บทบาทของความดันอุทกสถิตต่อระดับการแสดงออกของอินเตอร์ลิวคิน 6 ในเซลล์เนื้อเยื่อในของฟันมนุษย์

นางสาว สิริโฉม สาตราวาหะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

THE ROLE OF HYDROSTATIC PRESSURE ON INTERLEUKIN-6 EXPRESSION IN HUMAN DENTAL PULP CELLS

Miss Sirichom Satrawaha

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Oral Biology
Faculty of Dentistry
Chulalongkorn University
Academic year 2010

Thesis Title	THE ROLE OF HYDROSTATIC PRESSURE ON INTERLEUKIN-6 EXPRESSION IN HUMAN DENTAL PULP CELLS
By	Miss Sirichom Satrawaha
Field of Study	Oral Biology
Thesis Advisor	Associate Professor Piyamas Sumrejkanchanakij, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

W. Tasachan ..... Dean of the Faculty of Dentistry

(Associate Professor Watcharaporn Tasachan)

THESIS COMMITTEE

Prasit Pavasant ..... Chairman

(Associate Professor Prasit Pavasant, D.D.S., Ph.D.)

Piyamas Sumrejkanchanakij ..... Thesis Advisor

(Associate Professor Piyamas Sumrejkanchanakij, D.D.S., Ph.D.)

..... Examiner

(Assistant Professor Chootima Ratisoontorn, D.D.S., Ph.D.)

..... Examiner

(Assistant Professor Ruchanee Ampornaramveth, D.D.S., Ph.D.)

Peraya Surapipongpuntr ..... External Examiner

(Assistant Professor Peraya Surapipongpuntr, D.D.S., Ph.D.)

สิริโฉม สาตราวาหะ : บทบาทของความดันอุทกสถิตต่อระดับการแสดงออกของอินเตอร์ลิวคิน 6 ในเซลล์เนื้อเยื่อในของฟันมนุษย์. (THE ROLE OF HYDROSTATIC PRESSURE ON INTERLEUKIN-6 EXPRESSION IN HUMAN DENTAL PULP CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ทญ.ดร. ปิยมาศ สำเร็จกาญจนกิจ, 92 หน้า.

ความดันอุทกสถิตภายในโพรงฟันจะเพิ่มสูงขึ้น ในขณะที่มีการอักเสบภายในโพรงฟัน หรือจากวิธีการบูรณะฟันบางอย่าง แต่อย่างไรก็ตาม ยังไม่มีการศึกษาถึงบทบาทของความดันอุทกสถิตในเซลล์เนื้อเยื่อในของฟันมนุษย์ การวิจัยนี้จึงได้ศึกษาผลของความดันอุทกสถิตต่อการสร้างสารอินเตอร์ลิวคิน 6 ในเซลล์เนื้อเยื่อในของฟันมนุษย์ ผลการศึกษาพบว่า ความดันอุทกสถิตกระตุ้นการสร้างอินเตอร์ลิวคิน 6 ทั้งในระดับ เอ็มอาร์เอ็นเอ และโปรตีน ในลักษณะที่สัมพันธ์กับระยะเวลาและระดับของความดันอุทกสถิตที่เพิ่มขึ้น การเพิ่มขึ้นของอินเตอร์ลิวคิน 6 เมื่อกระตุ้นด้วยความดันอุทกสถิตจะถูกยับยั้งได้อย่างมีนัยสำคัญ ด้วยการใส่สารยับยั้งการส่งสัญญาณจากตัวรับที่ผิวเซลล์ในกลุ่ม P2Y ที่ไม่จำเพาะ (ซูรามิน) และสารที่จับกับแคลเซียมภายในเซลล์ (แบ็บตา-เอเอ็ม) ผลการทดลองนี้แสดงให้เห็นถึงความเกี่ยวข้องของการส่งสัญญาณผ่านตัวรับในกลุ่ม P2Y และระดับแคลเซียมภายในเซลล์ในกระบวนการส่งสัญญาณของความดันอุทกสถิต การขัดขวางการส่งสัญญาณจำเพาะต่อตัวรับที่ผิวเซลล์ชนิด P2Y6 ด้วยการใส่สารยับยั้ง MRS2578 และการใช้ siRNA ต่อตัวรับ P2Y6 สามารถลดการสร้างอินเตอร์ลิวคิน 6 ที่ถูกกระตุ้นด้วยความดันอุทกสถิต ในขณะที่การใส่สารยับยั้ง MRS2179 ซึ่งขัดขวางการส่งสัญญาณจำเพาะต่อตัวรับชนิด P2Y1 และ NF449 ซึ่งขัดขวางการส่งสัญญาณจำเพาะต่อตัวรับชนิด P2X1, P2X3, P2Y1 และ P2Y2 ไม่สามารถยับยั้งผลดังกล่าวได้ นอกจากนี้ การใส่อาหารเลี้ยงเซลล์ที่เก็บจากการกระตุ้นเซลล์เนื้อเยื่อในของฟันด้วยความดันอุทกสถิต และการใส่สาร UDP ซึ่งเป็นตัวกระตุ้นจำเพาะต่อตัวรับสัญญาณ P2Y6 สามารถกระตุ้นเซลล์เนื้อเยื่อในของฟันให้สร้างอินเตอร์ลิวคิน 6 เพิ่มขึ้นได้อย่างมีนัยสำคัญ โดยสรุปงานวิจัยนี้เป็นการศึกษาแรกที่แสดงให้เห็นว่า ความดันอุทกสถิตส่งเสริมการสร้างอินเตอร์ลิวคิน 6 ในเซลล์เนื้อเยื่อในของฟันมนุษย์ โดยการกระตุ้นผ่านตัวรับสัญญาณ P2Y6 ซึ่งผลการศึกษานี้จะทำให้เข้าใจถึงบทบาทของความดันอุทกสถิตต่อการหลั่งไซโตไคน์ที่เกิดขึ้นในขบวนการอักเสบภายในโพรงฟัน

สาขาวิชา .....ชีววิทยาช่องปาก.....ลายมือชื่อนิสิต.....สิริโฉม สาตราวาหะ.....

ปีการศึกษา.....2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....ปิยมาศ สำเร็จกาญจนกิจ

4876451832 : MAJOR ORAL BIOLOGY

KEYWORDS : HYDROSTATIC PRESSURE / INTERLEUKIN-6 / P2Y6 RECEPTOR / HUMAN DENTAL PULP CELLS

SIRICHOM SATRAWAHA : THE ROLE OF HYDROSTATIC PRESSURE ON INTERLEUKIN-6 EXPRESSION IN HUMAN DENTAL PULP CELLS. THESIS ADVISOR : ASSOC. PROF. PIYAMAS SUMREJKANCHANAKIJ, D.D.S., Ph.D., 92 pp.

An increase in intrapulpal hydrostatic pressure (HP) occurs during inflammation and restorative procedures. However, the role that HP plays in human dental pulp cell (HDPCs) is not well understood. In this study, we investigated the effect of HP on interleukin-6 (IL-6) expression in HDPCs. The results showed that HP up-regulated IL-6 HDPC mRNA expression and protein release in a time- and dose-dependent manner. The induction of IL-6 by HP was significantly inhibited by an antagonist for the non-specific purinergic receptor family (suramin) and an intracellular calcium chelator (BAPTA-AM). These results suggest the involvement of P2Y receptor and intracellular calcium in HP signaling pathway, respectively. Using loss of function experiments, we showed MRS2578 (a specific P2Y6 antagonist), as well as P2Y6 small interfering RNA, abolished HP-induced IL-6, while MRS2179 (a specific P2Y1 antagonist) and NF449 (a P2X1, P2X3, P2Y1, and P2Y2 antagonist) had no effect. Moreover, we demonstrated that either the conditioned medium collected from the culture after application of HP or addition of UDP, a selective agonist of P2Y6, up-regulated IL-6 expression in HDPCs. In conclusion, this study is the first demonstration that HP could induce IL-6 expression through the P2Y6 receptor in HDPCs, which provides new insight into the role of pressure on cytokine release during pulpal inflammatory process.

Field of Study : Oral Biology Student's Signature Sirichom Satrawaha

Academic Year : 2010 Advisor's Signature Piyamas Sumrejkanchanakij

ACKNOWLEDEMENTS

I would like to express my deepest gratitude and sincere appreciation to my advisor Associate Professor Piyamas Sumrejkanchanakij, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University for her supervision, suggestion on my work throughout the course. I sincerely appreciate Associate Professor Prasit Pavasant, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University for his helpful suggestion, guidance and kind support throughout my work.

I would like to thank my thesis committee members: Associate Professor Prasit Pavasant, Associate Professor Tussanee Yongchaitrakool, Assistant Professor Chootima Ratisoontorn, Assisstant Professor Peraya Surapipongpuntr, Assistant Professor Ruchanee Ampornaramveth for their valuable comment, suggestion and kindness.

I would like to thank Professor Vincent Everts and Dr. Kevin Thompkin for editing the manuscript. I would like to thank Associate Professor Oranart Matangkasombut, Associate Professor Chintana Sirichompun, Associate Professor Vachara Phetcharakupt for their encouragement and giving me an inspiration.

I would like to ackowledge Faculty of Dentistry, Naresuan University and Chulalongkorn University for financial support. This study was supported by the 90th anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University Centenary Academic Development Project and Mineralized Tissue Research Unit, Faculty of Dentistry, Chulalongkorn University.

Finallly, I express my sincere thanks to all my friends and my family for their support and encouragement during this work.

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BMP-2	bone morphogenetic protein-2
Ca^{2+}	calcium ion
CGRP	calcitonin gene-related peptide
CLC	cardiotrophin-like cytokine
CNTF	ciliary neurotrophic factor
COX-2	cyclooxygenase-2
CT-1	cardiotrophin-1
CX43	connexin 43
DAG	diacylglyceral
DKK1	Dickkopf 1
DMEM	Dulbecco's Modified Eagle Medium
DPSCs	dental pulp stem cells
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde 3 posphate dehydrogenase
gp130	glycoprotein 130
GPCRs	G protein-coupled receptors
HDPCs	human dental pulp cells
HP	hydrostatic pressure
HSP	heat shock protein
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IP-10	inducible-protein-10
IP3	inositol triphosphate

JAK	Janus kinase
KSHV-IL6	Kaposi's sarcoma-associated herpesvirus interleukin 6 like protein
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LTA	lipoteic acid
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MMP	matrix metalloproteinase
NF-κB	nuclear factor-kappa B
OPG	osteoprotegerin
OSCAR	osteoclast-associated receptors
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PKC	protein kinase C
PLC β	phospholipase β
PPADS	pyridoxal-5'-phosphate-6-azophenyl-2,4-disulfonate
PTHrP	parathyroid hormone-related protein
RANK	receptor activator of nuclear factor κB
RANKL	receptor activator of nuclear factor κB ligand
RT-PCR	reverse transcription polymerase chain reaction
sIL-6R	soluble interleukin-6 receptor
siRNA	small interfering ribonucleic acid
STATs	signal transducers and activators of transcriptions
TGF-β1	transforming growth factor- beta1
TNF-α	tumor necrosis factor-alpha
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate

CHAPTER I
INTRODUCTION

A unique feature of the dental pulp is that it is rigidly encased by the mineralized dentin wall, creating a so called “low-compliance environment”, wherein the pulp tissue has a limited opportunity to expand. Vasodilatation and increased vascular permeability evoked during an inflammatory reaction results in an increased pulpal hydrostatic pressure in the interstitial fluid surrounding the dental pulp cells *(1)*. It has been reported that restorative procedures such as high power laser cutting produced approximately a fourfold increase in intrapulpal pressure *(2)*. Moreover, dental trauma, uncontrolled orthodontic forces and chronic parafunctional forces could also cause pulpal inflammation and a chronic rise in intra-pulpal pressure *(3-5)*. However, little is known about the role of increased pulpal hydrostatic pressure on the biological response of human dental pulp cells.

Hydrostatic pressure (HP) is a mechanical stimulus, directing cellular activity in both physiological and pathological conditions. The significant role of hydrostatic pressure has been widely investigated in the tissues acquiring the loading forces such as bone, cartilage, intervertebral disc, renal glomeruli, and vasculature tissues *(6)*. Cells respond to hydrostatic pressure through several pathways including increased intracellular calcium concentration *(7)*, nitric oxide induction *(8)*, and nucleotide release *(9)*. Extracellular nucleotides are now recognized for their essential role in extracellular signaling and regulation of cellular activity. Once nucleotides are released from cells to the extracellular space, they bind to cell-surface purinergic receptors of the P2 class including 8 subtypes of the G-protien-coupled receptor P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) and 7 subtypes of the ligand-gated ion-conducting P2X (P2X1-P2X7) *(10)*. Evidences show the released of nucleotides in response to pressure changes, for example, increased intraocular pressure triggers an elevated release of adenosine-5'-triphosphate (ATP) leading to retinal cell damage *(11-12)*.

A number of studies have demonstrated that hydrostatic pressure influenced the expression of pro-inflammatory cytokines including interleukin (IL)-1β *(13-14)*, tumor

necrosis factor (TNF)-α *(15)*, and interleukin-6 (IL-6) *(16-17)*. IL-6, a pleiotropic cytokine regulating humoral and cellular responses, plays a central role in inflammation and tissue injury. It is rapidly released within dental pulp in response to infection, trauma or other stress conditions, and also has been detected in the pulp tissue of patient with pulpitis and periapical lesions. In the inflamed pulp, IL-6 might be involved in the formation of edema induced by the progressive penetration of Gram-positive oral bacteria into dentin. In the presence of IL-6, transforming growth factor (TGF)-β1 contributes to the development of a T helper 17 (Th-17) cell response, which mediates the attraction of neutrophils in the inflammatory process *(18-19)*. Apart from its role in immunomodulation, IL-6 also enhances bone resorption in pathological conditions *(20)*. It increases osteoclasts recruitment *(21)* and acts indirectly on osteoclastogenesis by stimulating the release of receptor activator of nuclear factor κB ligand (RANKL) from osteoblasts *(22)*.

To the best of our knowledge, no study has reported how dental pulp cells respond to HP in terms of cytokines production. In this study, we simulated increased intra-pulpal pressure condition by using a mechanical stress model and investigated the mechanisms involved in the regulation of IL-6 expression by performing several molecular approaches including reverse transcription–polymerase chain reaction (RT-PCR), Western Blot, enzyme-linked immunoabsorbent assay (ELISA) and small interfering RNA (siRNA) transfection. The results will be useful for identify the possible role of HP in the dental pulp inflammatory process.

Problems

1. What is the effect of hydrostatic pressure on the IL-6 expression in human dental pulp cells?
2. Which signaling pathway(s) is/are involved of HP-induced-IL-6 expressions in human dental pulp cells?

Specific aims

The specific aims of this present study are

1. To determine the effect of HP on of IL-6 expression in human dental pulp cells in
   - transcriptional level
   - protein synthesis level
   - cell morphology
2. To investigate the molecular mechanism involved in HP-induced IL-6 expression in human dental pulp cells.

Hypothesis

1. Hydrostatic pressure could induce IL-6 expression in human dental pulp cells.
2. P2Y signaling pathway may participate in the mechanism that HP modulates IL-6 expression in HDPCs.

Expected benefits

The findings will give a more understanding in the intracellular signaling of IL-6 expression after hydrostatic pressure stimulation, which may help to explain why during mild chronic pulpal inflammation an increased intra-pulpal pressure may cause a local immune response.

CHAPTER II
REVIEW LITERATURES

Hydrostatic pressure

Hydrostatic pressure (HP) is a mechanical stimulus, directing cellular activity in both physiological and pathological conditions. Among others, the cell of bone, cartilage, vascular tissues, tendon fibroblast, intervertebral disc, renal glomeruli, all experience and respond to HP *in vivo (6)*. The physiological HP appears to be an essential stimulus for proper direction of cellular activity. Obviously, HP is a major mechanical stimulus for bone remodeling throughout life to adapt bone mass and geometry to the functional demands (Wolff's law). Intermittent hydrostatic compression promotes the osteoblastic phenotype in bone organ cultures and in osteoblasts *(23)*. In addition, chondrocytes increase proteoglycan synthesis in response to physiologic levels of HP to maintain tissue homeostasis *(24)*. On the other hand, the pathological level of HP is involved in pathological diseases such as osteoarthritis *(25),* temporomandibular disorders *(26),* tendinopathy *(27)*, atherosclerosis *(28)*, renal pathologies *(29)* and glaucomatous optic neuropathy *(30)*. *In vitro* studies show that when the cells are subjected to high levels of HP (above 100 MPa), cell death will occur via necrotic/apoptotic pathways, induction of heat shock protein (Hsp) 70 and Hsp90β, increased in cytosolic levels of denatured protein and decreased protein synthesis, possibly by inhibiting factors necessary for initiation and elongation of translation process *(31)*.

It has been shown that the cells have differential response to different loading magnitudes. The study to compare the different loading magnitudes in intervertebral disc cells shows that low hydrostatic pressure (0.25 MPa) tended to increase collagen I and aggrecan expression, but no influence on the expression of matrix metalloproteinases (MMP1, MMP2, MMP3 and MMP13) of human nucleus cells. In contrast, high hydrostatic pressure (2.5 MPa) tended to decrease gene expression of all anabolic proteins with a significant effect on aggrecan expression but tended to increase the expression of MMP1, MMP3 and MMP13 of human nucleus cells. These findings suggest that low hydrostatic pressure has quite limiting effect with a tendency to anabolic effects,

whereas high hydrostatic pressure causes decreased the matrix protein expression with a tendency to increase some matrix-turnover enzymes *(32).* Therefore, the amplitude of HP is a critical factor to determine whatever the cells respond in physiologic or pathologic condition, particularly when evaluating the results of *in vitro* studies.

Currently, much attention has been given to the influences of mechanical stimuli on stem cell differentiation and proliferation, which provides a rational basis for tissue engineering and regeneration. For example, cyclic HP was effective in enhancing the accumulation of extracellular matrix and expression of genes indicative of chondrogenic differentiation by human adipose–derived stem cells *in vitro (33)*. HP promoted odontogenic differentiation, early *in vitro* mineralization, enhanced *in vivo* hard tissue regeneration and responsiveness to bone morphogenetic protein-2 (BMP-2) stimulation by human pulp stem cells *(34).* Furthermore, several other mediators such as growth factors and matrix environment (ECM substrates/scaffolds) were used in concert with mechanical loading to direct stem cell differentiation.

Hydrostatic pressure in the dental pulp

The dental pulp is a highly neurovascular connective tissue enclosed in the rigid mineralized dentin. It shares many similarities with other connective tissues of the body but it also has circulatory characteristics with physiological implications. The microcirculatory system in the dental pulp lacks collateral circulation. The limited ability to expand may severely compromise the circulation under conditions with increased fluid volume. Vasodilatation and the increase in vessel permeability are histological events commonly observed during acute inflammation *(35)*. These changes enhance both the blood volume and the interstitial fluid volume in the inflamed zone of connective tissues. When an inflammatory reaction takes place in the dental pulp, which is a connective tissue surrounded by mineralized dentine, it becomes vulnerable since this inflammatory process is occurring inside of a rigid chamber. Consequently, even a slight increase in the interstitial fluid volume may raise the pulpal hydrostatic pressure *(1).*

Compared to most other tissues, the interstitial fluid pressure in the dental pulp seems high. Several experimental techniques have been developed to measure pulpal interstitial pressure. Determination of this pressure during homeostasis and during inflammation is critical for understanding vascular responses to pulpal injury. Methods to determine pulpal interstitial pressure include photoelectric methods *(36)*, tonometric measurements *(37)* and micropuncture techniques *(38)*. The micropuncture technique is much less invasive and measurements with this method have provided values in the range of 6-10 mmHg in cat, ferret and rat *(39)*. Tonder and Kvinnsland (1983) showed the pulpal interstitial pressure in cat dental pulp was 5.5 mmHg in control teeth and 16.3 mmHg at the site of pulpal inflammation *(40)*. However, this technique is unable to measure human pulpal pressure because of requiring the direct exposure of pulpal soft tissue using drills. In the other way, Pashley (1981) measured pulpal tissue pressure through intact dentin by sealing a needle connected to a pressure transducer directly into cavities prepared, they obtained a mean value of 24.1 mmHg in normal dog molars *(41)*. Vongsavan and Matthews (1992) estimated pulpal tissue pressure in cats teeth by measuring fluid movement across dentin as a function of exogenous pressure. When no pressure was applied, they measured an outward fluid flow that slowed progressively as exogenous pressure was sequentially raised. No fluid flowed in either direction occurred when the mean exogenous pressure was 11 mmHg. This equilibrium pressure was thought to be equal to pulpal tissue pressure *(42)*. However, these approaches unable to show actually level of human pulpal hydrostatic pressure *in vivo*, therefore, a new combined approach is needed to better understand the intricate circulatory changes occurring during the development of pulpal inflammation.

Besides inflammation, It has been shown that restorative procedures cause increased intrapulpal pressure. For example, high power laser cutting produced approximately a fourfold increase in intrapulpal pressure *(2)*. Crown cementation created the measurable pressure, that transmitted to the pulp chamber *(43)*. Moreover, hot water and tactile stimuli produced an inward fluid movement *(44)*, which causes an increase in pulpal pressure *(45)*. Furthermore, dental trauma, uncontrolled orthodontic forces and

chronic parafunctional forces cause pulpal inflammation and a chronic rise in intra-pulpal pressure *(3-5)*.

Berggreen and Heyeraas (1999) have shown that calcitonin gene-related peptide (CGRP) seems to be mainly responsible for the increase in pulpal blood flow and interstitial fluid pressure during electrical tooth stimulation *(46).* Recent study demonstrated an increase in the blood vessel diameter occurs during the time course of the rat induced pulpitis and suggested that rennin-angiotensin system plays an important role in the regulation of vascular tone during the pulpal inflammation *(47).*

Cellular mechanotransduction of hydrostatic pressure

Up to date, numerous studies have investigated the cellular response to HP. However, the exact mechanisms by which cells sense HP and transduce into a cascade of cellular and molecular events are still unclear. Some possible cellular mechanotransduction mechanisms are shown in Figure 2.1. The early responses to HP including the opening of ion channels *(48),* increased intracellular calcium *(7),* nitric oxide induction *(8),* prostaglandin production *(49),* nucleotides release *(9)* have been described. Moreover, several membrane proteins have been shown to be mechano-sensitive to HP, including integrins *(50-51)* and G protein-coupled receptors (GPCRs) *(52-53)*. It has been shown that HP activates multiple intracellular signal transduction cascades such as those involving mitogen-activated protein kinase (MAPK)*(54)*, protein kinase C (PKC) *(55)* and nuclear factor-**κ**B (NF-**κ**B*) (56)*. For example, cultured human optic nerve head (ONH) astrocytes showed increased nuclear localization of c-Fos and c-Jun under exposure to HP. Immunohistochemistry demonstrated that the upstream regulators of c-Fos and c-Jun, extracellular signal-regulated kinase (ERK) and p38 MAPK localized to the nuclei of ONH astrocytes in monkeys with experimental glaucoma *(57).*

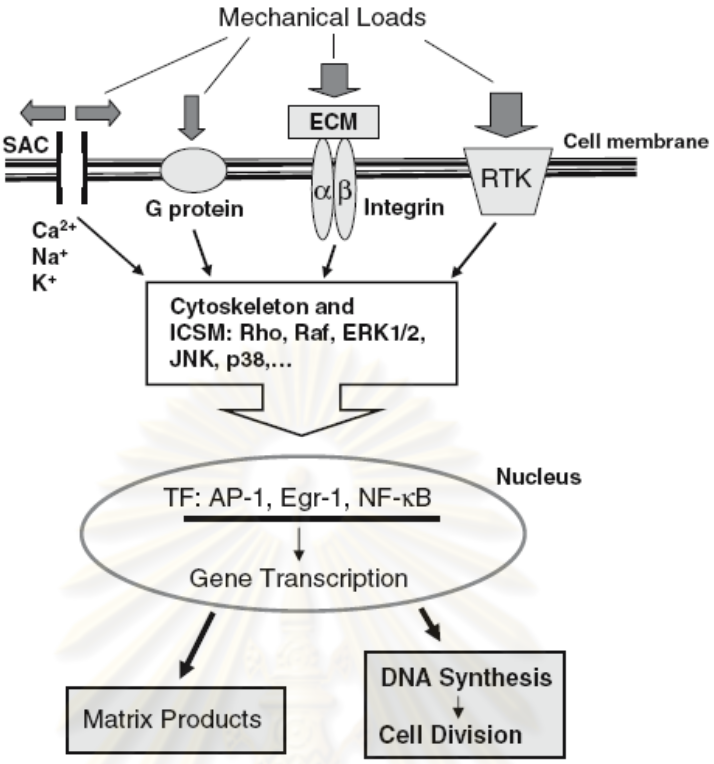


Figure 2.1 A conceptual illustration of cellular mechanotransduction mechanisms *(58)*. (ICSM: Intracellular signaling molecules; TF: Transcriptional factors)

It has been reported that HP stimulates extracellular matrix genes (e.g. collagen) and induces cellular inflammatory response (e.g., expression of COX-2 and production of prostaglandin E_2). For example, Reza and Nicoll observed increased production of collagen II in intervertebral disc cells from the outer annulus exposed to 5 MPa HP *(59)*. Almarza and Athanasiou demonstrated the increased collagen I gene expression and protein production when applying 10 MPa static HP to temporomandibular joint disc cells *(60)*. Additionally, a number of studies have demonstrated that HP influenced the expression of pro-inflammatory cytokines including interleukin-1β *(49)*, tumor necrosis factor-α *(16)*, and IL-6 *(55)*. Among them, IL-6 is one of the pro-inflammatory cytokines that received attention from researchers because IL-6 is a pleiotropic cytokine and implicated in the pathogenesis of rheumatoid arthritis, osteoporosis, and stress-induced pathological bone resorption.

Extensive studies were performed to explore the effect of HP and mechanical stress to IL-6 expression in various cell types. In human periodontal ligament cells treated with 1 MPa of HP for 60 minutes, enhancement of IL-6, IL-8, TNF-α mRNA expression was observed, which caused no morphological changes of cells and did not affect the cellular viability *(17).* The other study shows human dermal fibroblasts survive and to be active in producing IL-6 under high hydrostatic pressure, up to 40 MPa for 20 minutes, which involved the PKC signaling pathway *(55).* The exposure to elevated pressure (70 mmHg) induces a twofold increase in release by microglia *(61).* In addition, continuous compressive force (1.0- 3.0 g/cm^2) stimulates the production of IL-6 and IL-6 receptor (IL-6r) both mRNA and protein by human osteosarcoma cell line Saos-2 *(62).* In human dental pulp cells, it has been shown that mechanical strain activates IL-1β, TNF-α, IL-6, as well as antioxidant enzymes *(63)*. However, there is no direct evidence demonstrating the role of HP on inflammatory cytokines in dental pulp cells.

Interleukin-6

IL-6 is a 26-kDa glycopeptide that in humans is encoded by the *IL6* gene on chromosome 7. It has previously been known as hepatocyte-stimulating factor, cytotoxic T-cell differentiation factor, B-cell differentiation factor, B-cell stimulatory factor 2, hybridoma/plasmacytoma growth factor, monocyte granulocyte inducer type 2 and thrombopoietin. The variety of its name reflects the pleiotropism of IL-6. IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 is one of the most important mediators of fever and of the acute-phase response. It is capable of crossing the blood brain barrier and initiating synthesis of PGE_2 in the hypothalamus, thereby changing the body's temperature setpoint. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen associated molecular patterns (PAMPs). The role of IL-6 as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-α and IL-1, and activation of IL-1 receptor antogonist (IL-1RA) and IL-10. The IL-6-like family of cytokines has more than 10 members, including interleukin-11 (IL-11), interleukin-27 (IL-27), and leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1),

cardiotrophin-like cytokine (CLC), oncostatin M (OSM), Kaposi's sarcoma-associated herpesvirus interleukin 6 like protein (KSHV-IL6), because these cytokines that signal through receptors containing glycoprotein 130 (gp130).

Receptor binding and activation

IL-6 signals primarily through a protein complex including the membrane-bound, non-signaling a-receptor subunit (IL-6R) and two signal-transducing gp130 subunits. While gp130 is expressed ubiquitously, IL-6R is predominantly expressed on hepatocytes, neutrophils, monocytes, macrophages and lymphocytes. However, IL-6 can also signal via a soluble receptor (sIL-6R) that lacks transmembrane and cytoplasmic components. Activated sIL-6R binds to membranebound gp130 subunits in a process known as trans-signaling (Figure 2.2). sIL-6R is generated either by limited proteolysis of the membrane-bound IL-6R or by alternative mRNA splicing. sIL-6R is transported in bodily fluids and increases the variety of cells able to respond to IL-6.

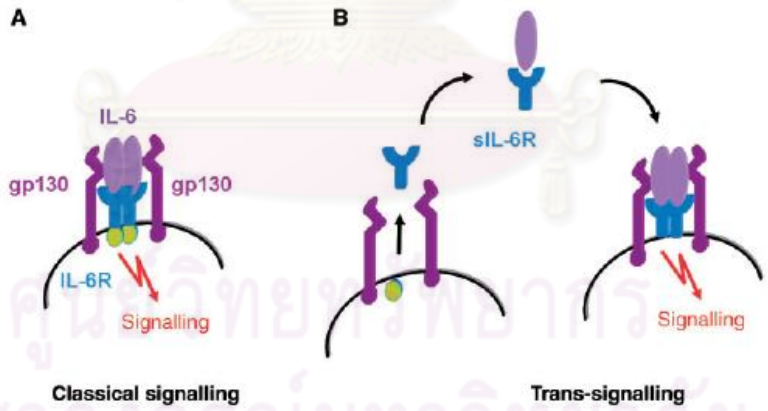


Figure 2.2 IL-6 signaling mechanism. IL-6-mediated signal transduction through classical (A) and trans-signaling (B) pathways. In IL-6 trans-signaling, sIL-6R is generated either by limited proteolysis of the membrane-bound IL-6R or by alternative mRNA splicing. In both classical and trans-signaling, responses are elicited through engagement with membrane-bound gp130 *(64)*.

Once IL-6 binds its receptor and gp130 homodimerization occurs, a signaling cascade is triggered. The Janus kinases (JAK) are activated, followed by the recruitment of signal transducers and activators of transcriptions (STATs). Phosphorylated STATs translocate to the nucleus where they activate gene transcription. IL-6 also activates the mitogen-activated protein kinase cascade, which is upstream of various molecules involved in cell survival and stress responses (Figure 2.3).

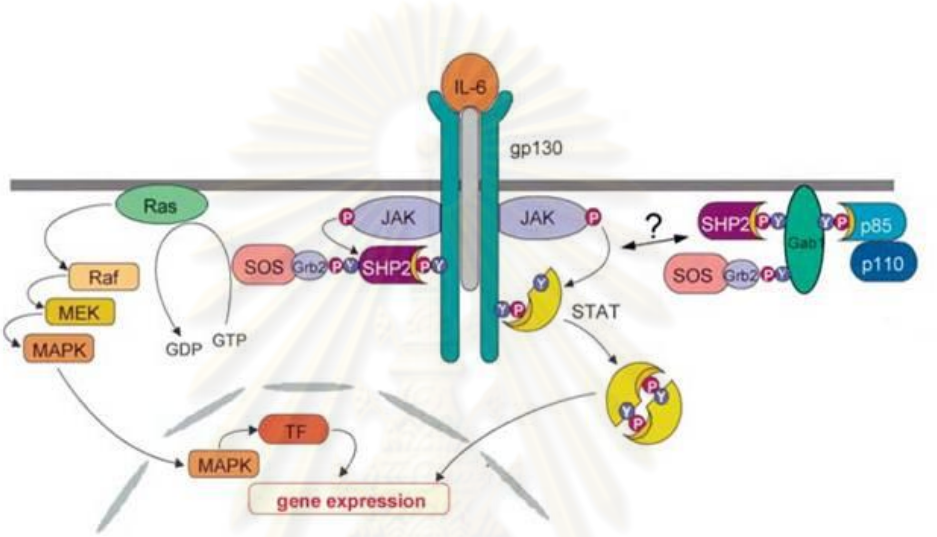


Figure 2.3 IL-6 activates the JAK/STAT pathway and the MAPK cascade *(65)*.

Function of Interleukin-6

(1) IL-6 and adaptive immune response

IL-6 was originally identified as a B-cell differentiation factor; it plays an important role in the development of antibody-producing plasma B cells. IL-6 induces B-cell differentiation through its action on plasmablasts and more recently has been shown to induce B-cell antibody production indirectly by promoting the B-cell helper properties of $CD4^+$ T cells via the production of IL-21.

In addition to B-cell development, IL-6 influences T-cell development. When activated, naive T cells develop into either effector or regulatory T cells. Effector T cells are further subdivided into Th-1, -2 and -17 cells, all of which have pro-inflammatory

properties. Animal studies have shown that Th-17 cells are important mediators in autoimmune diseases and the host defense against extracellular pathogens. Th-17 cells produce IL-17, -12 and -22. In murine models of autoimmune diseases, differentiation of Th-17 is regulated by the cytokine milieu. In the presence of transforming growth factor-β (TGF-β) alone, naive T cells differentiate into regulatory T cells and development of Th-17 cells is suppressed. When IL-6 is present together with TGF-β, naive T cells develop into Th-17 cells through activation of STAT3 and induction of the transcription factor retinoic acid-related orphan receptor (RORgammat) *(66)*. Th-17 cells are also involved in the host defense response against bacteria and fungi, suggesting that IL-6 may contribute indirectly to fighting infection through Th-17 cell development. Taken together, these findings demonstrate that IL-6 has an important role in the development of the adaptive immune response.

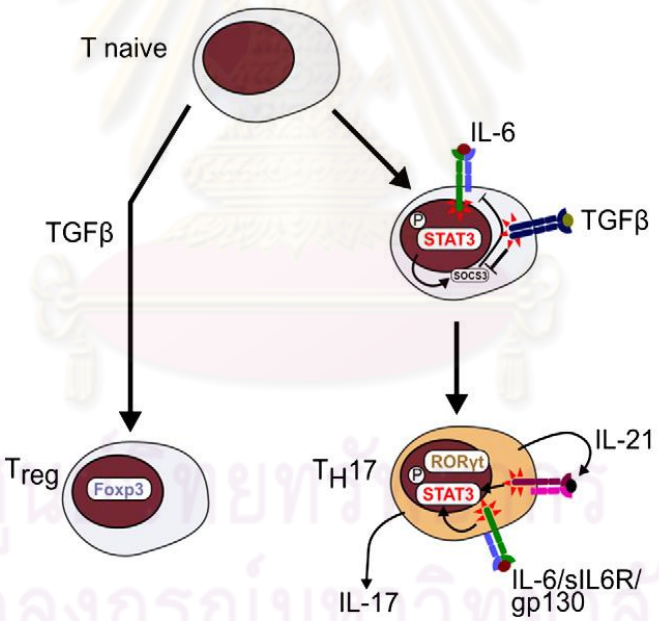


Figure 2.4 Role of IL-6 in T-cell differentiation *(66)*.

The presence of IL-17 was associated with an exacerbation of inflammatory response and bone resorption in both rat and human periapical lesions *(67)*. Recent study shows that *in situ* expression of IL-17 was increased in human radicular cysts and

periapical granulomas *(68)*. Moreover, IL-17 has a role in osteoclastogenesis through an interaction between osteoclast progenitors and osteoblasts *(69)*. Since IL-17 was involved both in PGE_2 synthesis and in RANKL mRNA expression in cultures of osteoblasts, Kotake *et al.* hypothesized that IL-17 may induce osteoblast-related COX-2-dependent PGE_2 synthesis, which is responsible for RANKL expression *(69)*. In addition, IL-17 upregulates receptor activator of nuclear factor **κ**B (RANK) on human osteoclast precursors *in vitro*, leading to increased sensitivity to RANKL signaling, osteoclast differentiation and bone loss *(70)*. Furthermore, it has been demonstrated that IL-17 is involved in joint degradation in juxta-articular bone and synovium explants from rheumatoid arthritis patients *(71)*.

(2) IL-6 and bone resorption in pathological conditions

IL-6 is produced by both stromal cells and osteoblasts in response to several stimuli such as lipopolysaccharides (LPS), parathyroid hormone, 1,25-dihydroxyvitamin D3, TGF-**β**, IL-1 and TNF-**α**. Once released IL-6 stimulates bone resorption and osteoclast formation *(72)*. Therefore, IL-6 has a pathogenetic role in the abnormal bone resorption associated with rheumatoid arthritis, multiple myeloma, Paget's disease and other bone diseases characterized by excessive osteoclastogenesis and focal osteolytic lesions.

IL-6 enhances bone resorption in many ways. First, it induces the production of RANKL by bone marrow mesenchymal cells and osteoblasts. Under physiological conditions, these cells express low levels of IL-6R, but in the presence of sIL-6R, STAT3 is activated and the expression of RANKL is induced *(73)*. The binding of RANKL to its receptor RANK activates NF-**κ**B, ERK1/2, and p38 MAPK signaling, and induces osteoclast maturation and the expression of osteoclast-associated receptors (OSCAR), an immunoglobulin-like surface receptor that acts as a co-stimulatory receptor for osteoclast differentiation *(74)*.

Second, IL-6 induces in tumor cells the expression of several proteins involved in bone resorption such as parathyroid hormone-related protein (PTHrP), IL-8, IL-11, RANKL, and COX-2. IL-6 and sIL-6R stimulate PTHrP production by osteoblastic stromal

cells via MEK/ERK1/2 pathways. PTHrP increases the expression of RANKL and downregulates the expression of osteoprotegerin (OPG), the decoy receptor for RANKL by osteoblasts tipping the bone metabolism toward osteolysis *(75-76)*.

Third, by stimulating the expression of Wnt-Signaling Antagonist, Dickkopf 1 (DKK1) in tumor cells, IL-6 inhibits Wnt-mediated osteogenesis and further imbalances the bone homeostasis toward excessive degradation *(77)*. IL-6 also increases the activity of estradiol 17 β-hydroxysteroid dehydrogenase, thereby inhibiting the anti-osteoclast activity of estrogens, promoting osteolysis and hypercalcemia in breast cancer patients *(78)*. In addition, IL-6 down-regulates the synthesis of genes like type II collagen and aggrecan, contributing to a decrease in new bone formation *(79-80)*.

(3) IL-6 and diseases

IL-6 is relevant to many disease processes such as diabetes *(81-82)*, atherosclerosis *(83)*, depression *(84)*, Alzheimer's disease *(85)*, systemic lupus erythematosus *(86)* , prostate cancer *(87)*, syetemic sclerosis *(88)* and rheumatoid arthritis *(89)*. Advanced/metastatic cancer patients also have higher levels of IL-6 in their blood. Hence there is an interest in developing anti-IL-6 agents as therapy against many of these diseases. The first such is tocilizumab which has been approved for rheumatoid arthritis *(90)*. Another, siltuximab (CNTO 328) is a monoclonal anti-IL-6 antibody which has been successfully applied in several models representing prostate cancer *(91)*.

(4) IL-6 and dental pulp

IL-6 is one of inflammatory cytokines that has been released within dental pulp during inflammation. Significant quantities of IL-6 have been detected in the pulp tissue of patient with pulpitis *(92)* and periapical lesions *(93)*. The production of IL-6 from human dental pulp cells is also stimulated by bacterial challenge, pro-inflammatory cytokines and sensory neuropeptides *(94-95)*. IL-6 is associated with periapical bone resorption *(96)*. Moreover, IL-6 elevated the mRNA expression of MMP-1,3,14,11, increased the plasminogen activator activity of dental pulp cell *(97)* and enhanced dental

pulp fibroblast mediated type I collagen degradation *(98)*, suggesting IL-6 may involve in pulp destruction during inflammation.

P2Y6 receptor

P2Y6 receptor is a member of purinergic receptor family and encoded by the *P2RY6* gene. The human *P2Y6 receptor* gene is localized in chromosome 11q13.3-13.5. cDNA cloning of P2Y6 receptor is identified in three cDNA isoforms, among which two isoforms have identical contiguous open reading frames (ORFs) but differ in their 5' untranslated regions (5' UTRs), originating probably from alternative splicing, and the third isoform represents a pseudogene. P2Y6 receptor is widely distributed in various tissues, including placenta, spleen, thymus, small intestine, blood, heart, blood vessels and brain. On the cellular level, P2Y6 receptor is expressed in many kinds of cells, including intestinal epithelial cells, T cells (affected T cells), monocytes, microglia, vascular endothelia cells, cardiomyocytes, smooth muscle cells and neurons in the guinea pig enteric nervous system. Its mRNA expression has also been detected in motor neurons and spinal sensory neurons.

Overview of purinergic receptor

Purinergic receptors are a family of newly characterized plasma membrane molecules, which respond to extracellular nucleotides. There are two main families of purine receptors, adenosine or P1 receptors, and P2 receptors, recognizing primarily to nucleotides. Adenosine/P1 receptors have been further subdivided, according to convergent molecular, biochemical, and pharmacological evidence into four subtypes, A1, A2A, A2B, and A3, all of which couple to G proteins. Based on differences in molecular structure and signal transduction mechanisms, P2 receptors divide naturally into two families of ligand gated ion channels and G protein-coupled receptors termed P2X and P2Y receptors, respectively. To date, seven mammalian P2X receptors (P2X1–7) and eight mammalian P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13

and P2Y14) have been cloned, characterized, and accepted as valid members of the P2 receptor family.

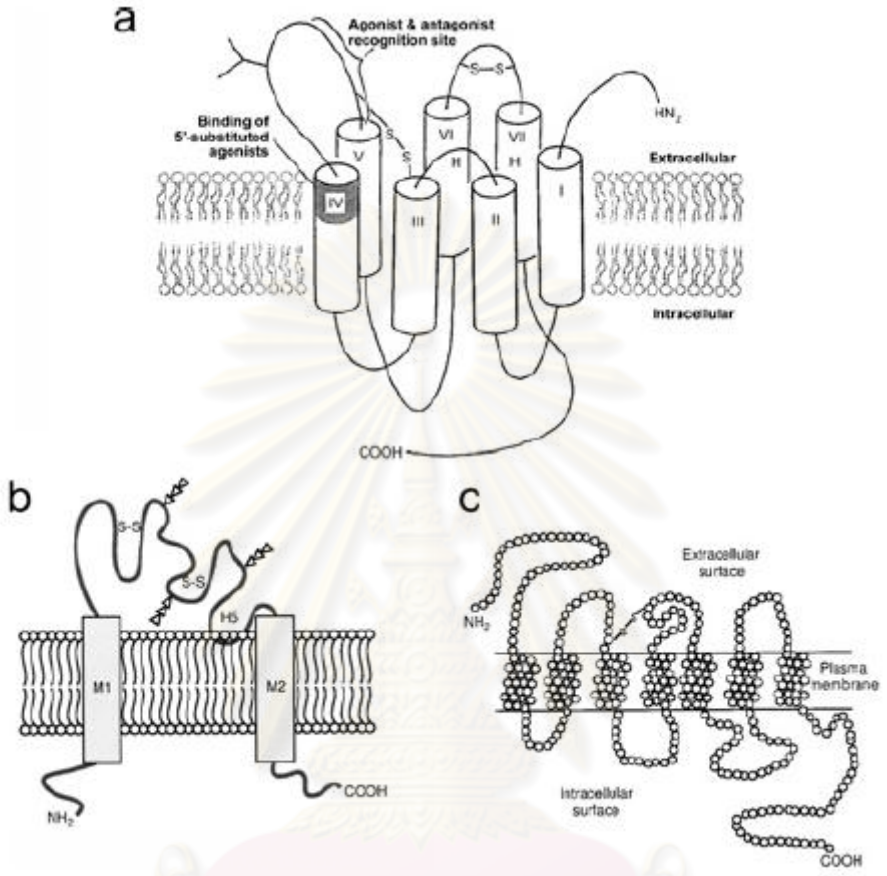


Figure 2.5 Membrane receptors for extracellular nucleotides. The P1 receptors for extracellular adenosine are G protein-coupled receptors that signal by inhibiting or activating adenylate cyclase (a). The P2 receptors comprise two types of receptors (P2X and P2Y). The P2X family of receptors are ligand-gated ion channels (b), and the P2Y family are G protein-coupled receptors (c) *(99)*.

Pharmacologically P2Y receptors can be broadly subdivided into (1) adenine nucleotide-preferring receptors mainly responding to ADP and ATP. This group includes human P2Y1, P2Y11, P2Y12, and P2Y13 (2) uracil nucleotide-preferring receptors. This group includes human P2Y4 and P2Y6 responding to either UTP or UDP (3) receptors of mixed selectivity (human P2Y2) and (4) receptors responding solely to the sugar nucleotides UDP-glucose and UDP-galactose (human P2Y14) *(10)*. On the other hand,

the P2Y family can be subdivided into two groups based on their coupling to specific G proteins. The P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptors couple to Gq, to activate phospholipase β (PLCβ), and the P2Y12, P2Y13 and P2Y14 receptors couple to Gi, to inhibit adenylyl cyclase. The P2Y11 receptor has the unique property to couple through both Gq and Gs (Table 2.1).

Table 2.1 The properties of P2Y receptors *(100)*.

Properties of P2Y receptors.[a]						
Group	Receptor	Chromosome (human)	Native agonist (human, pEC_{50})	Selective agonist (pEC_{50})	Selective antagonist (pIC_{50})	G protein
A	$P2Y_1$	3q24-25	ADP (5.09)	MRS2365 (9.40)	MRS2500 (9.02), MRS2179 (6.48)	G_q
	$P2Y_2$	11q13.5	UTP (8.10), ATP (7.07)	MRS2698 (8.10), MRS2768 (5.72)	PSB-716 (5.01), AR-C126313 (6)	G_q (+G_i)
	$P2Y_4$	Xq13	UTP (5.60)[b]	N/A[c]	N/A[c]	G_q (+ G_i)
	$P2Y_6$	11q13.5	UDP (6.52)[d]	PSB-0474 (7.15), 5-iodo-UDP (7.83)	MRS2578 (7.43) [non-competitive]	G_q
	$P2Y_{11}$	19p31	ATP (4.77)	NF546 (6.27)	NF340 (7.14)	G_q + G_s
B	$P2Y_{12}$	3q21-25	ADP (7.22)	N/A[c]	AZD6140 (7.90),	G_i
					AR-C69931MX	
					(9.40),	
					PSB-0739 (9.8)	
	$P2Y_{13}$	3q24-25	ADP (7.94)	N/A[c]	MRS2211 (5.97)	G_i
	$P2Y_{14}$	3q24-25	UDP-glucose (6.45), UDP (6.80)	MRS2690 (7.31), MRS2802 (7.20)	[e]	G_i

a: The missing numbers in the classification represent either nonmammalian orthologs or receptors having some sequence homology to P2Y receptors, but for which there is no functional evidence of responsiveness to nucleotides.

b: The pharmacology of some P2Y receptors exhibits species differences: whereas the human P2Y4 is a UTP receptor, the rat and mouse P2Y4 receptors are activated equipotently by ATP and UTP.

c: Selective ligands not yet available. Other useful nonselective agonists include (pEC50): INS365 (7.00 at P2Y2), 20-azido-20-deoxyUTP (7.14 at P2Y4), INS48823 (6.90 at P2Y6), AR-C67085 (5.05 at P2Y11) and 2-MeSADP (7.85 at P2Y12 and P2Y13). Other useful nonselective antagonists include (pIC50): PPADS (<5.0 at P2Y4) and 2-MeSAMP (4.00 at P2Y12).

d: UTP is also an agonist of the P2Y6 receptor.

e: Non-nucleotide antagonists have been reported.

Extracellular nucleotides are ubiquitous molecules that regulate a wide spectrum of biological effect via membrane-bound purinergic receptor. A nucleotide is composed of a nitrogenous base, a five-carbon sugar (either ribose or 2'-deoxyribose) and one to three phosphate groups. There are five nitrogenous bases. The so-called pyrimidines (cytosine, thymine, and uracil) are smaller, having only one ring structure. The larger purines (adenine and guanine) have two rings. There are five major native ligands of the P2Y receptors (Figure 2.6) are adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), and UDP-glucose. Figure 2.7 shows the correspondence of these nucleotides and the subtypes that they activate or antagonize, along with the diversity of representative biological effects induced by P2Y receptor activation. Additionally, evidences show the released of nucleotides such as ATP, UTP, UDP in response to mechanical stimuli including fluid flow *(101)*, cyclic compression *(102)*, stretch *(103-104)* and hydrostatic pressure *(11)*. Thus, mechanical stimulation of cells could result in the local release in nucleotide level, which then activates purinergic receptors in paracrine or autocrine fashion.

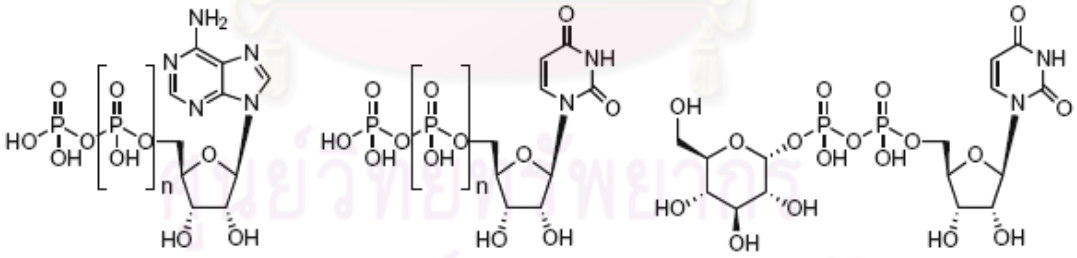


Figure 2.6 The molecular structures of five major naturally occurring P2Y receptor agonists *(105)*.

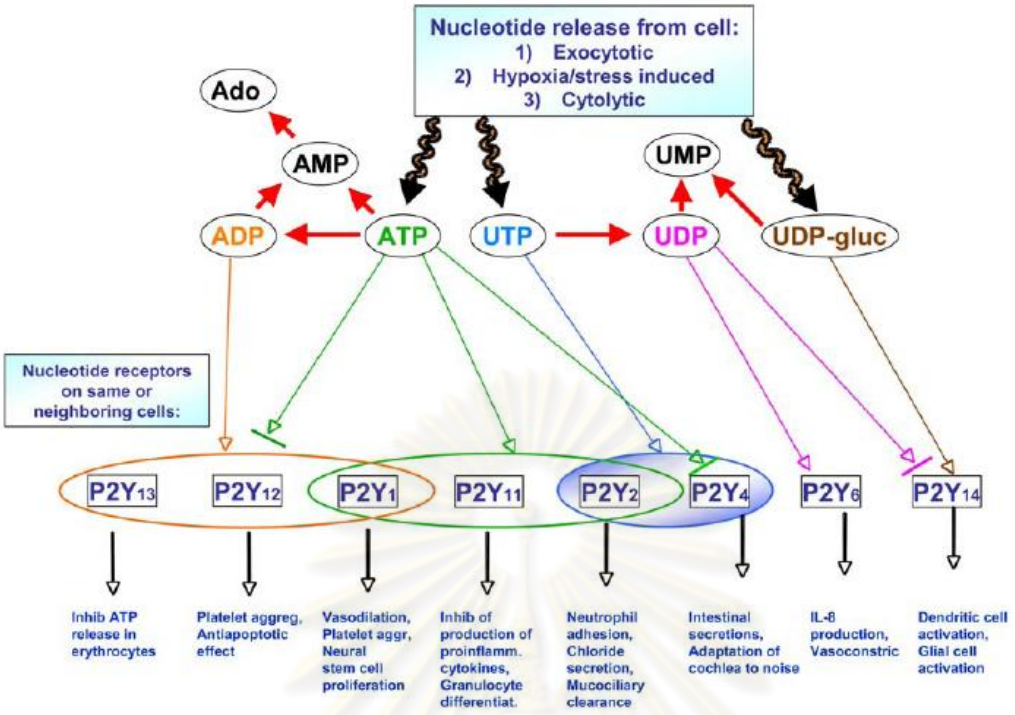


Figure 2.7 Correspondence of the five principal native ligands of the human P2Y receptors and the subtypes they activate *(105)*.

Structure of P2Y6 receptor

Like other receptors of the P2Y family, P2Y6 receptor has seven transmembrane (TM) domains. This receptor exhibits significant homology with the TM domains of other P2Y1-like receptors, such as P2Y1 (43.0%), P2Y2 (47.7%), P2Y4 (48.2%) and P2Y11 (31.6%) *(105)*. Chang *et al.* have demonstrated the isolation of a cDNA encoding P2Y6 receptor subtype from rat aortic smooth muscle cell cDNA library and analysed the function of this receptor *(106)*. Figure 2.8 demonstrates nucleotide and deduced amino acid sequences of P2Y6 receptor clone. An in-frame initiating codon (nucleotides 440–442) is in the context of the Kozak translation initiation consensus sequence and is preceded by an in-frame stop codon. The predicted molecular mass of 36.7 kDa of this protein is substantially low among G protein-coupled receptors and close to that of A1 adenosine receptor and several odorant receptors so far reported to have the smallest molecular weight. Hydropathy analysis of the clone reveals seven stretches of hydrophobic amino acids, predicted to represent membrane-spanning domains

characteristic of the G protein-coupled receptors. The amino-terminal region preceding the putative first transmembrane domain contains a single potential asparagine-linked glycosylation site, and the third intracellular loop and cytoplasmic tail have two recognition sites (Ser-235 and Thr-320) for phosphorylation by PKC. This protein also possesses a number of residues conserved in most of the G protein-coupled receptors such as Asp in the second transmembrane domain, Leu in the second and the seventh transmembrane domains, Arg-Tyr immediately behind the third transmembrane domain, and Pro in the fifth, the sixth, and the seventh transmembrane domains. A Cys in the carboxyl-terminal region conserved in many of the G protein-coupled receptors, which may be a membrane- anchoring palmitoylation site, and a Asp in the third transmembrane domain conserved in the G protein-coupled receptors for charged amines are absent in this protein.

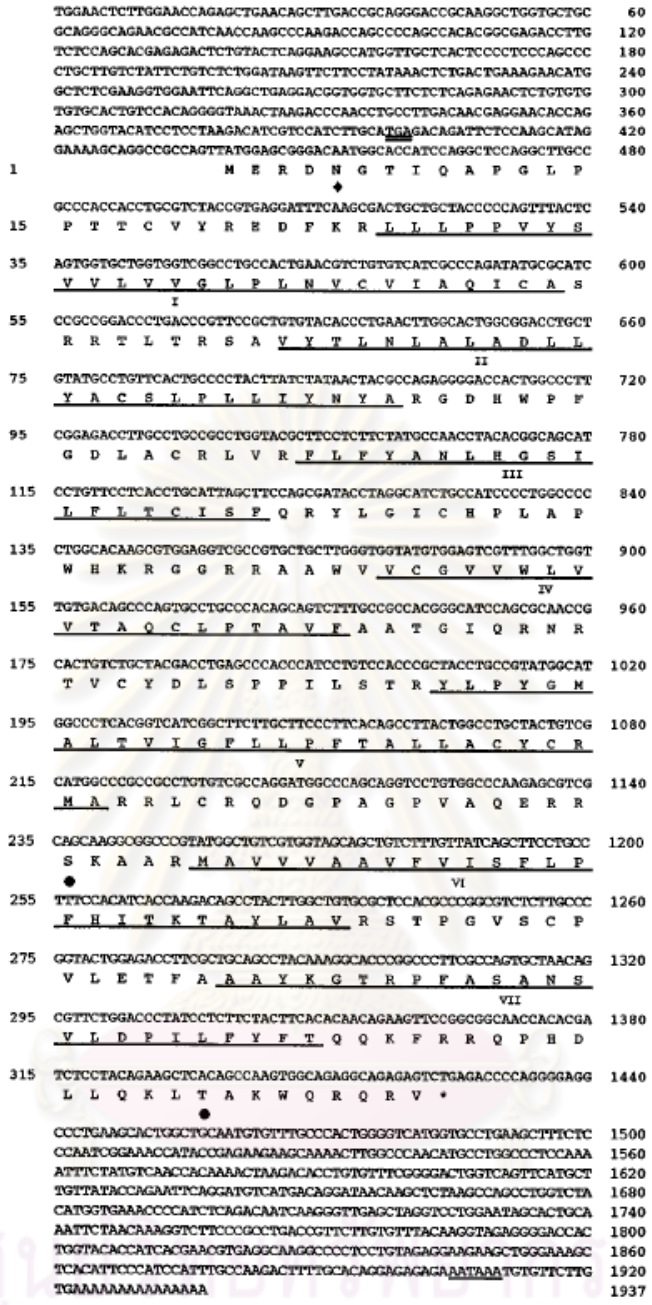


Figure 2.8 The nucleotide sequence and the predicted amino acid sequence of the cloned P2Y6 receptor. The *double underlining* indicates an in-frame stop codon preceding the initiating codon. Potential *N*-linked glycosylation site (♦) in the amino-terminal region and phosphorylation sites (●) for protein kinase C in the cytoplasmic loop and the carboxyl-terminal region are indicated. Both symbols are *below* the amino acids. The putative transmembrane domains I–VII assigned on the basis of the results of Kyte and Doolittle hydropathicity plot are indicated by *single underlining*. Poly(A)$^{+}$ signal AATAAA is indicated by *single underlining (106)*.

Agonist of P2Y6 receptor

As a ligand of P2Y6 receptor, UDP is approximately 100-fold more potent than UTP, whereas ADP, ATP and their 2-methylthio derivatives are almost inactive. Cells can synthesize UDP *de novo* or by salvage of uridine. The physiological role of UDP is less well understood compared to the other nucleotides. However, extracellular UDP promotes widespread and pronounce cellular responses. For example, UDP induces the production of proinflammatory cytokines/chemokines, including IL-8, TNF-α, interferon-inducible-protein-10 (IP-10), and monocyte chemotactic protein-1 (MCP-1) in human leukemic monocyte lymphoma cell line (U937) stably transfected with the human P2Y6 receptor *(107)*. Moreover, UDP reduced intraocular pressure by 82.9±2.6% compared to control acting probably on P2Y6 receptors present on the ciliary processes, which, by vasoconstriction, would significantly reduce the production of the aqueous humour. UDP therefore may be used for the treatment of ocular hypertension and glaucoma *(108).*

In the cardiovascular system, extracellular pyrimidines have several effects, as showed UDP and UTP induce not only vasoconstriction through P2Y receptor stimulation on vascular smooth muscle cells, but also dilatation on endothelial cells, thereby regulating vascular tone and blood pressure *(109-111)*. Extracellular UTP and UDP have also been shown to mediate growth stimulation and cell migration in vascular smooth muscle cells *(112-114)*. Patients with acute myocardial infarction had significantly higher UTP levels, indicating release during ischemia *(115)*. On the surface of cardiac cells, there are ectonucleotidases rapidly hydrolyzing nucleotides (UTP to UDP to UMP to uridine). Because UTP is rapidly degraded to UDP, these findings of cardiac UTP release indicate that UDP will be present in the circulation with possible actions on the heart via P2Y6 receptors. This rapid degradation also results in much lower plasma levels than the actual nucleotide concentrations at the cell surface. Inotropic effects of UDP, mediated by P2Y6 receptors via an IP3-dependent pathway may involved in the development of cardiac disease *(115).*

However, measurement of extracellular UDP released was difficult due to the complexity of measurement method and lack of a sensitive assay for the quantification of UDP mass since its rapidly degrade. Some report determined the extracellular UTP

levels by using enzymatic approach based on the high selectivity of UDP-glucose pyrophosphorylase for UTP as a co-substrate for the conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose *(116)*. The percent conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose was determined by high-performance liquid chromatography (HPLC). In most resting attached cultures, extracellular UTP concentrations were found in the low nanomolar range (1 ± 10 nM in 0.5 ml medium bathing 2.5 cm^2 dish). Up to a 20 fold increase in extracellular UTP levels was observed in cells subjected to a medium change. Extracellular UTP levels were 10 - 30% of the ATP levels in both resting and mechanically-stimulated cultured cells *(117)*.

Antagonist of P2Y6 receptor

Various nonselective antagonists of P2Y receptors including reactive blue 2, suramin and pyridoxal-5'-phosphate-6-azophenyl-2,4-disulfonate (PPADS) have been used for antagonize P2Y receptors. The P2Y6 receptor is blocked by reactive blue 2, PPADS and suramin with apparent antagonist potencies decreasing in this order *(118)*. Suramin is a polysulfonated naphthylurea, uncouples G-proteins from receptors, when given at high micromolar concentrations (e.g., 30–100 μM) affects all nucleotide-sensitive P2Y receptors with the exception of the P2Y4 receptor *(119)*.

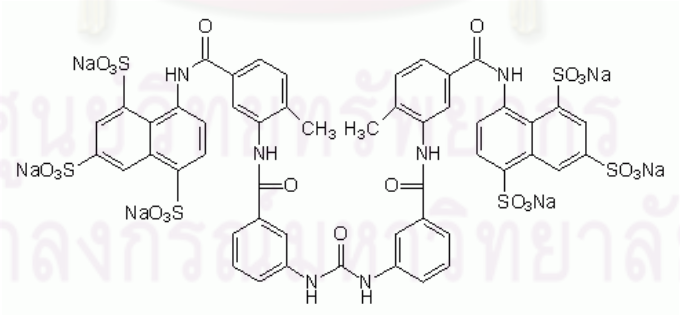


Figure 2.9 Structure of suramin.

Up to now, only one class of selective antagonists of the P2Y6 receptor has been reported. Mamedova *et al.* synthesized a series of symmetric aryl diisothiocyanate derivatives and showed that diisothiocyanate derivatives of 1,2-diphenylethane (MRS2567) and 1,4-di-(phenylthioureido) butane (MRS2578) inhibit UDP activated PLC

activity in concentration dependent, with IC_{50} values of 126 ± 15 nM and 37 ± 16 nM, respectively. Figure 2.11 shows the concentration-response curve for UDP acting at the P2Y6 receptor (EC_{50} 230 ± 83 nM) was inhibited by MRS2567 and MRS2578. Additionally, the selective antagonists MRS2567 and MRS2578 (1 μM) completely blocked the protection by UDP of cells undergoing TNFα-induced apoptosis. Moreover, MRS2567 and MRS2578 at 10 μM did not affect the UTP (100 nM)-induced responses of cells expressing P2Y2 and P2Y4 receptors, nor did they affect the 2-methylthio-ADP (30 nM)-induced responses at the P2Y1 receptor or the ATP (10 μM)-induced responses at the P2Y11 receptor *(120)*.

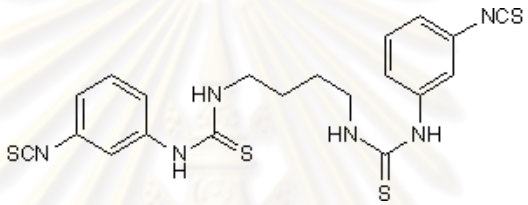


Figure 2.10 Structure of MRS2578.

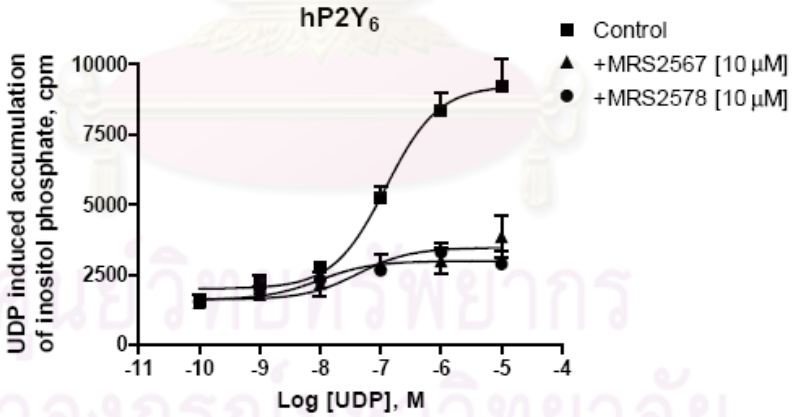


Figure 2.11 Inositol phosphate production in human P2Y6-transfected 1321N1 human astrocytes. After labeling with myo-[^{3}H]inositol (1 μCi/106 cells) for 24 hours, the cells were treated for 30 minutes at 37 °C with antagonists in the presence of lithium chloride, followed by addition of the agonist, UDP, for another 30 minutes. The quantity of inositol phosphates was analyzed after extraction though Dowex AG 1-X8 columns. Data shown are from the combined results of three independent experiments in triplicate.

Function of P2Y6 receptor

P2Y6 receptor is one of most P2Y receptor subtypes have been intensively investigated during the past decade. The major draw-back in studying the function of P2Y receptors is the presence of ectonucleotidases on the plasma membrane that degrade the native agonist and antagonist nucleotides. Products of the enzymatic hydrolysis might either be inactive or have activity at other P2Y receptor subtypes *(105)*. Additionally no reliable and sensitive assay is available to measure the extracellular concentration of UDP (the ligand of P2Y6 receptor). However, a growing body of evidence indicates that P2Y6 receptor play important roles in a wide range of biological processes including immunoinflammation, vasoconstriction, cytoprotection, counteract apoptosis induced by TNF-α and increase survival of osteoclasts.

(1) P2Y6 receptor and immunoinflammation

The presence of P2Y6 transcripts in human spleen, thymus and blood leukocytes suggest a possible role of P2Y6 receptor in the immune system. The study of a role for P2Y6 receptor in T-cell function show that activated peripheral T cells show increased levels of P2Y6 receptor mRNA. Together with, RT-PCR analysis of CD4+ and CD8+ subsets illustrates strong expression in both activated CD4+ and CD8+ T cells. Stimulation of resting and activated T cells with the P2Y6 ligand, UDP, caused a rise in the intracellular free calcium concentration in only the activated subset, indicating the presence of functional receptor. By in situ hybridization, P2Y6 receptor expression was detected in the T cells of the thymic medulla and spleen, whereas no signal was detected in the bone marrow, fetal liver, or lymph nodes. Interestingly, P2Y6 receptor is highly expressed in the T cells infiltrating active inflammatory bowel disease, whereas its expression was absent from the T cells of unaffected bowel *(121)*.

In the mucosa of the colon of chemical induced-colitic mice, showed increased the expression P2Y6 receptor mRNA by 3-fold after 7 days of treatment *(122)*. Consistent with *in vitro* study, revealed that the expression of P2Y6 receptor was increased by challenging intestinal epithelial cell-6 and colon carcinoma cell line (Caco-2/15) with

TNF-α and IFN-α. It also demonstrated that stimulation of epithelial cells by UDP results in an increased expression and release of IL-8 by an ERK1/2-dependent mechanism. The increase in IL-8 expression was associated with a transcriptional activation by the P2Y6 receptor. These data suggested P2Y6 receptor is involved in the pathogenesis of ulcerative colitis *(122).* Furthermore, another study reports that UDP stimulates IL-8 release via P2Y6 receptor in human monocytic cells induced by LPS *(123)*. Moreover, it has been shown that human neutrophil peptides (HNPs)-induced IL-8 production is predominantly regulated by the P2Y6 receptor in epithelial cells. The exact mechanisms by which HNPs signal through the P2Y6 receptor remain unknown *(124)*. These findings indicate a role for P2Y6 in inflammation and immune defenses.

UDP caused microglial phagocytosis through P2Y6 in a concentration-dependent manner, and that neuronal injury caused by kainic acid (KA) up-regulated P2Y6 receptors in microglia, the KA-evoked neuronal injury resulted in an increase in extracellular UTP, which was immediately metabolized into UDP in vivo and in vitro *(125).* P2Y6 receptor seems to trigger phagocytosis through the pathway(s) mediated by PLC-linked Ca^{2+} and PKC. UDP leaked from the injured neurons caused P2Y6 receptor-dependent phagocytosis *in vivo and in vitro*. Microglia expresses P2Y6 receptor that function as a sensor of phagocytosis. The activation of P2Y6 receptors by UDP would be a key event in initiating the clearance of dying cells or debris in the central nervous system. Thus, P2Y6 receptor agonist promises to be a therapeutic application in neurodegenerative conditions such as Alzheimer's disease, via an increased clearance of amyloid-β deposits.

The study of P2Y6 knockout (KO) mice show that these mice are viable and are not distinguishable from the wild-type (WT) mice in terms of growth, behavior fertility. However, the P2Y6 KO mice have a defective response to UDP in macrophages, endothelial cells, and vascular smooth muscle cells. The amount of IL-6 and macrophage-inflammatory protein-2 released in response to LPS stimulation was significantly enhanced in the presence of UDP, and this effect was lost in the P2Y6 KO macrophages. The endothelium-dependent relaxation of the aorta by UDP was abolished

in P2Y6 KO mice. The contractile effect of UDP on the aorta, observed when endothelial nitric-oxide synthase is blocked, was also abolished in P2Y6 KO mice *(126)*.

In addtion, UDP induces soluble TNF-**α** and IL-8 production in a promonocytic U937 cell line stably transfected with hP2Y6. Surprisingly, UDP induces the production of IL-8, but not TNF-**α**, in human astrocytoma 1321N1 cell lines stably transfected with hP2Y6. Therefore, the effect of UDP/P2Y6 signaling on the production of proinflammatory cytokines is selective and dependent on cell types. In addition, UDP can also induce the production of proinflammatory chemokines MCP-1 and IP-10 in hP2Y6 transfected promonocytic U937 cell lines, but not astrocytoma 1321N1 cell lines stably transfected with hP2Y6 *(107)*.

(2) P2Y6 receptor and vascular system

UDP stimulates mitogenesis of vascular smooth muscle cells through activation of P2Y6 receptors. The intracellular signal pathways are dependent on PLC, possibly PKC-**δ**, and a tyrosine kinase pathway but independent of Gi proteins, eicosanoids, and protein kinase A. Moreover, P2Y6 receptors play a prominent role in mediating contraction of human cerebral arteries *(127)*. The P2Y6 receptor is involved in both the direct contraction *(128)* and endothelium-dependent relaxation *(129)* of the aorta by UDP.

(3) P2Y6 receptor and cell survival

In 1321N1 human astrocytes, activation of P2Y6 receptors prevents apoptosis induced by tumor necrosis factor-**α** *(130)*, suggesting that the P2Y6 receptor play a role in cell survival. Consistent with that, treatment of rat osteoclasts with UDP increased osteoclast survival by initiate anti-apoptotic signals that enhance the life span of osteoclasts, thus increased bone resorption. Activation of P2Y6 receptors on osteoclasts induces transient increase of [Ca2+]*i* and translocation of NF-**χ**B through a proteasome-dependent mechanism *(131)*.

(4) P2Y6 receptor and mechanotranduction

Some evidences have been reported that P2Y6 receptor involved in the mechanotransduction of cells. For instance, mechanical stretch induces release of nucleotides (UDP) through pannexin-1 hemichannels from cardiac myocytes, which leading to stimulation of P2Y6 receptors. Nucleotide-bound P2Y6 receptor activates Gα12/13 proteins then induces the expression of fibrogenic factors, which activate cardiac fibroblasts in a paracrine manner. The activated fibroblasts produce excessive amount of collagen types I and III, leading to induction of cardiac fibrosis *(53)*.

High shear stress (25 dyn/cm^2) decreased P2X1 receptors, whereas P2Y2 and P2Y6 receptors were upregulated both at the mRNA level and protein level in smooth muscle cells (SMCs). Because of P2Y2 and P2Y6 receptors stimulate growth and migration of SMCs, increased expression of these receptors could promote vascular remodeling induced by shear stress *(132)*. Moreover, fluid shear stress (12 dyn/cm^2) regulates NFκB activity through the P2Y6 and P2X7 receptor in MC3T3-E1 osteoblasts *(133)*.

CHAPTER III
MATERIALS AND METHODS

1. Cell culture

HDPCs were obtained from caries-free lower third molars extracted for orthodontic reason with the patients' informed consent. The protocol was approved by the ethical committee, Faculty of Dentistry, Chulalongkorn University No. 07/2008. The teeth were immediately split using a hammer and the pulp tissues were harvested. The pulp tissues were gently removed by forceps, cut into pieces and placed in a 35-mm culture dish (Nunc, Naperville, IL, USA). The explants were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU ml^{-1} penicillin G, 100 IU ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B and incubated at 37°C in 5% CO_2. The medium and all supplements were from Gibco (BRL, Carlsbad, CA, USA). After the outgrowth cells reached confluence, they were subcultured into the new culture dishes. Cells from the third to the fifth passages were used. All experiments were performed in triplicate with cells prepared from three different donors.

2. Application of hydrostatic pressure, inhibitors and nucleotides

The method for hydrostatic pressure application was applied from Kanzaki et *al. (134)* and Wongkhantee *et al. (135)*. Briefly, cells were seeded in six-well plates at a density of 2×10^5 cells/well for 16 hours. A plastic cylinder containing metal coins was placed over a confluent cell layer in one well of a 6-well plate to generate compressive force at 0.7, 0.9, 1.4 g/cm^2 as shown in Figure 3.1.

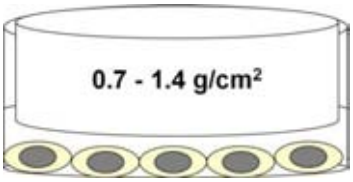


Figure 3.1 Model of Hydrostatic pressure.

For inhibition experiments, each inhibitor was added to the medium 30 minutes prior to application of hydrostatic pressure. The inhibitors used included 100 μM suramin, 0.18 μM NF449, 1 unit/mL apyrase, 5-10 μM MRS2179, 1-3 μM MRS2578 (All from Sigma-Aldrich Chemical, St. Louis, MO, USA), and 10-15 μM BAPTA-AM (Enzo Life Sciences International Inc, Plymouth Meeting, PA, USA). Exogenous UDP, UTP, or ATP (50-200 μM) (All from Sigma-Aldrich) was added to the culture for 16 hours.

3. Fluorescence microscopy

Rhodamine phalloidin was used to label cytoskeletal F-actin. Cells were fixed with 4% formalin for 10 minutes and then washed twice in phosphate buffer saline (PBS). PBS containing 0.1% Triton-X100 was used to permeate the membrane for 1 minute. Then cells were washed twice in PBS and incubated with Rhodamine-phalloidin (Invitrogen, Carlsbad, CA, USA) diluted 1:100 in PBS for 15 minutes. After labeling, the cells were washed three times in PBS, mounted for microscopy with Prolong® Gold antifade reagent (Invitrogen) and visualized with fluorescence microscope (Axiovert 40CFL, Carl Zeiss, Gottingen, Germany). The images were acquired using Axiocam MRc5 and the AxioVs40v4.7.2.0 software.

4. Cell viability

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermaeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of the detergent solution containing 1:9 of DMSO and glycine buffer (0.1 M glycine/ 0.1 M sodium chloride pH10) results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be measured spectromically used the absorbance at 570 nM

(Genesys UV scanning, Thermospectronic, Roche, NY, USA). All measurements were done in triplicate.

5. Transfection of siRNA

For siRNA transfection, the cells at 70-80% confluence were transfected with P2Y6R siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at various concentrations by using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. Silencer Negative Control siRNA (Invitrogen) was used as a negative control and was introduced into the cells using the same protocol. The cells were kept as static controls or subjected to HP experiments 16 hours after transfection.

6. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted with Tri-reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. One microgram of each RNA sample was converted to cDNA by the use of an avian myeloblastosis virus (AMV) for 1.5 hours at 42°C. Subsequently, PCR was performed to detect IL-6 and GAPDH was used as internal control. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers were:

GAPDH (NM002046.3), forward 5'-TGA AGG TCG GAG TCA ACG GAT-3'

reverse 5'-TCA CAC CCA TGA CGA ACA TGG-3'

IL-6 (NM000600.2), forward 5'-CCT GAA CCT TCC AAA GAT GGC-3'

reverse 5'-CTG ACC AGA AGA AGG AAT GCC-3'

P2Y1 (NM002563.2), forward 5'-CGG TCC GGG TTC GTC C-3'

reverse 5'-CGG ACC CCG GTA CCT-3'

P2Y2 (NM002564.2), forward 5'-CTA AAG CCA GCC TAC GGG AC-3'

reverse 5'-TCC TAT CCT CTG CAT GTC-3'

P2Y4 (NM_002565.3), forward 5'-AGT GAG GTG GAG CTG GAC TGT TGG-3'

reverse 5'-CCT AGG AGT AGA GCA GCT ACT GTC-3'

P2Y6 (NM_004154.3), forward 5' -CGT AAC CGC ACT GTC TGC TA-3'

reverse 5' -TGA GCT TCT GGG TCC TGT GA- 3'

P2Y11 (NM_002566.4), forward 5'- GGG TTG TGG CAC AAT GAG GA-3'

reverse 5'-CAG GTT GCA GGT GAA GAG GA-3'

P2Y12 (NM 176876.10), forward 5'-AGA AGA CCA CCA GGC CAT TT -3'

reverse 5'-CAC CTT TTT CCT GGG GAC TT-3'

P2Y13 (NM_176894.2), forward 5' AGC AAT GCC TTC CTG GAC CA -3'

reverse 5'-GTA GCT AGG AGG TAA GGC CAG A-3'

P2Y14 (NM_001081455.1), forward 5'-GCA GTG TTT CCC ACT GGT CA-3'

reverse 5'-AGC AAG GAG GAG CAT GAG CA-3'

The PCR was performed with Taq polymerase (Qiagen, Hilden, Germany) and a PCR reaction volume of 25 µL. The amplification profile for IL-6, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 was 1 cycle at 94°C for 1 minute, 35 cycles at 94°C for 1 minute, hybridization at 60°C for 1 minute, and extension at 72°C for 2 minutes, followed by 1 extension cycle at 72°C for 10 minutes. The same profile was also used for GAPDH with 22 cycles. The PCR was performed in a DNA thermal cycler (Biometra, Gottingen, Germany). The amplified DNA was subjected to electrophoresis on a 2% agarose gel and visualized by ethidium bromide fluorostaining. The relative intensities of the bands were measured by Scion image analysis software (Scion, Fredevick, Maryland, USA).

7. Enzyme-Linked Immunoabsorbent Assay (ELISA)

Cells were cultured and treated as indicated in DMEM without phenol red (Gibco-BRL, Carlsbad, CA, USA). The culture media were collected and assayed to quantify concentrations of IL-6 by ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacture instruction. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-6 has been pre-coated onto a microplate. Briefly, the media were first centrifuged at low speed to remove intact cells and were centrifuged again at a high speed to remove cellular debris. Add 100 µl of Assay Diluent into the microplate followed by adding 100 µl of

sample media and cover with the adhesive strip provided. Each sample was run in duplicate. Samples were incubated for 2 hours at room temperature, then aspirated and washed with wash buffer (400 µl) for four times. Add 200 µl of IL-6 conjugate to each well and incubate for 2 hours at room temperature. Then, repeat the washing process as described before. Add the substrated solution to each well and incubate at room temperature on the benchtop and protect from light. After incubation for 20 minutes, add the stop solution to each well. The color in the wells would change from blue to yellow. Determine the optical density of each well by using microplate reader set to 450 nm.

8. Protein extraction and Western Blot analysis

Protein was extracted with a radioimmunoprecipitation assay (RIPA). RIPA buffer was added into cells and left at 4°C for 5 minutes, then cells were scraped and transferred to 1.5 ml microcentrifuge tube and spin at 14,000g, 4°C, for 5 minutes. Protein concentrations were measured by means of a BCA protein assay kit (Pierce, Rockford, IL, USA) and measured at the absorption of 560 nm. Equal amounts of protein samples were mixed with 3X Laemmli buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 10% glycerol) and denatured by boiling for 10 minutes. The samples were separated by 12% SDS polyacrylamide gel and transferred onto nitrocellulose membrane. For blocking of non-specific binding, the membrane was incubated in 5% non-fat milk for 1 hour. Subsequently, the membranes were incubated with primary antibody against P2Y6R (1:100; Santa Cruz Biotechnology, Santa Cruz,CA, USA), or β-actin (1:1000; Chemicon International, Temecula, CA, USA). The membranes were then incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemoluminescence (Pierce Biotechnology). The relative intensity of bands was measured by Scion image analysis software (Scion, Frederick, MD, USA).

9. Luciferin-Luciferase bioluminescence assay

The extracellular ATP concentration was determined by means of an ENLITEN® ATP assay system bioluminescence detection kit for ATP measurement (Promega, Madison, WI, USA). The assay uses recombinant luciferase to catalyze the following reaction:

$$ATP + D\text{-}Luciferin + O_2 \longrightarrow Oxyluciferin + AMP + PPi + CO_2 + Light\ (560nm)$$

When ATP is the limiting component in the luciferase reaction, the intensity of the emitted light is proportional to ATP concentration. Measurement of the light intensity using a luminometer permits direct quantitation of ATP.

Cells were cultured and exposed to 0.7, 1.4, 2.5 g/cm^2 of HP or incubated at atmospheric pressure for 30 minutes in DMEM without phenol red. The culture media were collected and assayed to quantify concentrations of ATP according to the manufacture instruction. Briefly, add 100 µL of Enliten® Luciferase/Luciferin reagent and 100 µL of sample media into the microplate. The resulting light signal was immediately measured by a luminometer (Victor Light Luminescence Counter, PerkinElmer Ltd., Salem, MA, USA).

10. Statistical analysis

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

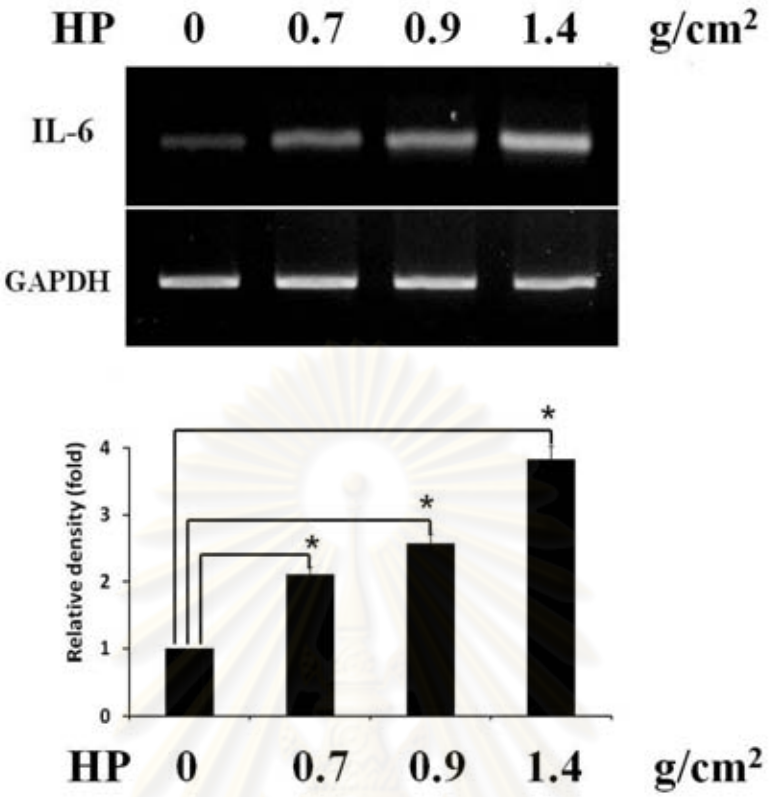
CHAPTER IV
RESULTS

Hydrostatic pressure induced IL-6 production in HDPCs

The effect of HP on IL-6 expression was determined by exposed HDPCs to HP (0.7, 0.9, 1.4 g/cm^2) or control condition (0 g/cm^2) for 16 hours and the cell extracts were collected. The levels of transcription and protein were detected by RT-PCR and ELISA, respectively. The result of RT-PCR analysis in Figure 4.1A and ELISA in Figure 4.1B showed that HP dose dependently induced IL-6 mRNA expression and protein level in HDPCs.

In the time-course experiments, a significant increase in IL-6 mRNA expression could be detected as early as 2 hours after pressure applied and maintained for 24 hours (Figure 4.2A). However, a significant increase of protein secretion was observed at 4 hours after stimulation (Figure 4.2B).

A.



B.

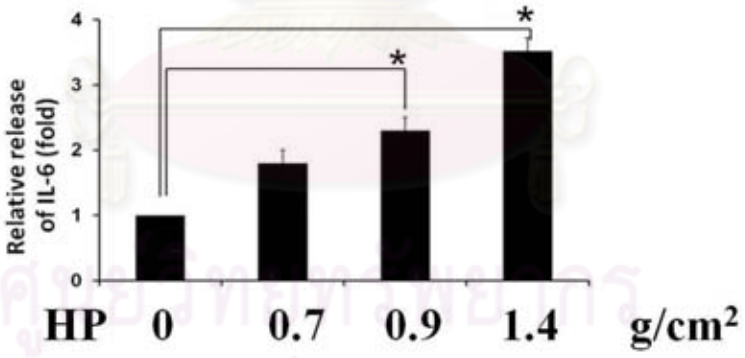
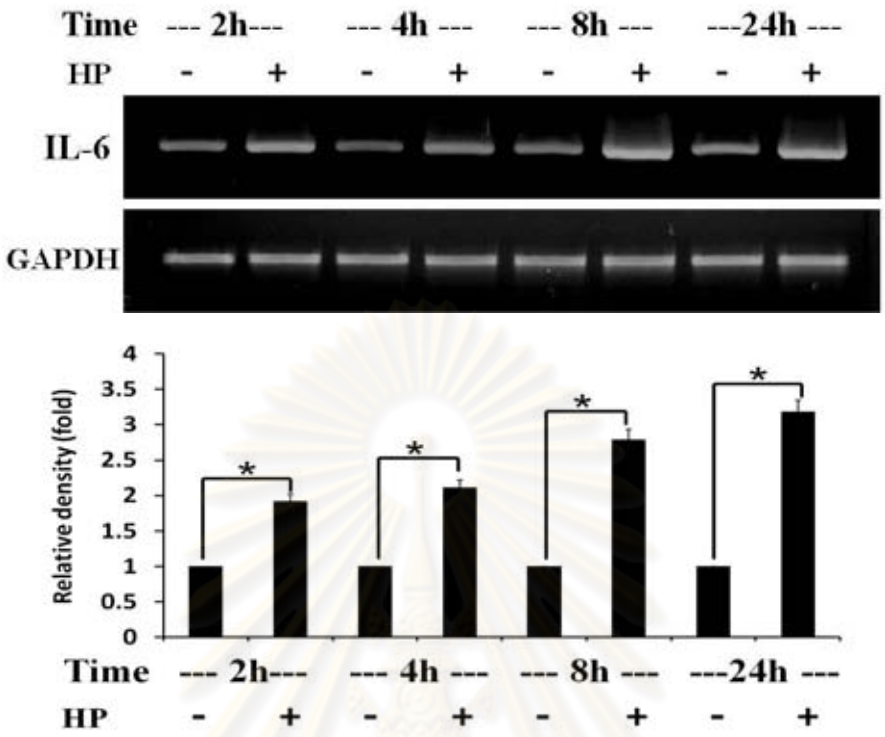


Figure 4.1 Effect of HP on IL-6 expression in HDPCs. HDPCs were subjected to HP (0.7, 0.9, 1.4 g/cm^2) or control condition (0 g/cm^2) for 16 hours. (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

A.



B.

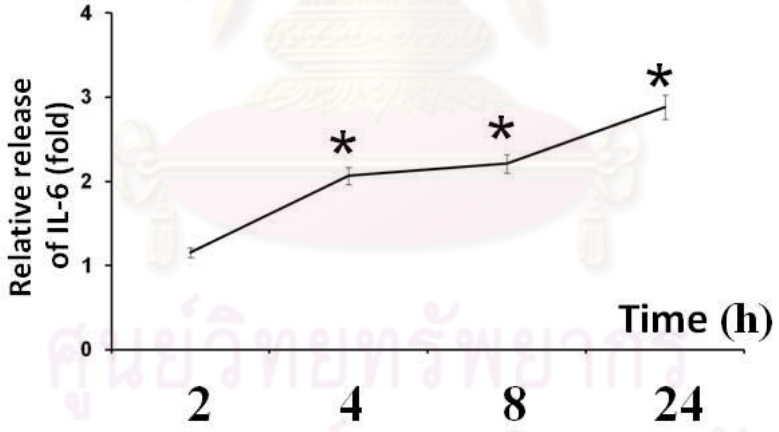


Figure 4.2 **Effect of HP on IL-6 expression at various time points.** HDPCs were exposed to HP (1.4 g/cm^2) or control condition for 2, 4, 8 and 24 hours. Increased IL-6 mRNA (A) and protein expression (B) was observed. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

The effect of hydrostatic pressure on cell viability and morphology of HDPCs

After loading different levels of HPs ranging from 0.7, 0.9, 1.4 g/cm^2 for 24 hours, the cell viability of pressurized cells were 91.1% $\pm$ 4.2, 90.1% $\pm$ 3.8 and 91.6% $\pm$ 5.4 respectively (Figure 4.3). Though the cell viability in HP treated groups were lower than 100%, there were no statistical differences on comparison of cells in the control condition. Results of multiple comparisons also showed no significant difference when HDPCs were exposed to different loading of HPs.

Actin cytoskeleton was stained with rhodamine phalloidin to observe cell morphology. The image in Figure 4.4 showed that neither morphological changes nor abnormal actin organization was observed in HP-treated cells even at the 1.4 g/cm^2 of HP. In both control and HP groups, HDPCs exhibited elongated shape, and thick and long F-actin filaments were centrally and peripherally observed. Based on these results, we used HP at 1.4 g/cm^2 in the subsequent experiments.

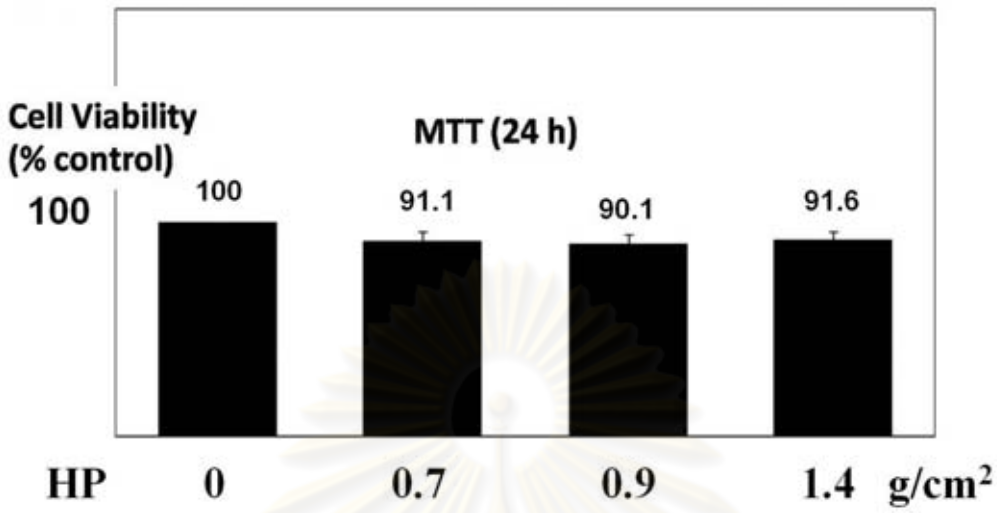


Figure 4.3 **Effect of hydrostatic pressure on cell viability of HDPCs.** HDPCs were exposed to HP (0.7, 0.9, 1.4 g/cm^2) or control condition for 24 hours. Cell viability was determined by using MTT assay. Results are expressed as percentage of the control and mean ± S.D. from three different experiments. *P < 0.05 compared with the control.

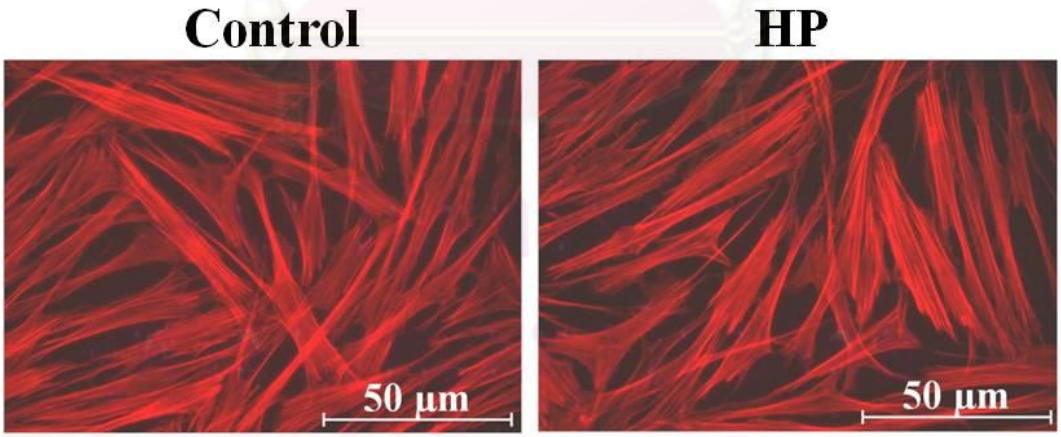


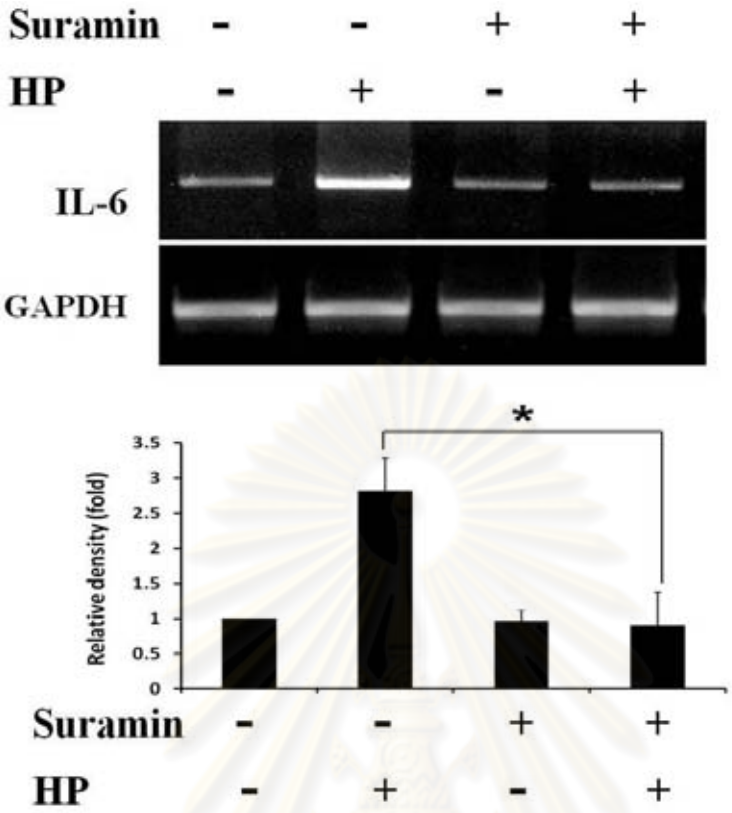
Figure 4.4 **The morphology of HDPCs after HP treatment.** Fluorescence micrographs of cells labeled with rhodamine-phalloidin showed the cells under control (left), showed the cells under 1.4 g/cm^2 HP (right) for 16 hours. Scale bar = 50 µm.

Involvement of P2Y receptor in HP-induced IL-6 expression

To identify the mechanotransduction pathways involved in HDPCs response to HP, we first examined the effect of suramin, an agent that uncouples G-proteins from receptors and broad spectrum antagonist at P2Y purinergic receptors. Figure 4.5 showed that pretreatment with suramin caused a significant reduction in the HP-induced both of IL-6 expression and secretion. Since most P2Y-receptors couple with Gq-protein to stimulation of phospholipase C followed by increase in inositol phosphates and mobilization of Ca^{2+} from intracellular storage to promote its physiological effects, thus an intracellular Ca^{2+} chelator, BAPTA-AM was used to pretreatment the cells before HP application. Then cells extracted were collected at 16 hours, RT-PCR and ELISA were performed. Figure 4.6 showed pretreatment of HDPCs with BAPTA-AM at 15 μM resulted in significantly decrease of HP-induced IL-6 expression, which indicate that the mechanism of HP-induced IL-6 expression involves intracellular calcium signaling pathway.

Because the quick response as early as 2 hours of HP on IL-6 induction and the inhibitory effect of suramin, give us a clue that HP may transduce signal through nucleotides release into the conditioned media and then in-turn activate P2Y receptor of HDPCs.

A.



B.

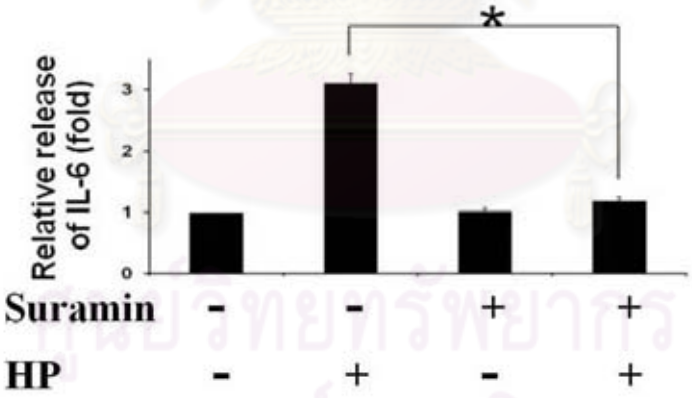
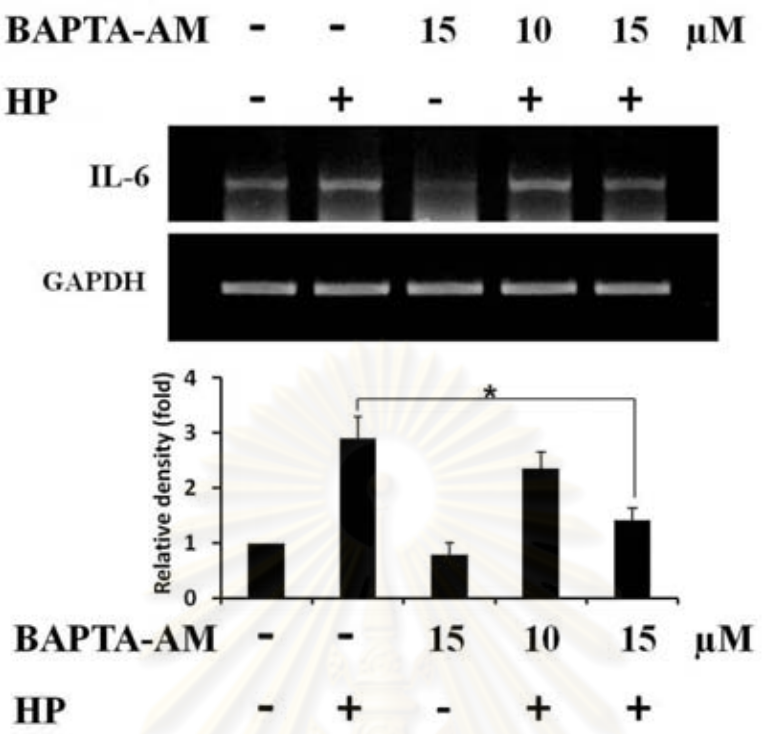


Figure 4.5 **Suramin could block HP-induced IL-6 expression.** HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the suramin (100 μM). (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the HP-treated sample.

A.



B.

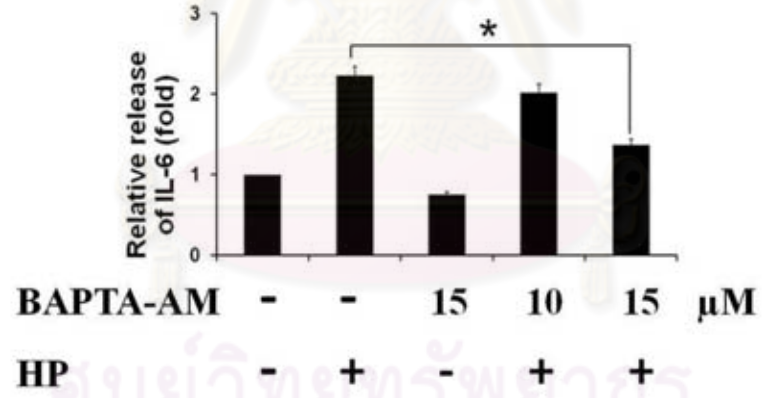


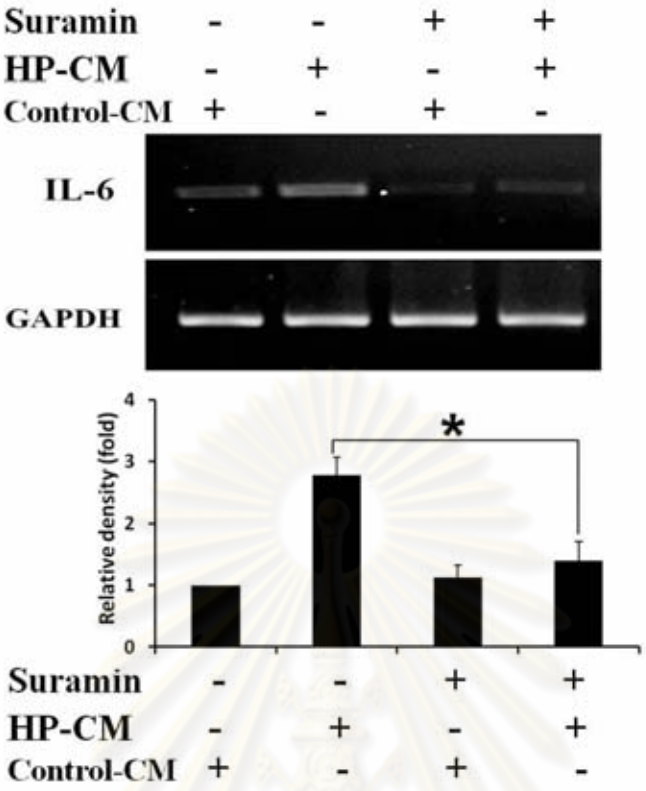
Figure 4.6 BAPTA-AM attenuated pressure-induced IL-6 expression. HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the BAPTA-AM (10, 15 μM). (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the HP-treated sample.

HP-induced conditioned medium could stimulate IL-6 expression in HDPCs

To confirm the role of nucleotides release from HDPCs due to HP, the conditioned medium was collected after application of HP (HP-induced conditioned medium; HP-CM) for 2 hours or non-stimulated (Control-CM) and treated another set of cells. The data in Figure 4.7 revealed that the cells incubated with the HP-CM significantly increased IL-6 expression when compared with those incubated with Control-CM. Consistent with the result in Figure 4.5, the inductive effect of HP-CM was significantly abolished by pretreatment of the cells with suramin.

As we hypothesized that HP transduce signal through nucleotide release, we next determined the level of ATP in the conditioned medium. The conditioned medium was collected to measure the amount of ATP by Luciferin-Luciferase bioluminescence assay. Figure 4.8 showed that the amount of ATP in the medium collected from HP-stimulated cultures was significantly increased when compare to the control.

A.



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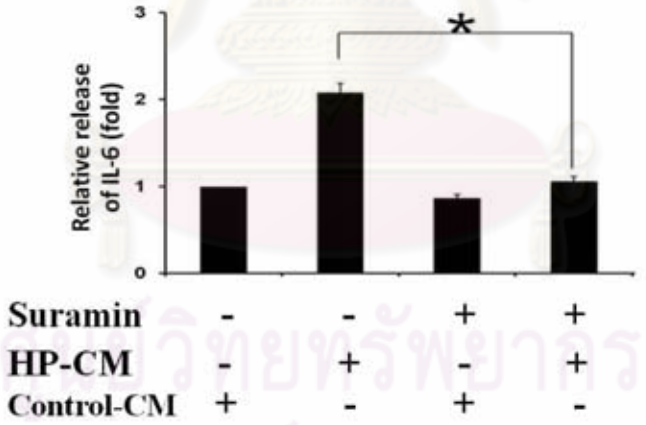


Figure 4.7 HP-induced conditioned medium stimulated IL-6 expression in HDPCs. HDPCs were incubated with the medium transferred from the HP-stimulated (HP-CM) or non-stimulated (Control-CM) cultures in the absence or presence of suramin (100 μM) for 16 hours. (A) IL-6 mRNA expression was determined by RT-PCR. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments.*P < 0.05 compared with the HP- CM treated sample.

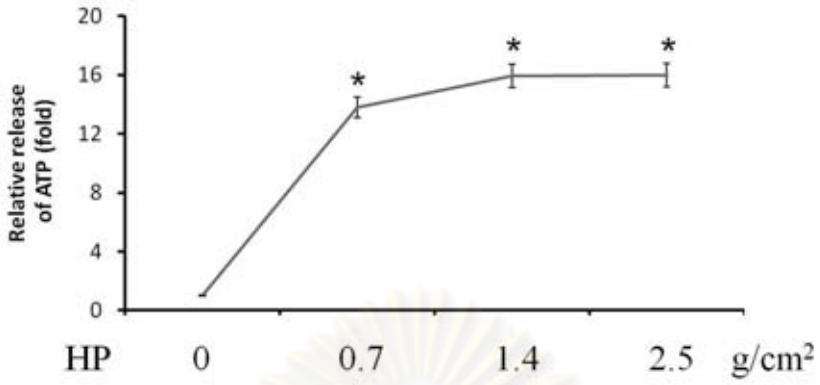


Figure 4.8 Measurement of ATP levels in culture medium after HP stimulation. HDPCs were exposed to 0.7, 1.4, 2.5 g/cm^2 of HP or control condition for 30 minutes. Then, the condition medium was collected and assay by Luciferin-Luciferase bioluminescence assay. Data from three independent experiments were shown by graph as mean ± S.D. (*P < 0.05) as a fold-induction compared to the control.

The expression profile of P2Y-receptors in HDPCs

The expression of P2Y-receptors in cultured HDPCs from 5 donors was determined by RT-PCR. RT-PCR analysis showed that HDPCs expressed mRNAs coding all subtypes of P2Y receptors. HDPCs expressed P2Y-1, 4, 6, 12, 13 mRNAs whereas P2Y-2, 11, 14 mRNAs were hardly detected as shown in Figure 4.9.

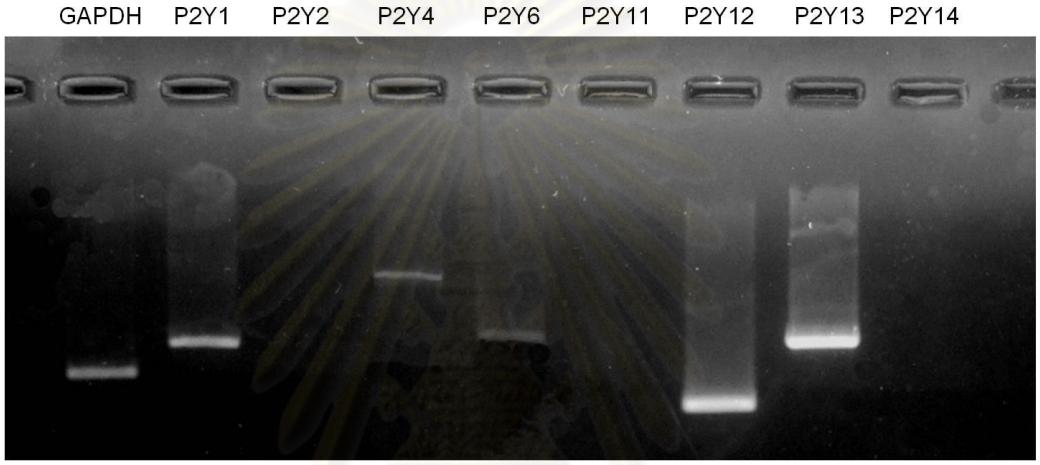


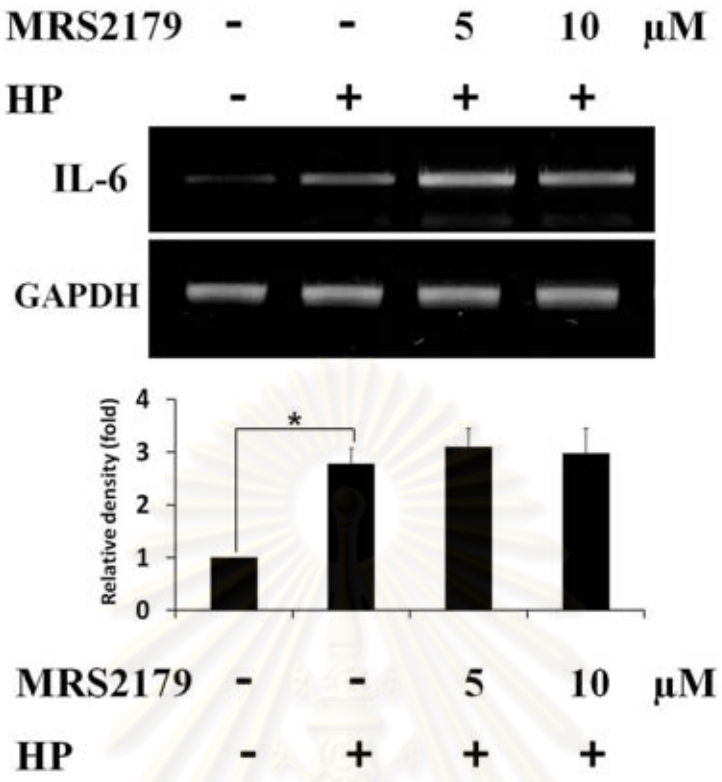
Figure 4.9 RT-PCR analysis of P2Y-receptors expression profile. RNA was extracted from cultured HDPCs, reverse transcribed and amplified with specific primers for P2Y-1,2,4,6,11,12,13,14 receptors (Lane: 2-9). GAPDH was used as a control housekeeping gene (Lane: 1).

HDPCs responded to HP using a non-P2Y1 receptor dependent pathway

We further investigated which P2Y receptor subtype(s) is involved in HP-induced IL-6 expression. As we have shown in Figure 4.8 that ATP was significantly released by HDPCs after HP stimulation, therefore we first clarify the pathway involved P2Y1 receptor, which is selective for adenine nucleotides. HDPCs were pretreatment with MRS2179 (a selective P2Y1 receptor antagonist) 30 minutes before HP stimulation for another 16 hours. Surprisingly, MRS2179 did not suppress HP-induced IL-6 expression in HDPCs both at mRNA and protein level (Figure 4.10).

To confirm the result of MRS2179, HDPCs were incubated with NF449 (a P2X1, P2X3, P2Y1, and P2Y2 antagonist) for 30 minutes before HP stimulation. Figure 4.11 demonstrated that NF449 could not reduce HP-induced IL-6 mRNA expression in HDPCs, which consistent with the result from MRS2179 (Figure 4.10).

A.



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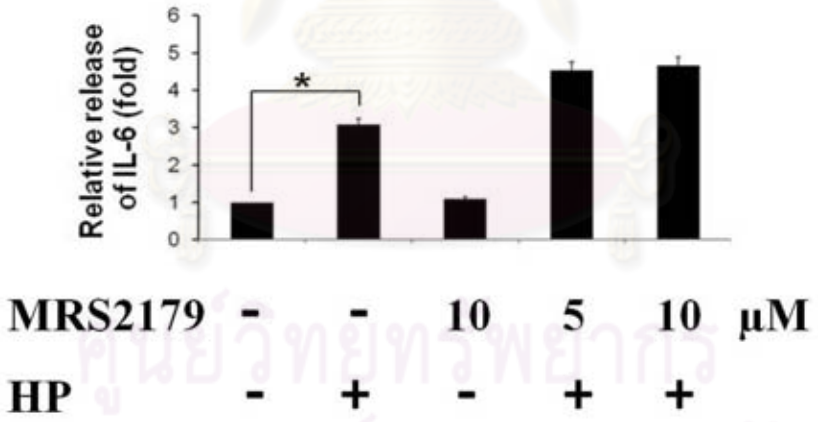


Figure 4.10 **Effect of MRS2179 on HP-induced IL-6 expression.** HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the MRS2179 (5, 10 μM). (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

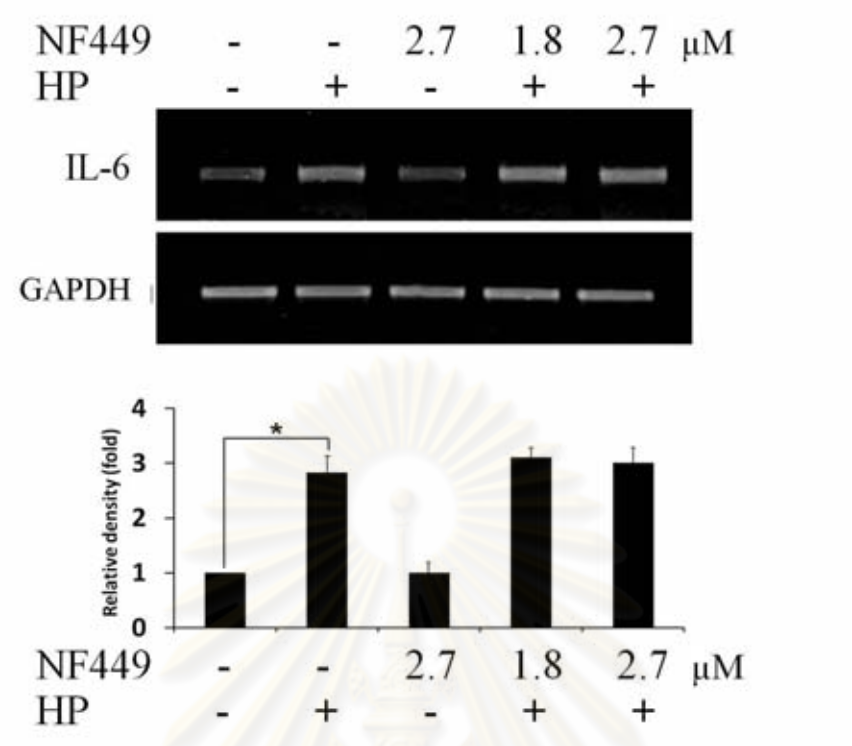


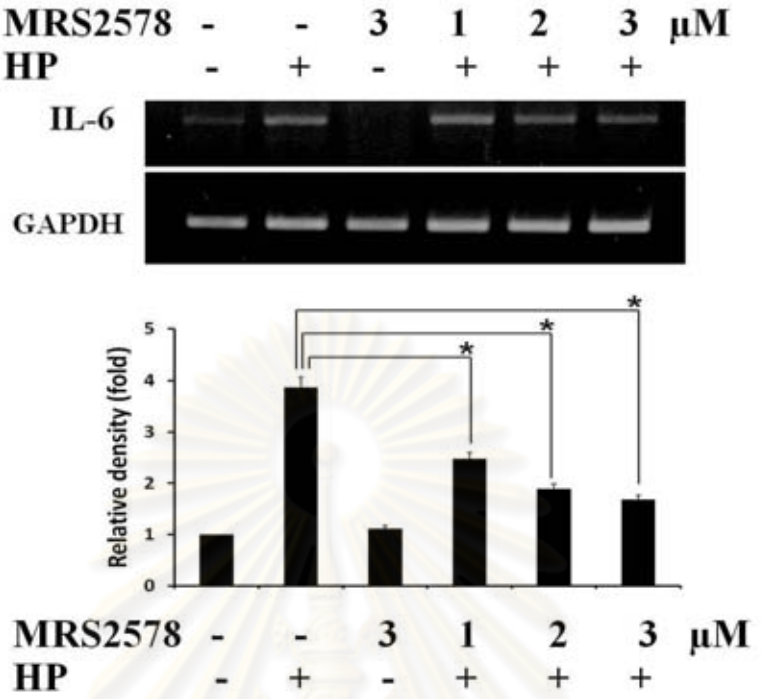
Figure 4.11 Effect of NF449 on HP-induced IL-6 expression. HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the NF449 (1.8, 2.7 μM). IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

Inhibition of P2Y6 receptor abolished HP-induced IL-6 expression

We next determined the other possible P2Y receptors which participate in the pathway of HP-induced IL-6 expression by HDPCs. P2Y6 receptor is one of the target because its specific agonist is uracil, not adenine nucleotides. Strikingly, pretreatment the cells with MRS2578 (a selective P2Y6 receptor antagonist) before HP stimulation, significantly diminished HP-induced IL-6 expression and secretion in a concentration-dependent manner (Figure 4.12).

To confirm the involvement of P2Y6 receptor, HDPCs were transfected with a small-interfering RNA (siRNA) specific for the P2Y6 receptor gene and then exposed to HP for 16 hours. The treatment with P2Y6R-specific siRNAs (40-80 nM) successfully abolished the P2Y6 mRNA (Figure 4.13A) and decreased the P2Y6 receptor protein level by about 50% (Figure 4.13B). Consistent with the result in Figure 4.12, P2Y6R-specific siRNAs at 80 nM significantly suppressed HP-induced IL-6 expression in HDPCs both at mRNA and protein level compared to the cells transfected with control siRNA (Figure 4.14).

A.



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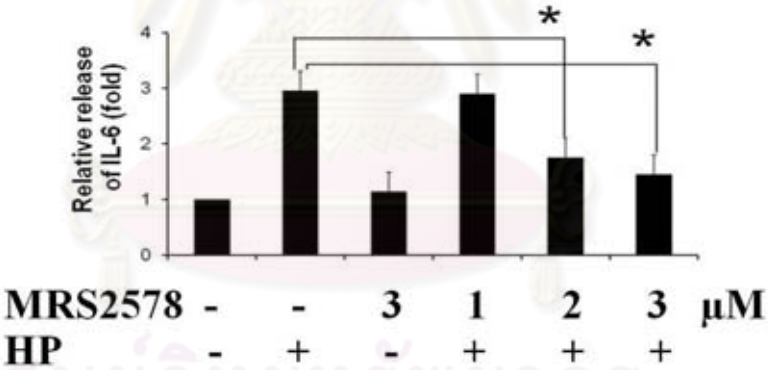


Figure 4.12 **Inhibitory effect of MRS2578 on HP-induced IL-6 expression.** HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the MRS2578 (1-3 μM). (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold induction compared to the control. (B) IL-6 protein release was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the HP-treated sample.

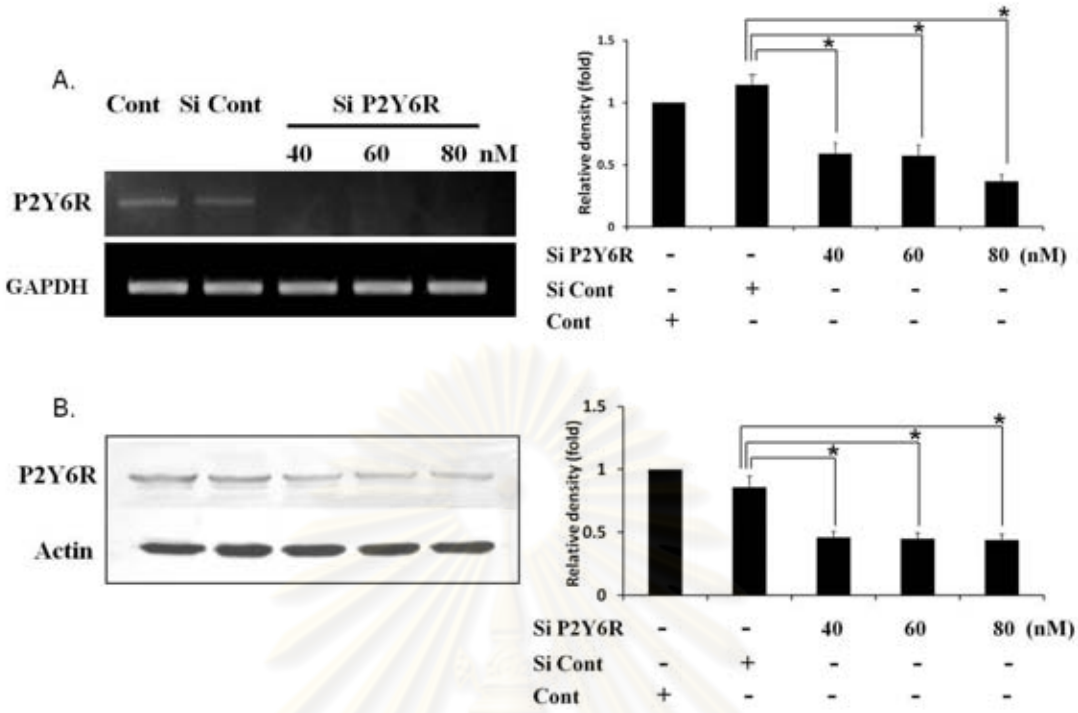
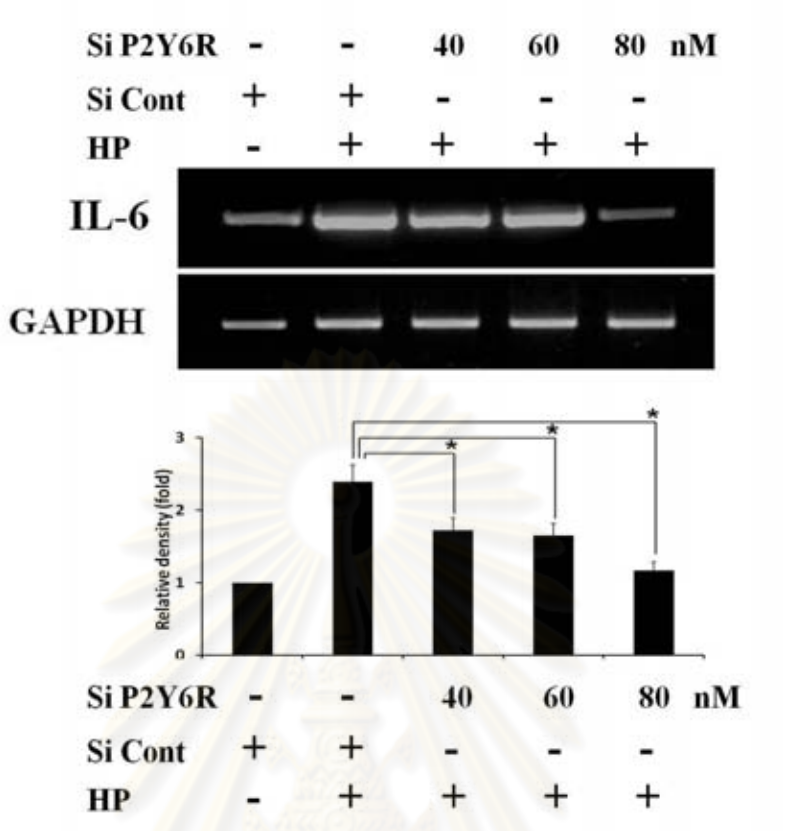


Figure 4.13 **P2Y6R-siRNAs decreased the P2Y6 receptor mRNA and protein level.** HDPCs were transfected with a siRNA oligonucleotide sequence specific for P2Y6R (Lane: 3, 4, 5) or a siRNA control (Lane 2) for 16 hours. (A) P2Y6R mRNA expression was determined by RT-PCR. GAPDH served as internal control. (B) P2Y6R protein expression was determined by Western Blotting. β-actin used for protein normalization. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the Si Control sample.

A.



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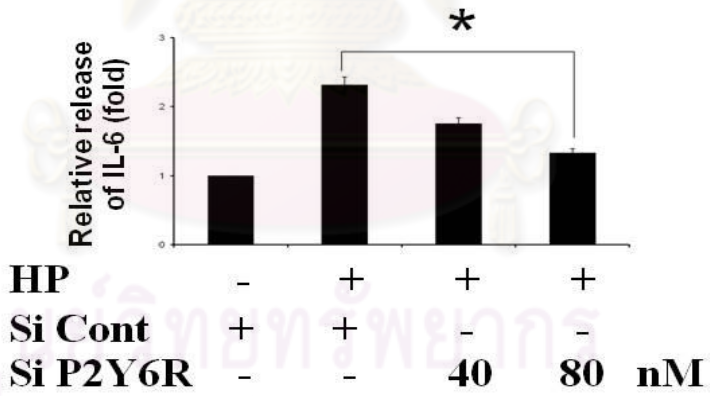
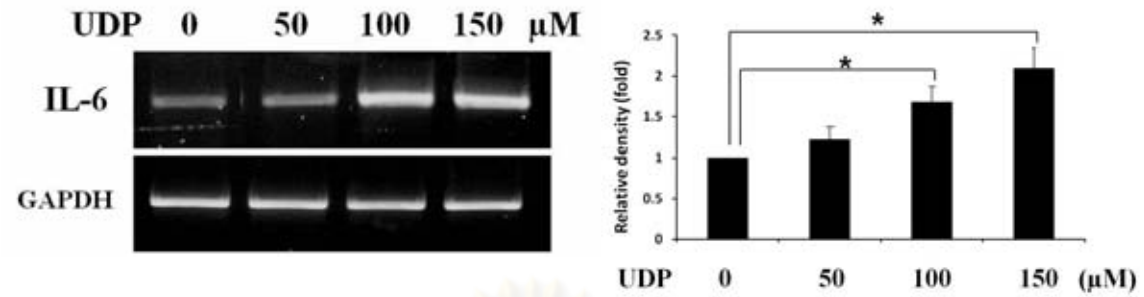


Figure 4.14 P2Y6-siRNAs attenuated HP-induced IL-6 expression in HDPCs. HDPCs were transfected with control siRNA or P2Y6R siRNA, following treatment with HP (1.4 g/cm^2) for 16 hours. (A) IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. (B) IL-6 protein expression was determined by ELISA. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the HP-treated sample.

The effect of nucleotides on IL-6 expression in HDPCs

As the P2Y6 receptor is mainly activated by UDP, which is approximately 100-fold more potent than UTP, and ADP and ATP are almost inactive, we also examined the effects of nucleotides UDP, UTP and ATP on IL-6 expression by HDPCs. Figure 4.15 and 4.16 showed UDP but not ATP and UTP, could increase IL-6 both at mRNA and protein level in a concentration-dependent manner. Collectively, these findings imply that UDP released from HP stimulated cells and then in turn activate the P2Y6 receptor on the cell surface of HDPCs and subsequently induce of IL-6 expression.

A.



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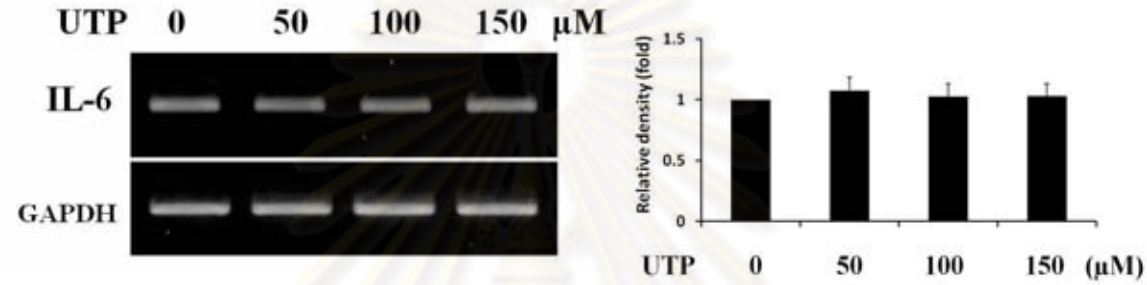

C.

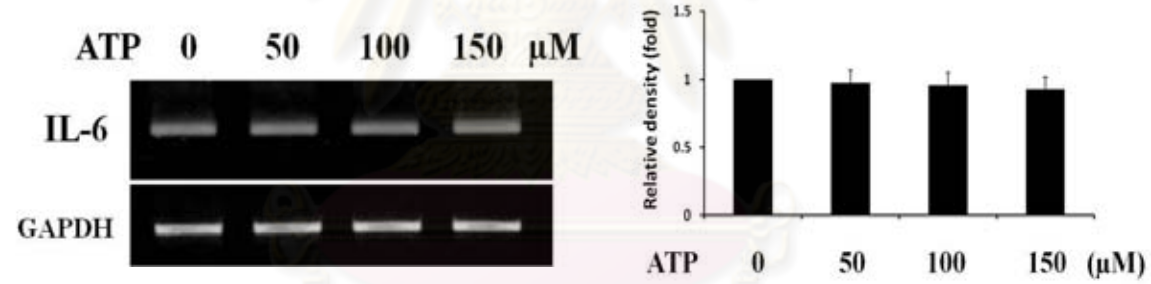

Figure 4.15 **Effect of nucleotides on IL-6 expression in HDPCs.** Exogenous UDP (A) or UTP (B) or ATP (C) 50, 100, 150 μM was added to HDPCs cultures for 16 hours. IL-6 mRNA synthesis was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

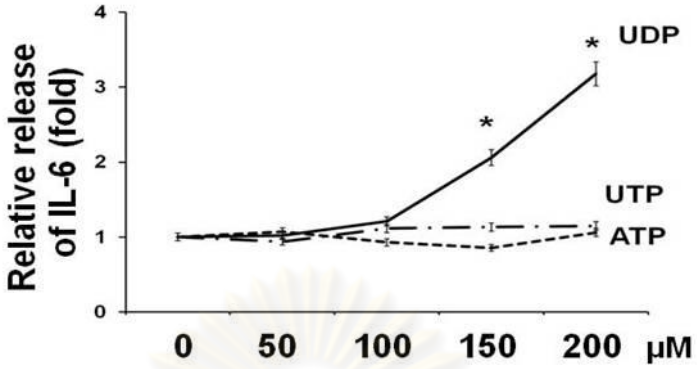

Figure 4.16 **Effect of nucleotides on IL-6 release by HDPCs.** IL-6 protein release after treatment with UDP, UTP, ATP (0-200 μM) 16 hours was determined by ELISA. Data represent the mean ± S.D. of three independent experiments.*P < 0.05 compared with the control.

HP-induced IL-6 expression was not depended on COX-pathway in HDPCs.

Finally, we examined the other pathway which may mediate HP-induced IL-6 expression. Several in vitro studies have demonstrated increased cyclooxygenase-2 (COX-2) expression and PGE_2 level in numerous cell culture models, which have been subjected to hydrostatic pressure *(49)*. Therefore, we use NS398, a selective inhibitor of COX-2, to determine whether COX-2 involved in HP-induced IL-6 expression in HDPC.

HDPCs were incubated with NS398 (1-10 μM) for 30 minutes before HP stimulation. Cells extracted were collected at 16 hours and RT-PCR was performed. Figure 4.17 showed that NS398 could not exert any inhibitory effect on HP-induced IL-6 expression.

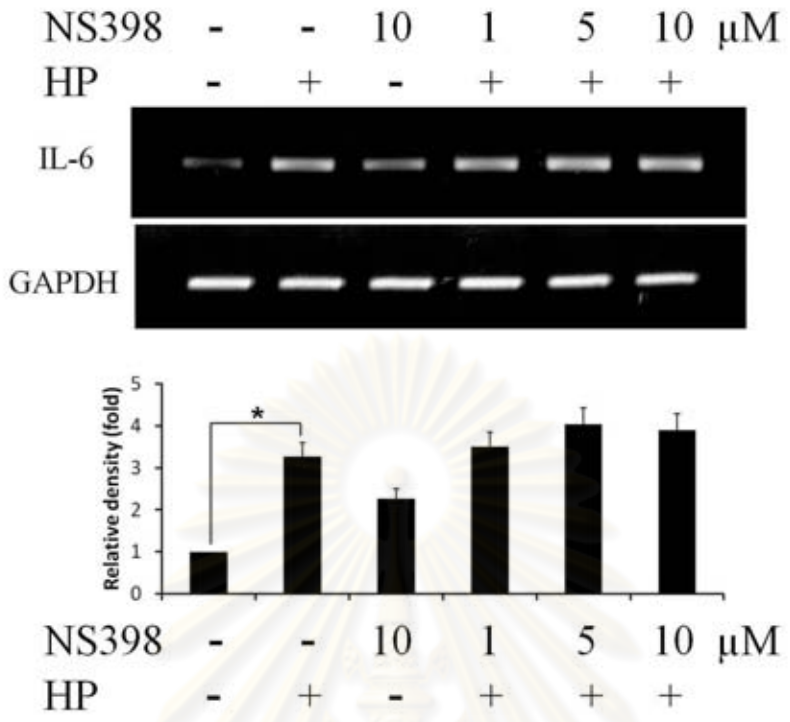

Figure 4.17 Effect of NS398 on HP-induced IL-6 expression in HDPCs. HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the NS398 (1, 5, 10 μM). IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

Beta 1 integrin was not involved in HP-induced IL-6 expression in HDPCs.

Another possible pathway we investigated is integrins, which have been identified as mechanosensory molecules in osteoblasts and osteocytes *(136)*, myocytes *(137),* fibroblasts *(138),* endothelial cells *(139)* and chondrocytes *(140).* Since beta 1 integrin is the major group which expressed in HDPCs *(141)*, we were treated HDPCs with inhibitory antibody to beta 1 integrin for 30 minutes prior to application of HP for another 16 hours, then RNA was collected and RT-PCR was performed. Figure 4.18 showed that beta 1 integrin had no effect on HP-induced IL-6 expression in HDPCs.

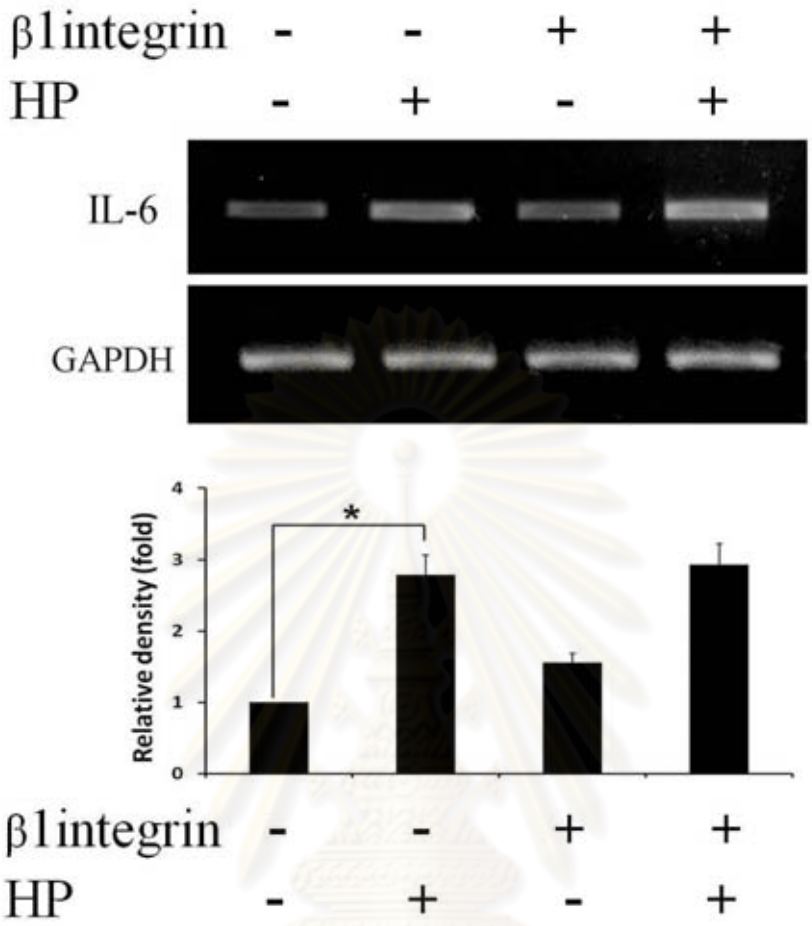

Figure 4.18 Effect of β1 integrin on HP-induced IL-6 expression in HDPCs. HDPCs were subjected to HP (1.4 g/cm^2) or control condition for 16 hours in the absence or presence of the β1 integrin (1 ng/ml). IL-6 mRNA expression was determined by RT-PCR. GAPDH served as internal control. The relative density was presented by graph as a fold-induction compared to the control. Data represent the mean ± S.D. of three independent experiments. *P < 0.05 compared with the control.

CHAPTER V
DISCUSSION AND CONCLUSION

To date, our results are the first to demonstrate that HDPCs produce IL-6 in response to hydrostatic pressure via the P2Y6 receptor. We simulated increased intra-pulpal pressure condition by using a mechanical stress model to examine the effect of HP on the inflammatory cytokine production and clarify the underlying mechanism. Although it has been reported that HP could promote hard tissue mineralization in human dental pulp stem cells (DPSCs) *(34)*, we provided the other role of HP on immunomodulation by regulating IL-6 expression. In addition, this model is a potentially useful tool to study the role of HP change as occurs during mild or chronic pulpal inflammation.

Differing loading amplitudes of HP cause responses of the cell in different ways and the difference may be cell type-specific. For example, dynamic hydrostatic pressure at 2.5 MPa, 0.5 Hz decreased DPSCs viability within 12 hours post-stimulation, but viability moderately improved within 24 hours of re-introduction to regular culture conditions *(34)*. Moreover, dynamic hydrostatic pressure showed marked morphological changes and decreased DPSCs attachment when the loading was greater than 1.5 MPa, 0.5 Hz and exposure time longer than 2 hours *(34)*. The exposure to 6 MPa of static hydrostatic pressure 60 minutes caused no morphological changes of human periodontal ligament cells, and did not affect the cellular viability *(17)*. Normal human dermal fibroblasts remained alive for 60 minutes at up to 40 MPa *(55)*. Additionally, some evidences revealed that HP stimulates cell proliferation in various cell types. Static pressure stimulated rat aortic smooth muscle cells proliferation in pressure- and time-dependent manners, by the maximal increase was observed at 120 mmHg after 48 hours treatment *(142)*. Exposure of bladder smooth muscle cells to sustained HP (7.5 cmH_2O) for 48 hours led to a significant increase in cell proliferation when compared with the control *(143)*.

Up to date, there is no data on absolute magnitude of physiologic and pathologic HP level within human dental pulp cavity *in vivo*. By experimental studies in animals, the

interstitial fluid pressure was suggested about 6-10 mmHg in the normal dental pulp and 16 mmHg in inflamed pulp *(40, 144, 145)*. The magnitude of HP which we applied in this study seems low compared to the previous studies. In our model, the pressure is directly transmit to the dental pulp fibroblasts, unlike *in vivo* conditions, which dental pulp fibroblasts is surrounded by a number of tissue elements including connective tissue fibers, ground substance, interstitial fluid and vascular tissue. Although, this is the limitation for 2D monolayer cultures to mimic the physiological situation *in vivo*, the majority of cell culture studies have been performed in 2D model because of the ease and convenience. These conventional 2D culture systems have notably improved the understanding of basic cell biology before further investigate using the 3D culture conditions.

A number of studies have demonstrated that HP influenced the expression of many pro-inflammatory cytokines including IL-1β, TNF-α and IL-6 by various cell types *(13, 16, 55)*. These cytokines are widely accepted to be important in the pathogenesis of pulpitis. High levels of IL-1β and TNF-α have been detected in the inflamed dental pulps and periapical lesions *(146-147)*. TNF-α and IL-1 act on vascular endothelial cells at the site of infection to induce the expression of adhesion molecules that promote extravasation of phagocytes during inflammation *(148)*. We also found that HP induced IL-1β and TNF-α mRNA expression by HDPCs. However, the level of IL-1β and TNF-α protein release was hardly detected in culture supernatants using a highly sensitive ELISA kit (data not shown). This finding is in agreement with Coil *et al.* (2004) reported that HDPCs are not capable of releasing high amounts of IL-1β and TNF-α in vitro in response to *Escherichia coli* LPS, whereas, LPS significantly stimulated IL-6 release, and this production was augmented when methyl mercaptan was present *(149)*. A similar increase in TNF-α gene expression without TNF-α secretion was observed in human dental pulp fibroblasts stimulated with lipoteichoic acid *(150)*. The inability to secrete IL-1β and TNF-α in vitro can be supported by the fact that fibroblasts lack the convertase needed for the IL-1β precursor polypeptide to be cleared and secreted extracellularly *(151)*. Conversely, another finding has observed IL-1β release in human pulp cells stimulated with *Porphyromonas endodontalis* LPS *(152)*.

IL-6 is a prototypical pleiotropic cytokine produced by a variety of immune and non-immune cells, which regulates immune response, induces inflammation, and supports hematopoiesis and malignancy. In inflammatory organs, IL-6 induces local infiltration of immunocompetent cells through adhesion molecule and matrix metalloprotease up-regulation, and angiogenesis by augmenting vascular endothelial growth factor production and by stimulating endothelial progenitor cell proliferation and migration *(153-155)*. It also increases vascular permeability and leads to inflammatory edema. IL-6 might also orient T cell response as it is essential for the induction of Th-17 cell differentiation when associated with the TGF-β1 *(18-19)*. Th-17 cells, initially characterized by IL-17 production, have a crucial role in the clearance of extracellular pathogens during host defence reactions, in the induction of tissue inflammation and in osteoclastogenesis *(69, 156, 157)* Their presence, as well as the production of IL-17, has been reported in periapical lesions *(67-68)*.

Regarding the relationship between HP and IL-6, our data corresponds with previous studies reporting HP induced IL-6 expression in periodontal ligament cells *(17),* skin fibroblasts *(55),* microglial *(61)*, chondrocyte-like cell line *(16)* and osteoblasts *(158),* thus it is convincing that IL-6 is mechano-sensitive cytokine. The induction of IL-6 by mechanical stimulation is implicated in osteoporosis, stress-induced pathological bone resorption, and chronic inflammatory diseases such as rheumatoid arthritis *(20, 159)*. IL-6 acts in the biomechanical control of bone remodeling in osteoarthritis by regulating the OPG/RANKL ratio *(158)*. Moreover, IL-6 elevated MMP-1, 3, 14, 11 expression, increased the plasminogen activator activity and enhanced dental pulp fibroblasts cells mediated collagen I degradation *(97-98)*. In the inflamed pulp, IL-6 might be involved in the formation of edema induced by the progressive penetration of Gram-positive oral bacteria into dentin. Therefore, it is supporting that the up-regulated expression of IL-6 in response to increased HP could involve in activation and modulation of the immune response and may contribute to pulp tissue destruction or bone resorption in case of pulpo-periapical inflammation.

We showed in the present study that HP increased IL-6 protein about three-fold over control, which had been markedly suppressed by pre-treatment with suramin.

Moreover, the experiments using conditioned medium from the culture after applied HP, showed similar results. Suramin is an agent that uncouples G-proteins from receptors presumably by blocking their interaction with intracellular receptor domains, when given at high micromole concentration, it affects all nucleotide-sensitive P2Y-receptors with the exception of the P2Y4 receptor *(10)*. These findings suggest that there are some nucleotide(s) released from HP treated cells and then in turn activate the P2Y-receptor on the cell surface of HDPCs and subsequently produce of IL-6.

To date, little is known about the role of P2 receptors in dental pulp cells. There are only few studies investigating the existence and the role of P2X receptors in dental pulp tissue. For example, Alavi *et al.* show P2X3 receptors are present on both myelinated and unmyelinated nerve fibers in human dental pulp tissue and may play a role in dental pain mechanisms *(160)*. Recent study demonstrate P2X3 and P2X2/3 receptors may be involved in the activation of tooth pulpal nerve fibers following tooth pulp injury, resulting in central sensitization of trigeminal spinal subnucleus caudalis (Vc), trigeminal spinal subnucleus interpolaris (Vi), upper cervical spinal cord (C1/C2) and paratrigeminal nucleus (Pa5) neurons through the intracellular MAP kinase cascade, which underscore the importance of purinergic receptor mechanisms in tooth pulp nociceptive processes *(161)*. Our result of RT–PCR analysis revealed that HDPCs express mRNAs coding all P2Y receptors as well as P2X1, P2X3, P2X7 (data not shown). However, the expression of P2Y-1, 12, 13 mRNA were abundant compared to P2Y-2, 4, 6, 14. One explanation for the discrepancy of P2Y subtypes expression is the different level of its specific agonist, which the first group response mainly for purine nucleotides (ATP/ADP), the latter for pyrimidine nucleotides (UTP/UDP). There are evidences supported that the physiologic extracellular UTP: ATP level ratio were about 1:3-5 *(117, 162)*.

A large number of studies have been performed to investigate the biological effects of P2Y receptors particularly P2Y1 receptor. P2Y1 receptor is selectively activated by adenine-based nucleotides (ATP and ADP) and important for platelet shape change, aggregation, thromboxane A2 generation, procoagulant activity, adhesion to immobilized fibrinogen and thrombus formation under shear conditions *(163-164)*. The distribution of P2Y1 receptors on vascular endothelium and smooth muscle cells implies

a role in the regulation of vascular tone. In most blood vessels, P2Y1 receptors are present on the endothelium and mediate vasodilatation by Ca^{2+}-dependent activation of endothelial NOS and generation of endothelium-derived relaxing factor (EDRF) and by generation of endothelium-derived hyperpolarizing factor (EDHF) *(165)*. The fact that ATP and ADP are released locally from endothelial cells during shear stress and hypoxia and from platelets during aggregation, identifies a possible role for endothelial P2Y1 receptors in modulation of vascular tone under normal conditions and during thrombosis *(166-167)*. Additionally, P2Y1 receptor is also associated with the mechanotransduction in many types of cells. For example, stretch induced ATP release and elicited Ca^{2+}-wave propagation through P2Y1 activation by subepithelial fibroblasts form the gastrointestinal tract *(168)*. Another study from our laboratory demonstrated that mechanical stress induces release of ATP, which in turn activates through the P2Y1 receptor, resulting in the up-regulation of osteopontin by human periodontal ligament cells and suggested that stress-induced ATP could play a significant role in alveolar bone resorption *(135)*.

Our finding showed that HP increased the amount of ATP in the conditioned medium, therefore we first hypothesized that ATP could mediated HP-induced IL-6 expression via P2Y1 activation. To help discriminating among P2Y-receptors subtypes affects, we used specific antagonists to P2Y receptors and siRNA method and found that pre-treatment with MRS2179, NF449 could not abolish HP induced IL-6 expression in HDPCs. In contrast, MRS2578 and P2Y6R siRNA significantly diminished HP induced IL-6 expression. These results suggest that the P2Y6 receptor predominantly regulates the HP-induced IL-6 expression in HDPCs.

In this study, we demonstrated that HDPCs express both P2Y6 mRNA and protein. Despite no direct evidence demonstrating the role of P2Y6 receptor in HDPCs, plausible roles for the P2Y6 receptor in immunoinflammation have been described in microglial phagocytosis and inflammatory bowel diseases *(121, 125, 169)*. Inflammation up-regulates P2Y6 receptor expression in the mucosa of the colon of colitic mice *(122)*. P2Y6 receptor also mediates a novel release of proinflammatory cytokines and chemokines in monocytic cells stimulated with UDP *(107)*. It has been shown that extracellular nucleotides, via P2Y2 and P2Y6 receptors, regulate neutrophil migration by

controlling Toll-like receptor (TLR) 2-induced IL-8 release from human monocytes *(170)*. UDP and UTP induce vasoconstriction through P2Y6 receptor stimulation on vascular smooth muscle cells, but also dilatation on endothelial cells, thereby regulating vascular tone and blood pressure. Also, it was reported that extracellular nucleotides stimulated contractions of human cerebral arteries primarily by activation of the P2Y6 receptor *(171)*. Taken together, we hypothesized the role of P2Y6 receptor in dental pulp is involved in inflammation, immune responses and regulation of intrapulpal pressure. However, the immune effect of UDP/P2Y6 signaling on the production of proinflammatory cytokines may selective and dependent on cell types.

Mechanical stress appeared to be the major stimulus for nucleotide release. It has been reported that mechanical stretch increased extracellular UDP concentration three-fold over control and activate P2Y6 receptor in rat cardiomyocytes, which has a role in cardiac fibrosis resulting from pressure overload *(53)*. Many line of evidences showed the release of UTP and UDP from resting or mechanically stimulated cells in concentrations capable of stimulating their respective receptors *(53, 116, 172, 173)*. For example, mechanical stimulation of 1321N1 human astrocytoma cells resulted in a 10- to 20-fold increase in extracellular UTP concentrations *(116)*. In addition, stressed and dying cells, activated leukocytes, and the acidic and hypoxic microenvironment provided by inflammation favor the release of nucleotides such as UTP and UDP *(122, 174, 175, 176)*. Although the extracellular levels of UDP in HDPCs after pressure applied have not been measured in this study due to the complexity of measurement method and lack of a sensitive assay for the quantification of UDP mass *(117),* but recent studies suggest that measurements of nucleotide levels in bulk medium samples may be underestimate the local accumulation of nucleotides in the vicinity of P2 cell surface receptors compared to that of anchor to the outer cell surface *(117, 122, 162)*. Unfortunately, there is no such system to quantify the level of UTP or UDP at the cell surface level.

Several mechanisms are proposed to be involved in the release of nucleotides, given the diversity of conditions and cell types in which extracellular nucleotides have been detected *(177)*. ATP binding cassette transporters *(9)*, multidrug resistance protein transporters *(178)*, connexin/pannexin hemichannels *(179-181)*, cystic fibrosis

transmembrane conductance regulator *(182)*, and stretch-activated channels *(183)* all have been postulated as pathways, regulators, or sensors of nucleotide release, whereas other studies have suggested that nucleotides are released during cargo-vesicle trafficking *(184)* and/or via exocytotic granule secretion *(185)*. Of all these mechansims, connexin/pannexin hemichannels is the good candidate because of its property of gap junction proteins that connecting the intracellular and extracellular space, allowing the passage of ions and small molecules between these compartments such as ATP, arachidonic acid derivatives and sulforhodamine B *(186)*. There are studies supported that mechanical stimuli initiate ATP and UDP release through pannexin-1 hemichannels from cardiac myocytes *(53)* and retina *(11)*.

The investigations on the expression and localization of connexin 43 (CX43) in rat incisor odontoblasts and pulp cells have demonstrated that CX43 is highly expressed in young odontoblasts *(187-190)*. Furthermore, CX43 has been shown to be a marker of viability in dental pulp tissue *(191)*. Chung *et al.* (2007) showed that CX43 involved in cell growth, mineralization, and differentiation to odontoblasts in rat pulp cells *(192)*. CX43 appeared to be upregulated in odontoblasts facing carious lesions therefore CX43 may participate in the processes of dentin formation and pathology *(193)*. Additionally, the presence of other connexins, such as CX32 and CX26, has been reported *(189, 194)*. The expression of CX32 was found in the entire region of the dental pulp, however, there was strong expression only beneath the cell-rich zone. Therefore, it may be hypothesized that CX32 associated with odontoblast differentiation from the cells in the cell-rich zone *(194)*. The CX26 has been known to express on the perineurium *(195)*, and the region expressing CX26 in pulp tissue section coincided with that of CD56, which has been known to express in mature human normal peripheral nerves *(196)*. Therefore, Cx26 expression in the subodontoblastic regions of tissue sections was thought to appear on the perineurium of the nerve plexus of Raschkow *(194)*. However, the functional significance of connexins in dental pulp cells remains to be elucidated. Although there is no study investigating the presence of pannexins in human dental pulp cells, the expression of pannexin in taste bud cells suggests that action potentials in taste cells responsive to sweet, bitter, or umami tastants enhance ATP release through pannexin 1.

Because the downstream signaling pathway activated by P2Y6 receptor is involved in calcium mobilization by releasing of Ca^{2+} from intracellular stores *(10)*, therefore we investigated the role of calcium in HP-induced IL-6 by using BAPTA-AM, which is the membrane permeable form of the Ca^{2+}-chelating agent and useful for establishing an intracellular zero free calcium level. Loading cells with BAPTA-AM is an analytical tool which has been used to suppress a rise in cytoplasmic calcium activity under various stimuli. Our results of Ca^{2+} involvement as show by pretreatment HDPCs with BAPTA-AM significantly decrease of HP-induced IL-6 expression, was supported by a study in microglial cells which HP-induced IL-6 was mediated through G_q activation followed by IP_3-induced Ca^{2+} release from the endoplasmic reticulum *(61)*. In addition, Korcok *et al.* show that P2Y6 receptors on osteoclasts induce transient increase of $[Ca^{2+}]i$ and translocation of NF-**κ**B through a proteasome-dependent mechanism *(131)*. It was showed BAPTA-AM reduced UTP-induced ERK phosphorylation and IL-6 mRNA expression in HaCaT keratinocytes cells *(197)*. The ability of BAPTA-AM to partially suppress HP-induced IL-6 suggests alternative pathways other than intracellular Ca^{2+} concentration are involved in HP signal transduction. The mechanisms responsible for HP or mechanical stimuli induced IL-6 expression were intensively investigated. For example, in skin fibroblasts, IL-6 production induced by HP was dependent on PKC *(55)*. Cyclic tensile stress induces IL-6 expression in smooth muscle cells via Ras/Rac1-p38 MAPK-NF-**κ**B signaling pathways *(198)*. Shear stress induced IL-6 synthesis in chondrocytes via cAMP/PKA and PI3-K/Akt-dependent NF-**κ**B activation *(199)*. Fluid shear stress regulates NF-**κ**B activity through the P2Y6 and P2X7 receptor in osteoblasts *(133)*. Further studies are required to elucidate the precise mechanism of HP/P2Y6 mediate IL-6 expression signaling pathway in HDPCs.

A number of studies have revealed the role of PGE_2 plays in early response to mechanical stimuli, but in HP stimulation still not much been investigated. Prostaglandins are eicosanoids synthesized by cyclooxygenase (COX). Constitutive COX-1 and inducible COX-2 metabolize arachidonic acid to PGG_2 and PGH_2. PGH_2 is subsequently metabolized to PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ or thromboxane A2 *(200)*. A recent study present that PGE_2 production in the renal medullary interstitial cells was increased when

cells were subjected to 60 mmHg pressure for 6 hours and was prevented by a selective COX-2 inhibitor. Moreover, COX-2 mRNA and protein were induced following 60 mmHg pressure for 4 and 6 hours, respectively, whereas COX-1 mRNA and protein levels were unchanged *(201)*. We found slightly increased level of PGE_2 release and COX-2 mRNA expression following HP stimulation (data not shown). However, pretreatment the cells with COX-2 inhibitor (NS398) could inhibit HP-induced PGE_2 release but not IL-6 production, suggest that HP-induced IL-6 expression is not required COX-pathway.

Beside prostaglandin, another possible pathway we investigated is integrins, which are a family of heterodimeric transmembrane glycoproteins that bind many components of extracellular matrix. Integrin consists of an α and a β subunit. At least 18 different α subunits and 8 different β subunits have been identified with more than 24 different members of integrins. They mediate cell attachment to extracellular matrix and cell-cell adhesive interactions. Cell-extracellular matrix interaction through integrins regulates cell growth, adhesion, differentiation, and cell morphology. Apart from cell-extracellular matrix signaling, integrins have been identified as mechanosensory molecules in various cell types. For example, pressure stimulates fibroblast phagocytosis via increasing β1-integrin threonine789 (T789) phosphorylation. Interventions that target β1-integrin T789 phosphorylation may modulate phagocytic function *(202)*. Min Zhang *et al.* show crosstalk between integrin and G protein pathways involved in mechanotransduction in mandibular condylar chondrocytes under pressure *(203)*. Moreover, mechanical strain stimulates conformational activation of integrin αvβ3 in NIH3T3 cells. Mechanical stretch stimulation of JNK was dependent on new integrin binding to extracellular matrix *(204)*. HDPCs expressed α1, α3, α5, α6, αv and β1 integrin subunits, which β1 integrin is the most expression subunit. The adhesion of HDPCs to laminin and fibronectin was not inhibited by monoclonal antibody to any subunit, except that anti-β1 antibody inhibited pulp cells adhesion on laminin, suggest that integrin β1 plays a major role in regulating HDPCs adhesion to laminin *(141, 205)*. Another study demonstrates the presence of an αvβ3 integrin in the cell membrane of human odontoblasts *in vivo* by *in situ* hybridization and immunohistochemistry *(206)*. αvβ3 integrin gene expression was also detected *in vitro* in human dental pulp stem cells

(207). The αvβ3 integrin could play a role in interodontoblast adhesion and odontoblast binding to the surrounding predentin/dentin/pulp matrix, possibly through osteoadherin *(206)*. However, our result show beta 1 integrin could not attenuate HP-induced IL-6 expression in HDPCs.

In conclusion, our present study represent HP stimulates the mRNA expression and protein release of the cytokine IL-6 by human dental pulp cells. Suramin, a specific P2Y6R antagonist and P2Y6R siRNA could inhibit HP-induced IL-6 expression, whereas UDP, a selective agonist of P2Y6, could up-regulate IL-6 expression. These results suggest that HP mediated IL-6 expression via UDP/P2Y6 receptor of these cells. Our findings may help to explain why during mild chronic pulpal inflammation an increased HP may cause a local immune response.

Future perspectives

1. Further investigate the function of P2Y6 receptor in HDPCs.
2. Further investigate the level of UDP release, the mechanisms for UDP release after HP stimulation and its biological effects in HDPCs.
3. Further investigate the effects of HP on intracellular calcium mobilization in HDPCs.
4. Further investigate the mechanism of downstream signaling by HP/P2Y6 receptor in HDPCs.

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BIOGRAPHY

I, Sirichom Satrawaha was born on November 11, 1977 in Bangkok, Thailand. I received Doctor of Dental Surgery from Mahidol University in 2000. I worked as an instructor at Faculty of Dentistry, Naresuan University from 2000-2002. I received Certificate specialty training in Orthodontics from Chulalongkorn University in 2005. In June 2005, I entered the program of Doctor of Philosophy in Oral Biology and I received granted by the 90th anniversary of Chulalongkorn university fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University Centenary Academic Development Project, and Mineralized Tissue Research Unit, Faculty of Dentistry, Chulalongkorn University.