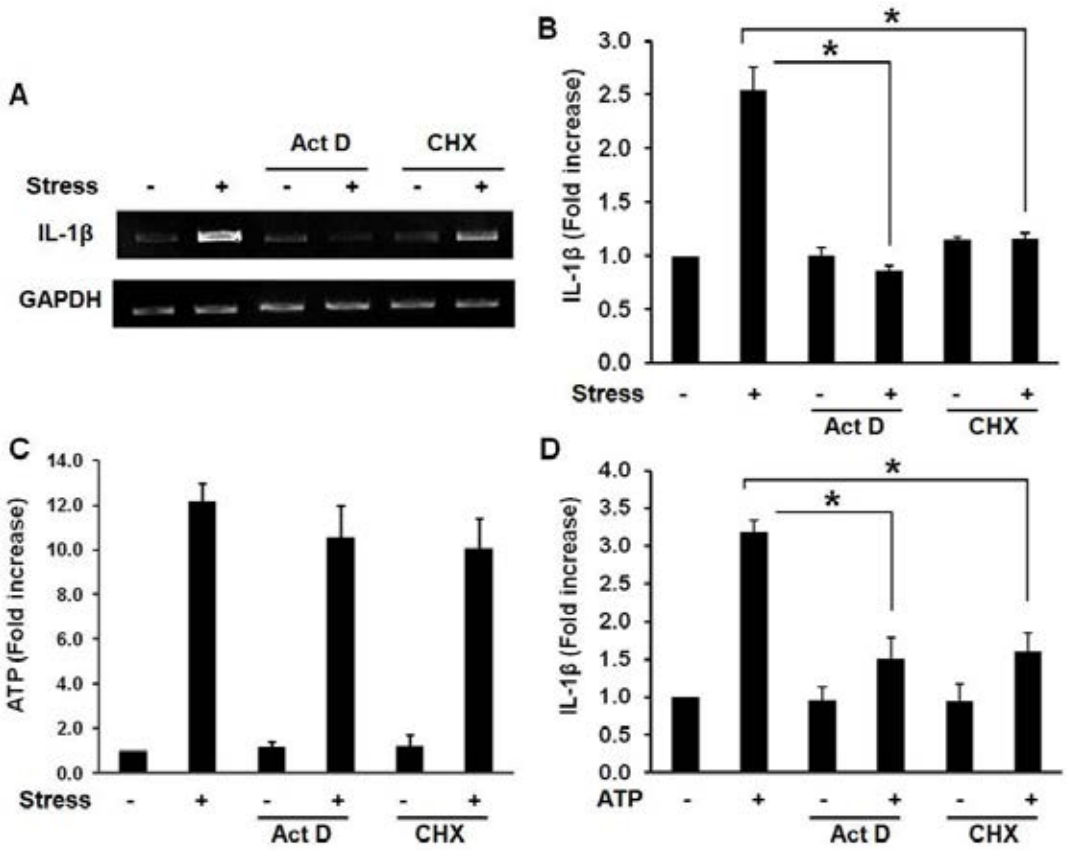

Figure 4.4 IL-1β mRNA induction in mechanically-stimulated HPDL cells.

Mechanical stress (2 g/cm^2, 3 h) or ATP (40 μM, 30 minutes) was applied on cultured HPDL cells to stimulate IL-1β expression. Act D and CHX strongly attenuated stress- or ATP-induced IL-1β expression at both mRNA and protein levels (A, B, D), while no significant effect was observed in stress-induced ATP release (C). Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

Mechanical stress induced gap junction hemichannels opening in HPDL cells

Several types of gap junction proteins, including Cx40, Cx43 as well as Panx1 were presented in HPDL cells (Fig. 4.5 A). To preliminarily examine the role of gap junctions in mechanically-stimulated HPDL cells, dye uptake assay was used. Increased fluorescent signaling of carboxyfluorescein dye was observed in stress-stimulated HPDL cells, compared to control non-treated cells (Fig. 4.5 B), suggesting the opening of gap junctions upon mechanical stimulation. This incident was attenuated in the presence of a nonspecific gap junction inhibitor, carbenoxolone, and the specific Panx1 and Cx43 inhibitors, probenecid and meclofenamic acid respectively. A specific Cx40 inhibitor, spermine, and a specific Cx50 inhibitor, quinine, showed no significant effect. These data indicated an involvement of hemichannels in cellular response to mechanical stimulation.

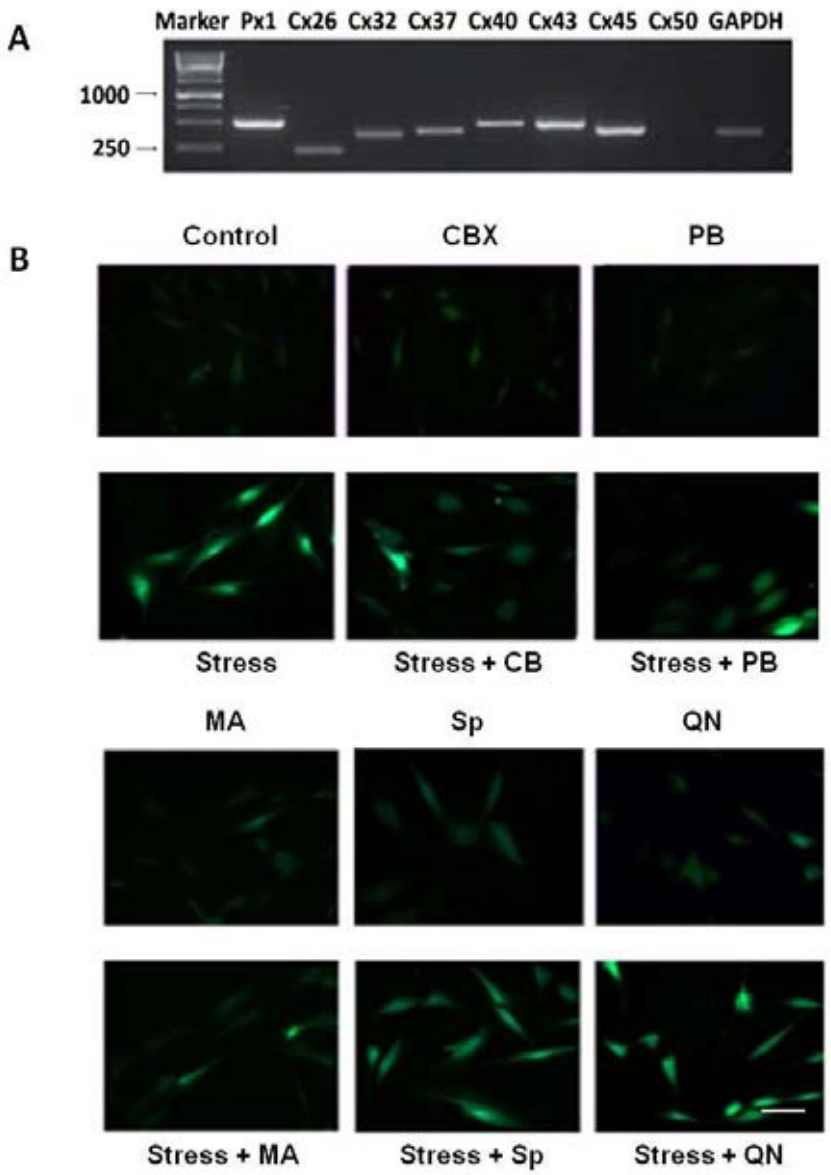

Figure 4.5 Gap junctions opening upon mechanical stimulation. RT-PCR (A) showed several types of gap junction protein expression in HPDL cells. HPDL were treated with mechanical stress (2 g/cm^2, 3 h), prior to 10 minutes incubation in carboxyfluorescein. Mechanical stress could induce the opening of gap junction as observed by increased fluorescent signaling, which indicated an influx of carboxyfluorescein dye. Carbenoxolone (CBX), probenecid (PB) and meclofenamic

acid (MA) significantly inhibited carboxyfluorescein uptake, while spermine (Sp) and quinine (QN) seemed to have no significant effect. Scale bar = 50 μm.

Panx1 and Cx43 hemichannels participated in mechanical stress-induced IL-1β expression

To further determine the involvement of gap junctions in mechanical stress-induced IL-1β expression, gap junction inhibitors were used. Carbenoxolone, a non-specific gap junction blocker, and probenecid, a specific Panx1 inhibitor, significantly inhibited IL-1β upregulation upon stress application at both mRNA and protein levels, as shown by RT-PCR (Fig. 4.6 A) and ELISA (Fig. 4.6 B). A specific Cx43 inhibitor, meclofenamic acid, was also able to decrease IL-1β induction, but with less effect, whereas spermine (a specific Cx40 inhibitor) and quinine (a specific Cx50 inhibitor) had no significant effects (Fig. 4.6 A, 4.6 B). Selective inhibition of Panx1 by its mimetic peptide, 10Panx, also resulted in significant decreased IL-1β secretion from stimulated HPDL cells (Fig. 4.6 C).

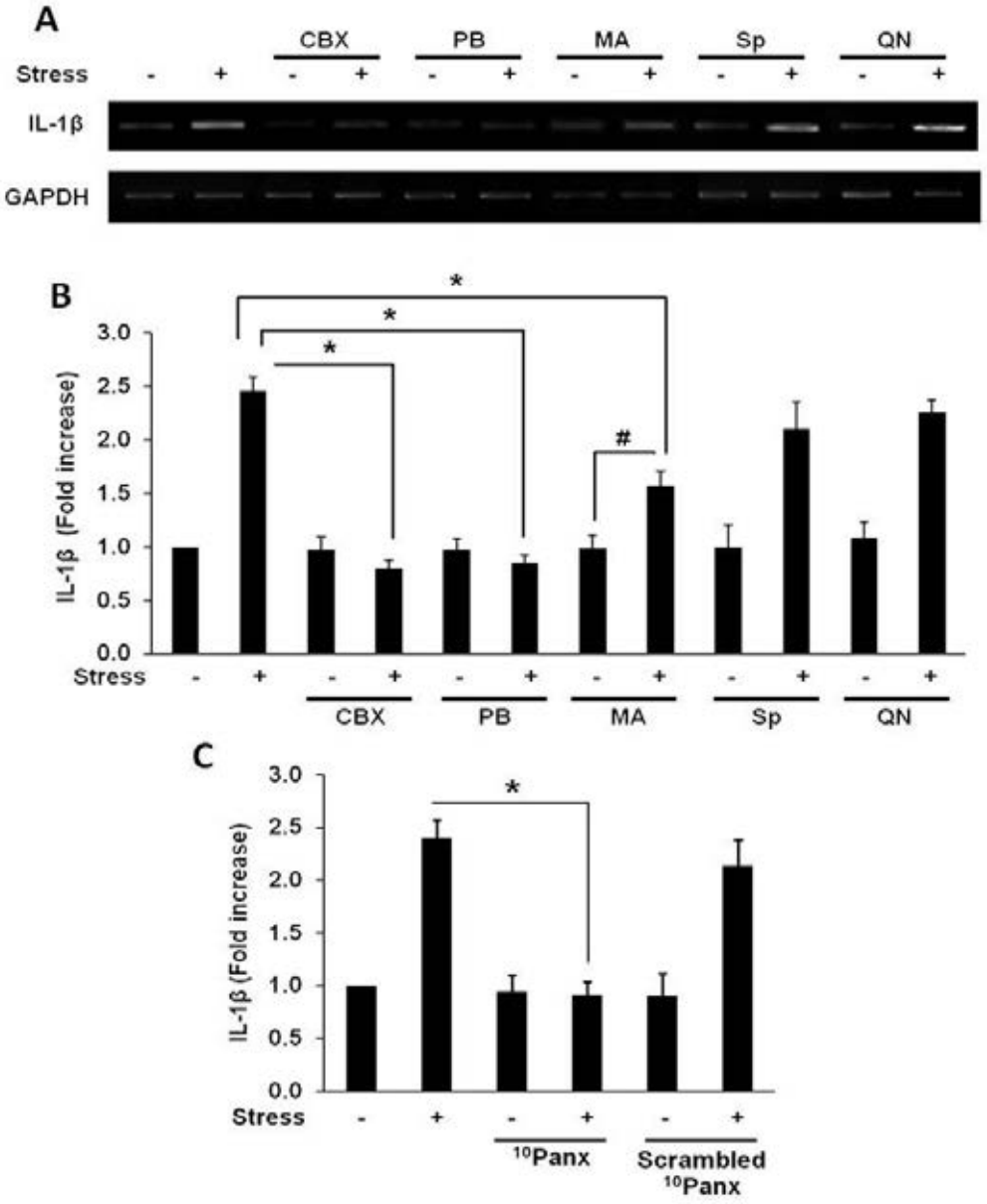

Figure 4.6 Role of gap junction hemichannels in mechanical stress-induced IL-1β expression. HPDL cells were treated with compressive loading (0-2.5/cm^2) for 3 h to stimulate IL-1β expression, which was measured by ELISA (A). Carbenoxolone (CBX), probenecid (PB) and meclofenamic acid (MA) significantly inhibited mechanical stress-induced IL-1β mRNA expression (B) and release (C), while spermine (Sp) and quinine (QN) had no significant effect. Similar results were

obtained from pretreatment of HPDL cells with ^{10}Panx (D). Results expressed as means ± SD from three different experiments. *, $^{\#}$ Significant difference, $p<0.05$. C, control.

The role of Panx1 and Cx43 were further investigated using siRNA transfection. Treatment with specific siRNA targeting Panx1 and Cx43 yielded approximately 80% knockdown of their mRNA and protein expression (calculated by Scion Image software, data not shown), compared to non-treated cells, as shown by RT-PCT (Fig. 4.7 A, 4.7 D) and western blot analysis (Fig. 4.7 B, 4.7 E). Transient knockdown of Panx1 (Fig. 4.7 C) and Cx43 (Fig. 4.7F) markedly decreased IL-1β release from mechanical-stimulated HPDL cells. However, similar to the inhibitory effect of Cx43 specific inhibitor, significant elevation of IL-1β level was still observed in Cx43 knockout group. Moreover, knockdown of Panx1 could suppress ATP release from stress-stimulated cells, as reported by luciferin-luciferase assay (Fig. 4.8 A). Besides, treatment with ATP was not able to stimulate IL-1β release in the absence of Panx1 (Fig. 4.8 B). While in Cx43 knockout group, even though the release of ATP was significantly decreased (Fig. 4.8 C), the induction of IL-1β upon ATP application was not much altered (Fig. 4.8 D). These data indicated a crucial function of Panx1 in IL-1β induction mechanism.

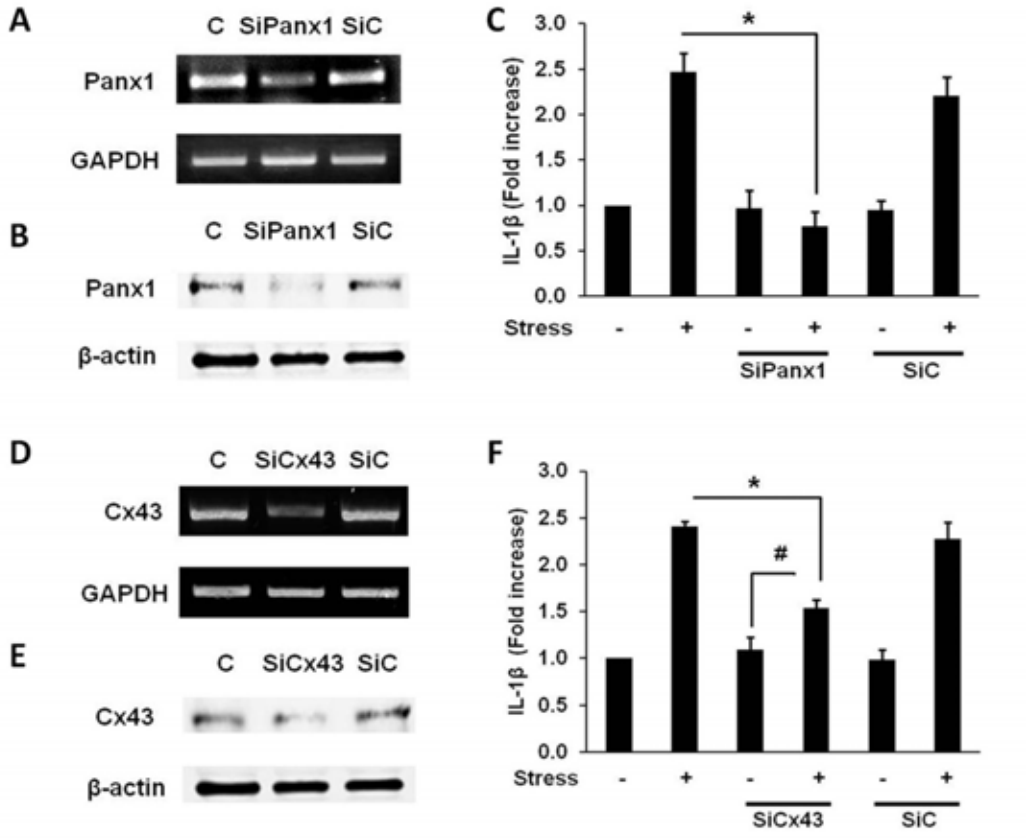

Figure 4.7 Function of Panx1 and Cx43 hemichannels is important in stress-induced IL-1β release in HPDL cells. Transient knockdown of Panx1 (A, B) and Cx43 (D, E) by siRNA resulted in marked decrease in their mRNA and protein expression, as shown in RT-PCR (A, D) and western blotting (B, E). ELISA showed knockdown of Panx1 (C) and Cx43 (F) strongly attenuated IL-1β release from stress-stimulated cells. Note the stronger effect in Panx1-knockout group. Results expressed as means ± SD from three different experiments. *,# Significant difference, $p<0.05$. C, control; siC, siRNA control.

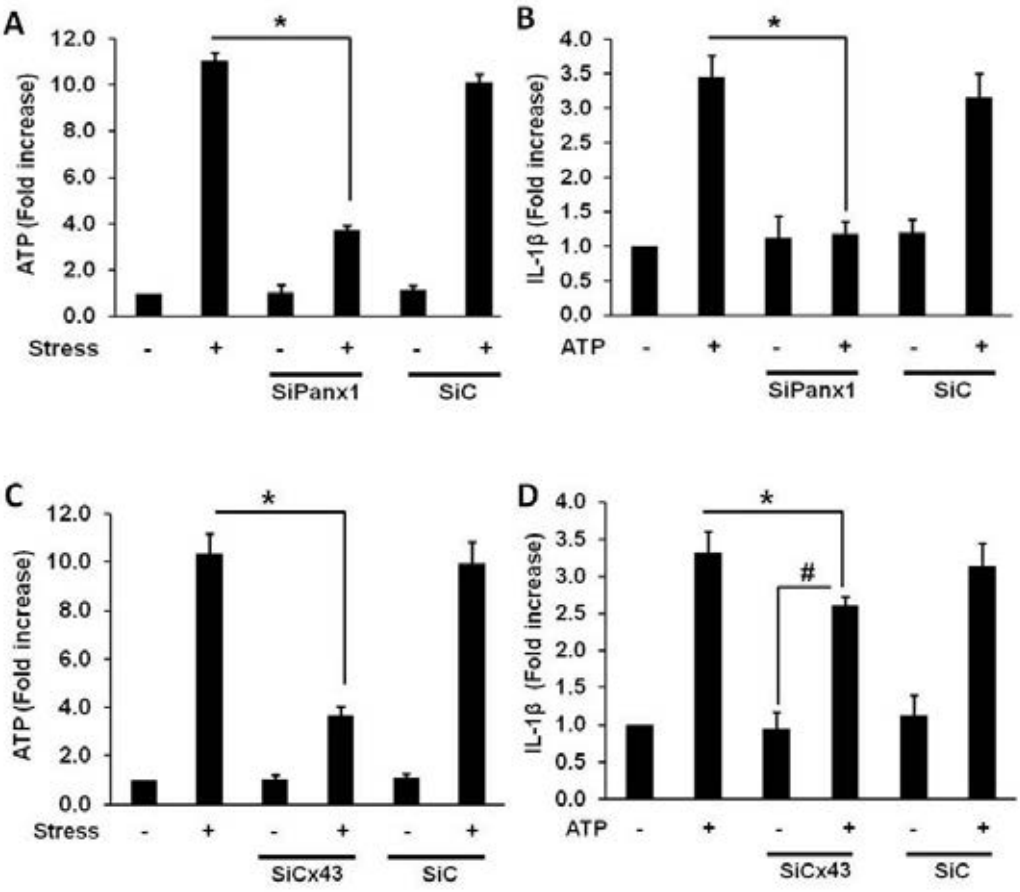

Figure 4.8 Roles of Panx1 and Cx43 hemichannels in stress-induced ATP release and ATP-induced IL-1β secretion in HPDL cells. Knockdown of Panx1 by siRNA significantly decreased ATP release upon mechanical stimulation (A), as well as IL-1β release upon ATP application (B) in HPDL cells. On the contrary, knockdown of Cx43 by siRNA could only suppress stress-induced ATP release (C), with no significant effect on ATP-stimulated IL-1β secretion (D). Results expressed as means ± SD from three different experiments. *,# Significant difference, $p<0.05$. siC, siRNA control.

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

Mechanical stress induced ATP release, which later stimulated IL-1β expression in HPDL cells. In order to determine specific P2 receptor subtype involved in ATP-induced IL-1β release in stress activated HPDL cells, several P2 receptor antagonists were used. A specific P2X7 receptor antagonist KN-62, in the same way as suramin, significantly diminished ATP-induced IL-1β release, while no or less significant effect was observed when using MRS2179 (a specific P2Y1 receptor antagonist), NF449 (P2X1, P2X3, P2Y1, P2Y2 receptor antagonist), and NF023 (P2X1, P2X2, P2X3, P2X4 antagonist) (Fig. 4.9). Similar inhibitory effects of these antagonists on stress-induced IL-1β expression were also observed (data not shown).

Function of P2X7 receptor was additionally confirmed using siRNA transfection. Treatment with siRNA targeting P2X7 receptor for 24 h yielded approximately 75-80% knockdown of P2X7 receptor mRNA and protein expression in HPDL cells (calculated by Scion Image Software, data not shown), as demonstrated by RT-PCR (Fig. 4.10 A) and western blot analysis (Fig. 4.10 B). After transfected with siRNA, mechanical stress or ATP was applied on cultured HPDL cells. IL-1β expression was detected using RT-PCR (Fig. 4.10 C, 4.10 D) and ELISA (Fig. 4.10 E, 4.10 F). Knockdown of P2X7 receptor resulted in dramatically decreased IL-1β expression in stimulated HPDL cells, while elevated IL-1β level was still observed in all control groups.

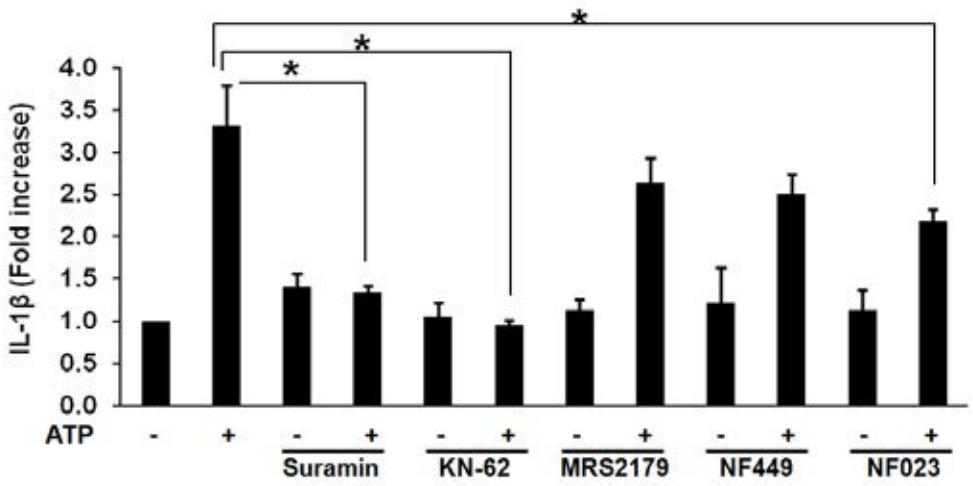

Figure 4.9 ATP-induced IL-1β release involved P2 purinergic signaling.

HPDL cells were treated with P2 receptor antagonists prior to ATP application. ELISA showed suramin and KN-62 markedly inhibited ATP-induced IL-1β release, while MRS2179, NF449 and NF023 had no or less significant effect. Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

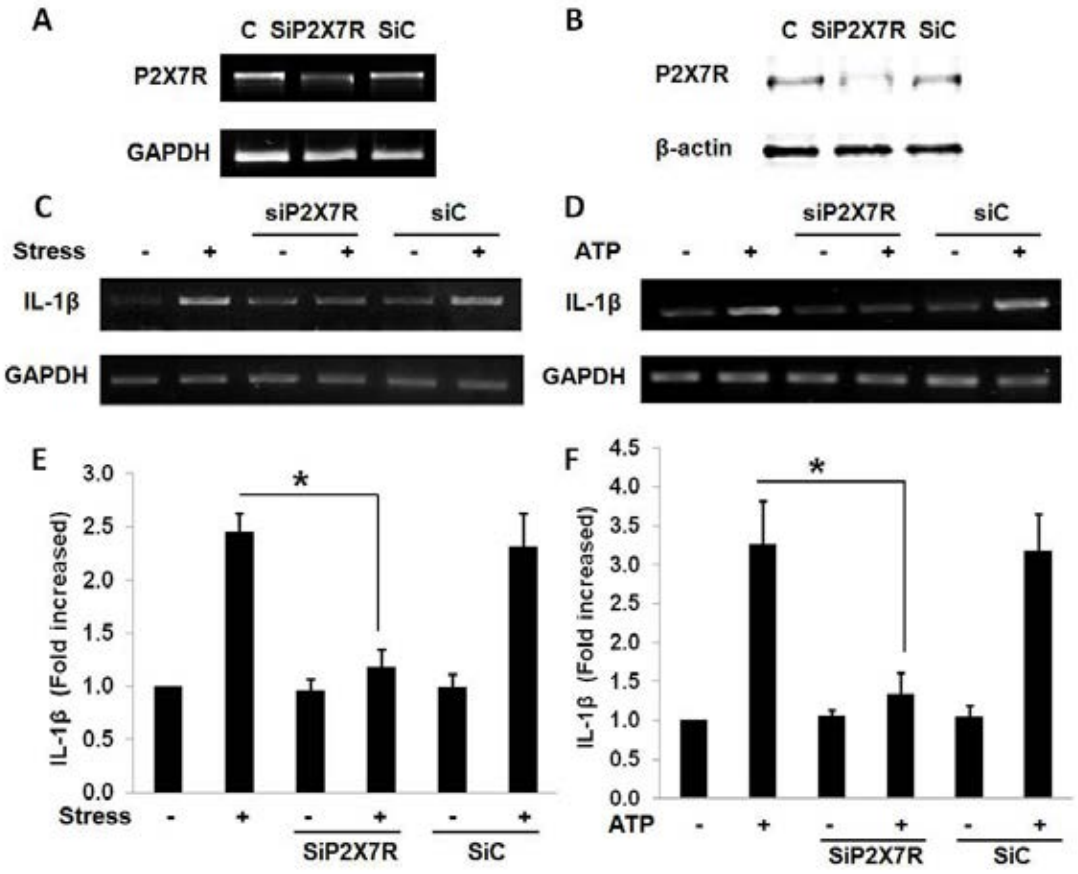

Figure 4.10 P2X7 receptor is a major P2 subtype responsible for IL-1β induction. Transient knockdown of P2X7 receptor by siRNA resulted in drastically decreased P2X7 receptor expression as shown in RT-PCR (A) and Western blot analysis (B). RT-PCR and ELISA showed siRNA targeting P2X7 receptor dramatically decreased IL-1β expression upon mechanical stress (C, E) or ATP (D, F) application, while no significant effects were observed on control groups. Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

The IL-1β induction is calcium-dependent

Since P2X7 receptor is well known as a cationic-channel highly permeable to calcium, the role of calcium was examined by using calcium inhibitors. An intracellular calcium chelator, BAPTA, a calcium-ATPase inhibitor, thapsigargin, and an intracellular calcium antagonist, TMB8, were able to diminish IL-1β induction in HPDL cells (Fig. 4.11 A, 4.11 B), no matter from stress or ATP application.

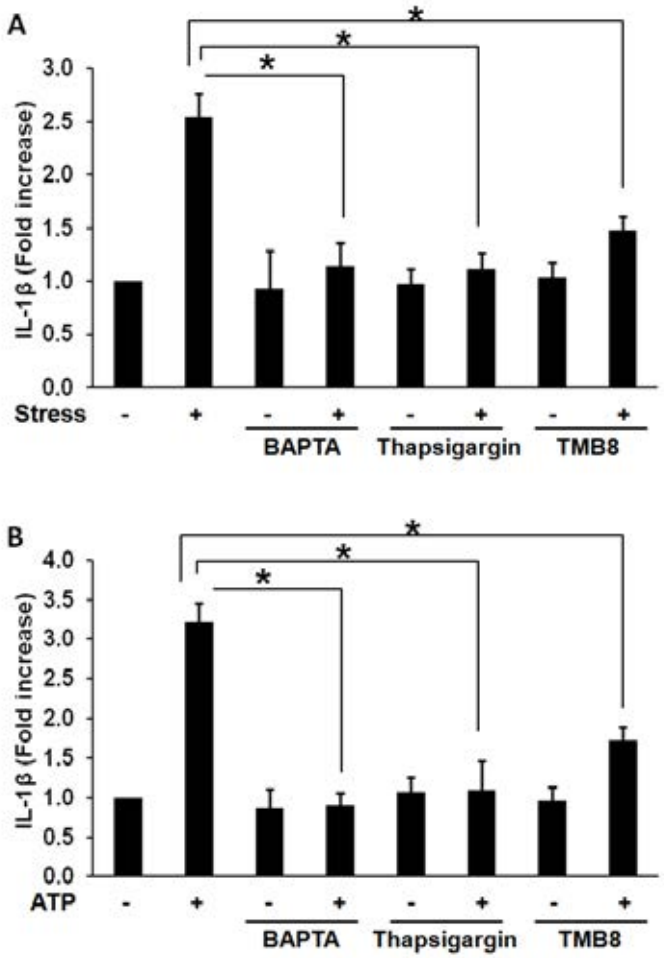

Figure 4.11 Calcium-dependent IL-1β release in HPDL cells. HPDL cells were treated with intracellular calcium antagonists prior to stress or ATP application. ELISA showed treatment of cells with BAPTA, thapsigargin and TMB8 significantly diminished IL-1β release in stress- (A) or ATP- (B) stimulated HPDL cells. Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

Co-expression of Panx1 and P2X7 receptor in HPDL cells

Panx1 hemichannel and P2X7 receptor have been reported to form complex in various cells, including macrophages. To determine the association of P2X7 receptor and Panx1 in HPDL cells, immunocytochemistry and co-immunoprecipitation were used. Fluorescence staining revealed that these two proteins were located at HPDL cells membrane (Fig. 4.12 A). Most of them were found co-localized with each other, suggesting they might as well form Panx1/P2X7 receptor complex in HPDL cells. Co-expression of P2X7 receptor and Panx1 was confirmed using co-IP and immunoblotting (Fig. 4.12 B). Upregulation of Panx1 and P2X7 receptor after mechanical stimulation was observed in western blot analysis (Fig. 4.12 B). Interestingly, in co-IP samples, their co-expression was extremely increased in stress-stimulated cells.

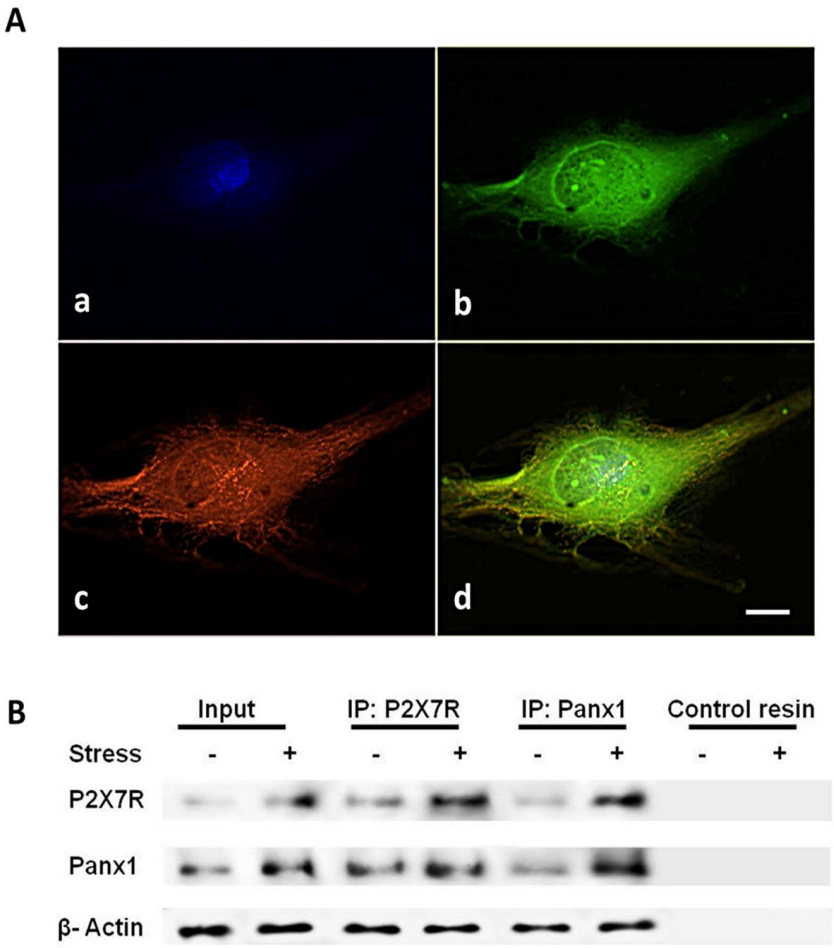

Figure 4.12 Co-localization of Panx1 and P2X7 receptor in HPDL cells. Immunostaining (A) showed expression of Panx1 hemichannel (A; b, green) and P2X7 receptor (A; c, red) in HPDL cells. Membrane co-localization of these two membrane channels was observed (A; d, merged). DAPI (A; a, blue) was used for nuclear staining. Scale bars = 10 µm. Western blot (B) showed protein expression of Panx1 (45 kDa) and P2X7 receptor (70 kDa) in HPDL cells, which was elevated after mechanical stimulation (Input, lane 1-2). Co-immunoprecipitation confirmed the association of Panx1 and P2X7 receptor. Co-expression of Panx1 and P2X7 receptor was significantly increased after mechanical stimulation (lane 3-4, 5-6). Control resin

without antibody was used as negative control (lane 7-8). IP: P2X7R and IP: Panx1 refer to immunoprecipates undergoing co-IP steps using resin tagged with antibody against P2X7 receptor and Panx1, respectively.

IL-1β release mechanism in HPDL cells

To investigate whether IL-1β could be released through vesicle, vesicular trafficking inhibitors monensin, N-ethylmaleimide (NEM) and brefeldin A (BFA) were used. Added 30 minutes prior to stress or ATP application, all inhibitors strongly inhibited the release of IL-1β from HPDL cells, as observed by ELISA (Fig. 4.13 A, 4.13 B), while mRNA expression of IL-1β remained unaffected (Fig. 4.13 C, 4.13 D). In addition, treatment of HPDL cells with mechanical stress or ATP resulted in increased fluorescent signal of SNAP-25, a specific protein appeared in the core of vesicular-membrane fusion complex, compared to non-treated cells (data not shown). Interestingly, SNAP-25 localization appeared to be associated with P2X7 receptor, which was co-localized with Panx1 (Fig. 4.14).

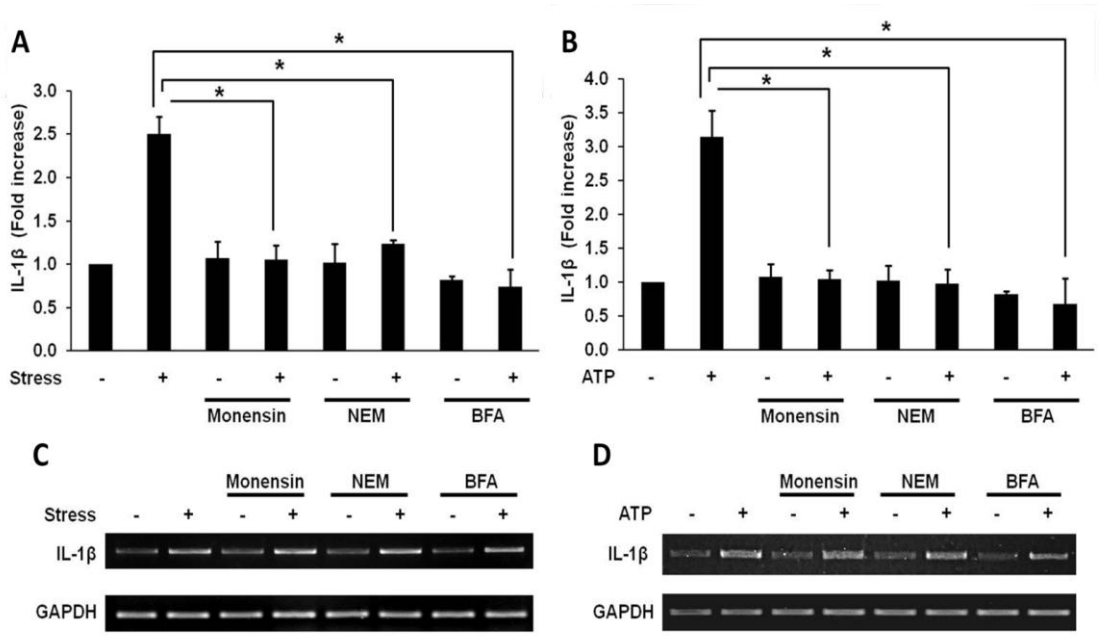

Figure 4.13 IL-1β release occurred through vesicles. Mechanical stress (2 g/cm^2, 3 h) or ATP (40 μM, 30 min) was applied on cultured HPDL cells in order to induce IL-1β expression. ELISA showed monensin, NEM and BFA significantly decreased IL-1β release (Fig. 4A, 4B), while RT-PCR showed no effect on IL-1β mRNA expression (Fig. 4C-4D). Results expressed as means ± SD from three different experiments. *Significant difference, $p<0.05$

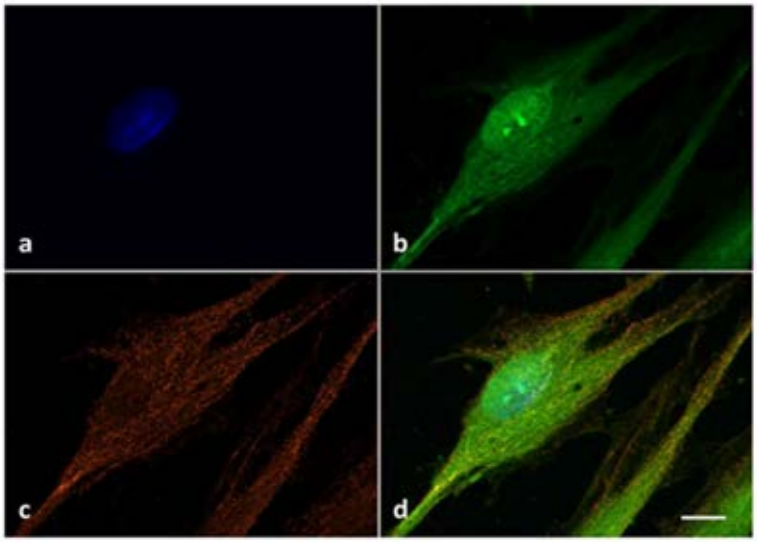

Figure 4.14 Panx1 / P2X7 receptor might act as docking site for IL-1β release in HPDL cells. HPDL cells were treated with compressive force (2 g/cm^2, 3 h) to stimulate IL-1β release. Immunostaining revealed the positive staining of SNAP-25 (b, green), which was found associated with membrane localization of P2X7 receptor (c, red - d, merged). Scale bars = 10 µm

CHAPTER V

DISCUSSION AND CONCLUSION

It is well acknowledged that mechanical stress could induce tissue inflammation. Increased proinflammatory cytokines including IL-1β, IL-6, IL-8, as well as TNF-α have been reported in many cell types e.g. chondrocytes (Fujisawa et al., 1999; Lee et al., 2008), osteoblasts (Koyama et al., 2008), TMJ synovial cells (Muroi et al., 2007) and human dental pulp cells (Lee et al., 2008) receiving various kinds of stress. In our study, compressive stress induced inflammation in HPDL cells by increasing a major proinflammatory cytokine IL-1β expression. IL-1β, presented in early phase of inflammation, accounts for the activation of downstream inflammatory mediators such as COX-2, PGE2 and MMPs (Archambault et al., 2002a; Fermor et al., 2002; Tsuzaki et al., 2003a; Tsuzaki et al., 2003b), resulting in tissue damages.

We previously reported that mechanical stress-induced RANKL and osteopontin in HPDL cells through the release of a potent inflammatory mediator ATP (Luckprom et al., 2010; Wongkhantee et al., 2008). By using ATPase as well as P2 receptor antagonists, we found IL-1β induction upon mechanical stimulation also occurred through the release of ATP. In addition, ATP itself could act as IL-1β inducer and IL-1β expression level was related to the applied force, ATP concentration as well as duration of treatments.

ATP could be released from various cells receiving stress or trauma (Fredholm, 2007; Gourine et al., 2007; Hazama et al., 1999; Praetorius and Leipziger, 2009; Skaper et al., 2010; Wongkhantee et al., 2008). Treatment of stress- or ATP-stimulated HPDL cells with a transcription blocker and translation blocker markedly decreased stress-induced IL-1β expression, suggesting IL-1β induction occurred at mRNA level. However, the level of ATP release from HPDL cells receiving mechanical stress was slightly affected by these two inhibitors, implying ATP was most likely released from an intracellular storage, not mainly by new protein synthesis. In our study, rising in ATP level could be detected within 5 min after stress application in HPDL cells. These results suggested the rather rapid response of HPDL cells and supported the idea that ATP might play a role as an early inflammatory signaling molecule responding to mechanical stress, especially in HPDL cells.

The released ATP subsequently activates P2 receptors. We formerly reported that ATP could stimulate osteopontin and RANKL expression via activation of P2Y1 receptor (Luckprom et al., 2010). In the present study, by using P2 receptor antagonists and siRNA silencing experiments, we found that P2X7 receptor is a main P2 subtype responsible for ATP-induced IL-1β expression in HPDL cells. ATP-induced IL-1β processing and release via activation of P2X7 receptor has been illustrated in immune cells including macrophages and monocytes (Ferrari et al., 1997a; Solle et al., 2001) as well as in microglia (Bianco et al., 2005; Sanz and Di Virgilio, 2000). In genetically engineered human embryonic kidney cells HEK-293 overexpressing P2X7 receptor, activation of P2X7 receptor resulted in rapid secretion of IL-1β (Gudipaty et

al., 2003), while P2X7 receptor-deficient macrophages in P2X7R$^{-/-}$ mice were not able to generate IL-1β in response to ATP activation (Solle et al., 2001). The activation of P2X7 receptor is therefore undoubtedly correlated with inflammation.

In orthodontic treatment, the induction of IL-1β mRNA was observed after an application of orthodontic force in rats (Alhashimi et al., 2001). Besides, experiments in P2X7R$^{-/-}$ mice revealed a significant role of P2X7 receptor in mechanotransduction in orthodontic tooth movement (Viecilli et al., 2009). In knockout mice, decreased bone resorption along bone/PDL interface was observed. Moreover, severe external root resorption in treated tooth was described as a consequent of an incompetent immune response to remove necrotic tissue around stress area, due to the deficient levels of P2X7 and IL-1β. Our data clarified the role of P2X7 receptor and ATP in stress-induced IL-1β expression in HPDL cells, which could explain the phenomena observed in animal model. Despite that, blocking other P2 subtypes also resulted in slightly decreased IL-1β level upon HPDL cells activation, suggesting an alternative signaling pathway aside from P2X7 receptor. Besides, since there are several types of ATP receptors, it is possible that ATP could induce other cellular responses via activation of other types of P2 receptors.

ATP-induced IL-1β expression mechanisms have been proposed in macrophages and monocytes. Activation of P2X7 receptor by ATP could activate caspase-1, an enzyme function in converting inactive precursor of IL-1β into its active mature form (Dinarello, 2009; Sanz and Di Virgilio, 2000). Caspase-1 activation by P2X7 receptor stimulation possibly occurred through rapid decrease in intracellular

potassium (K^+) level, which activates caspase-1 from its pro-form to active-form (Dinarello, 2009). Though it still remains unclear in HPDL cells, we demonstrated the involvement of intracellular calcium (Ca^{2+}). With high affinity binding to Ca^{2+}, BAPTA acts as an intracellular calcium chelator, thapsigargin depletes intracellular calcium storage, while TMB 8 acts as an intracellular Ca^{2+} antagonist, calcium channel blocker and Ca^{2+} mobilization inhibitor. Treatment of HPDL cells receiving mechanical stress or ATP with these inhibitors, in order to diminish intracellular calcium, resulted in vividly decrease in IL-1β release. Together with our previous study stated that mechanical stress-induced ATP release is calcium-dependent (Luckprom, Kanjanamekanant and Pavasant, 2011), these finding revealed a significant participation of intracellular calcium signaling in response to mechanical stimulation in HPDL cells.

The involvement of Ca^{2+} in P2X7 receptor-dependent IL-1β secretion was also found in macrophage and monocytes (Ferrari et al., 2006; Gudipaty et al., 2003). Moreover, considering that all P2X receptors could act as ion channel, it is possible that they function in similar way. This could also explain the slight inhibitory effect of NF449 and NF023 on IL-1β induction due to their specificity to P2X subtypes.

We also reported gap junction hemichannels function in mechanical stress-induced IL-1β expression. In dye-uptake experiment, we found mechanical stress could activate the opening of gap junction, as observed by increased fluorescent signaling. Nonspecific gap junction blocker, as well as specific Panx1 and Cx43 blockers, could inhibit carboxyfluorescein uptake, suggesting these two hemichannel

might play a role in cellular response to mechanical stimulation. Pannexins represent a new family of gap junction proteins consisting of three members, Panx1, Panx2 and Panx3. Among these, a ubiquitously expressed Panx1 has been widely studied and its unique functional role as hemichannel has been proposed. Panx1 is found to be mechanosensitive and permeable to ATP (Bao et al., 2004a; Huang et al., 2007; Iglesias et al., 2009). The close relationship between Panx1 and P2X7 purinergic receptor has been established. The involvement of Panx1 in P2X7R-dependent IL-1β release has been stated in ATP- or endotoxin-primed macrophages (Ferrari et al., 1997a; Pelegrin, Barroso-Gutierrez and Surprenant, 2008; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009). In our study, a nonspecific gap junction blocker (CBX), Panx1 specific inhibitor (probenecid), Panx1 mimetic peptide (^{10}Panx) and siRNA transfection could strongly inhibit IL-1β induction, both from stress and ATP stimulation. Our results are in agreement with Pelegrin and Surprenant study (2006), which stated that blocking of Panx1 by CBX, ^{10}Panx and siRNA could inhibit P2X7 receptor-mediated IL-1β release from endotoxin-primed macrophage (Pelegrin and Surprenant, 2006). Beside from Panx1 hemichannel, another gap junction hemichannel involved in mechanical stress-induced IL-1β expression in HPDL cells is Cx43. Selective blocking of Cx43 by its specific inhibitor meclofenamic acid and siRNA transfection resulted in decreased IL-1β expression. However, the inhibitory effects of Cx43 inhibitor and siRNA targeting Cx43 on IL-1β expression were less than those of Panx1 inhibitors.

Since Panx1 and Cx43 hemichannels are permeable to ATP (Bao et al., 2004a; Huang et al., 2007; Iglesias et al., 2009; Kang et al., 2008), and mechanical stress-induced IL-1β expression occurred through ATP secretion in HPDL cells, we then examined the involvement of hemichannels in ATP release. We found that blocking Panx1 or Cx43 hemichannels by inhibitors or siRNA transfection significantly decreased ATP release from mechanical-stimulated HPDL cells. These results indicate that Panx1, as well as Cx43, could serve as ATP releasing channels in HPDL cells. The role of Panx1 in ATP release has been demonstrated in macrophages (Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009), erythrocytes (Locovei et al., 2006), astrocytes (Iglesias et al., 2009), air way epithelia (Ransford et al., 2009), and taste buds (Huang et al., 2007). Studies in neutrophils (Eltzschig et al., 2006), endothelial cells (Faigle et al., 2008), and articular chondrocytes(Knight et al., 2009) also indicated Cx43 as ATP releasing channel.

In this study, although blocking Cx43 or Panx1 by its specific inhibitor and siRNA could strongly inhibit mechanical stress-induced IL-1β expression, some significant elevation in IL-1β level was still observed in Cx43 knockout group. Furthermore, in the absence of Panx1, ATP-induced IL-1β expression was almost abolished, while no significant effect was observed in the absence of Cx43. These finding signify the role of Panx1 hemichannel both in stress-induced ATP release, and in ATP-induced IL-1β expression, while Cx43 hemichannel only plays an important role as one of the ATP releasing channels in HPDL cells. In all, these evidences

indicated an important function of gap junction hemichannel, particularly Panx1 and Cx43, in cellular response to mechanical stimulation.

Increasing reports have demonstrated the association of Panx1 and P2X7 receptor. Studies in J774 macrophage cell lines provided the evidences that these two membrane proteins were immunoprecipitated (Iglesias et al., 2008; Pelegrin and Surprenant, 2006). Upregulation of P2X7 receptor and Panx1 have been identified in lipopolysaccharide (LPS)-primed macrophages and monocytes (Humphreys and Dubyak, 1996; Humphreys and Dubyak, 1998; Pelegrin and Surprenant, 2006). Panx1 has been proposed to be part of P2X7 receptor and involves in ATP-gated P2X7 receptor activation, which results in the opening of plasma membrane pore responsible for IL-1β release (Pelegrin and Surprenant, 2006). By using co-immunoprecipitation along with immunocytochemistry, we directly illustrated the membrane co-localization of P2X7 receptor and Panx1 in HPDL cells. We also found an increase in protein expressions of Panx1 and P2X7 receptors upon mechanical stimulation. Significantly, the level of their co-expression was also increased in stress-stimulated cells, indicating that they might re-locate themselves to each other after receiving stress. This finding supported the idea that these two membrane channels might be working together in response to stress or ATP, as well as in IL-1β release mechanism.

ATP-induced P2X7 receptor activation causes a formation of plasma membrane pore allowing transmembrane fluxes of hydrophilic molecules of up to 900 Da (Di Virgilio, 1995; Ferrari et al., 2006; Sluyter et al., 2004; Surprenant et al.,

1996), while mature IL-1β itself has a molecular weight of around 17 kDa (Dinarello, 2009; Ferrari et al., 2006). It is therefore unlikely to speculate that IL-1β could be released through this membrane pore. Although detailed IL-1β release mechanism is not fully understood, studies in immune cells proposed several IL-1β release pathways including exocytosis of secretory lysosome, secretion via microvesicles shedded from plasma membrane, or caspase-1 gated transmembrane pore (Dinarello, 2009; Le Feuvre et al., 2002). In this study, three well-known vesicular trafficking inhibitors, monensin, NEM and BFA, were used. Monensin is capable of blocking vesicular formation; NEM prevents vesicular-membrane fusion, while BFA inhibits protein transportation during cell activation. Pretreatment with these inhibitors in stress- or ATP-stimulated cells significantly decreased IL-1β release, while the mRNA activation of IL-1β still remained unchanged, strongly indicated that IL-1β release was most likely occurred through vesicles. This is similar to Bianco et al. study (2005), which illustrates the ATP/P2X7 receptor-dependent vesicular shedding of IL-1β in microglia (Bianco et al., 2005).

In order for intracellular vesicles to be released from cells, fusion of vesicular membrane and plasma membrane is needed. SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) is a group of proteins mediating vesicle-membrane fusion. They form SNARE complex, which function in bridging cytoplasmic vesicle and plasma membrane, allowing vesicular-membrane fusion and, afterwards, the release of vesicle (Chen and Scheller, 2001; Hay, 2001; Ungar and Hughson, 2003). Immunostaining showed plasma membrane co-localization of SNAP-

25, an important core protein of the SNARE complex, and P2X7 receptor/Panx1 in stress-stimulated HPDL cells. Collectively, we postulate that Panx1/P2X7 receptor could function in direct docking or regulating IL-1β vesicles to cell membrane, where they merged and, subsequently, IL-1β is released to extracellular space. However, the underlying mechanism is still unknown and requires further investigation.

In conclusion, we clarified the role of ATP as an early-released signaling molecule in mechanically-stimulated HPDL cells. The released ATP, which mainly occurs through Cx43 and Panx1 hemichannels, subsequently activates P2X7 receptor, which found to be co-localized with Panx1, resulting in the upregulation of a potent inflammatory cytokine IL-1β. Function of Panx1 / P2X7 receptor might be required for IL-1β induction and vesicular release (Figure 5.1). These findings suggest the possible novel role of Panx1 / P2X7 receptor in cellular response to mechanical stimulation or injury, not only in macrophages or immune cells, but in other cell types as well. Our work provides an insight of how mechanical stress regulates inflammation in HPDL cells, which might benefit clinical treatment in the future. Nevertheless, cells surface receptors or proteins that function in receiving or conferring stress to cellular signaling and response, as well as the mechanotransduction pathway is yet to be investigated.

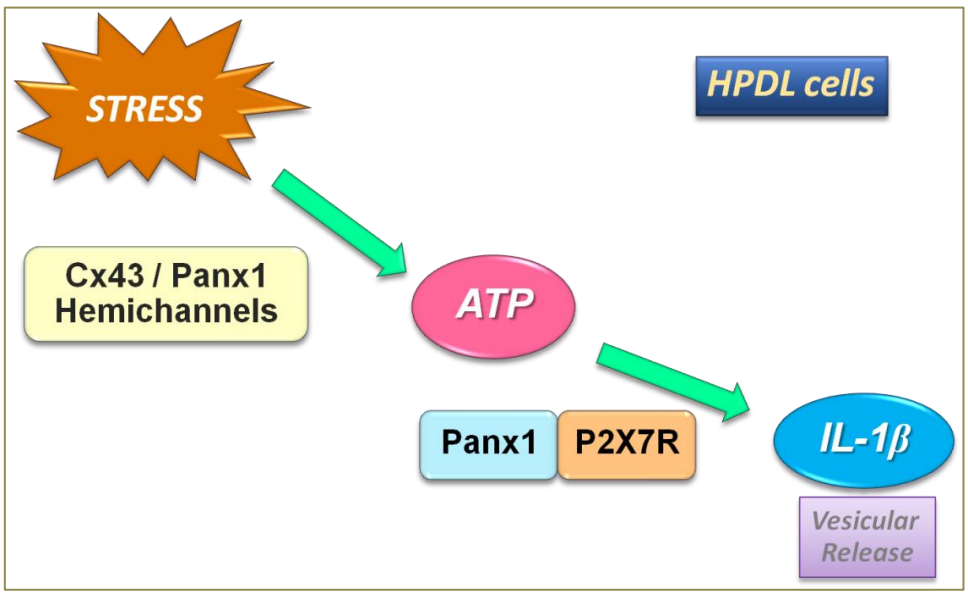

Figure 5.1 Roles of ATP and gap junction hemichannels in mechanically-stimulated HPDL cells.

Future studies

1. To examine the underlying mechanism in IL-1β processing and vesicular release in HPDL cells
2. To study the effects of other types of stress, including tension and fluid sheer stress, on inflammation in HPDL cells
3. To find other pathological roles of ATP via other P2 subtypes activation in HPDL cells
4. To determine other physiological roles of hemichannels, particularly Panx1, in HPDL cells
5. To identify the mechano-sensors or cell surface receptors that initiate the signaling cascades in mechanical stimulated cells

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