P2Y2 Receptor Biased Signaling in Control of Vascular Inflammation

by

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Abstract

Vascular inflammation plays a critical role in the pathogenesis of endotoxic shock and sepsis. Bacterial endotoxins like lipopolysaccharide (LPS) are biologically active substances that initiate the inflammatory response in vascular endothelial cells (ECs) and blood cells including monocytes/macrophages. Progression of sepsis involves the amplification of the inflammatory response due to the overwhelming production of many inflammatory mediators including GM-CSF, TNF-α, and extracellular nucleotides. One of the potential new therapeutic strategies for sepsis is to limit the degree of endothelial inflammation. Although the pro-inflammatory effect of the nucleotide P2Y2 receptor (P2Y2R) has been intensively investigated, accumulating evidence indicates that P2Y2R may also play a protective role in inflammation. For example, the P2Y receptor ligand 2-MeS-ATP was reported to protect mice from endotoxic death, the exact receptor mechanism(s) remains unclear. In addition, our previous founding in HCAEC show that P2Y2R mediated different anti-inflammatory signaling to control TF transcription, which inspired us to find ligands to select target anti-inflammatory pathway. Further, we presented interesting data indicating that activation of the P2Y2R induced a hypothetical new effector protein termed p25 in HCAEC. Based on these original findings, therefore we hypothesized that manipulating P2Y2R biased signaling (tissue bias and pathway bias) is a new strategy in control of pro-inflammatory and pro-thrombotic gene expression through transcriptional and translational mechanisms. In this dissertation, we assessed signaling pathway bias for P2Y2R function in suppression of inflammatory gene transcription in cultured primary human cells and cells isolated from P2Y2R-null mice; determined tissue/cell-specific bias for P2Y2R signaling in control of inflammatory gene expression; and characterized a putative new post-P2Y2R signaling molecule p25. The dissertation revealed P2Y2R biased signaling, a new mechanism that has the potential to produce new therapeutic drugs in combating inflammatory and thrombotic diseases such as sepsis. In addition, identification strategies of a new signaling or effector protein p25 may open a new

window in the field of membrane receptor biology. Further understanding the role of p25 may also establish a new therapeutic target for relevant diseases.

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List of Abbreviations

CFTR	Cystic fibrosis transmembrane regulator
cAMP	Cyclic adenosine monophosphate
CREB	cAMP-responsive element binding protein
DMEM	Dulbecco's essential modified medium
DIC	Disseminated intravascular coagulation
DREADDs	Designer receptors exclusively activated by a designer drug
EC	Endothelial cells
ERK	Extracellular-regulated kinase
EGFR	Epidermal growth factor receptor
E-NTPase	Ecto-nucleoside triphosphate diphosphohydrolase
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCAECs	Human coronary artery endothelial cells
IL-1β	Interleukin-1β
MAPKs	Mitogen activated protein kinase
mDCs	Myeloid dendritic cells
MODS	Multiple organ dysfunction syndrome
NO	Nitric oxide
NTP	Nucleoside triphosphate
NTPDase	Nucleoside triphosphate diphosphydrolase
PKB	Protein kinase B
PKC	Protein kinase C
P2Y2R	P2Y2 nucleotide receptor
RAS	Renin-angiotensin aldosterone-system
ROS	Reactive oxygen species

TF	Tissue factor
TNF-α	Tumor necrosis factor-α
VEC	Vascular endothelial cell
VEGF	Vascular adhesion molecule-1
VNUT	Vesicular nucleotide transporter

Chapter 1. Introduction

1.1 Sepsis

Sepsis is one type of the Systemic Inflammatory Response Syndrome (Schoenberg, Weiss et al., 1988) which can be triggered by infection and other causes like trauma, serious burn, surgery, etc. Patients with sepsis often present multiple organ dysfunction syndrome, which includes acute circulatory failure, shock, and respiratory failure (Aird 2003, Hotchkiss and Karl 2003, Treacher and Brown (2009)).

In the United States alone, there are at least 500,000 episodes of sepsis annually even with intensive medical care and the resultant mortality rate ranges from 30 to 50 percent (Hotchkiss and Karl 2003). Other studies suggest that 28-day mortality rates of severe sepsis may be 50% or greater (Schoenberg, Weiss et al. 1998). According to a national inpatient hospital cost report, septicemia ranked among the four most costly conditions in the hospital costs with the annual cost of 23.7 billion (Torio and Moore 2006).

The pathophysiology of sepsis is under constant research. Most cases of sepsis are initiated by pathogen invasion such as Gram-positive or Gram-negative bacteria infection. Even though the majority of bacterial infections can be controlled by antibiotics, the major complication is the uncontrolled immune response involving multiple cell types, inflammatory mediators, and coagulation factors (Aird 2003). Endothelial cells, monocytes, tissue macrophages, myeloid-derived neutrophils, and macrophages mediate the host response by initiation of inflammation and coagulation cascade. The clinical laboratory markers of inflammation include high circulating levels of interleukin 6 (Douillet, Robinson et al.), IL-8, and tumor necrosis factor (TNF-α) (Pinsky, Vincent et al. 1993, Thijs and Hack 1995, Damas, Canivet et al. 1997).

1.1.1 Vascular inflammation

The endothelium is one of the largest organs, and plays a pivotal role in maintaining the homeostasis of the vascular system. During the disease progress of sepsis, the innate host response is activated to eradicate pathogens and necrotic tissue; endothelium plays a vital role in the process. One of the important hallmarks in sepsis is endothelium dysfunction. The function of vascular ECs to maintain fluidity of blood is disrupted by the activities of pro-inflammatory cytokines which promote upregulation of pro-inflammatory and pro-coagulant mediators (Wilcox, Smith et al.,2012) on the endothelial cell surface.

Leucocytes produce reactive oxygen species (ROS), lipid mediators, peptides, cytokines, and proteinases to fight infection, however, uncontrolled the immune response turned against the host and promotes inflammation (Libby 2015). Meanwhile, dysfunctional endothelium attracts platelets, monocytes, and neutrophils that are capable of initiating or amplifying inflammation and coagulation. The infiltrating neutrophils entry into the dysfunctional endothelium and insult multiple organ failures. Sepsis also causes pro-thrombotic environment which generates thrombin that converts fibrinogen into fibrin, leads to acute disseminated intravascular coagulation (DIC) (Colucci, Balconi et al.) with hemorrhagic and thrombotic complications.

Besides vascular inflammation, patients with sepsis were often accompanied with the progressive subcutaneous edema which suggests increased vascular permeability and loss of endothelial integrity. Cytokine and inflammatory mediators induce gaps between endothelial cells by altering the cellular cytoskeleton, resulting in microvascular leak and tissue edema. An in vitro model of sepsis study show that endothelium permeability is increased caused by endotoxin activity and cytokines. Nitric oxide (NO) is overproduced due to L-arginine catalyzed by enzyme nitric oxide synthase (eNOS, nNOS, iNOS). The endothelial permeability in sepsis is increased. The gram

negative bacteria cell wall component lipopolysaccharide (Colucci, Balconi et al., 1983) or other pro-inflammatory stimuli such as cytokines and chemokines lead to the induction of iNOS and initiate an activation cascade for nitric oxide production in endothelial cells (Sprague and Khalil, 2009) and thus impaired vascular homeostasis (Ait-Oufella, Maury et al. 2012).

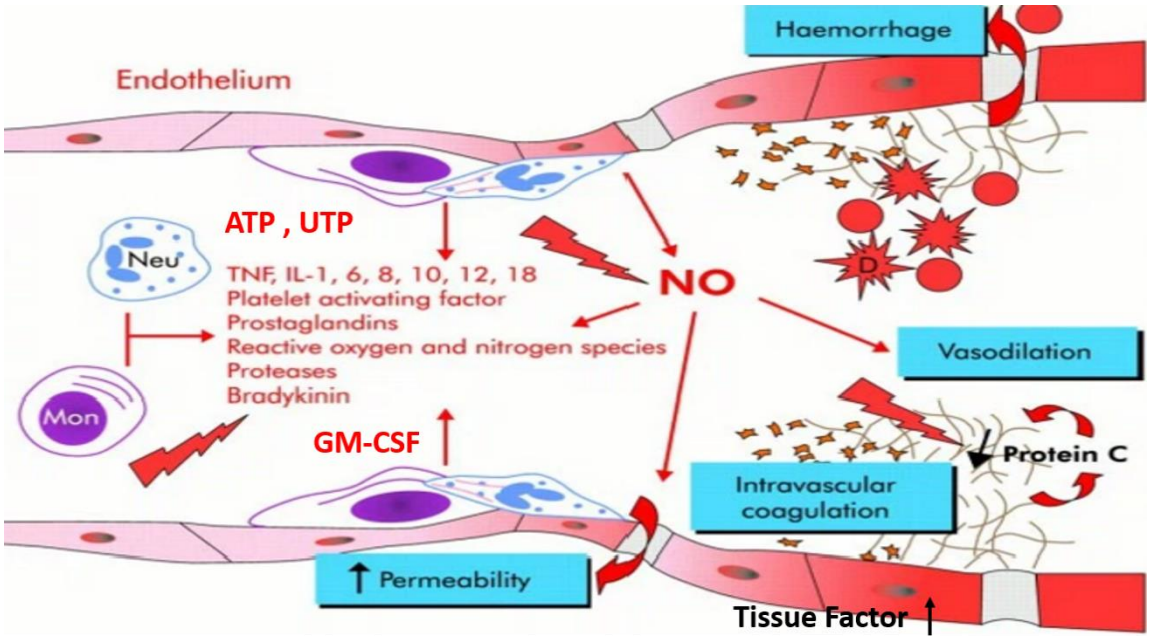


Figure1.1. Scheme of pathophysiology of endothelium dysfunction in sepsis.

Adapted from Chris Snowden et al. The pathophysiology of sepsis. CEACCP 2002 2:11-14.

1.1.2 Adhesion molecule

The endothelium-leukocyte interaction is crucial to sustain an inflammatory response, the leukocyte margination and rolling along the endothelial cells are regulated by a group of glycoproteins known as selectins (P- and E- selectins) on the surface of endothelial cells (Snowden and Kirkman 2002). E-Selectin is an adhesion molecule not expressed by unstimulated endothelium, but the temporal expression of E-selectin which can be induced by lipopolysaccharide, TNF-α, interleukin (IL-1), histamine, or other cytokines stimulated endothelial cells culture (Drake, Cheng

et al. 1993). Inflammatory mediators signaling in the vasculature also promotes the expression of endothelial VCAM-1, E-selectin and recruits monocyte adhesion to endothelial cells (Eklund and Saharinen 2013). Increased expression of adhesion molecule and endothelial-leukocyte interaction injured both endothelial cells and induced neutrophil produce more cytokines, or oxygen free radicals ($O_2^{\cdot}$ and $OH^{\cdot}$) leading to apoptosis and thus injuring the underlying tissue. In addition, activated endothelial cells also recruit platelets to the vessel well (Sheu, Hung et al. 1999, Tsujikawa, Kiryu et al. 2000). Thrombin signaling can induce the expression of adhesion molecules such as E-selectin, P-selectin or ICAM-1 on endothelium. The cross talk between the inflammatory and coagulation pathways interact with other to amplify the inflammation response.

1.1.3 Tissue factor

Infection aggravated endothelial dysfunction and inflammation also influence hematological balance, due to the impairment of anti-coagulant system (antithrombin III and thrombomodulin) and cytokine induction of extrinsic coagulation system (Snowden and Kirkman 2002). Tissue factor (TF) (Wilcox, Smith et al.) is a membrane-bound and lipid-dependent glycoprotein with a molecular weight (47kd) that facilitate blood coagulation and signaling (Wilcox, Smith et al. 1989). TF is the primary initiator the coagulation cascade, formation of the TF: FVIIa complex thus activating both factor IX and X triggering extrinsic pathways; FVIIIa: FIXa complex of the intrinsic route generates FXa, which facilitate FVa: FXa complex converts prothrombin to thrombin. The generation of thrombin activates various proteases and cofactor, and leads to fibrin deposition and activation of the platelet and eventually leads to the coagulation.TF mRNA has been identified presents in the vascular adventitia, adipose tissue, placenta, renal glomeruli, and cerebral cortex, et al (Maynard, Dreyer et al. 1977, Drake, Morrissey et al. 1989). Several studies show TF are increased in various disease states, such as atherosclerosis and diabetes.

In the vascular system, fibroblast and vascular smooth muscle cells have higher levels of TF expression and coagulant activity than endothelial cells (Maynard, Dreyer et al. 1977). Quiescent endothelial cells produce low levels of TF mRNA, however, endotoxin, cytokines interleukin-1β (IL-1β), tumor necrosis factor-α, thrombin or nucleotide can stimulate endothelial cells generate more TF (Colucci, Balconi et al. 1983). In chronic vascular disease, several investigators reported that TF localizes in normal vessel and atherosclerotic plaque. In septic shock model, the disruption of organ microvasculature induced more TF expression and circulation monocytes also produce more TF (Oeth, Parry et al. 1994). TF also has a role in the tumor associated vascular endothelial cell (VEC) thrombosis and neovascular response, by controlling the balance of angiogenic and antiangiogenic to modulate angiogenesis in breast cancer cells (Zhang, Deng et al. 1994).

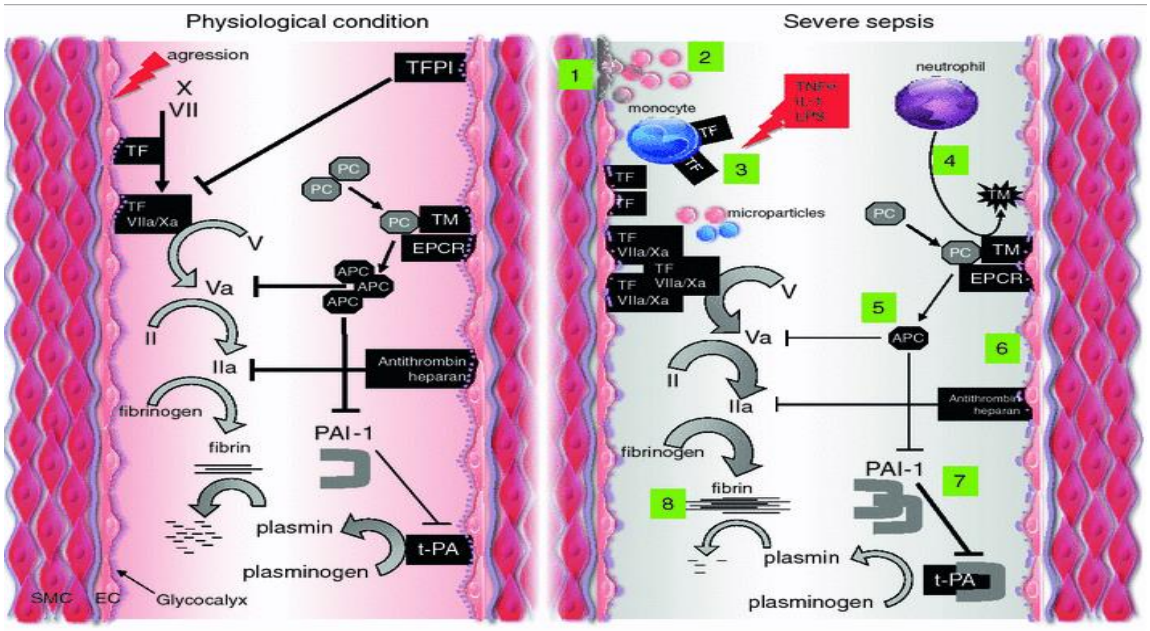


Figure 1.2. Role of tissue factor in severe sepsis.

Adapted from Aird, William C. "The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome." Blood 101.10 (2003): 3765-3777.

1.1.4 GM-CSF

The role of vascular inflammation in the initiation and progression of acute or chronic vascular disease is increasingly recognized. A consensus opinion regarding sepsis is the systemic inflammatory response syndrome induced by 'cytokine storm' that occurs during infection. Cytokines such as IL-6, TNF-α, and cell adhesion molecules such as ICAM-1, P-selectin, and E-selectin are associated with inflammation by activated ECs, VSMCs, monocytes/macrophages (Sprague and Khalil 2009). These vascular cells contribute to the inflammatory response by inducing more cytokine production or eliciting response to those cytokines, which accelerates the progress of sepsis.

Granulocyte-macrophage colony stimulating factor GM-CSF (Flohe, Borgermann et al. ,1999) is a pro-inflammatory cytokine involved in endotoxic shock. It has a short half-life, but promotes the production of secondary cytokines (IFN-γ, IL-1a, and IL-6) and induces the release of neutrophils from the bone marrow and contributes to the pathogenesis of vascular disease (Flohe, Borgermann et al. 1999, LeVine, Reed et al. 1999). Therefore, we chose GM-CSF as a model of inflammatory gene regulation in part of this study.

1.1.5 Current therapy

The administration of anti-endotoxin antibodies in randomized controlled trial did not improve the survival rate of gram-negative sepsis (McCloskey, Straube et al. 1994, Angus, Birmingham et al. 2000). Endogenous activated protein C, which promotes fibrinolysis, inhibits thrombosis and inflammation, has a protective role in sepsis. Drotrecoginalfa (a human recombinant activated protein C) is a component of the natural anticoagulant system and the only licensed product specifically indicated for the treatment of sepsis (Hosac 2002). Activated protein C was investigated in the Phase 3 Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) trail,

but has been withdrawn by the manufacturer because of a failure to confirm the first positive efficacy study.

Etanercept is a soluble TNF-α receptor decoy that binds circulating TNF-α, other chimeric monoclonal anti-TNF-α monoclonal antibodies including Infliximab, Adalimumab all shown to worsen outcomes from septic patients resulted from suppressing the immune function and were associated with increased rates of side effects such as tuberculosis, and intracellular bacterial infection (Schoenberg, Weiss et al. 1998, Boyd 2012, Wu, Lin et al. 2012, Ikumi, Matsukawa et al. 2013). Corticosteroids have potent and widespread anti-inflammatory therefore they are frequently used adjunctive therapy in sepsis, but their use has undesired side effects such as hypertension, insulin insensitivity, and obesity.

Obviously, sepsis studies need a new direction. One of the possible therapeutic strategies is to target endothelial components, surface receptors, and signaling pathways to fine-tune the expression of inflammatory mediators, adhesion molecules, and coagulant factors.

1.2 The Release and breakdown of nucleotides

Virtually all tissues and cell types exhibit regulated release of nucleotide of ATP, which in many cases is accompanied by the release of other nucleotides such as uridine triphosphate (UTP) and related molecules such as uridine diphosphate (UDP), which are sugars that have actions similar to ATP. The release of nucleotides under basal conditions and other mechanical stimulation or other chemical stimuli results in the induction of autocrine and paracrine response in cultured or isolated cells or tissues (Corriden and Insel 2010). ATP and UTP, purine and pyrimidine nucleotides, not only function in the synthesis of DNA and RNA or energy metabolism, but also as extracellular signaling molecules and have roles in regulation of the cell physiology and pathophysiology.

In many cell lines, the mechanisms of ATP release is exocytotic. Under apoptosis, cellular stress or inflammatory stimuli, nucleotide ATP can be released by lytic or non-lytic mechanisms by various cell types, including endothelial cells, platelets, and inflammatory dendritic cells.

Nucleotide and breakdown products are part of feedforward and feedback control mechanisms in controlling coronary blood flow (Gorman, Rooke et al. 2010), regulation of water transport, and body water homeostasis (Pochynyuk, Bugaj et al. 2008). In sepsis, intracellular purine and pyrimidine nucleotides are also released from vascular ECs in response to a variety of stimuli including ischemia/hypoxia, and various pro-inflammatory receptor agonists (Abbracchio, Burnstock et al. 2006). Released nucleotides serve as important extracellular signaling molecules and trigger the alteration of a series of intracellular events by activating purinergic or nucleotide P2 receptors (Erlinge and Burnstock 2008). For example, ATP release via exocytosis mediated by SLC17A9 vesicular nucleotide transporter (VNUT) from human monocyte THP-1 cells after LPS treatment and followed activation of P2Y11 has pro-inflammatory roles in mice peritoneal /spleen macrophage M1-type polarization and IL-6 production (Sakaki, Tsukimoto et al. 2013).

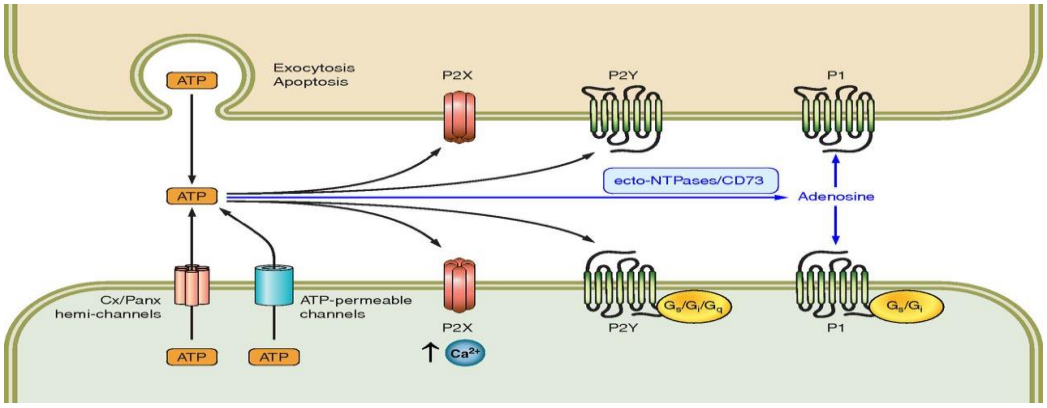


Figure 1.3 Pathways for ATP release.

Adapted from Lazarowski, Eduardo R., and Richard C. Boucher. "Purinergic receptors in airway epithelia." Current opinion in pharmacology 9.3 (2009): 262-267.

Several scenarios possibly account for the basal and stimulated the release of nucleotides from airway epithelial cells. (i) ATP entering the Golgi lumen via specific transporters may be released as a residual cargo product of the constitutive secretory pathway (Makita, Sato et al.). Secretory granules (e.g., mucin granules) containing ATP may be competent for Ca^{2+}-regulated exocytosis. (iii) Not yet identified ATP conductance effluxes cytosolic ATP out of the cells (Lazarowski and Boucher 2009).

Ectonucleotidases are ectoenzymes that hydrolyze extracellular nucleotides to the respective nucleosides. The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase/CD39) family (NTPDase1,2,3, and 8) located on the cell surface (Robson, Sévigny et al. 2006). NTPDase 1 is the major ectonucleotidase in the vasculature and immune cells which has been identified on B cells, subsets of activated NK cells, T lymphocytes and endothelial cells (ECs) (Maliszewski, Delespesse et al. 1994, Robson, Sévigny et al. 2006). Therefore, local UTP or ATP increase is transient because of the diffusion and the activity of ecto-nulceotidase and UTP to their respective monophosphates (Lazarowski, Watt et al. 1995, Lazarowski, Homolya et al. 1997).

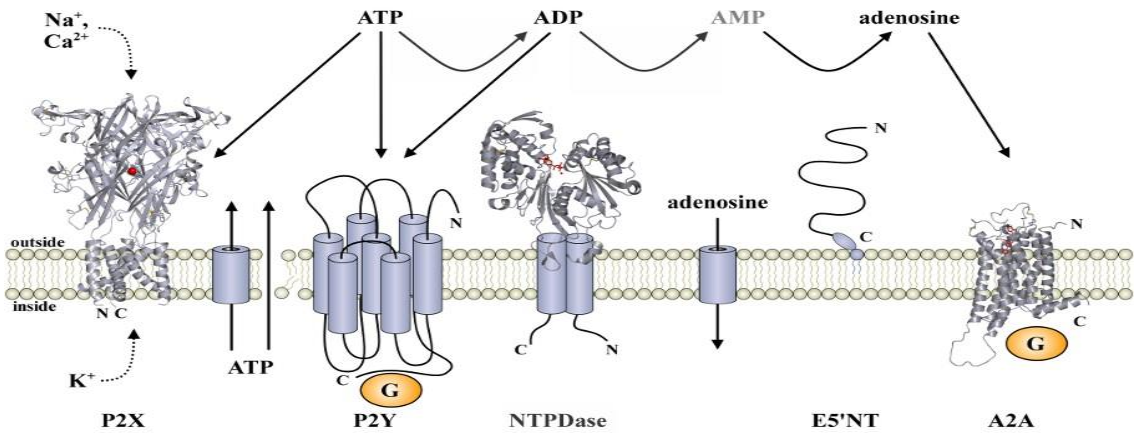


Figure 1.4. The role of ecto-nucleotidase in nucleotide hydrolyzation.

Adapted from Robson, Simon C. et. al. 2006.

1.3 Purinergic Receptor

P2 receptors are widely expressed in multiple cell types or tissue, such as hippocampus, liver, kidney, lung, vascular and epithelial cells and the immune system including T-cells and monocytes (Zhu and Kimelberg 2001, Jacobson, Ivanov et al. 2009). P2 nucleotide receptors are further divided into a P2Y family of G-protein-coupled receptors (P2Y1, 2, 4, 6, 11, 12, 13 and 14) and a ligand-gated ion channel family (Kaebisch, Schipper et al. 2015) .The platelet P2Y12 receptor antagonist Clopidogrel (Plavix) is used to prevent blood clotting, heart attacks, and strokes in patients.

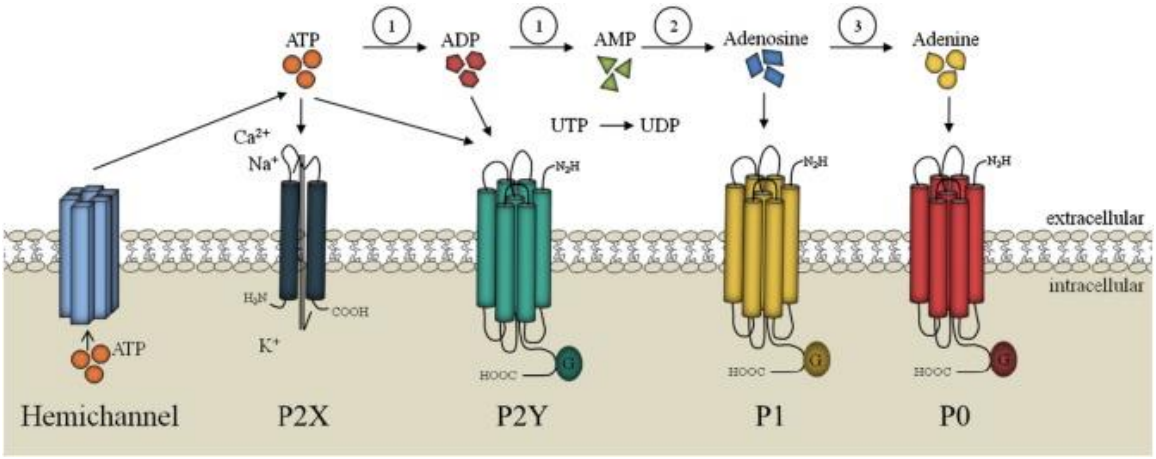


Figure1.5. Classification of Nucleotide Receptor.

Adapted from (Constanze Kaebisch et al,2015)

P2Y receptors are members of the GPCR superfamily coupled to heterotrimeric G proteins, which consist of Gα subunit that is associated with Gβγ subunits. Upon receptor activation, guanine nucleotide exchange factors (GEFs) active GTPases by stimulating the conversion of guanosine diphosphate (GDP) to allow the binding of guanosine triphosphate (GTP), and dissociate G protein

form the activated receptor as well as dissociation of the Gα subunit from the Gβγ complex. According to the sequence similarity of the G protein subunits, Gα subunits are divided into four subfamilies (Gq, Gs, Gi/o, and $G_{12/13}$). Gq-coupled receptors include P2Y1, 2, 4, 6, 11, and the Gi-coupled receptors include P2Y12, 13, 14. The activation of G subunits defines receptor activation or effector couplings, such as the activation or deactivation of the adapter protein or second messenger such as Ca^{2+}, cyclic AMP (cAMP), inositol phosphate, etc. Gαs activates adenylyl cyclase, resulting in the increased cellular cyclic adenosine monophosphate (cAMP) levels, while Gαi inhibits adenylyl cyclase activity, leading to decreased cellular cAMP levels. Gαq activates phospholipase C, increasing cytoplasmic Ca^{2+} levels which is released from intracellular stores. Inhibitor pertussis toxin (PTX) is commonly used to measure the sensitivity of P2Y signaling to the Gi/o protein. Nucleotide activation of P2Y receptors result in activation of the small GTPases G proteins, Rho family (RhoA) and Rac (Sauzeau, le Jeune et al. 2000). The nucleotides participate GTP hydrolysis or generation of second messengers. Nucleotides regulate P2Y signal transduction through the heterotrimeric G proteins families are shown in Table 1.1 (Abbracchio, Burnstock et al. 2006).

Table 1.1. Native agonists for P2X and P2Y receptors

Receptor	Native Agonist	G protein- Main Effector(s)
P2X receptors		
P2X1-P2X7	ATP	ATP-gated cation channel
P2Y receptors		
P2Y1	ADP	Gq/PLCβ → Ca2+/PKC
P2Y2	ATP, UTP	Gq/PLCβ → Ca2+/PKC Go/PLCβ →Rac activation G12 → Rho activation
P2Y4	UTP	Gq/PLCβ → Ca2+/PKC Go/PLCβ
P2Y6	UTP, UDP	Gq/PLCβ → Ca2+/PKC G12/13 - Rho activation
P2Y11	ATP	Gq/PLCβ → Ca2+/PKC Gs→ AC
P2Y12	ADP	Gi/o→↓AC/↓cAMP
P2Y13	ADP	Gi→ ↓AC/↓cAMP
P2Y14	UDP/UDP-glucose	Gi→ ↓AC/↓cAMP
Adenosine receptors		
A1, A_3	Adenosine	Gi→ ↓AC/↓cAMP
A_{2A}, A_{2B}		Gs → AC/↓cAMP

Adapted from (Jacobson, Ivanov et al. 2009)

1.4 P2Y2 receptor

1.4.1 P2Y2R receptor signaling

P2Y2 mRNA has a wide tissue distribution and is found at high levels in the lung, heart, skeletal muscle, spleen, kidney, liver, and epithelia cells (Lustig, Shiau et al. 1993, Parr, Sullivan et al. 1994, von Kügelgen 2006). Depending on the P2Y receptor subtype, P2Y receptor can be activated by ADP, ATP, UDP, UTP, or UDP-sugars. P2Y2R are typically equipotently activated by ATP and UTP (Lazarowski and Boucher 2009). P2Y2R couples to Gαq, induces calcium signaling as determined by response to agonist stimulation in 1321N1 astrocytoma cells transfected P2Y2R. Studies also indicated that the P2Y2R coupled Gαi/o and G12 protein interacts αv integrins via integrin-binding domain, Arg-Gly-Asp (RGD) in the first extracellular loop of the P2Y2 receptor, which is independent of Gαq coupling (Erb, Liu et al. 2001, Bagchi, Liao et al. 2005, Liao, Seye et al. 2007). P2Y2 receptors regulate through cellular functions through the formation of different signaling complexes independently or interaction with other receptors, ion channels, adapter proteins and monomeric G proteins such as Rac and Rho(Soulet, Hechler et al. 2005). The P2Y2R in multiple tissues has functional roles in regulating cardiovascular, hemodynamic, neurological, lung and renal functions.

In sensory neurons, the P2Y2 receptor is expressed widespread and participates in nociceptive signal transduction, both ATP- and UTP- induced action potential evoked Ca^{2+} entry, which contributes to cAMP-responsive element binding protein (CREB) phosphorylation to regulate cytokine transcription (Molliver, Cook et al. 2002). Activation of P2Y2 receptor on the ocular surface, increases the intracellular free calcium levels, net chloride secretion, and plasma membrane ionic permeability. Diquafosol, derivatives of UTP (Santen Pharmaceutical Co, Ltd, Osaka, Japan), a selective P2Y2 agonist that has been used for the treatment of dry eye disease and Sjögren's syndrome and has been approved in Japan (El-Tayeb, 2011). Also, nucleotides and nucleosides regulate two purinergic receptor subtypes, the Gαq-coupled P2Y2R and the A_{2b} adenosine receptor in the airways which mediate innate defense mechanisms that remove particles and pathogens from the airway. Activation of the P2Y2R promotes inhibition of Na^+ absorption as well as cystic fibrosis

transmembrane regulator (CFTR)-dependent and CFTR-independent Cl^- secretion to regulate mucociliary clearance (MCC). Denufosol (INS7217) is currently in phase III clinical trial to assess the safety and tolerability in mild to moderate cystic fibrosis, which is caused by defective in gene results in abnormal ion and liquid volume in the airway surface (Deterding, Retsch-Bogart et al. 2005). P2Y2R receptors have been identified to possess significant roles in blood pressure regulation in the renal system deficiency of P2Y2 receptor impairs renal reabsorption of Na^+ and fluid excretion, resulting in the suppression of the renin-angiotensin aldosterone-system (RAS) and increase in blood pressure (BP) (Rieg, Bundey et al. 2007). Vascular endothelial cells express P2Y2 receptors, upon sheer stress and hypoxia, endothelial cells release ATP and UTP, which binds to P2Y2R and can elicit nitric oxide and subsequent vasodilation (Burnstock 2006). However, it has been reported that ATP induce vasoconstriction on P2 receptors on smooth muscle cells of the isolated rabbit artery was reported (Kennedy and Burnstock 1985). Gorman et al, (Gorman, Rooke et al. 2010) recently reported that the selective but weak P2Y2 receptor agonist MRS2768 produces coronary vasodilation in a dose-dependent manner from *in vivo* study in the canine heart. Wihlborg et al, found UTP levels increase in myocardial infarction patients and UTP is likely to be an inotropic factor by activation of P2Y2 receptor that involved in the development of cardiac disease (Wihlborg, Balogh et al. 2006).

1.4.2 Synthesized nucleotide analogs and structure

A series of derivatives and analogs of nucleotides with modifications in various position of the ribose and/or the phosphate chain, the uracil residue was synthesized as previously reported (El-Tayeb, 2011). For example, the triphosphate residue was modified to obtain stability versus ecto-nucleotidase such as UTPγS (Sauer, El-Tayeb et al. 2009). Ali El-Tayeb et al, synthesized the UTP derivatives (modified at 2-positions of the uracil moiety, 2-thio group enhances potency and/or selectivity of UTP analogues. P2Y2 receptor agonist 2-thio-UTP presents (EC_{50}) 50 nM, 30-fold selective vs P2Y4 and P2Y6 (El-Tayeb, Qi et al. 2006). Ko, Hyojin et al, investigated the effects

of substitution of aryl phosphate ester at the terminal position resulted in high selectivity for the P2Y2 receptor (MRS2768) (Ko, Carter et al. 2008). Later, PSB1114, 4-Thio Uridine with the Pβ, Pγ-oxygen bridge was replaced by a difluoromethylene moiety was synthesized as the most potent P2Y2 agonist with an EC_{50} value of 0.134 μM and >60-fold selectivity versus P2Y4 and P2Y6 receptors. Because of its high potency, selectivity, and expected metabolic stability toward ectonucleotidases, in particular NTPDases, PSB1114 could be an important pharmacology tool to characterize P2Y2R (El-Tayeb, Qi et al. 2011). Sugar modified nucleoside triphosphates with bulky 2'-substituent (2'-O-Me), can be incorporated as non-canonical NMPs into RNA for a variety of applications (Padilla and Sousa 2002). We will also test this set of 2'-O-Me modified NTPs signaling behavior on P2Y2R.

Table 1. 2. Synthesis agonists for P2Y receptors

Receptor Subtype	Synthetic agonist	Chemical name
P2Y	2-MeS ATP	2-Methylthioadenosine-5'-triphosphate tetrasodium salt
P2Y2 P2Y4	UTPγS	Uridine-5'-(γ-thio)-triphosphate trisodium salt
P2Y2 P2Y4 P2Y6	2-ThioUTP	2-Thiouridine 5'-triphosphate tetrasodium salt
P2Y2	MRS2768	Uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt
P2Y2	PSB1114	4-Thiouridine-5'-O- (β,γ- difluoromethylene)triphosphate tetrasodium salt
To be determined	2'-O-Me ATP	2'-(O-Methyl)-Adenosine-5'-Triphosphate lithium salt
To be determined	2'-O-Me UTP	2'-(O-Methyl)-Uridine-5'-triphosphate lithium salt
To be determined	AA-UTP	5-[3-Aminoallyl]-Uridine-5'-Triphosphate
To be determined	Alpha-thio-UTP	Uridine-5'-(1-Thiotriphosphate)

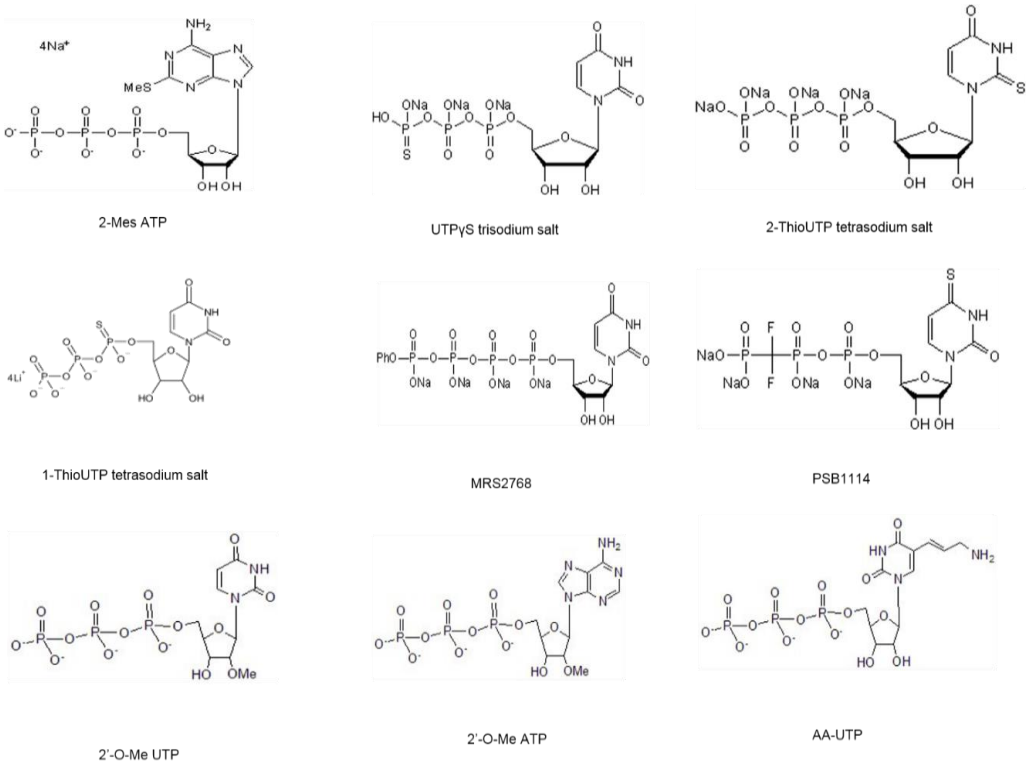


Figure 1.6. Structure of synthesized nucleotide analogs.

1.4.3 P2Y2R in signaling, inflammation and thrombosis

Extracellular ATP serves as a danger or 'find me signaling' in the immune system (Elliott, Chekeni et al. 2009). In allergen-driven lung inflammation, nucleotide receptor P2Y2R shows higher expression in patients than healthy individuals (Idzko, Hammad et al. 2007). Nucleotide ATP released into airway as an important mediator by controlling P2Y2R purinergic signaling, induces migration of myeloid dendritic cells (mDCs) and eosinophils, and reactive oxygen species (ROS) production (Müller, Robaye et al. 2010). In a mouse model of sepsis, A3 adenosine receptors and P2Y2R are involved in the recruitment of neutrophil and sequestration to the sites of infection such as lungs (Inoue, Chen et al. 2008). Extracellular nucleotides were investigated as important mediators of a variety of processes including vascular inflammation and thrombosis. Activation of

P2Y2R can involve signaling downstream of the mitogen- activated protein kinases (MAPK), the protein kinase B (Akt/PkB) family of serine/threonine protein kinases and a number of protein kinase C (PKC) isoforms. In endothelial cells, P2Y2R utilize Src kinase to transactivate the vascular endothelial growth factor receptor-2 (VEGRR-2), leading to the up-regulated expression of vascular cell adhesion molecule (VCAM-1) which enables the adhesion of leukocytes to the vascular wall (Seye, Yu et al. 2004). For the in vivo model, $ApoE^{-/-}/P2Y2R^{-/-}$ mice model exhibit low VCAM-1 expression in endothelial cells and delayed the onset and pivotal to the early stage of atherosclerosis (Qian, Hoggatt et al. 2016).

P2Y12 receptor antagonist Plavix also can lead to easy bleeding, stomach upset, and CYP2C19 poor metabolizers associated with diminished antiplatelet response to Plavix (Ma, Lam et al. 2011, Wang and Han 2013). The other non-platelet nucleotide receptor, particularly P2Y2 receptor (P2Y2R), is widely expressed in immune cells, epithelial and ECs, kidney tubules, and osteoblasts, etc. (Guzman-Aranguez and Pintor 2012). It turns out that P2Y2R is up-regulated in vascular injury models in a variety of tissues in response to inflammation or injury, including balloon-injured rat aorta, collared rabbit carotid arteries, and stented coronary arteries (Seye, Gadeau et al. 1997, Seye, Kong et al. 2002, Shen, Seye et al. 2004). In addition, our previous study has demonstrated that the P2Y2R is the dominant P2Y subtype receptor expressed in human coronary artery endothelial cells (HCAEC) (Ding, Ma et al. 2011). Our lab has also shown that activation of the P2Y2R induces tissue factor (TF) expression in HCAEC through two independent pro-inflammatory signaling pathways, Rho/JNK and Src/p38 (Ding, Ma et al. 2011). Activation of p38 MAPK has been reported as implicated in myocyte death during myocardial ischemia (Ma, Kumar et al. 1999) and the development of cardiac fibrosis and dysfunction in post-infarct remodeling and heart failure (Petrich and Wang 2004, Ren, Zhang et al. 2005).

Our recent follow-up study brings up the new knowledge that P2Y2R-ERK1/2-Fra-1 pathway functions as a negative pathway, fine-tuning TF expression, which suggests that P2Y2R may have anti-inflammatory effect by signaling to defined pathway(s) (Liu, Zhang et al. 2016). Indeed, there exists other evidence that P2Y2R may have a protective role in inflammation. For example, 2-methylthio-ATP (2-MeS-ATP) was reported to protect mice from endotoxic death (Proctor, Denlinger et al. 1994). P2Y2R knockout mice are more susceptible to lung bacteria (Geary, Akinbi et al. 2005) and virus infection (Vanderstocken, Van de Paar et al. 2012).In addition, UTP reduces infarct size and improves mouse heart function after myocardial infarction via P2Y2R (Cohen, Shainberg et al. 2011), and the UTP analog 2-thio-UTP protects heart from ischemic damage *in vitro* and *in vivo* (Hochhauser, Cohen et al. 2013). 2-thio-UTP also induces regression of aortic valve stenosis in obese diabetic mice (Le Quang, Bouchareb et al. 2013). Furthermore, P2Y2R deficiency aggravates chronic kidney disease progression and early pathology in Alzheimer's disease (Potthoff, Stegbauer et al. 2013, Ajit, Woods et al. 2014). However, the cellular and molecular mechanism(s) underlying P2Y2R-mediated pro-inflammatory versus anti-inflammatory effect remains completely unknown.

1.4.4 Transcription regulation of tissue factor

The regulatory regions involved in TF gene expression was established, and the putative promotor and first exon are located within a 1.2 kbp region (Mackman, Morrissey et al. 1989). In different cell types, TF promoter can be regulated in a constitutive or inducible manner. Later, the transcription factor binding sites in the human TF promoter that regulate expression of the TF gene was reported. Specificity protein 1 (Sp1) regulated constitutive TF transcription while epidermal growth response-1 (Erg-1), activated protein -1 (AP-1) and NF-κB are in charge of induced TF transcription. Induction of TF gene in human monocyte cells and endothelial cells with LPS treatment is regulated by distal enhancer (-227 to -172 bp) containing two AP-1 sites and constitutively bind c-Fos /c-Jun heterodimer (Oeth, Parry et al. 1994, Mackman

1995). Liu et al. (Liu, Zhang et al. 2016) identify a new distal activate protein 1 (AP-1) at -1363 bp has significant impact on TF gene transcription when activated by UTP through P2Y2R in endothelial cells. We also show that AP-1 subunits c-Jun, and ATF-2 are new positive regulator of TF transcription, but Fra-1 as a negative regulator fine-tuning the overall transcription activity of TF gene promoter activity.

1.5 Biased signaling

1.5.1 ligand biased

Biased agonism refers to different ligands stabilizing distinct conformations of a certain receptor that only one or a subset of signaling pathways mediated by the receptor are activated at the relative exclusion of others (Wootten, Christopoulos et al. 2013). Biased ligands for certain receptor exerts different efficacies for different signaling pathways, ligand bias, receptor bias, and cell-types, or tissue types bias (Steen, Larsen et al. 2014). Biased signaling poses unique challenges to the current pharmacologic nomenclature of agonist and antagonist. This biased ligand-receptor activation mechanism is also described as functional selectivity that biased ligand stabilize receptor conformation that are distinct from those induced by balanced agonists thus change the propensity of GPCR coupling to various effectors go on to selectively interact with different coupling proteins to induce collateral activation of cellular pathways (Kenakin 2013). As GPCR constitute the largest family of cell surface receptors with the most druggable target for drug discovery (Lefkowitz 2007), Biased Agonists are increasing being identified for a range of GPCRs, and this phenomenon is likely to be widespread.

It was subsequently discovered that different ligands can mediate GPCR signaling bias between different G protein subunits (Gαs, Gαi/o, Gαq). For example, muscarinic acetylcholine receptor agonist pilocarpine. Unlike Carbachol, mediated balanced response on both Gαs and Gαq, pilocarpine does not lead to Gαs-directed adenylyl cyclase stimulation but lead to phospholipase C

(PLC) activity that is mediated by Gαq (Fisher, Heldman et al. 1993, Gurwitz, Haring et al. 1994). The calcium-sensing receptor (CaSR) and allosterically modulates the calcium-mediated potentiation of Gαq signaling, while inhibiting the Gαi response (Makita, Sato et al. 2007). The metabotropic glutamate receptor 1α, glutamate coupled both to Gs and Gq proteins whereas the Gd3-induced active conformation state preferred Gq to Gs, suggesting that mGluR1α serves not only as a simple on-off switch but also as a multiple signaling path regulator (Tateyama and Kubo 2006).

In addition, biased signaling on GPCRs is not only limited to G proteins vs G proteins signaling but also occur between G proteins and other signaling effector, such as β-arrestin and leads to unique biochemical and physiological effects. β-arrestin is expressed throughout the body and has roles in receptor desensitization internalization (Ferguson, Downey et al. 1996), translocation (Kovacs, Whalen et al. 2008), exocytosis (Imamura, Huang et al. 2001) trafficking by binding to the G-protein coupled receptor kinases (GRKs) phosphorylated receptor (Perry and Lefkowitz 2002). β-arrestin can also act as multifunctional adaptor proteins or clarthrin protein that scaffold a diverse group of signaling protein at the seven transmembrane recetpors and modulate the downstream activity of a number of signaling network, regulating MAPKs, AKT, or other cellular processes such as the interaction with epidermal growth factor receptor (EGFR) (Goodman, Krupnick et al. 1996, Bhola and Grandis 2008). Several studies found some receptors upon activation of certain ligands can mediate signaling independent of G-protein but through β-arrestin dependent signaling. Therefore, some antagonist originally described as antagonists but found later to recruit β-arrestin independent of G protein activation currently be defined as biased agonist/antagonists.

Biased agonists/ antagonist for GPCR receptors including the angiotensin β1, β2-adrenoceptor and the μ-opioid receptor (Luan, Zhang et al. 2005), a short lipidated peptide of the ICL1 of the

chemokine receptors CXCR4 (Balabanian, Lagane et al. 2005), and D1 dopamine receptor (Beaulieu, Gainetdinov et al. 2009), 5-HT receptor (Berg, Maayani et al. 1998), proteinase Activated Receptor-2 (Ramachandran, Mihara et al. 2009) , the angiotensin II peptide analog for AT1 receptor (Domazet, Holleran et al. 2015), show the ability to bias towards β-arrestin while having no effect on GPCR or activate both pathways with different degrees. JNJ7777120 has been described as a selective antagonist at the H4 receptor but later be found to recruit β-arrestin to the H4 receptor, independent of G protein activation (Rosethorne and Charlton 2011).

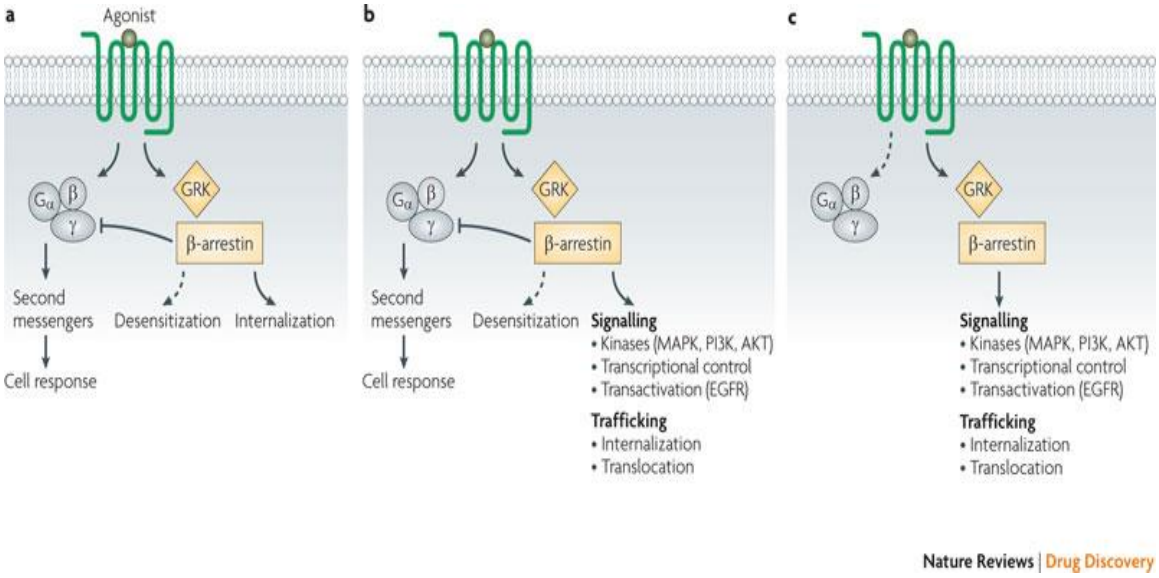


Figure 1.7 Scheme of biased signaling.

Adapted from (Rajagopal, S.et al. 2010).

1.5.2 Cell-type or species biased signaling

Tissue bias (also referred to as system or cell bias) covers the phenomenon in which a ligand for a given receptor activates different pathways in a tissue/cell-specific manner – or in a species-specific manner. AMD3100 has been reported to act as a partial agonist on WT CXCR4 in a Ca^{2+} release assay, and with even higher efficacy on a constitutively active CXCR4 mutant in THP-1 cells (Zhang, Navenot et al. 2002). Human P2Y4 receptor can be only activated by UTP; the rat

orthologue is potently activated by UTP as well as ATP (Brunschweiger, 2006, Jacobson, 2009). Therefore, it is important to dissect the relative physiological relevance of different G protein-mediated signaling in different cell types/tissues, which is important in particular for drug discovery within 7TM receptors.

1.5.3 Quantifying bias

The quantification of the relative levels of bias is important in the identification of compounds and the optimization of drug screening for biased agonists (Kenakin 2007). The detection of molecular bias of GPCR for compound screening including G protein signaling and second-messenger production, β-arrestin recruitment, receptor internalization. For the characterization of G protein signaling, performing high-throughput assays in the presence of inhibitors of G protein signaling (such as pertussis toxin for Gαi), or second messengers such as cAMP or calcium (Karimian, Buist-Homan et al. 2012). Ligand-directed signaling (i.e., biased signaling) can occur through changes the balance of signaling between the effectors as compared to a ligand of reference. Formation of receptors complexes, such as dimers as well as reorientation of extra/intracellular domains of receptors can also lead to conformational rearrangements or targeting for biased signaling (Khoury, Clément et al. 2014).Several approached directed towards addressing β-arrestin recruitment and signaling have been developed over recent years (Verkaar, van Rosmalen et al. 2008).

In proximity assays, the proximity between the β-arrestin and the receptor is monitored using some strategies, such as resonance energy transfer (RET) assays, a donor probe, and an acceptor probe can be attached to the receptor and β-arrestin, respectively (Bertrand, Parent et al. 2002). Upon recruitment of the β-arrestin, emission of the donor probe, by excitation with light in fluorescence RET (FRET) or with a chemiluminescent substrate in bioluminescence RET (BRET), results in energy transfer to the acceptor probe. Proximity can also be assessed using reporter-based assays, such as the Tango assay (Bertrand, Parent et al. 2002, Krasel, Bunemann et al. 2005). A protease

is covalently linked to the β-arrestin, upon recruitment of the β-arrestin results in cleavage and releases a transcription factor that was attached to a modified receptor. This transcription factor then translocates to the nucleus resulting in activation of a luciferase. In assays based on enzyme complementation, the β-arrestin and the receptor are modified with mutant fragment of β-galactosidase, which upon recruitment of the β-arrestin, results in the formation of a functional enzyme (Wehrman, Raab et al. 2006, van der Lee, Blomenrohr et al. 2009).

In conformation assays, changes in the receptor or β-arrestin conformation are used to assess ligand activity and binding. In assays of receptor conformation, site-specific probes are incorporated in areas of the receptor that are thought to undergo a significant structural change after ligand binding. Monitoring of FRET between these two fluorescent probes shows distinct changes associated with binding of the ligand.

Cell-based assay for β-arrestin biased signaling are currently commercially available to measure β-arrestin translocation, such as Transfluor®, β-arrestin redistribution-based technology (Invitrogen Tango™), the proximity test by depending on enzyme fragment complementation between ProLink Tm and EA to form active β-galatosidase or (EFC) to monitor β-arrestin translocation based on bioluminescence intensity (Hu, Stern et al. 2016).

DREADDs (designer receptors exclusively activated by a designer drug) are designer G protein-coupled receptors (GPCRs) that is a powerful novel chemogenetic tools to study GPCR signaling pathways in specific cell types or tissues. DREADDs can only activate heterotrimeric G proteins or trigger β-arrestin-dependent (G protein-independent) signaling (Rosethorne and Charlton 2011).

A number of analytical methods have been developed that build on the seminal operational model of agonism proposed by Black and Leff (Black and Leff 1983). The key drug–receptor parameters

in these models are the equilibrium dissociation constant of the agonist for the receptor (KA) and an operational measure of signaling efficacy (τ) that subsumes both receptor density and coupling efficiency to a given pathway. The τ/KA ratio ('transducer ratio') can thus be used as an index of the relative intrinsic activity of an agonist at a given pathway and represents a starting point for quantification of biased agonism (Klein Herenbrink, Sykes et al. 2016).

To gauge signaling pathway toward or away from a defined or reference signaling pathway, kenakin et al. developed an approach to the statistical comparison of multiple agonists to yield ΔΔLog (τ/KA) values, which proposed by Kenakin group (Kenakin, Watson et al. 2012).

Sudarshan Rajagopal et al. normalize this value for a test agonist relative to a reference agonist, biased factor has been proposed β, which is equal to the distance from the point (σ_{path1}, σ_{path2}) for a ligand as compared to balanced ligands, can then be calculated as the difference between the effective signaling factors (σ_{lig}) to nullify the impact of system and observational bias (Rajagopal, Ahn et al. 2011).

$$\sigma lig = \log\left(\frac{\tau lig}{\tau ref}\right) = \log\left(\frac{\varepsilon lig}{\varepsilon ref}\right)$$

$$\beta lig = \frac{\sigma path1 - \sigma path2}{\sqrt{2}}$$

1.5.4 The impact of functional selectivity on receptor pharmacology

GPCR functional selectivity has therapeutic impact (Lazarowski, Homolya et al. 1997), the overall goal of studying stimulus bias is the ability to design ligands that selectively engage therapeutically relevant signaling pathways while sparing those that contribute to undesirable side effects; drug discovery emphasis has shifted from a traditional merely agonist/antagonist -receptor view toward a receptor-pathway selective view (Valant, Robert Lane et al. 2012)**.** An excellent example of the translational study of a known biased ligand in vivo the testing of TRV120027, (Sar-Arg-Val-Tyr-

Ile-His-Pro-*D*-Ala-OH) competitively antagonizes angiotensin II-stimulated G protein signaling, but stimulates β-arrestin recruitment and activates several kinase pathways(Violin, DeWire et al. 2010), which reduces arterial blood pressure but increase cardiac performance. Adensine A1 receptor atypical A1AR agonist VCP746 and capadenoson significant bias away from calcium mobilization relative to other pathways (ERK1/2, cAMP, pAKT1/2/3), thus retain cytoprotective signaling in the absence of bradycardia (Baltos, Gregory et al. 2016).

Therefore, it is useful to discuss the impact of pluridimensional efficacies on P2Y2R pharmacology and drug discovery; as P2Y2R has dual effects on inflammation response. To determine the functional selectivity by evaluating the downstream effects such as gene transcriptional or translational regulation induced by biased signaling. Biased signaling offers the potential to emphasize beneficial signals, deemphasize harmful signals while blocking the harmful signal produced by endogenous agonists (Kenakin 2015). To gain further insight into the role of the individual ligand directed biased signaling, a new P2Y2R model based on the crystal structure and performed mutagenesis to probe the UTP analog mediated activation of key signaling pathways.

1.6 Aim of study

Vascular inflammation is commonly observed in sepsis, which results in high morbidity and mortality worldwide (Treacher and Brown 2009). Current medication for sepsis mainly depends on antibiotics with limited options. Death rate for sepsis is as high as 30%, with severe sepsis being ~50% and septic shock being ~80% (Schoenberg, Weiss et al. 1998). The influence of various risk factors including LPS leads to the alteration in inflammatory and thrombotic gene expression in the endothelium (Remick 2007, Schouten, Wiersinga et al. 2008, Fink 2014). One of the potential new therapeutic strategies for sepsis is to target endothelial receptors to regulate the expression of inflammatory mediators, adhesion molecules and coagulant factors, etc. Our previous study showed that the principal P2Y nucleotide receptor subtype in human coronary artery endothelial cells

(HCAEC) is the P2Y2 receptor (P2Y2R) (Ding, Ma et al. 2011). P2Y2R has been shown to induce key inflammatory and thrombotic gene expression including VCAM-1 and TF, suggesting that unveiling the molecular mechanism of P2Y2R-mediated inflammatory response may provide new target for sepsis treatment (Seye, Yu et al. 2004, Ding, Ma et al. 2011).

Indeed, nucleotide analog, 2-methylthiol ATP (2-MeS-ATP) was reported to rescue mice from endotoxin shock and death nearly 100%, but the exact mechanism remains unclear (Proctor, Denlinger et al. 1994). Our preliminary study found that 2-MeS-ATP activates AMPK and MAPKs pathways in naïve cells transfected with human or mouse P2Y2R, but it has no agonistic effect on the endothelial P2Y2R. Thus, 2-MeS-ATP may be a biased P2Y2R ligand, signaling in a tissue/cell-specific manner. In addition, we found that the UTP derivative, PSB1114, activates endothelial P2Y2R, leading to AMPK and ERK1/2 activation, without affecting JNK and p38 pathways, though all of which are activated by UTP. Furthermore, we also found that even the same ligand like UTP can induce both positive and negative pathways to fine-tune the expression of pro-thrombotic and inflammatory TF gene through the P2Y2R (Liu, Zhang et al. 2016). Our preliminary data also showed that endothelial P2Y2R signaling can mediate translational suppression for some key inflammatory genes such as GM-CSF, despite a significant induction of GM-CSF mRNA. These findings suggest that the P2Y2R can be manipulated by different ligands to induce a preferred signaling profile that suppresses the inflammatory response. Therefore, the major objective of this project is to determine P2Y2R biased signaling (tissue bias and pathway bias) in control of pro-inflammatory and pro-thrombotic gene expression through transcriptional and translational mechanisms.

During the quest for new evidence in support of P2Y2R activation of the AMPK pathway, we unexpectedly found that in HCAEC, P2Y2R activation not only induced phosphorylation of the anticipated substrate ACC, but also significantly induced phosphorylation of a 25 kD unknown

protein that we termed p25. Further study showed that P2Y2R-induced p25 phosphorylation can be mimicked by other receptor agonists including thrombin, histamine, and VEGF, but not by TNFα, SDF-1 and adenosine. These interesting findings prompt us to hypothesize that p25 may be a general and important signaling or effector protein for a group of membrane receptors.

It has been demonstrated that vascular inflammation due to endothelium dysfunction is responsible for roughly fifty percent of ongoing arterial damage seen after transplant, angiogenesis in cancer, and kidney disease, which is obviously not limited to endotoxin shock (Brasier, Recinos et al. 2002, Wu, Antony et al. 2014). This thesis is innovative for the following reasons: 1) current treatment on sepsis or endotoxin shock is not ideal, and this study proved P2Y2R being a new anti-sepsis drug target; 2) this study revealed new molecular mechanism underlying the reported anti-sepsis effect for 2-MeS-ATP; 3) identification of new UTP derivatives that prompt P2Y2R signaling to one exclusive ERK1/2 pathway will change the current paradigm of GPCR signaling research and provide new avenue of potential drug discovery in relevant inflammation and thrombotic diseases; 4) discovery of a new post-receptor signaling protein p25 will open a new window in the general field of membrane receptor biology, and finally 5) from a pharmacological perspective, our proposed study on P2Y2R biased signaling (tissue/cell bias and pathway bias) will prompt us to propose a new concept termed “precision receptor pharmacology”, which is in line with the precision medicine initiative worldwide.

Chapter 2. Identification of Biased Agonists for P2Y2 Nucleotide Receptor to Suppress Pro-inflammatory Gene Expression

2.1 Abstract

Recently we reported that in human coronary artery endothelial cells (HCAEC), the P2Y2 nucleotide receptor (P2Y2R) regulates the pro-thrombotic gene tissue factor (TF) expression through positive and negative signaling mechanism. Here, we report the identification of a P2Y2R ligand PSB1114 which selectively activates the negative, but not the positive pathways, leading to decreased TF expression. Unlike the endogenous agonist UTP, PSB1114 alone had no effect on TF mRNA transcription, but it unexpectedly inhibited UTP-induced TF expression. The mechanistic study showed that PSB1114 induced intracellular Ca^{2+} mobilization in HCAEC which exhibited 2/3 efficacy as UTP and was inhibited by AR-C118925, a P2Y2R-selective antagonist (Rafehi, Burbiel et al. 2016). In contrast to UTP, which activated both Gαq and Gαi/o, PSB1114 exhibit biased agonism via Gαi/o coupled P2Y2R. When evaluated in their activation of the MAPK pathways, PSB1114 only activated the ERK1/2, but not JNK and p38, whereas UTP activated all the three, suggesting PSB1114 as a biased ligand for the P2Y2R. This notion was supported by the fact that PSB1114-induced ERK1/2 activation was inhibited by AR-C1189258 and it had no effect on ERK1/2 in P2Y2R-null cells. In addition, PSB1114 selectively activated the TF gene repressor Fra-1, but not the positive AP-1 subunits c-Jun and ATF-2, whereas UTP-activated all of them. Furthermore, luciferase activity assay indicates that PSB1114 suppressed TF gene promoter activity via ERK1/2 activation, while UTP increased overall TF promoter activity. These findings reveal that PSB1114 acts as a biased agonist in human endothelial P2Y2R, in which it selectively activates the ERK1/2-Fra1 pathway, leading to suppression of TF gene transcription. Thus,

PSB1114 represents the first identified biased ligand in the P2Y receptor family, opening a new avenue for potential drug discovery in relevant inflammatory and thrombotic diseases.

2.2 Introduction

The extracellular nucleotides (e.g. ATP and UTP) regulate intracellular processes in the vascular system under physiological and pathological conditions, including cell proliferation, differentiation, apoptosis, and migration. It has been well established that the cellular functions of extracellular nucleotides are mediated through the P2 nucleotide receptors, some of which have been implicated in cardiovascular diseases, such as atherosclerosis and thrombosis (Höpfner, Maaser et al. 2001, Inoue, Chen et al. 2008, Elliott, Chekeni et al. 2009). There are two different families of P2 receptors: P2X (1-7) are ionotropic receptors and the heterotrimeric G protein-coupled P2Y receptors, which are subdivided into eight subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) (Erb and Weisman 2012). Stimulation of different P2Y receptor leads to activation of various G-protein (Gs, Gi/o, Gq/11, and G12/13), where different G protein activation prompts the generation of second messengers (Ca^{2+}, cyclic AMP, inositol phosphates) to modulate downstream events (Erb, Liu et al. 2001). In the past decade, the platelet P2Y12 receptor antagonist clopidogrel (Plavix) has been successfully used to prevent blood clotting, heart attack and stroke in patients. Among other non-platelet P2Y receptors, The P2Y2 receptor (P2Y2R) is widely expressed in immune cells, epithelial and endothelial cells, vascular smooth muscle cells, and osteoblasts, etc. It turns out that the P2Y2R in the vessel wall is upregulated in vascular injury models, including balloon-injured rat aorta (Seye, Gadeau et al. 1997), collared rabbit carotid arteries (Seye, Kong et al. 2002), and stented coronary arteries (Hou, Moller et al. 2000, Shen, Seye et al. 2004). The P2Y2R is typically activated nearly equal-potently by ATP and UTP (Bagchi, Liao et al. 2005). Upon activation, the P2Y2R mediates Ca^{2+} mobilization primarily through Gαq, but may also through Gαi/o subunit in some cells (Bagchi, Liao et al. 2005), leading to activation of various signaling pathways including the mitogen-activated protein kinases (MAPKs) such as ERK1/2,

JNK, and p38 (Hou, Moller et al. 2000, Erb, Liu et al. 2001, Höpfner, Maaser et al. 2001, Erb, Liao et al. 2006).

It has been known that P2Y2R mediates inflammatory response in the vasculature (Seye, Yu et al. 2004, Douillet, Robinson et al. 2006). As endothelium plays a pivotal role in maintaining the homeostasis of the vascular system, inflammatory stimuli or stress initiate an activation cascade in endothelial cells (ECs), leading to impaired vascular homeostasis (Sprague and Khalil 2009, Ding, Ma et al. 2011). The function of vascular ECs to maintain fluidity of blood is disrupted by the activities of various pro-inflammatory cytokines, including tissue factor (TF) (Steffel, Luscher et al. 2006). TF is an important initiator for thrombi formation, and the quiescent ECs express a negligible amount of TF (Steffel, Luscher et al. 2006, Ding, Ma et al. 2011). Under inflammatory condition, however, TF is upregulated on the EC surface, which generates thrombin that converts fibrinogen into fibrin, ultimately leading to coagulation and disseminated intravascular coagulation in sepsis (Schouten, Wiersinga et al. 2008, Ding, Ma et al. 2011). We initially reported that the P2Y2R is the predominant P2Y subtype expressed on human coronary artery ECs (HCAEC) and it can be activated by endogenous ligand UTP or ATP to induce TF expression (Ding, Ma et al. 2011). Further, we identified the role of post-P2Y2R signaling including the JNK and p38 MAPK pathways responsible for TF induction in HCAEC (Ding, Ma et al. 2011).

In contrast, new information has accrued that P2Y2R may have anti-inflammatory effects, though the mechanism underlying this process are not well understood. P2Y2R knockout mice are more susceptible to lung bacterial and virus infection (Geary, Akinbi et al. 2005). In addition, activation of P2Y2R by UTP reduces infarct size and improves mouse heart function after myocardial infarction, and the UTP analog 2-thio-UTP protects heart from ischemic damage both in vitro and in vivo (Le Quang, Bouchareb et al. , Cohen, Shainberg et al. 2011, Hochhauser, Cohen et al. 2013). Furthermore, P2Y2R deficiency aggravates chronic kidney disease progression and early pathology

in Alzheimer's disease (Weisman, Ajit et al. 2012, Ajit, Woods et al. 2014). These studies indicate that the P2Y2R can play a protective role in inflammation. Currently, the mechanism to reconcile the pro- vs. anti- inflammatory response of the P2Y2R is not fully understood. Interestingly, our recent study indicated that activation of the P2Y2R in HCAEC induces both pro-inflammatory and anti-inflammatory signaling pathways, fine-tuning TF expression (Liu, Zhang et al. 2016). Specifically, we found that the P2Y2R-mediated ERK1/2-Fra-1 signaling axis functioning as a negative regulator, while JNK and p38-controlled c-Jun and ATF-2 act as positive regulators on TF transcription (Liu, Zhang et al. 2016).Thus, targeting the negative regulatory axis, P2Y2R/ERK1/2-Fra-1, could be a new option for controlling vascular inflammation and thrombogenicity. Ideally, one possible strategy to exploit this anti-inflammatory pathway on HCAEC is to seek for a new P2Y2R ligand which can activate the negative ERK1/2 pathway but circumvent the pro-inflammatory JNK and p38 pathways. Indeed, it has been increasingly noted that receptor biased signaling could apply in drug discovery. Biased agonists can preferentially activate one pathway or certain pathways over another to achieve functional selectivity. For some GPCRs, such as those, for example, angiotensin II, adrenoceptor, dopamine, opioid and chemokine receptors, are now accepted as receptors that can elicit bias signaling (DeWire and Violin 2011, Liu, Horst et al. 2012, Rajagopal, Bassoni et al. 2013, Keenan, Lew et al. 2016, Yang and Tao 2016). However, similar ligands in the purinergic or nucleotide receptor field have not been reported.

Thus, the principle objective of this study was to determine whether the P2Y2R can be pharmacologically manipulated to induce a biased signaling, and if so, which receptor signaling pathway(s) is selectively activated by such a biased ligand and its potential underlying mechanism. Secondly, we sought to determine the functional significance of P2Y2R biased signaling in control of the pro-thrombotic TF gene expression in ECs and macrophages. Here we report that PSB1114, a UTP derivative (El-Tayeb, Qi et al. 2011), functions as a first identified biased agonist on P2Y2R,

as evidenced by its selective activation of the Ca^{2+} and ERK1/2 signaling pathways through Gi/o protein, leading to a suppression of TF transcription in ECs and macrophages.

2.3 Material and Methods

2.3.1 Mice

Wild-type (C57BL/6) and P2Y2R KO ($P2Y2R^{-/-}$) on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor) and bred at the animal facility at Auburn University. DNA extraction from mouse tail and PCR genotyping were routinely performed according to the instruction of the animal supplier. Mouse P2Y2R genotyping primers are the following: wild-type forward: 5'-AGC CAC CCG GCG GGC ATA AC-3'; mutant forward: 5'- AAA TGC CTG CTC TTT ACT GAA GG-3'; common reverse: 5'-GAG GGG GAC GAA CTG GGA TAC-3'. Mice were housed in Auburn University's Biological Research Facility in a controlled and pathogen-free environment (25 °C; 12:12-h light-dark cycle) with free access to water and standard chow diet.

2.3.2 Regents and materials

The sources of the chemicals and recombinant proteins used in this study are listed in Table 2.1.

Table 2.1 Chemicals

Items	Catalog Number	Company
Uridine 5'- triphosphate tris salt	U6875	Sigma-Aldrich
2-thioUTP tetrasodium salt	3280	Tocris
UTPγS trisodium salt	3279	Tocris
EcoRV	1042A	Takara
Recombinant human TNF-α	210-TA	R&D Systems
LPS (E. coli O111: B4)	L4391	Sigma-Aldrich
Murine M-CSF	315-02	Peprotech
Bordetella pertussis toxin	BML-G101-0050	Enzo Life Sciences
2'-O-Me ATP	CC-4008	Chemcyte Incorporated
2'-O-Me CTP	CC-4009	Chemcyte Incorporated
2'-O-Me GTP	CC-4009	Chemcyte Incorporated
2'-O-Me UTP	CC-4009	Chemcyte Incorporated
dNTP set (100 mM)	TQAC135	Omega(bio-tek)
AA-UTP	CC-2004	Chemcyte Incorporated
Alpha-thio-UTP	CC-3008	Chemcyte Incorporated
Human GM-CSF Elisa Kit	900-M55	Peprotech
Dulbecco's Modified Eagle Medium	12-702F	Lonza
Fetal Bovine Serum (FBS)	SH38396.03	Hyclone

2.3.3 Cell Culture

2.3.3.1 Human coronary artery endothelial cells

HCAEC were cultured in EBM-2 medium supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA1000 (Lonza), and 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. HCAEC were

used between the third and eighth passages. Before stimulation, cells were seeded to grow for 24 h and starved overnight. Where inhibitor or antagonist was used, cells were pretreated with the inhibitor/antagonist for 40 minutes before cell stimulation.

2.3.3.2 Human and mouse transfected -1321N1 cells

Wild-type and P2Y2R-transfected 1321N1 cell lines were kindly offered by Dr. Gary Weisman (The University of Missouri-Columbia). Wild-type and P2Y2R-transfected 1321N1 cells were cultured in DMEM supplemented with 10% FBS with or without G418 (0.5 mg/mL). All experiments on transfected receptors are run in parallel with WT controls.

2.3.3.3 Isolation of murine peritoneal macrophages

All animal study protocols were approved in advance by the Institutional Animal Care Use Committee at Auburn University. Details on the isolation of mouse peritoneal macrophages can be found in our previous study(Ma, Liu et al. 2014) and others (del Rey, Renigunta et al. 2006). Briefly, mice were injected with thioglycollate medium (3% in PBS, 1mL, i.p.) into their peritoneal cavity three days prior to cell harvest. Mice were anesthetized and sacrificed by cervical dislocation, and the peritoneal cells were collected by lavage with cold PBS. After centrifugation of peritoneal fluid, the supernatant was discarded, and the cell pellet was re-suspended in cold DMEM containing 10% FBS. A total of 2X106 cells/well were seeded into the 6-well plate and cultured for 3 hours at 37°C. Non-adherent cells were removed by gently washing three times with the medium. Before cell stimulation, macrophages were grown in DMEM and starved overnight. For the P2Y2R-/- cells, stimulation of the cells with 10% FBS for 5 min was used as a positive control.

2.3.3.4 Generation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages were obtained according to the literature (Manzanero 2012). Briefly, bone marrow plugs were flushed from femurs and tibiae of mice with serum-free DMEM

medium, after filtration and centrifugation, the harvested cells were resuspended in DMEM medium containing 10% FBS, penicillin (50 U/ml), streptomycin (50 μg/ml), and M-CSF (10 ng/ml). Bone marrow cells (2 x 10^6) were seeded in 6-well plate with 2ml DMEM medium and incubated at 37°C. On day 3, medium was removed and replaced with fresh DMEM medium plus new M-CSF. On day 5, the cells were starved overnight in serum-free DMEM medium, after which the macrophages were used for further experiments.

2.3.4 Long-term Storage

For long-time storage, cells should be healthy with a viability of >90% and no signs of microbial contamination. Cells were collected by centrifuge at ~ 1100 rpm for 5 mins. Cells at concentration of ~3x 10^6 were resuspended with cryoprotectant dimethyl sulphoxide (DMSO) at a final concentration of 10% in 2 ml (Greiner Bio One). The vials were transferred to isopropanol freezing container(VWR) to reach 1°C/min cooling rate and were kept in -80°C overnight. The next day, vials were stored in liquid phase nitrogen allows the lowest possible storage temperature to be maintained with absolute consistency. Each vials were individually labelled with cell line, lot number and data of freezing and recorded on electronic database and spreadsheet.

2.3.5 Intracellular Ca^{2+} mobilization assay

Cells were seeded at a density of 4x10^4 cells per well into 96-well culture plates and cultured for one day. On day two, the original medium was removed, and the assay medium from FluoForte™ kit (Enzo Life Sciences) containing the Ca^{2+} dye was added and receptor-mediated Ca^{2+} mobilization was determined as previously described (Ding, Ma et al. 2011). Fluorescence was determined immediately after adding of different reagents, with an excitation wavelength set to 485 nm and an emission wavelength set to 525 nm, and readings were taken every 1s for 500s. For antagonist inhibition experiment, cells were pre-incubated with the antagonist for 30 min before agonist addition. Measurement of Ca^{2+} signal was performed with the fluorometer plate reader

(BMG FLUOstar) with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU).

2.3.6 PCR analysis

2.3.6.1 Isolation of and measurement of RNA and DNA

The total RNA and DNA were extracted from cells according to manufacturer's protocol for the RNeasy and DNeasy kits. RNA was extracted from macrophages using RNAzol® RT Kit. For the synthesis of the first strand of cDNA, 1 μg of total RNA after DNase (Ambion) treatment or BAN purification. For the determination of RNA an DNA yield, the concentration was measured by UV spectrophotometry with NanoDrop 2000 (Thermo Scientific). The quotient of the 260nm/280nm absorption determines the degree of RNA/DNA purity, a ratio of 1.8 or higher indicted sufficient DNA/RNA purity for following procedures.

2.3.6.2 cDNA Synthesis

For the synthesis of the complementary DNA(cDNA), 0.5μg of total RNA after Dnase (Ambion) treatment was reverse-transcribed using Taqman reverse transcription reagents (Applied Biosystems) using the recipe as in Table 2.2.

Table 2.2 cDNA synthesis

Component	Volume/reaction
10X Taq RT buffer	5µL
25mM $MgCl_2$	11µL
10mM dNTP	10µL
Oligo dT	2.5µL
RNase inhibitor	1µL
Reverse Transcriptase or RNase free H_2O	1.25µL
RNA	variable
RNase free H_2O	variable
Total Volume	50µL

The mixture of all components was incubated at PCR system (Bio-Rad) at 25°C for 10 min; 48°C for 20 min; 95°C for 5 min and then held at 4°C.

2.3.6.3 RT-PCR for genotyping

The DNA samples were amplified by PCR using HotStart Taq DNA Polymerase (Biotool) as in the Table.2.3.

Table 2.3. RT-PCR reaction

Component	Volume/reaction
2X PCR buffer	10µL
Forward WT primer	0.5µL
Forward Mutant primer	0.5µL
Common Reverse Primer	1µL
RNase free H_2O	7µL
DNA template	1µL
Total Volume	20µL

PCR was performed on an iCycler iQ5 detection system (Bio-Rad) with pre-mixed PCR reagents (Bio-tool), with jump start for 2 min at 94 °C, followed by 40 thermal cycles for denaturation for 1 min at 94°C, annealing for 1 min at 66°C, and extension at 72°C for 1min with a final extension at 72°C for 10 min. The resulting PCR products were resolved on a 1% agarose (Amresco) gel with 0.01 % ethidium bromide (Sigma) in gel electrophoresis system (Minicell primo, Thermo), and the bands were visualized with universal hood imaging system (Bio-Rad) under UV light as we previously described.

2.3.6.4 Real-time PCR

The DNA samples were amplified by PCR using SYBR® Green PCR Master Mix (Biotool/Applied Biosystem) as in Table 2.4.

Table 2.4. Real-time PCR reaction

Component	Volume/reaction
2X PCR buffer	12.5µL
Forward primer	0.5µL
Forward primer	0.5µL
RNase free H_2O	6.5µL
cDNA template	5µL
Total Volume	25µL

PCR was performed on an iCycler iQ5 detection system (Bio-Rad) with pre-mixed PCR reagents (Bio-tool), with jump start for 5 min at 95 °C, followed by 40 thermal cycles for denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension at 72°C for 2 min with a final extension at 72°C for 10 min. Fold-increases in mRNA expression in treated cells relative to control cells calculated with 2-ΔΔCt method, and GAPDH or β-actin mRNA were used for internal control.

The fold change in the target genen relative to the endogenous control gene is determined by:

Fold change=2-Δ(ΔCt)

ΔCt=Ct(target genes)- Ct(internal controls)

and Δ(ΔCt) = ΔCt(stimulated)- ΔCt(controls)

All valuses for mRNA expression were presented as difference (n-fold) between the sample mRNA and the control mRNA.

The resulting PCR products were resolved on a 1% agarose (Amresco) gel with 0.01 % ethidium bromide (Sigma) in electrophoresis gel system (Minicell primo, Thermo), and the bands were

visualized with universal hood imaging system (Bio-Rad) under UV light as we previously described(Ding, Ma et al. 2011).

2.3.6.5 Primers for PCR assay

Table 2.5. Primers for PCR assay

Gene	Forward Primer	Reverse Primer
Human TF hnRNA	5’-CCCCTGGGTTGCTATGAGG-3’	5’-CCTGGCTGTGGTGTTCTGTGC-3’
Human TFmRNA	5’-ACGCTCCTGCTCGGCTGGGT-3’	5’-CGTCTGCTTCACATCCTTCA-3’
Human GM-CSF	5’AGCATGTGAATGCCATCCAG-3’	5’- AGGGGATGABAAGCAGAAAG-3’.
Human GAPDH	5’-TCAACAGCGACACCCACTCC-3’	5’-TGAGGTCCACCACCCCTGTTG-3’;
Human P2Y2R	5’-GTGCTCTACTTCCTGGCT-3’	5’-CTGAAGTGTTCTGCTCCTAC-3’
Human GM-CSF	5’-AGCATGTGAATGCCATCCAG-3’	5’-AGGGGATGACAAGCAGAAAG-3’
Mouse TF mRNA	5’- ATGGCGATCCTCGTGCGCCCG-3’	5’-CTATGCCAAGCGCGACGGGGTG-3’
Mouse β-actin	5’-ATGGATGACGA-TATCGCTGCG-3’	5’CTAGAAGCACTTGCGGTGCAC-3’

2.3.7 Western Blotting Assay.

2.3.7.1 Sampling

After stimulation, adherent cells were lysed in Laemmli sample buffer (Sigma-Aldrich) and scratched with cell lifter. For suspension cells, after centrifugation, the cell pallets were resuspended in Laemmli sample buffer. All lysates were collected on ice followed by being heated in boiling water for 5 min. The buffers used for Western blot and chemicals used in this study for Western blotting are listed in Table 2.6

Table 2.6 Buffers used for Western blot

Items	Catalog Number	Company
10X Tris-Glycine Buffer	75894	USB Affymetrix
10X Tris/Glycine/SDS buffer	161-0732	Bio-Rad
10X Tris-buffered Saline	CAS-77-86-1	Alfa Aesar
Tween-20	P7949	Sigma
Western Lightning® Plus-ECL	NEL105001EA	PerkinElmer
Non-fact dry milk	170-640A	Bio-Rad
Albumin, Bovine Fraction	0332-100G	Amresco

Electrophoresis buffer contain 25mM Tris, 192 mM Glycine and 0.1%(w/v) SDS at pH 8.3. Transfer buffer contains 25mN Tris, 192mM Glycine with 20%(v/v) methanol at pH8.3 Tris-Buffered Saline and Tween 20 was made from 10X TBS-T buffer and containing 500mM Tris,60 mM KCl, 2.8mM NaCl, and 1.0% Tween-20. Blocking buffer was made with 5% non-fat dry milk in TBS-T buffer. Primary antibody was diluted in 5% bovine serum albumin(BSA) in TBST buffer.

2.3.7.2 Blotting

Sample was loaded and separated on 10% Mimi-Protean®TGX^{TM} Precast Gel (Bio-Rad) in a SDS-PAGE gel chamber (Bio-Rad) in electrophoresis buffer for 45 min with the voltage of 110V. After running the gel, assemble the stack in the order of two layers of absorbent paper, which was thoroughly soaked in transfer buffer; the SDS-PAGE gel chamber(Bio-Rad) in electophoresis buffer for 45 min with voltage of 110V. After running the gel, assemble the stack in the order of two layers of absorbent paper, SDS-PAGE gel, a wet polyvinylidene difluoride (PVDF) membrane (Thermo); two layers of filter paper in transfer buffer using semi-dry blotting apparatus (Bio-Rad) for 35 min with the voltage of 20V. After transfer, the membrane was blocked with 5% non-fat milk for 1h (room temperature) on shaking rocker. After 10min TBST wash for 4 times, the membrane was probed with the primary antibody overnight in 5% BSA in TBS-T buffer. The primary antibodies used for Western for this study are listed in Table 2.7.

After washing by TBS-T 10 mins for four times. The secondary antibody conjugated with horseradish was incubated for 1 h at room temperature with 5% non-fat dry milk in TBS-T. After washing out the unbound antibodies. The membrane was incubated briefly in Western Lightning® Plus-ECL(PerkinElmer). The image was developed on a photographic film (Research products international corp.), placed against the membrane, and visualized by medical film processor (Konica Minolta Medical &graphic Inc).

Table2.7. The primary antibodies used for Western blot

Items	Catalog Number	Company
p-AMPKα (Thr172) (40H9) Rabbit mAb	2535	Cell Signaling
AMPKα Rabbit mAb (23A3)	2603	Cell Signaling
p-p44/42 MAPK(Thr202/Tyr204) Rabbit mAb	4370	Cell Signaling
p44/42 MAPK	9102	Cell Signaling
p-p38 MAPK(Thr180/Thr182) Rabbit mAb	4511	Cell Signaling
p-SAPK/JNK(Thr183/Thr185) Rabbit mAb	4511	Cell Signaling
SAPK/JNK Rabbit mAb	9252	Cell Signaling
p-c-Jun(Ser63) (54B3) Rabbit mAb	2361	Cell Signaling
p-Fra-1(Ser265) (D22B1) Rabbit mAb	5841	Cell Signaling
p-ATF-2(Thr71) Rabbit mAb	5112	Cell Signaling
p-c-Jun(Ser63) Rabbit mAb	2361	Cell Signaling
Fra-1(R-20) Rabbit Ab	sc-605	Santa Cruz
c-Jun (H-79) Rabbit mAb	sc-1694	Santa Cruz
ATF-2(20F1) Rabbit mAb	9226	Cell Signaling
GAPDH(14C10) Rabbit mAb	2118	Cell Signaling
β-tubulin(9F3) Rabbit mAb	2128	Cell Signaling
Mouse tissue factor (TF9-10H10) mAb	12161	Calbiochem
Anti-rabbit IgG, HRP-linked Ab	7074	Cell Signaling
Anti-mourse IgG, HRP-linked Ab	7074	Millopore

2.3.7.3 Imaging Analysis

The developed images on the files were scanned by greyscale and converted to the digital image. The intensity of signals was analyzed using densitometric software, Quantity one. Data output were calibrated against the background and given in relative density units.

2.3.7.4 Stripping and Re-probing

Equal protein loading was verified by stripping off the original antibodies and re-probed with the primary antibodies. Blots were rinsed with TBS-T buffer and incubated for 20 min at room temperature in Restore PLUS Western blot stripping buffer (Thermo). After which, the membrane was briefly rinsed with TBS-T for three times and blocked for 1h in 5% non-fat dry milk in TBS-T buffer. Subsequently, the blots were reporbed with desired primary antibodies.

2.3.8 Luciferase assay

2.3.8.1 Tissue Factor Promoter Constructs.

pGL4.12[luc2CP] Vector (Promega) was used as transcription activity vector. The pGL4.12[luc2CP] vector (4.4kb) encodes the luciferase reporter gene luc2CP (Photinus Pyralis) which yield 61kDa protein and contain multiple cloning regions can be recognized by EcoRV restriction endonuclease.

Table2.8. Restriction enzyme reaction using EcoRV endonuclease

Component	Volume/reaction
EcoRV	1µL
10X H Buffer	2µL
Vector	1µL
Sterilized distilled water	up to 20µL
Total Volume	20µL

Promoter constructs encompassing the region from -1427 to +207 relative to the transcription starting site of the human TF gene were amplified from human genomic DNA using specifically designed forward and reverse primers containing the EcoRV restriction enzyme site as previously described (Liu, Zhang et al. 2016). Forward primer: 5'- GCT AGC CTC GAG GAT ATC CTA CCT TCA ATC CCA GAG-3'; Reserve primer: 5'-AGG CCA GAT CTT GAT ATC TCC ATG TCT ACC AGT TGG CG-3'.

The resulting PCR products were resolved on a 1% agarose (amresco) gel with 0.01 % ethidium bromide (Sigma) in gel electrophoresis system (Minicell primo, Thermo), and the bands were visualized with universal hood imaging system (Bio-Rad) under UV light. Visible bands were cut form gel and the PCR products were recovered from agarose gel with QIAquick Gel Extraction Kit (Qiagen). The insert fragment was mixed with linearized vector at the ratio of 2:1 and ligated with linearized vector at the ratio of 2:1 and ligated with In-Fusion HD EcoDry Cloning kit (Clonetech) at 15 min at 37 ºC, followed by 15 min at 50 ºC. Thus, the ligate vector is used to transform electrocompetent E.coli HST 08 strain (Clontech). Ligation product and E.coli were mixed and heat shocked at 42 ºC for 45 sec in SOC medium (Clontech). Transformed E.coli was spread on LB agar plate with amipicllin, incubated at 37 ºC overnight. Monoclonal E.coli were collected and inoculated into 5ml of LB culturing medium(with ampicillin) each and cultured in a 37 ºC incubator for 18h. E.coli were collected and lysed, and plasmids inside were purified with QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's manual. To verify inserted fragments, plasmids were sequenced by (Eurofins) as described in (Liu, Zhang et al. 2016).

2.3.8.2 Plasmid Transfection and Luciferase Activity Assay

HCAEC were seeded into 96-well plates and transiently transfected with the pGL4.12 vector construct with Xfect Transfection Reagent (Clontech) following the manufacturer's instruction. Transfection efficiency was normalized by co-transfection with a plasmid containing the β-

galactosidase gene driven by the SV 40 promoter (Promega). β-galactosidase activity was measured by β-galactosidase Enzyme Assay System (Promega). Luciferase activity was measured for reporter expression in triplicate according to the instruction provided in the Luciferase Reporter Assay System (Promega).

2.3.9 Immunofluorescence

HCAEC were seeded into 8-chamber glass slides (Nunc), starved overnight, and then treated with vehicle, PSB1114 or UTP. After the 30-minute treatment, the medium was aspirated, and cells were then fixed with 4% paraformaldehyde for 15 minutes and washed with PBS for three times and blocked with 5% horse serum for 1h at room temperature. Then the cells were incubated with rabbit monoclonal antibodies to human p-Fra-1, p-c-Jun, or p-ATF-2 (1:50) (Cell Signaling Technology), overnight at 4°C followed by incubation with FITC-conjugated anti-rabbit IgG (1:200) (KPL) for 90 minutes at room temperature in darkness. For negative controls, cells were incubated with non-immune rabbit IgG in place of specific primary antibody or only the FITC-conjugated secondary antibody. Cells were then washed with PBS three times and incubated with Phalloidin Red 594 (BioLegend) for 40 min. Finally, mounting medium containing DAPI was added to seal the slides. Images with fluorescent signals in random fields were acquired and captured using an AMG EVOS digital inverted multi-functional microscope (AMG).

2.3.10 Enzyme-linked immunosorbent assay's (ELISA)

HCAECs supernatant was collected, and the level of cytokines was further evaluated with ELISA (human granulocyte-macrophage colony-stimulating factor (GM-CSF) according to the manufacturer's protocol (Peprotech). Absorbance was measured within 30 minutes at 450nm. Data were normalized by protein concentration.

2.3.11 Data Analysis.

All data were analyzed by Prism 4 (Graphpad Software Inc.). Data are expressed as the mean ± S.E.M. The means of two groups were compared using Student's t-test (unpaired, two-tailed), and one-way analysis of variance was used for comparison of more than 2 groups with $p<0.05$ considered to be statistically significant. Unless otherwise indicated, all experiments were repeated at least three times.

2.4 Results

Similarity and difference of PSB1114 versus UTP on P2Y2R-induced Ca^{2+} signaling

To identify potential biased ligands on the P2Y2R, we initially performed a small scale screening assay on a handful of commercially available analogs or derivatives of ATP and UTP, the two endogenous P2Y2R agonists. We found ARC-118925 is selective antagonize UTP induced signaling such as p-38 and p-AMPK and not induce toxicity from 0.1 to 100 μM on HCAEC (**Fig. 2.1**). We found that PSB1114, a derivative of UTP (Ko, Carter et al. 2008), induced an increase of intracellular Ca^{2+} with a similar efficacy as UTP in 1321N1 human astrocytoma cell line stably transfected with human P2Y2R gene (**Fig. 2.2B**), but it had no effect on the wild-type 1321N1 cells (**Fig. 2.2A**). However, in HCAEC in which P2Y2R is the only functional P2Y subtype receptor (Ding, Ma et al. 2011), PSB1114 triggered a Ca^{2+} signaling with about 70% of the efficacy of UTP, even at 100 μM (**Fig. 2.2C**), suggesting that on the endogenous P2Y2R in EC, PSB1114 may act as a partial agonist. To validate the specificity, HCAEC was pretreated with ARC-118925 (3 μM) for 30 min, a selective P2Y2R antagonist (Jacobson, Ivanov et al. 2009). As expected, both UTP- and PSB1114-induced Ca^{2+} signals were abolished by ARC-118925 (**Fig. 2.2C**), suggesting a P2Y2R-mediated effect in HCAEC. Next, we tested the role of Gi/o protein in PSB1114-induced P2Y2R-mediated Ca^{2+} signaling, Figure 2.2D shows that pretreatment of HCAEC with pertussis toxin (PTX, 100ng/ml, overnight), an inhibitor for Gi/o protein, abolished PSB1114-induced Ca^{2+} signaling, suggesting that the endogenous P2Y2R in HCAEC is primarily coupled to Gi/o proteins in response to PSB1114 stimulation.

PSB1114 and UTP activation of AMPK pathway through P2Y2R

One of the downstream events of Ca^{2+} signaling is activation of AMPK. Figure 2.3A shows that stimulation of HCAEC with PSB1114 induced a rapid AMPK phosphorylation from 1 to 5 mins, and that the AMPK substrate Acetyl-CoA Carboxylase (ACC) was also activated from 2 to 5 mins. In addition, the effect of PSB1114 on AMPK activation was dose-dependent in HCAEC (**Fig. 2.3B**). Further, pretreatment of HCAEC with 3μM ARC-118725 abolished UTP- and PSB1114-induced AMPK phosphorylation (**Fig. 2.3C & D**), indicating a role of the P2Y2R.

Differential effects of PSB1114 and UTP on MAPKs signaling

To further compare the difference of PSB1114 and UTP on P2Y2R signaling, we focused on the MAPKs pathways. Figure 2.4A shows that stimulation of HCAEC with PSB1114 induced ERK1/2 phosphorylation from 2 to 20 mins, but it had a negligible effect on JNK and p38, whereas UTP significantly activated all the three MAPKs, suggesting a biased signaling for PSB1114. The phosphorylation of ERK1/2 by PSB1114 stimulation was also dose dependent, with no effect on JNK and p38 at all the tested doses while the effects of UTP were robust (**Fig. 2.4B**). Furthermore, we observed that the increased ERK1/2 phosphorylation in response to PSB1114 was abolished by ARC-118725 (**Fig. 2.4C**), suggesting the contribution of the P2Y2R.

PSB1114 inhibition of P2Y2R-induced TF gene transcription

Since our previous studies demonstrated that activation of the P2Y2R induces TF gene transcription in HCAEC(Ding, Ma et al. 2011, Liu, Zhang et al. 2016), we sought to determine how PSB1114 affects TF expression. Figure 2.5A shows that two pairs of primers were designed to selectively amplify the non-spliced TF pre-mRNA (also termed hnRNA) and the mature TF mRNA as we previously reported (Liu, Zhang et al. 2016). Interestingly, we found that activation of HCAEC by PSB1114 did not increase the levels of TF pre-mRNA and mature mRNA; instead, it significantly

inhibited UTP-induced upregulation of both TF pre-mRNA and mature mRNA by ~40% in HCAEC (**Fig. 2.5B & C**).

The inhibition of PSB1114 on TF promoter activity

To confirm the effect of PSB1114 on TF transcription, we assessed the impact of PSB1114 treatment on the TF promoter activity. HCAEC were transfected with the luciferase plasmid harboring the full length human TF promoter as we recently reported (Liu, Zhang et al. 2016). As shown in Figure. 2.6A, UTP stimulation promoted a ~4-fold increase in TF promoter activity in HCAEC. In contrast, PSB1114 treatment alone inhibited TF promoter activity and it also significantly suppressed UTP-induced TF promoter activity (**Fig. 2.6B**), suggesting that PSB1114 and UTP exhibit an opposite effect on TF transcription after activation of the endothelial P2Y2R.

PSB1114 activation of TF promoter repressor Fra-1, but not AP-1 activators

Our recent study demonstrated that P2Y2R utilizes different signaling pathways to control the activity of the three AP-1 components c-Jun, ATF-2 and Fra-1, fine-tuning TF expression in HCAEC (Liu, Zhang et al. 2016). Fra-1, activated by ERK1/2, functions as a new repressor on the TF promoter by competing with the two activators c-Jun and ATF-2, downstream of JNK and p38 pathways (Liu, Zhang et al. 2016). Thus, we explored which AP-1 subunits can be activated by PSB1114. As shown in Figure. 2.7B, while UTP activated all three AP-1 subunits, PSB1114 only activated Fra-1 in a time-dependent manner. Immunofluorescent study further indicates that PSB1114 stimulation of HCAEC led to increased staining of phosphorylated Fra-1 in the cell nuclei without affecting phosphorylated c-Jun and ATF-2, whereas UTP treatment increased the positive staining for all the three AP-1 subunits (**Fig. 2.7A**). These data suggest that PSB1114-activated P2Y2R biased signaling leads to a selective activation of the ERK1/2-Fra-1 negative pathway that suppresses TF transcription and TF protein expression (**Fig. 2.7C & D**).

PSB1114-induced P2Y2R signaling bias in macrophages from WT and P2Y2R-null mice

It was reported that although the endothelial TF plays a pivotal role in vascular inflammation, TF expressed in monocytes/macrophages is more important in inflammation-induced thrombosis (Drake, Cheng et al. 1993, Oeth, Parry et al. 1994). Therefore, we further examined whether the biased P2Y2R signaling mechanism we observed in HCAEC could be extended to macrophages. Figure. 2.8 A shows that by crossing the wild-type C57BL/6 mice with the P2Y2R-null mice, we successfully obtained littermate controlled male mice with or without the P2Y2R gene. We then isolated peritoneal macrophages from these mice and stimulated them with PSB1114. Figure. 2.8B shows that PSB1114 treatment significantly increased the phosphorylation level of ERK1/2, with no effect on the JNK and p38 pathways, while UTP activated all the three MAPK pathways, consistent with what we observed in HCAEC. Importantly, we found that both PSB1114 and UTP had no effect on the three MAPK pathways in macrophages isolated from the P2Y2R-null mice (**Fig. 2.8C**), indicating a specific role of the P2Y2R. In addition, we found that PSB1114 treatment alone did not induce TF mRNA expression, but it significantly suppressed UTP-induced TF mRNA expression (**Fig. 2.8D**), consistent with what we observed in HCAEC. These data suggest that PSB1114 can mediate biased signaling through the P2Y2R on different cells and species, leading to functional selectivity in receptor-controlled inflammatory gene expression.

Potential mechanism of PSB1114-induced P2Y2R biased signaling

To explore potential mechanism of PSB1114-induced P2Y2R signaling bias, we focused on the role of Gq and Gi/o proteins, which had been reported to be coupled with the P2Y2R (Liao, Seye et al. 2007). As shown in Figure. 2.9A, pretreatment of HCAEC with PTX, an inhibitor for Gi/o protein almost abolished PSB1114-induced Ca^{2+} mobilization, but it only had a ~10% inhibition on UTP-induced Ca^{2+} mobilization HCAEC, suggesting a differential contribution of Gi/o protein. In addition, we found that PTX pre-treatment also abolished PSB1114-induced ERK activation, with a minor impact on UTP-induced ERK activation (**Fig. 2.9B & C**). These data indicate that the

P2Y2R activated by UTP predominantly promotes Gq coupling, whereas PSB1114 may prompt P2Y2R selectively coupled to the Gi/o protein.

In fact, HCAECs pretreated with PTX virtually abolished PSB1114 induced ERK activation (**Fig. 2.9B & C**). From these results, we infer that PSB1114 activation of ERK1/2 mainly depends on Gαi/o protein, as PTX (100ng/ml) caused 80% reduction in ERK phosphorylation. In summary, unlike UTP, predominantly coupled to Gαq, which activates both pro- and anti-inflammatory pathways. A P2Y2R biased agonist, PSB1114 primarily coupled to Gαi/o can selectively active the anti-inflammatory ERK1/2 and AMPK pathways. Thus, PSB1114 selectively recruits Fra-1 enables the activation of P2Y2R-ERK1/2-Fra-1 axis to suppress TF gene expression in HAEACs, as shown in schematic description from (**Fig. 2.9D)**. Macrophages are important player in inflammation and coagulation, in isolated peritoneal or bone-marrow derived macrophage, PTX virtually abolished PSB1114 induced ERK activation, suggests PSB1114 mainly depends on Gαi/o on macrophages (**Fig. 2.10**).

Figures and legends

Figure 2.1

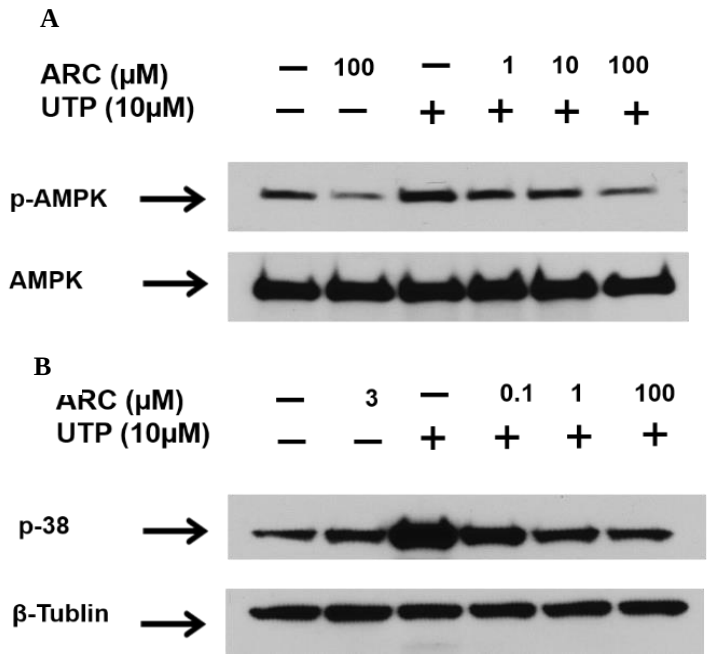


Figure 2.1. P2Y2R selective antagonist ARC-118925 antagonize UTP induced signaling.

UTP-induced AMPK phosphorylation was analyzed after HCAEC were pretreated with P2Y2R-selective antagonist ARC-118925(1-100μM) for 30 min before cell stimulation by UTP and was subjected to western blotting (**A**). UTP-induced p38 phosphorylation was analyzed after HCAEC were pretreated with P2Y2R-selective antagonist ARC-118925(0.1-100μM) for 30 min before cell stimulation by UTP and p38 activity was detected by western blot (**B**).

Figure 2.2

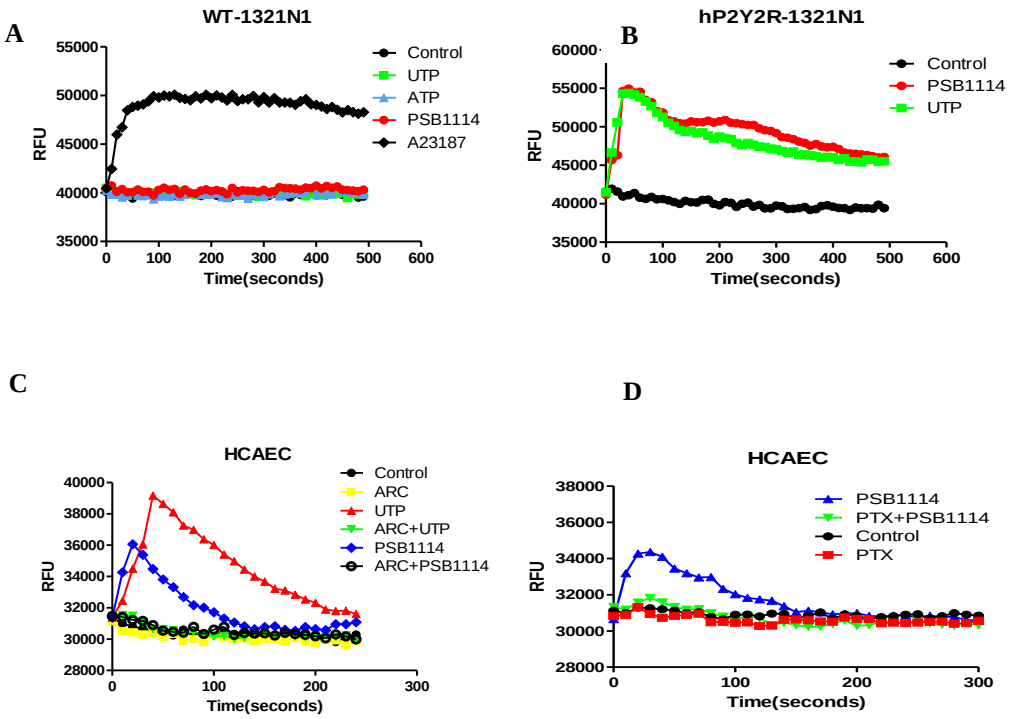


Figure 2.2. PSB1114 activation of P2Y2R mediated Ca^{2+} signaling.

P2Y2R agonist UTP (100μM)- or PSB1114 (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in wildtype 1321N1 cells which lack of any P2Y receptors. Calcium ionophore A23187 (1 μM) was used as positive control (**A**). UTP- or PSB1114-induced [Ca^{2+}] increase in 1321N1 cells transfected with human P2Y2R gene (**B**). UTP or PSB1114 -induced [Ca^{2+}] increase was determined after HCAEC were pretreated with or without P2Y2R selective antagonist ARC-118925 (3μM) for 30 min (**C**). UTP- or PSB1114- induced [Ca^{2+}] increase were determined after HCAEC were pretreated with or without PTX(100ng/ml) (**D**). Measurement of [Ca^{2+}] signal was performed by a fluorometer plate reader with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU). N=3.

Figure 2.3

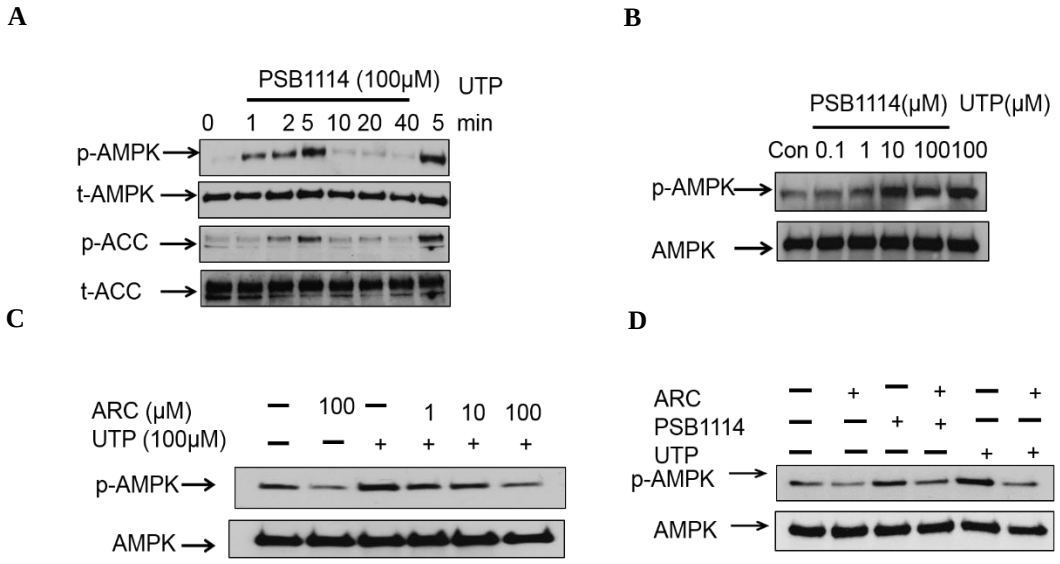


Figure 2.3. Effect of UTP and PSB1114 on P2Y2R-mediated AMPK activation.

Time-course of AMPK and its substrate ACC phosphorylation after PSB1114 stimulation of P2Y2R in HCAEC were determined by Western blotting (**A**). Dose-dependent of AMPK phosphorylation after PSB1114 stimulation of P2Y2R in HCAEC were determined by Western blotting (**B**). UTP-induced AMPK phosphorylation was analyzed after HCAEC were pretreated with P2Y2R-selective antagonist ARC-118925(1-100μM) for 30 min before cell stimulation by UTP (**C**). AMPK phosphorylation after ARC-118925 (3μM) treatment were detected and compared to that induced by classical agonist UTP (**D**). Total AMPK or ACC was used as loading controls. N=4.

Figure 2.4

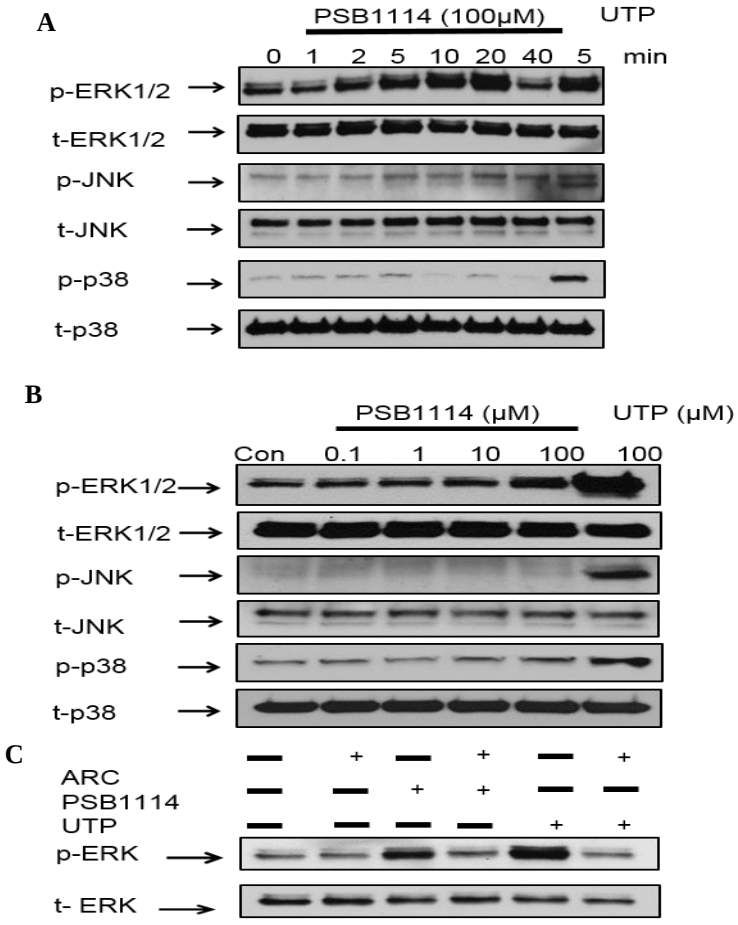


Figure 2.4. Differential effects of PSB1114 and UTP on MAPKs signaling.

Phosphorylation levels of ERK1/2, p38, and JNK after HCAEC were stimulated by PSB1114 (0.1-100μM) for 5 min (**A**). Time course (0-40min) for ERK1/2, p38, and JNK phosphorylation after HCAEC were stimulated by PSB1114 (100μM) for 1-5 min and compared to the effect induced by UTP (100 μM) treatment for 5 min (**B**). ERK1/2 phosphorylation after ARC-118925 (3μM) treatment were detected and compared to that induced by classical agonist UTP (**C**) N=3.

Figure 2.5

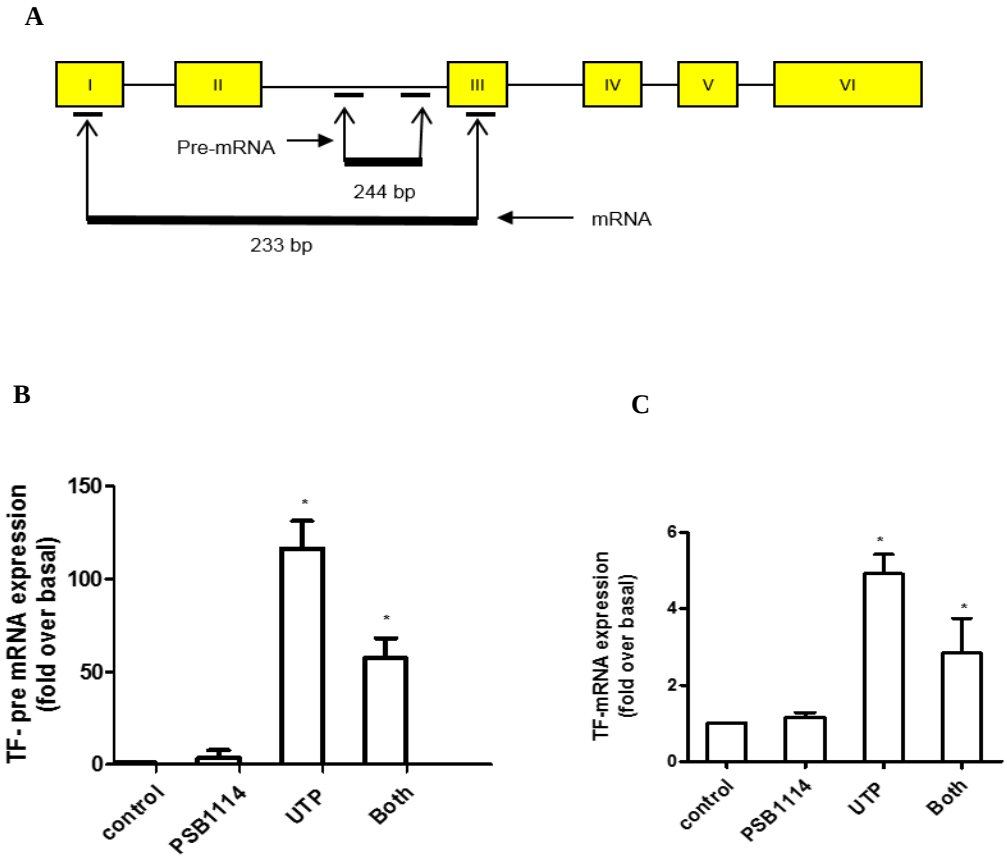


Figure 2.5. PSB1114 inhibits TF gene transcription induced by UTP in HCAEC.

Two pairs of primers were designed to quantify human TF pre-mRNA (hnRNA) and TF mature mRNA by Real-time RT-PCR respectively. Relative position of primers of the TF gene is depicted in (**A**). Effect of PSB1114 (10μM), UTP (10μM) or their co-treatment on TF pre-mRNA and mature mRNA expression in HCAEC were determined by Real-time RT-PCR. (n=5, *, $p<0.05$) (**B**).

Figure 2.6

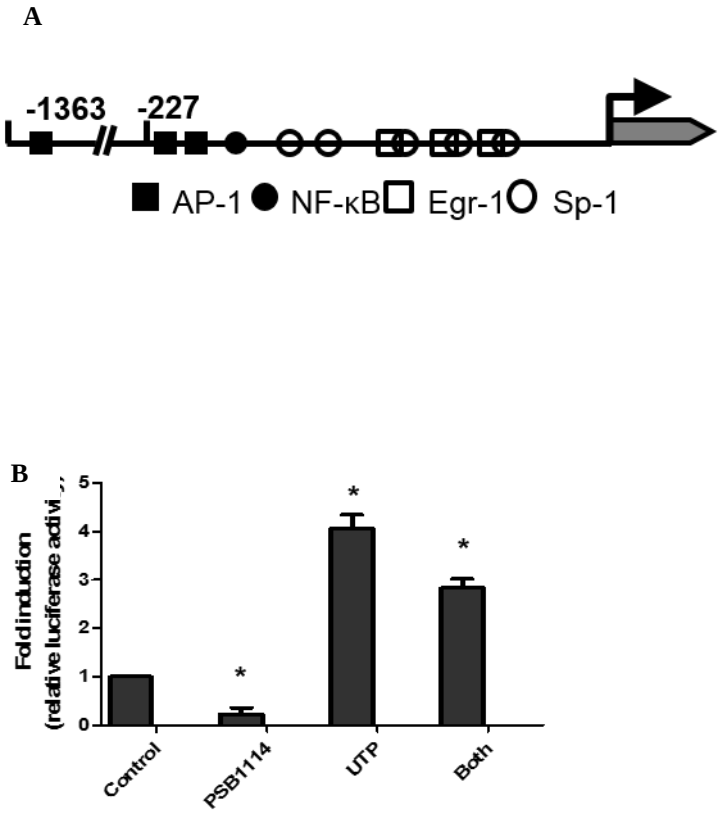
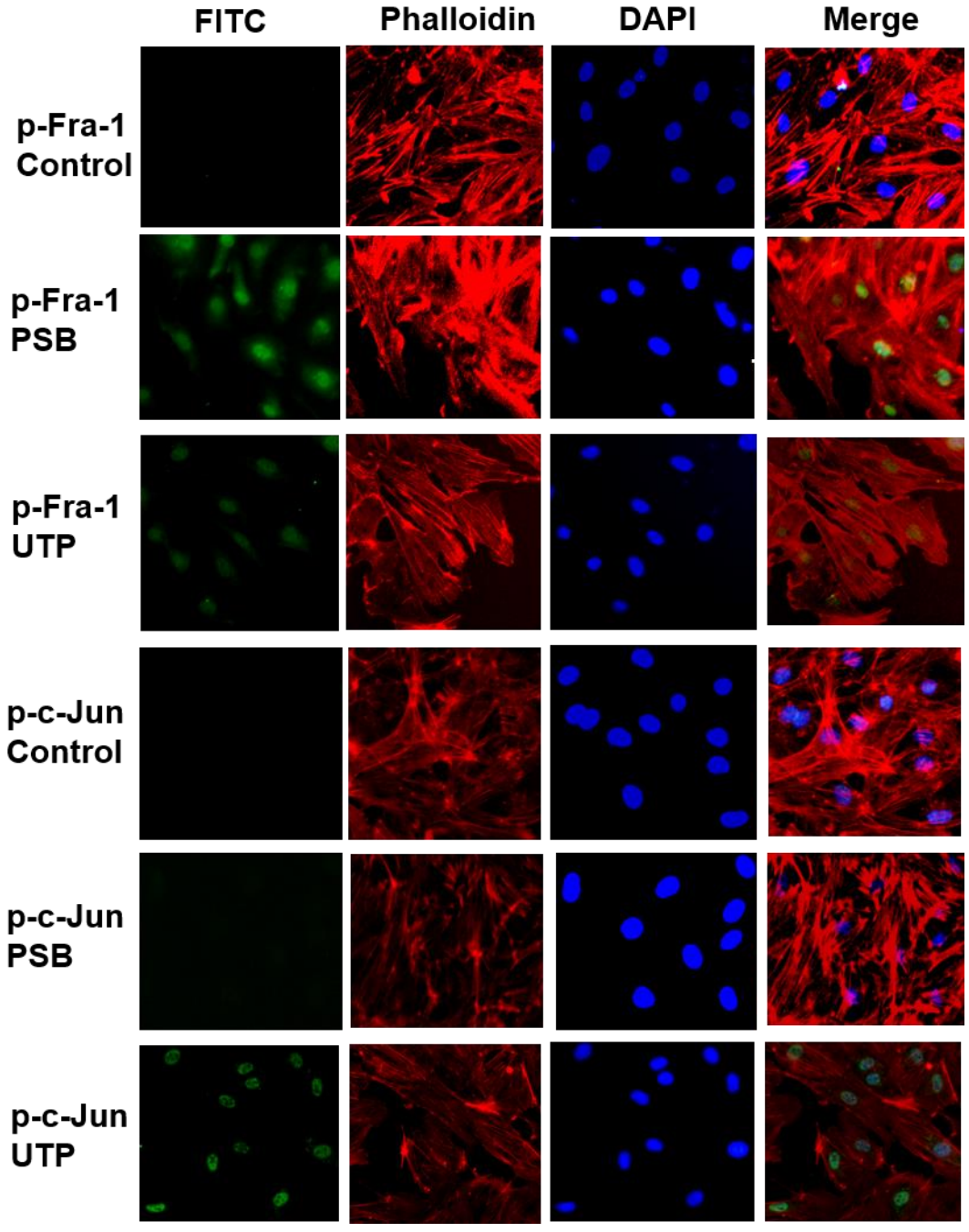
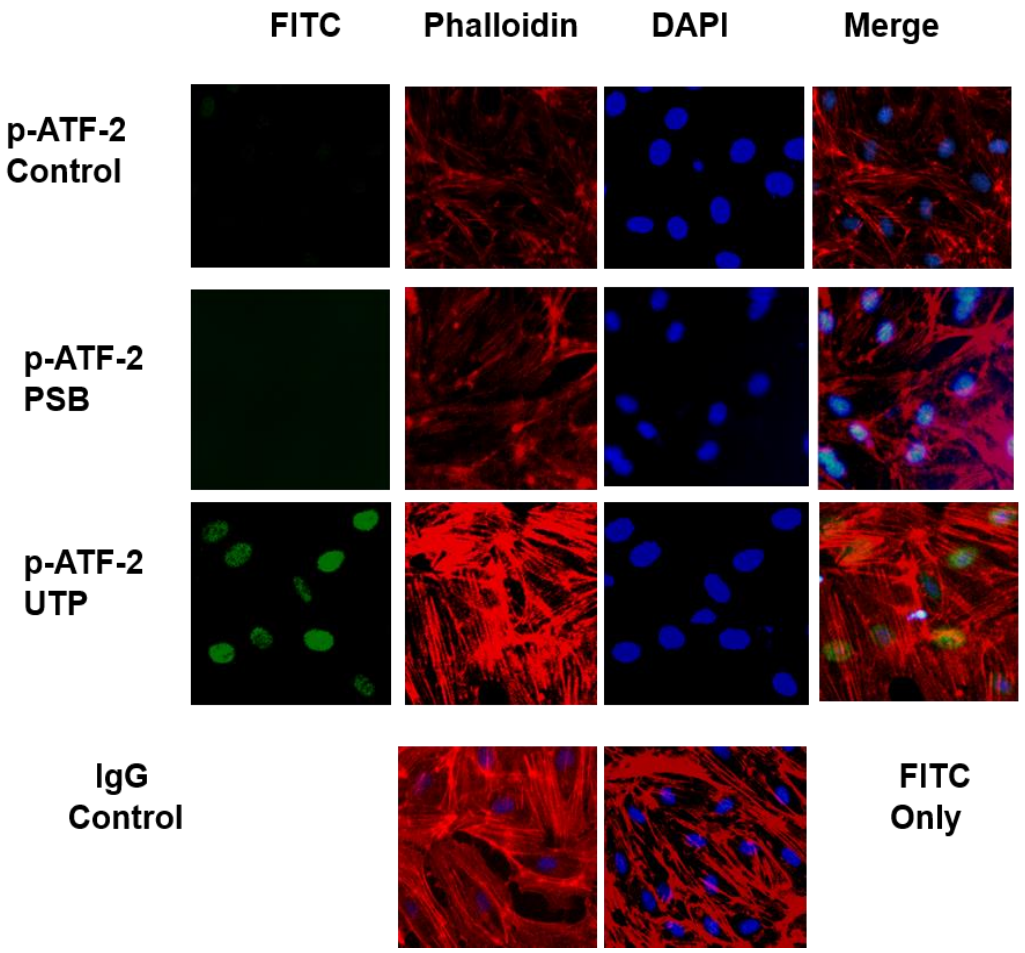


Figure 2.6. Differential effect of PSB1114 and UTP on TF promoter activity in HCAEC. Schematic description of human TF gene promoter and relevant transcription factor binding sites. A new distal AP-1 binding site at -1363bp in TF promoter was identified in our previous study (**A**). HCACE were transfected with luciferase plasmid constructs and stimulated with UTP (10μM), PSB1114 (10μM) or both for 40min, after which levels of luciferase activity relative to non-stimulated control were determined and summarized data are shown in (**B**). (n=4, *,$p<0.05$)

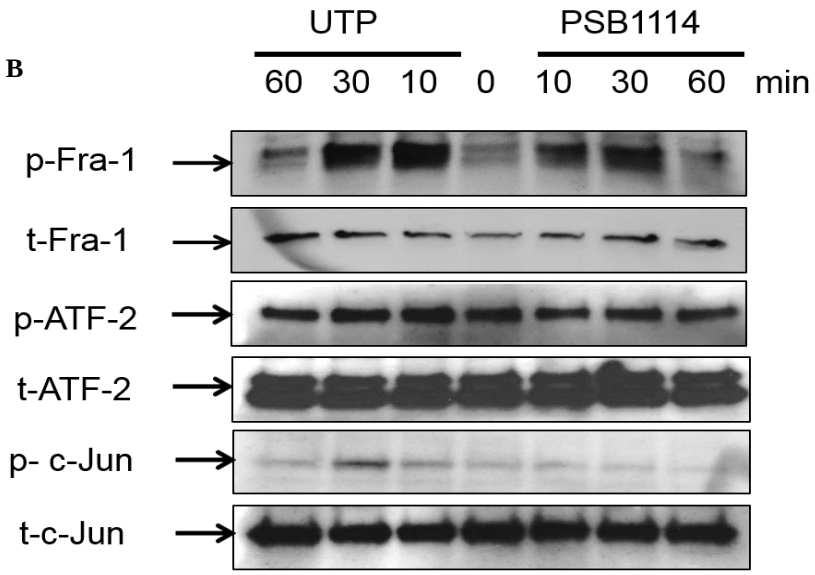
Figure 2.7

A

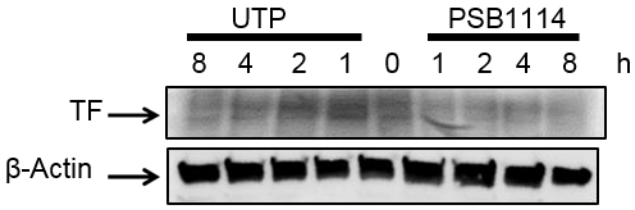

FITC
Phalloidin
DAPI
Merge
p-Fra-1 Control
p-Fra-1 PSB
p-Fra-1 UTP
p-c-Jun Control
p-c-Jun PSB
p-c-Jun UTP


FITC
Phalloidin
DAPI
Merge
p-ATF-2
Control
p-ATF-2
PSB
p-ATF-2
UTP
IgG
Control
FITC
Only

B


UTP
PSB1114
60 30 10 0 10 30 60 min
p-Fra-1
t-Fra-1
p-ATF-2
t-ATF-2
p- c-Jun
t-c-Jun

C


UTP
PSB1114
8 4 2 1 0 1 2 4 8 h
TF
β-Actin

D

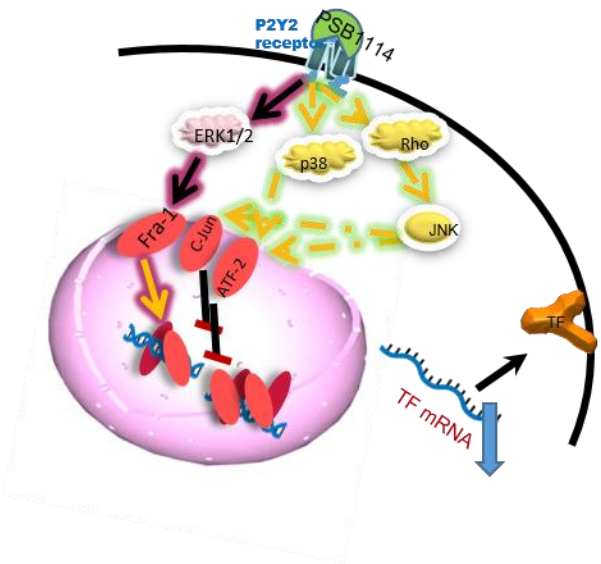

P2Y2 receptor
PSB1114
ERK1/2
p38
Rho
JNK
Fra-1
C-Jun
ATF-2
TF
TF mRNA

Figure 2.7 PSB1114 selectively activates TF promoter repressor Fra-1 in HCAEC.

HCAEC were stimulated with vehicle (control) or PSB1114 (100μM) or UTP (100μM) for 30 min before fixed for stained immunofluorescence assays. Locations of p-Fra-1, p-c-Jun, and p-ATF-2 were indicated by FITC (green). Cytoskeleton was stained with phalloidin (red) and cell nuclei were counterstained with DAPI (blue). Isotype-matched primary antibody or FITC-conjugated secondary antibody were used for negative controls. Scale bar=20μm (**A).** The time course of AP-1 subunits phosphorylation in response to UTP (10μM) or PSB1114 (10μM) were determined by Western blotting (**B**). TF protein expression in HCAEC stimulated by UTP or PSB1114 for the indicated times were shown in (**C**). A diagram showing PSB1114-mediated biased signaling via P2Y2R to the negative ERK1/2-Fra-1 pathway, leading to suppression of TF expression in HCAEC (**D**). Data represent three independent experiments.

Figure 2.8

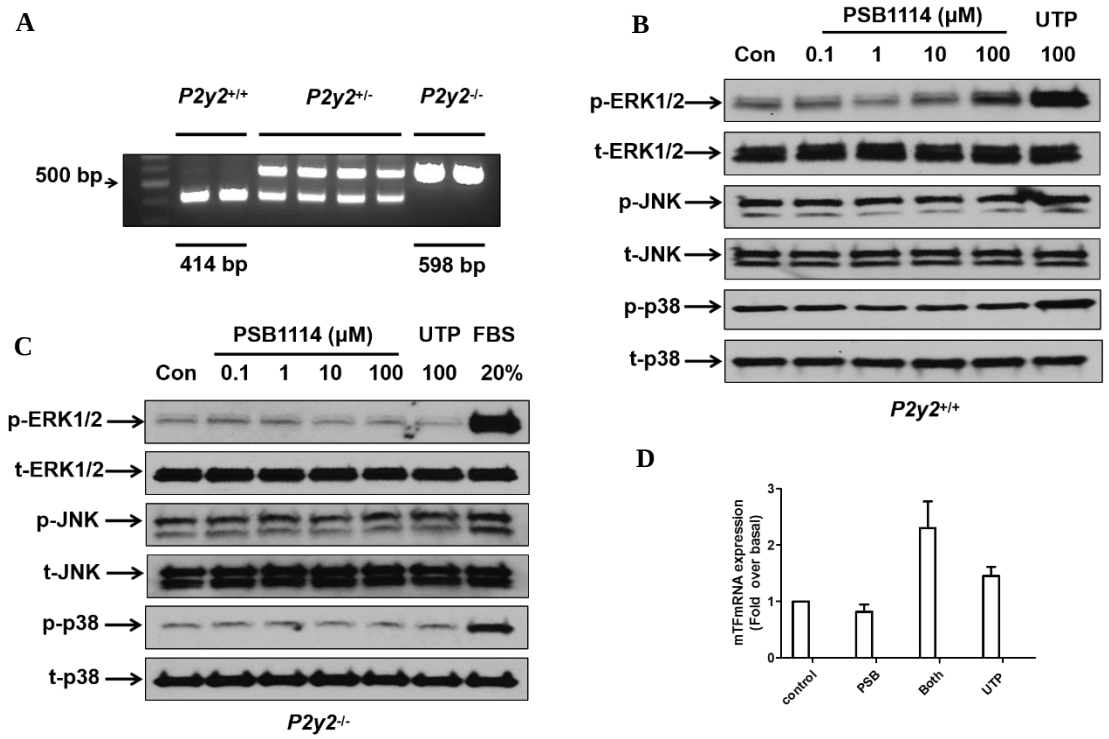


Figure 2.8 Role of P2Y2 receptor in PSB1114-induced biased signaling.

The genotypes of wide-type, heterozygous and P2Y2-null mice were determined by PCR as shown in image (**A**). Peritoneal macrophages were isolated from WT and P2Y2-null mice respectively. Phosphorylation levels of ERK1/2, p38, and JNK were determined by Western blotting after the P2Y2-positive macrophages were stimulated by PSB1114 (0.1-100 μM) for 5 min. UTP (100 μM) was used as a positive control **(B**). Phosphorylation levels of MAPKs were determined by Western blotting after P2Y2-null macrophages were stimulated by PSB1114 (0.1-100 μM) or UTP (100 μM) for 5 min. 20% FBS was used as a positive control (**C**). Effect of PSB1114 (10μM), UTP (10μM) or their co-treatment on mouse TF mature mRNA expression in HCAEC were determined by Real-time RT-PCR. (n=5, *, $p<0.05$) (**D**).

Figure 2.9

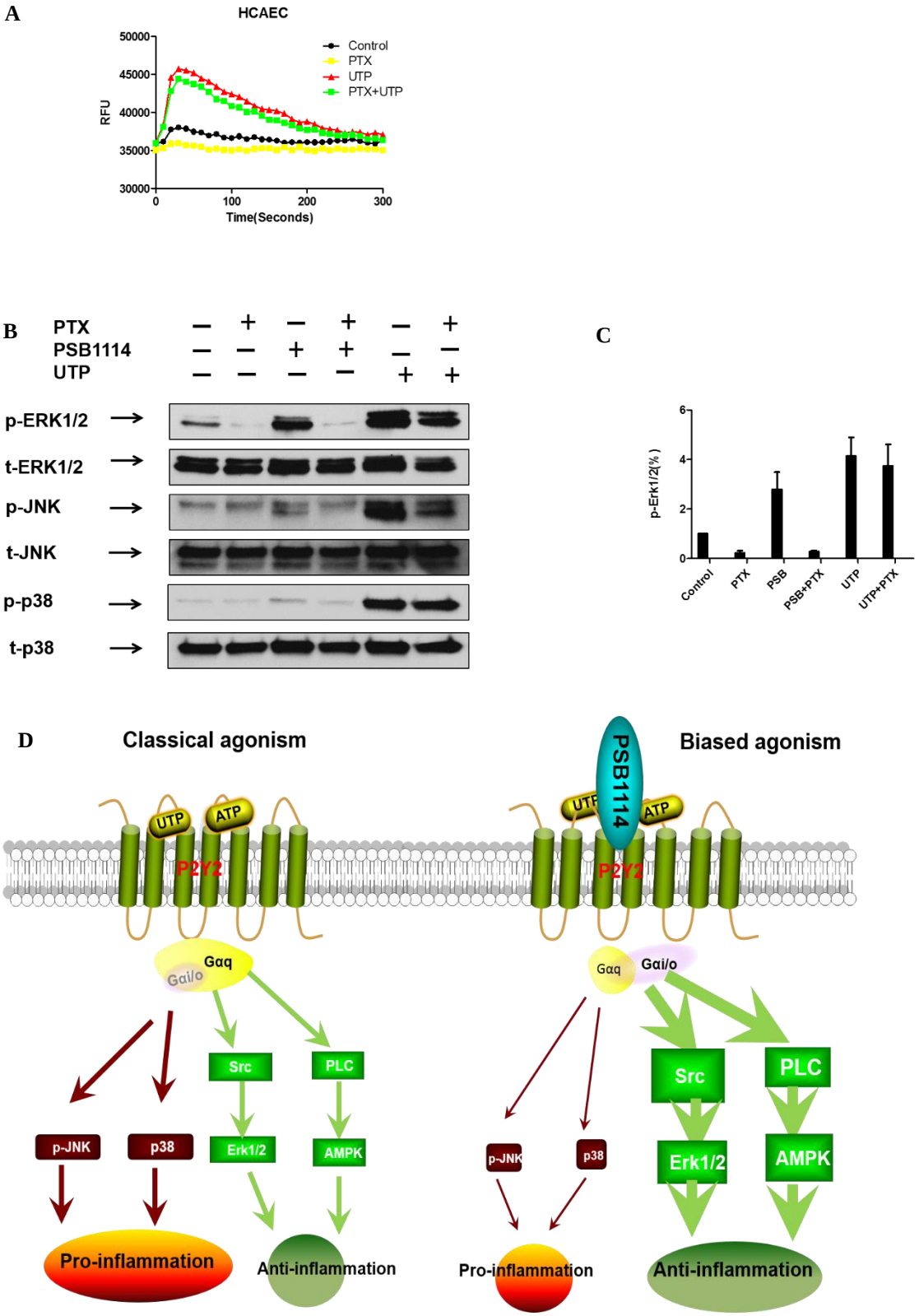

A
HCAEC
Control
PTX
UTP
PTX+UTP
RFU
Time(Seconds)
B
PTX
PSB1114
UTP
p-ERK1/2
t-ERK1/2
p-JNK
t-JNK
p-p38
t-p38
C
p-Erk1/2(%)
Control
PTX
PSB
PSB+PTX
UTP
UTP+PTX
D
Classical agonism
Biased agonism
UTP
ATP
P2Y2
PSB1114
Gαq
Gαi/o
Src
PLC
p-JNK
p38
Erk1/2
AMPK
Pro-inflammation
Anti-inflammation

Figure 2.9 Schematic description of classical signaling versus biased signaling for the P2Y2R. UTP or PSB1114 -induced [Ca^{2+}] increase was determined after HCAECs were pretreated with or without PTX(100ng/ml) (**A**). For the classical agonists such as UTP/ATP, they can induce all the signaling pathways through P2Y2R activation, but the pro-inflammatory JNK and p38 pathways may be dominant (**B**) Densitometry of above western blotting bands were quantified (n=3) (**C**). However, as a biased ligand, PSB1114 preferentially activates P2Y2R Gαi/o-mediated AMPK and ERK1/2 pathways with a minimal or no effect on the JNK and p38 pathways, leading to anti-inflammation in vascular endothelium (**D**).

Figure 2.10

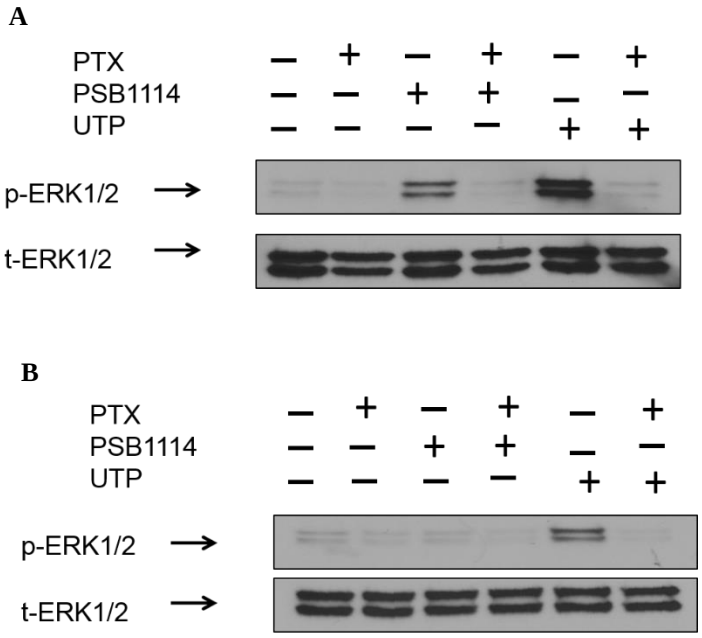


Figure 2.10 PSB1114 induced ERK1/2 signaling mechanism on mice macrophages

UTP or PSB1114 -induced ERK phosphorylation was determined after peritoneal macrophages were pretreated with or without PTX(100ng/ml) (**A**). UTP or PSB1114 -induced ERK phosphorylation was determined after bone marrow derived macrophages were pretreated with or without PTX(100ng/ml) (**B**)

Figure 2.11

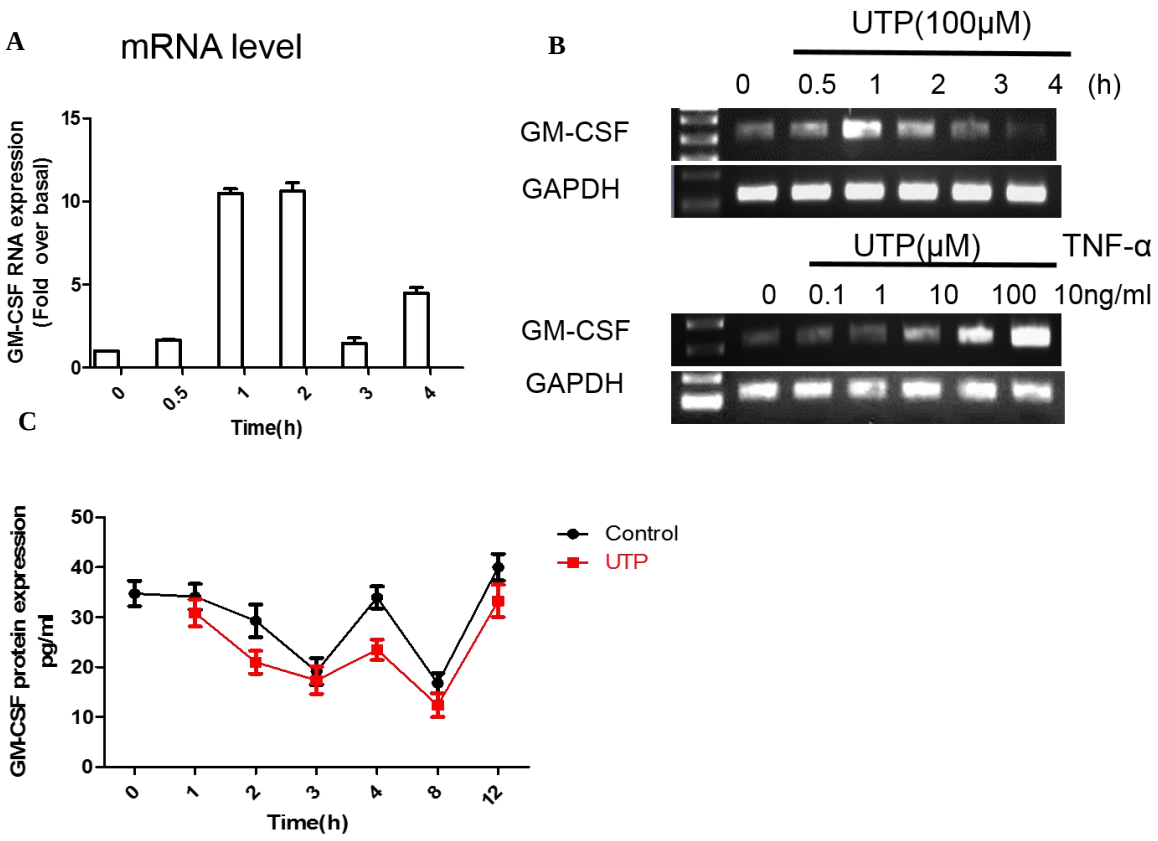


Fig. 2.11 Effect of P2Y2R activation on GM-CSF expression in HCAEC.

GM-CSF mRNA expression after UTP (100μM) stimulation of P2Y2R in HCAEC was determined by quantitative PCR relative to respective GAPDH controls (**A**, **B**). Effect of P2Y2R activation on GM-CSF protein expression in HCAEC was determined by ELISA (**C**). N=8.* $P < 0.05$.

Figure 2.12

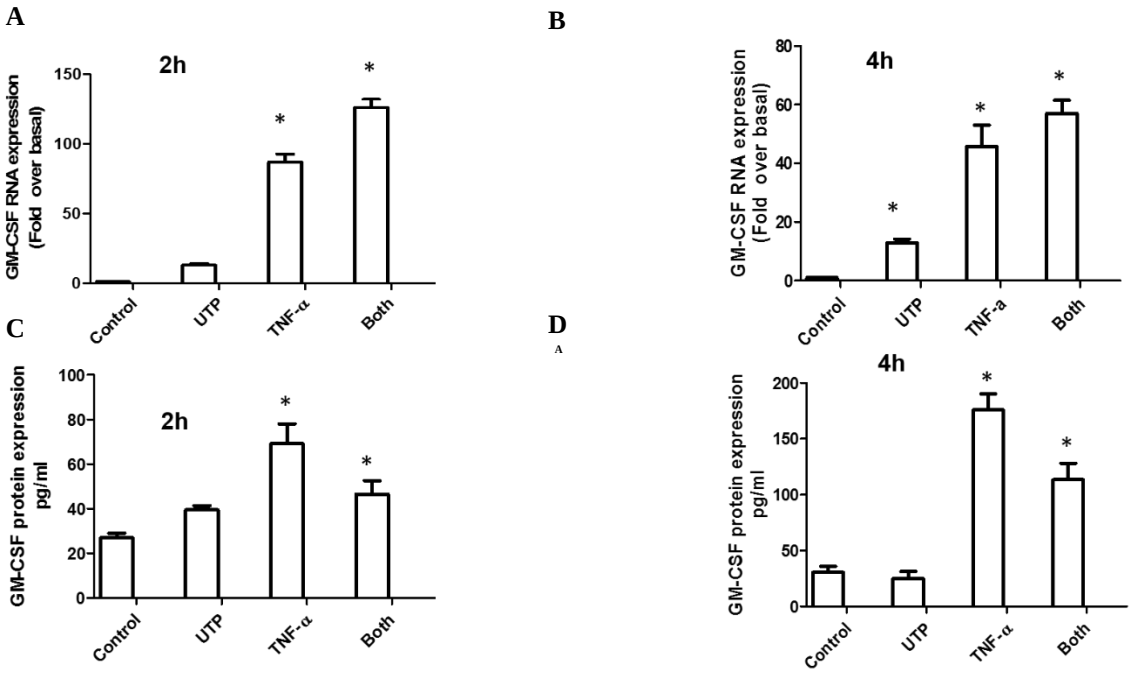


Figure 2.12 Effects of UTP and TNF-α on the mRNA levels of GM-CSF

After UTP (100μM) or TNF-α(1ng/mL) alone or both stimulation of P2Y2R in HCAEC , the effect on GM-CSF mRNA expression was determined by PCR (**A**). Effects of UTP or TNF-α alone or both on stimulation of the protein levels of GM-CSF was determined by Elisa (**B**). The data are expressed as means ± SD (n = 3) * $P < 0.05$, vs control groups.

Figure 2.13

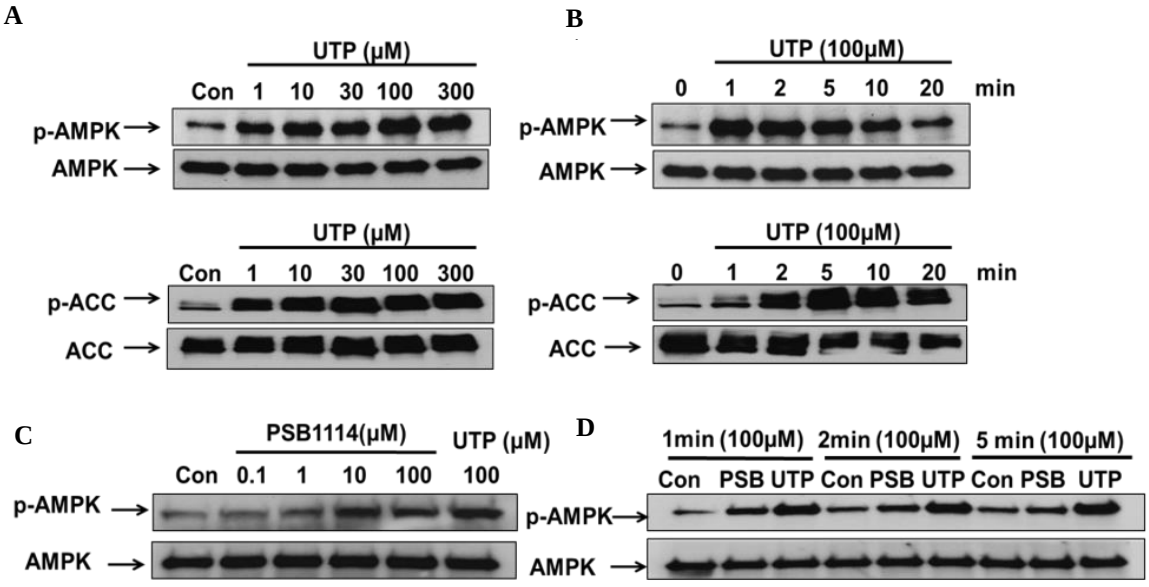


Figure2.13 Effect of UTP and PSB1114 on P2Y2R-mediated AMPK activation in HCAEC. Dose-response and time-course of phosphorylation for AMPK and its substrate ACC after UTP stimulation of P2Y2R in HCAEC was determined by Western blotting (**A&B**). AMPK phosphorylation after PSB1114 treatment were detected and compared to that induced by classical agonist UTP (**C&D**). Total AMPK and ACC as loading controls. N=4.

Figure 2.14

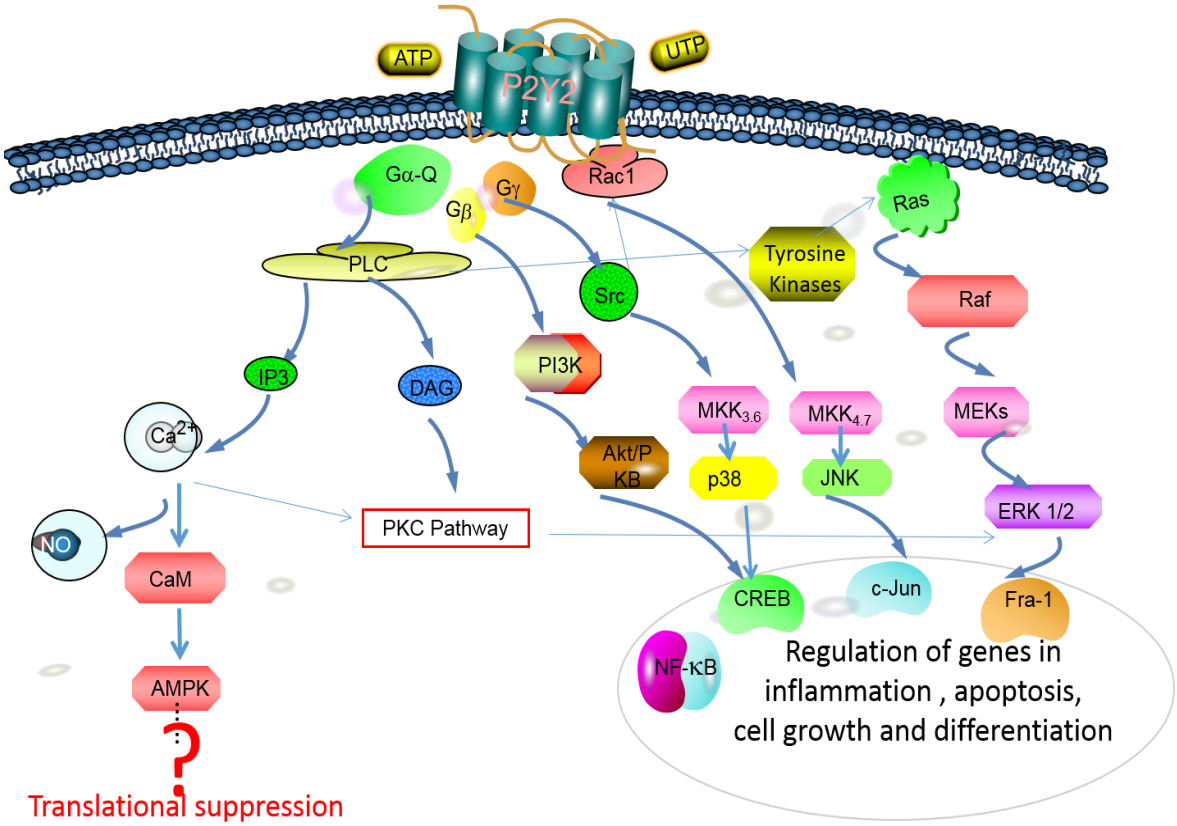


Figure2.14 Proposed mechanism of P2Y2R-AMPK axis translational suppression of inflammatory genes. Adapted from (Erb, Liao et al. 2006).

To date, P2Y receptor regulation of AMPK has not been fully studied, especially in the context of vascular inflammation evoked by P2Y2R. Therefore, we hypothesize that the P2Y2R/AMPK axis is a previously unappreciated anti-inflammatory pathway that leads to translational suppression of a set of key inflammatory proteins

2.5 Discussion

The role of P2Y2R in inflammation is still under debate, both pro-inflammation and anti-inflammation mechanisms are proposed, but previous studies hardly determine whether the P2Y2R is a 'friend or foe' (Inoue, Chen et al. 2008, Elliott, Chekeni et al. 2009). Though some studies have suggested that P2Y2R has anti-inflammation, the underlying mechanism remains unclear. Therefore, carefully dissecting post-P2Y2R signaling is critical to give us some insight on P2Y2R in control of inflammation. In HCAEC, activation of P2Y2R has been linked to signaling cascades which include IP3 accumulation, Ca^{2+} influx, and MAPKs activities. Calcium signaling downstream AMPK kinase has been associated with lower the inflammatory response, though to date, P2Y receptor regulation of AMPK has not been fully studied, especially in the context of vascular inflammation evoked by P2Y2R (Cacicedo, Yagihashi et al. 2004). Still, P2Y2R/AMPK could be an important anti-inflammatory pathway that leads to suppression of a set of key inflammatory proteins. Also, we previously discussed the multiple regulatory mechanisms of P2Y2R-MAPKs signaling and its importance in the regulation of inflammatory gene expression, such as tissue factor. P2Y2R-ERK1/2, MAPK signaling has recently been suggested as a negative regulator of TF expression through the recruitment of transcription factor Fra-1 to repress TF transcription (Liu, Zhang et al. 2016).

In order to better understand the role of the P2Y2R in inflammation and in the hope of searching for whether there exists P2Y2R biased ligands has functional selectivity. We screened several UTP analogs with modification on the ribose sugar group or phosphoric group and we found a UTP analog, PSB1114 (El-Tayeb, Qi et al. 2011). It is a novel selective agonist at the P2Y2R, but the signaling behaviors and functional characterization of which are not fully investigated. In this paper, we delineated PSB1114 biased signaling profiles and its impact on functional selectivity leads to anti-inflammatory effects via the purinergic system. Firstly, Ca^{2+} mobilization assay in wildtype or human P2Y2R-expressing 1321N1 cells and HCAEC revealed that PSB11114 is a truly P2Y2R

selective agonist and induces a dramatic increase in Ca^{2+} influx upon G protein activation. PSB1114 has similar potency and efficacy on P2Y2R Ca^{2+} signaling as UTP in transfected P2Y2R, however, it has 2/3 efficacy as UTP in HCAECs. Above data demonstrated that PSB1114 is an agonist with regards to P2Y2R Ca^{2+} signaling, but suggests a different mechanism as compared to UTP. More importantly, PSB1114 has a differential effect on MAPKs signaling. It also appears that pathway bias occurs at P2Y2R/MAPKs with PSB1114 treatment and leads to different functional outcomes. We provide lines of evidence that in contrast to UTP, PSB1114 mediated P2Y2R signaling bias with preferential activation of the ERK1/2 pathway and circumvent p38 and JNK pathways in HCAEC. Thus, in the transcriptional mechanism study by luciferase construct and immunofluorescence, PSB1114 recruits represser Fra-1 but not ATF-2 or c-Jun upon P2Y2R activation; negatively regulates TF promoter activity and inhibits TF mRNA transcription. Since PSB1114 is more prone to activate anti-inflammatory pathway and reduce pro-inflammatory signaling and this sets the first example of ligand-dependently pathway biased agonism on P2Y2R. As discussed above, our results strongly suggest that PSB1114 is a P2Y2R biased agonist and could be a new parental compound for the P2Y receptor in vascular inflammation control.

The understanding of GPCR biased signaling is ever increasing as more and more GPCR biased ligands are identified and spurred our investigation on G-protein coupled nucleotide receptors. Biased signaling can occur on other GPCRs is either G-protein dependent or G-protein independent (Rajagopal, Rajagopal et al. 2010, Smith, Bennett et al. 2011, Kenakin 2012). For example, TRV1200027, a peptide antagonist of AT1R-can induce β-arrestin- mediated but G-protein independent biased signaling, lower blood pressure but without impartment of cardiac output thus benefits acute heart failure treatment (DeWire and Violin 2011).Previous studies have shown that signaling toward the β-arrestin system have independent or reciprocal effects on GPCR-mediated activation of the MAPKs but in a low-level, longer time frame (Shenoy, Drake et al. 2006, Whalen, Rajagopal et al. 2011, Coggins, Trakimas et al. 2014). However, in our case, it is not likely

PSB1114 is a β-arrestin mediated response since the ERK1/2 phosphorylation is in a fast and transit pattern from 2min-20min. The phosphorylation of ERK1/2 by PSB1114 may be the result of joint signaling involves both Gαq and Gαi/o or independent of each other (Liao, Lu et al. 2016). Therefore, we investigated the possible involvement of P2Y2R- Gαi/o when activated by PSB1114 in HCAEC. As we demonstrated in Figure 2.9, that PSB1114 selectively is coupled to P2Y2R Gαi/o subunit in HCAEC and triggers pathway bias, induces PTX sensitive ERK signaling, but induces negligible JNK and p-38 signaling. In comparison, UTP is primarily coupled to Gαq and predominately activates pro-inflammatory p-JNK and p-38 signaling. Here, our data showing that PSB1114 is selectively targeting on P2Y2R Gαi/o-ERK/Fra-1 contributing to a significant reduction of the TF promoter activity and gene expression. This supports the notion that P2Y2R does have a protective role in inflammation; P2Y2R-Gαq and P2Y2R-Gαi/o act in opposition to modulate inflammatory signaling. PSB1114, ligand-dependently induced biased agonism to P2Y2R-Gαi/o, avoid of the pro-inflammatory effects and reserve the anti-inflammatory potential, which manipulates P2Y2R to exert beneficial effect.

Endothelial P2Y2R appears to exhibit anti-inflammatory effect by modulating Gαi/o signaling in response to PSB1114 activation as it also reduces inflammatory UTP-Gαq signaling, this may be due to its competition of binding site with UTP. Thus, PSB1114 only selectively couples to a portion of downstream signaling pathways, implying that it must induce or stabilize a receptor conformation that is distinct from the one induced by a balanced agonist UTP (Kenakin 2011, Wootten, Christopoulos et al. 2013). Such a mechanism may result in more potent P2Y2R biased ligand based on the tailored ligand over this signaling process in the future. However, there is still a lot to learn about how PSB1114 binds to the P2Y2R and induces distinct signaling behavior compared to balance agonist UTP. The replacement of the β,γ-oxygen bridge with a β,γ-difluoromethylene moiety may indicate this analog may have a different mode of binding to the P2Y2 receptor. In addition, due to the complexity of GPCR structure, makes it difficult to

understand the molecular level of P2Y2R signaling. In the future, if the transmembrane crystal structure of P2Y2R receptor is solved, we may gain novel insight from crystal structures of the P2Y2R to predict ligand mediated key molecular interaction for biased agonism (Kenakin and Miller 2010, Rajagopal, Rajagopal et al. 2010). By discovering the key residue in P2Y2R by PSB1114 mediated biased agonism, we can generate key residue mutation to probe the role of Gαi/o or Gαq of P2Y2R in driving calcium or MAPKs signaling. PSB1114 has not been studied in vivo to elucidate the impact of P2Y2 biased agonism, due to the uncertainty of PSB1114 against hydrolyzation. As a pilot study, our analyses of WT and P2Y2-null mice isolated macrophages stimulated by PSB1114 shed light on the post-P2Y2R biased signaling and functional selectivity on the primary cells. Also, the use of gene knockout mice is helpful to substantiate P2Y2R truly mediated PSB1114 induced ERK1/2 signaling. As our results suggest, P2Y2R biased agonism could be a global phenomenon in different cell/tissue types, which leads to functional selectivity.

In conclusion, we recognize for the first time that P2Y2R biased agonist PSB1114 elicits distinct profiles of signaling and inflammatory response compared to endogenous agonist UTP. Also, our studies document the transcriptional repression of TF expression by PSB1114 in endothelial cells and macrophages. This biased ligand for P2Y2R could be a template for selecting more compounds targeting the P2Y2R-ERK1/2-Fra-1 axis for therapeutic control of inflammation and spare unwanted effects. Furthermore, this work expands our understanding that the P2YR can be targeted by biased agonist against different G protein subunits and its functional outcome in inflammation control and suggests new strategies for the design of more efficient drugs targeting the P2Y receptor family. In summary, the therapeutic approach that through P2Y2 biased agonism inhibits pro-inflammatory pathways and bias toward anti-inflammatory pathways will fine-tune many inflammatory responses in health and disease.

Chapter 3. Identification of a novel biased ligand for P2Y2 Receptor to suppress pro-thrombotic gene expression by transcriptional mechanism

3.1 Introduction

We recently reported that P2Y2R is the primary endogenous nucleotide (UTP/ATP) receptor expressed in human coronary artery endothelial cells (HCAEC), and its dual role in facilitating thrombosis by up-regulation of tissue factor (TF) expression in HCAC. Interestingly, we previously found selective P2Y2R agonist PSB1114 also as a biased agonist preferentially activate the represser pathway ERK1/2-Fra-1 and transcriptional repress TF expression. There has been accumulating lines of evidence suggests that P2Y2 receptor can also elicit anti-inflammatory effect, understanding the underlying mechanism is helpful for screening other promising biased ligands for P2Y2R to exert its anti-inflammation role. Here, we for the first time report that 2'-O-methyl UTP, as a novel P2Y2R ligand also functions as biased agonist suppresses TF transcription through P2Y2 receptor. Firstly, we found treatment of 2'-O-methyl UTP on HCAEC not increase non-spliced TF pre-mRNA expression but also ameliorate TF pre-mRNA induced by UTP in HCAECs. In addition, we found that, unlike P2Y2R agonist UTP or selective agonist PSB1114, 2'-O-methyl UTP does not induce calcium mobilization but can antagonize UTP induced P2Y2R downstream Gαq signaling. Interestingly, using molecular pharmacological method to carefully probe 2'-O-methyl UTP signaling profiles, we observed it confers a bias toward ERK1/2 signaling over Gαq signaling in a time- and dose- dependent manner, with a 60% potency as compared to UTP. Importantly, ERK1/2 phosphorylation can be abolished by selective P2Y2R antagonist AR-C118925 to the basal level, which further validated that 2'-O-methyl UTP exerts its action through P2Y2R. The activation of ERK1/2 following recruits the represser AP-1 subunit Fra-1. In addition, we dissected the effect of 2'-O-methyl UTP on other pro-inflammatory MAPKs, Rho/JNK and

Src/p38 and AP-1 subunits, c-Jun and ATF-2 positive regulators of TF transcription are not activated on HCAECs. Therefore, 2'-O-methyl UTP is not just a simple classical P2Y2 agonist, but a novel biased ligand for P2Y2R, and can lead to functional selectivity. It turns out that 2'-O-methyl UTP suppress TF promotor activity and mRNA transcription induced by UTP on both HACECs and murine macrophages. To further delineate the mechanism of 2'-O-methyl UTP-P2Y2R biased agonism, we found 2'-O-methyl UTP coupled to P2Y2R-Gαi/o-ERK axis to regulate TF expression regulation on HCAECs. In summary, we found 2'-O-methyl UTP as a novel P2Y2R biased agonist to be a promising parental compound for P2Y2R receptor drug discovery and understanding the biased agonism of P2Y2R.

3.2 Material and methods

3.2.1 Materials

Dulbecco's Modified Eagle medium (DMEM) and EBM-2 were purchased from Lonza. FluoForte™ Kit was purchased from Enzo Life Sciences. Fetal Bovine Serum (FBS) was purchased from Thermo Fisher Scientific. DNA primers were purchased from Integrated DNA Technologies. EcoRV was purchased from Takara Bio Inc. PSB1114 and AR-C118925 were purchased from Tocris Bioscience. Purified UTP was obtained from Sigma. Triton X-100 was obtained from Sigma.

3.2.2 Knock-out (KO) mice.

Wild-type (C57BL/6) and P2Y2R KO ($P2Y2R^{-/-}$) on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor) and bred at the animal facility at Auburn University. DNA extraction from mouse tail and PCR genotyping were routinely performed according the instruction of the animal supplier. Mouse P2Y2R genotyping primers are the following: wild-type forward: 5'-AGC CAC CCG GCG GGC ATA AC-3'; mutant forward: 5'- AAA TGC CTG CTC TTT ACT GAA GG-3'; common reverse: 5'-GAG GGG GAC GAA CTG GGA TAC-3'. Mice were housed

in Auburn University's Biological Research Facility in a controlled and pathogen-free environment (25 °C; 12:12-h light-dark cycle) with free access to water and standard chow diet.

3.2.3 Cell culture.

HCAEC were cultured in EBM-2 medium supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA1000 (Lonza), and 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. HCAEC were used between the third and eighth passages. Before stimulation, cells were seeded to grow for 24 h and starved overnight. Where inhibitor or antagonist was used, cells were pretreated with the inhibitor/antagonist for 40 minutes before cell stimulation. Wild-type and P2Y2R-transfected 1321N1 cell lines were kindly offered by Dr. Gary Weisman (The University of Missouri-Columbia). Wild-type and P2Y2R-transfected 1321N1 cells were cultured in DMEM supplemented with 10% FBS with or without G418 (0.5 mg/mL).

3.2.3.1 Isolation of murine peritoneal macrophages.

All animal study protocols were approved in advance by the Institutional Animal Care Use Committee at Auburn University. Details on the isolation of mouse peritoneal macrophages can be found in our previous study(Ma, Liu et al. 2014) and others (del Rey, Renigunta et al. 2006). Briefly, mice were injected with thioglycollate medium (3% in PBS, 1mL, i.p.) into their peritoneal cavity three days prior to cell harvest. Mice were anesthetized and sacrificed by cervical dislocation, and the peritoneal cells were collected by lavage with cold PBS. After centrifugation of peritoneal fluid, the supernatant was discarded and the cell pellet was re-suspended in cold DMEM containing 10% FBS. A total of $2X10^6$ cells/well were seeded into the 6-well plate and cultured for 3 hours at 37°C. Non-adherent cells were removed by gently washing three times with the medium. Before cell stimulation, macrophages were grown in DMEM and starved overnight. For the $P2Y2R^{-/-}$ cells, stimulation of the cells with 10% FBS for 5 min was used as a positive control.

3.2.3.2 Generation and culture of bone marrow-derived macrophages

All animal study protocols were approved in advance by the Institutional Animal Care Use Committee at Auburn University. Details on the isolation of mouse peritoneal macrophages can be found in our previous study(Ma, Liu et al. 2014) and others (del Rey, Renigunta et al. 2006). Briefly, mice were injected with thioglycollate medium (3% in PBS, 1mL, i.p.) into their peritoneal cavity three days prior to cell harvest. Mice were anesthetized and sacrificed by cervical dislocation, and the peritoneal cells were collected by lavage with cold PBS. After centrifugation of peritoneal fluid, the supernatant was discarded and the cell pellet was re-suspended in cold DMEM containing 10% FBS. A total of 2 X 10^6 cells/well were seeded into the 6-well plate and cultured for 3 hours at 37°C. Non-adherent cells were removed by gently washing three times with the medium. Before cell stimulation, macrophages were grown in DMEM and starved overnight. For the P2Y2R$^{-/-}$ cells, stimulation of the cells with 10% FBS for 5 min was used as a positive control.

3.2.4 Intracellular Ca^{2+} mobilization assay

Cells were seeded at a density of 4x10^4 cells per well into 96-well culture plates and cultured for one day. On day two, the original medium was removed and the assay medium from FluoForte™ kit (Enzo Life Sciences) containing the Ca^{2+} dye was added and receptor-mediated Ca^{2+} mobilization was determined as previously described (Ding, Ma et al. 2011). Fluorescence was determined immediately after adding of different reagents, with an excitation wavelength set to 485 nm and an emission wavelength set to 525 nm, and readings were taken every 1s for 500s. For antagonist inhibition experiment, cells were pre-incubated with the antagonist for 30 min before agonist addition. Measurement of Ca^{2+} signal was performed with the fluorometer plate reader (BMG FLUOstar) with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU).

3.3.5 General and RT-PCR Analysis.

Total RNA and DNA were extracted from HCAEC and macrophages using the RNeasy and DNeasy kits, respectively (Qiagen). For the synthesis of the first strand of cDNA, 1 μg of total RNA after DNase (Ambion) treatment was reverse transcribed using a cDNA synthesis kit (Applied Biosystems). The cDNA samples were then amplified by PCR using 2.5 units of TaqDNA polymerase (Qiagen). Real time PCR was performed on an iCycler iQ5 detection system (Bio-Rad) with SYBR Green reagents (Qiagen). The sequences of primers are : human TF mature mRNA : Forward: 5'-ACGCTCCTGCTCGGCTGGGT-3', and Reverse : 5'-CGTCTGCTTCACATCCTTCA-3'; human TF pre-mRNA: Forward :5'-CCCCTGGGTTGC-TATGAGG-3'; Reverse : GCGT5'CTTGGTCCGGCTGTGTGC-3' ; human GAPDH: Forward: 5'-TCAACAGCGACACCCACTCC-3', Reverse :5'-TGAGGTCCACCACCCCTGTTG-3'; mouse TF mature mRNA : Forward: 5'- ATGGCGATCCTCGTGCGCCCG-3'; Reverse: 5'-CTATGCCAAGCGCGACGGGGTG-3' ; mouse β-actin : Forward:5'-ATGGATGACGA-TATCGCTGCG-3' ; Reverse: 5'- CTAGAAGCACTTGCGGTGCAC-3'.

3.2.6 Western Blotting Assay.

After stimulation, cells were lysed, and standard Western blotting assay was performed as previously described (Ding, Ma et al. 2011). The individual primary antibodies used were anti-p-AMPKα, anti-p-ERK1/2, anti-p-p38, anti-p-SAPK/JNK, anti-p-c-Jun, anti-p-Fra-1, anti-p-ATF-2, and anti-AMPKα, anti-ERK1/2, anti-p38, anti-SAPK/JNK, anti-c-Jun, anti-Fra-1, anti-ATF-2 (Cell Signaling Technology). Equal protein loading was verified by stripping off the original antibodies and re-probing the membranes with a different primary antibody such as anti-β-actin (Cell Signaling Technology). Pertussis toxin (PTX) treatment (100 ng/ml, overnight) was used to suppress Gαi/o activity, after which cells were stimulated and lysed for Western blotting assay.

3.2.7 Tissue Factor Promoter Constructs.

Promoter constructs encompassing the region from -1427 to +207 relative to the transcription starting site of the human TF gene were amplified from human genomic DNA using specifically designed forward and reverse primers containing the EcoRV restriction enzyme site as we previously described (Liu, Zhang et al. 2016). Forward primer: 5'- GCT AGC CTC GAG GAT ATC CTA CCT TCA ATC CCA GAG-3'; Reserve primer: 5'-AGG CCA GAT CTT GAT ATC TCC ATG TCT ACC AGT TGG CG-3'. The promoter fragment was cloned into the luciferase vector pGL4.12 (Promega) with the purchased In-Fusion HD EcoDry Cloning kit (Clontech). The plasmid was amplified in and purified from Escherichia coli, after which successful DNA insertion was verified by sequencing.

3.2.8 Plasmid Transfection and Luciferase Activity Assay

HCAEC were seeded into 96-well plates and transiently transfected with the pGL4.12 vector construct with Xfect Transfection Reagent (Clontech) following the manufacturer's instruction. Transfection efficiency was normalized by co-transfection with a plasmid containing the β-galactosidase gene driven by the SV 40 promoter (Promega). β-galactosidase activity was measured by β-galactosidase Enzyme Assay System (Promega). Luciferase activity was measured for reporter expression in triplicate according to the instruction provided in the Luciferase Reporter Assay System (Promega).

3.2.9 Immunofluorescence

HCAEC were seeded into 8-chamber glass slides (Nunc), starved overnight, and then treated with vehicle, PSB1114 or UTP. After the 30-minute treatment, the medium was aspirated, and cells were then fixed with 4% paraformaldehyde for 15 minutes and washed with PBS for three times and blocked with 5% horse serum for 1h at room temperature. Then the cells were incubated with rabbit monoclonal antibodies to human p-Fra-1, p-c-Jun, or p-ATF-2 (1:50) (Cell Signaling Technology), overnight at 4°C followed by incubation with FITC-conjugated anti-rabbit IgG (1:200) (KPL) for

90 minutes at room temperature in darkness. For negative controls, cells were incubated with non-immune rabbit IgG in place of specific primary antibody or only the FITC-conjugated secondary antibody. Cells were then washed with PBS three times and incubated with Phalloidin Red 594 (BioLegend) for 40 min. Finally, mounting medium containing DAPI was added to seal the slides. Images with fluorescent signals in random fields were acquired and captured using an AMG EVOS digital inverted multi-functional microscope (AMG).

3.2.10. Data analysis

All data were analyzed by Prism 4 (Graphpad Software Inc.). Data are expressed as the mean ± S.E.M. The means of two groups were compared using Student's t-test (unpaired, two-tailed), and one-way analysis of variance was used for comparison of more than 2 groups with $p<0.05$ considered to be statistically significant. Unless otherwise indicated, all experiments were repeated at least three times.

3.3 Results

2'-O-ME UTP actions through P2Y2R and mediates MAPK-ERK1/2 signaling.

To investigate whether 2'-O-ME UTP is a ligand for P2Y2R and is capable of increasing Ca^{2+} mobilization in HCAECs. Calcium signaling of 2'-O-ME UTP was compared to the endogenous agonist UTP. As our data suggested in HCAEC, 2'-O-ME UTP does not induce Ca^{2+} signaling as UTP but can inhibit UTP induced Ca^{2+} signaling to 50% (**Fig. 3.1A**). Though 2'-O-Me-UTP does not induce Ca^{2+} signaling, we unexpectedly found that 2'-O-Me-UTP activated ERK1/2 as evidenced by increased ERK1/2 phosphorylation. ARC-118725 has been described as a selective antagonist at the P2Y2 receptor and is used to characterize the functional role of 2'-O-ME UTP on P2Y2R (Rafehi, Burbiel et al. 2016). Further study confirmed that the stimulatory action of ERK1/2

by 2'-O-Me-UTP is through P2Y2R, since this effect was abolished by selective P2Y2R antagonist ARC-118925 (**Fig. 3.1B**).

Differential effects of 2'-O-Me-UTP and UTP on MAPKs signaling in HCAEC.

However, though 2'-O-Me UTP can activate ERK1/2 in a concentration- and time- dependent manner, it had no effect on the phosphorylation of JNK and p38 pathways (**Fig. 3.2A & B**). These results indicate that 2'-O-Me-UTP is a novel biased ligand of P2Y2R, which can selective activate the anti-inflammatory ERK1/2 pathway but not pro-inflammatory JNK and p38 MAPKs in HCAEC.

Biased agonism of 2'-O-ME UTP - mainly coupled to P2Y2R-Gαi/o subunit

UTP can mediate both Gαq and Gαi/o protein-dependent signaling activities at the P2Y2 receptor, to fully characterize the mechanism of 2'-O-ME UTP biased agonism on P2Y2R and its engagement of different heterotrimeric G proteins, we pretreated HCAECs with PTX (100ng/ml) to inhibit the Gαi/o signaling. As shown in (**Fig. 3.3A**), PTX only inhibits 10% ERK1/2 signaling when stimulated by UTP in HCAEC. In fact, HCAECs pretreated with PTX virtually abolished 2'-O-ME UTP induced ERK activation, 90% ERK1/2 activation mediated by the 2'-O-ME UTP was inhibited. (**Fig. 3.3B & C**). In summary, 2'-O-ME UTP primarily coupled to Gαi/o can selectively active the anti-inflammatory ERK1/2 pathway and antagonize UTP induced Ca^{2+} signaling.

2'-O-ME UTP inhibits the expression of TF-induced by UTP

The activation of P2Y2 receptor triggers multiple signaling cascades in HCAEC, and the physiological significance of each pathway needs to be elucidated. As we have recently identified that P2Y2/ERK1/2 is an important negative repressor pathway to suppress TF mRNA transcription, and P2Y2R-JNK, p38 pathways have been established as major positive regulator pathways for TF transcription in HCAEC (Liu, Zhang et al. 2016). As our aforementioned assays indicated that 2'-

O-ME UTP mediated pathway bias for ERK1/2-MAPKs via P2Y2R, to further determine whether this pathway bias among MAPKs can be translated into a functional response, we set out to test effects of 2'-O-ME UTP on the TF mRNA transcription as compared to UTP. In Figure 3.4B shows that activation of HCAEC by 2'-O-ME UTP does not increase TF expression levels of pre-mRNA and mature RNA, whereas induction of both TF pre-mRNA and mature mRNA by UTP stimulation of the P2Y2R is inhibited 60% by 2'-O-ME UTP treatment. Above data suggest that 2'-O-ME UTP as a biased ligand on P2Y2R leads to functional selectivity.

The inhibition of 2'-O-ME UTP on TF promoter activity.

To confirm the effect of 2'-O-ME UTP on TF transcription, we assessed the impact of PSB1114 treatment on the TF promoter activity. HCAEC were transfected with the luciferase plasmid harboring the full length human TF promoter as we recently reported (Liu, Zhang et al. 2016). As shown in Figure. 3.5B, UTP stimulation promoted a ~2-fold increase in TF promoter activity in HCAEC. In contrast, PSB1114 treatment alone inhibited TF promoter activity and it also significantly suppressed UTP-induced TF promoter activity, suggesting that 2'-O-ME UTP and UTP exhibit an opposite effect on TF transcription after activation of the endothelial P2Y2R.

2'-O-ME UTP/P2Y2R differentially activation on AP-1 subunits repress TF gene transcription

Our recent study suggests that P2Y2R utilizes different signaling pathways to control the activity of the three AP-1 components. Fra-1 negatively repress TF transcription while c-Jun and ATF-2, downstream of p38 and JNK positively regulate TF transcription (Liu, Zhang et al. 2016). As shown in Figure 3.6B, compared to UTP activating all three of AP-1 subunits, 2'-O-ME UTP only activated Fra-1 after 30 min treatment but let alone either c-Jun or ATF-2. compared the immunofluorescent images to determine the location of Fra-1, c-Jun, and ATF-2 between no treatment, 2'-O-ME UTP, or UTP treatment for 30min. In control groups without stimulation, very

little FITC signal was detected for three phosphorylated transcription factors. In UTP treatment group, high level of phosphorylated c-Jun, Fra-1, and ATF-2 all emitted intense FITC signals. However, in the 2'-O-ME UTP treatment group, we observed strong phosphorylated Fra-1 signals compared to UTP treatment group, but less intense phosphorylated c-Jun or ATF-2 signal than UTP. As shown in the merge images, overlaying with DAPI and Phalloidin, the majority of the phosphorylated Fra-1 accumulated in nucleus but not p-c-Jun or p-ATF-2 **(Fig. 3.6A**).

Ligand-mediated signaling bias at the P2Y2R in macrophages from WT and P2Y2-/- mice

It is noteworthy that proposed biased ligand 2'-O-ME UTP unbalanced activate post-P2Y2R signaling in HCAEC and inhibits inflammatory progress by regulating TF mRNA expression. To further explore the contribution of P2Y2 biased signaling in vivo relevance, we studied the macrophages which are of importance in inflammation process by isolated peritoneal macrophages from WT mice and mice lacking P2Y2R (**Fig. 3.7A**). By using the P2Y2 receptor deficient mice and RT-PCR analyses, we show that P2Y2 receptor is expressed in resident peritoneal macrophages and bone-marrow derived macrophage and also as reported in previous research(del Rey, Renigunta et al. 2006). We applied 2'-O-ME UTP (100μM) on isolated peritoneal macrophages, this caused a significant enhancement of ERK1/2 phosphorylation in wildtype but not in $P2Y2^{-/-}$ macrophages. In contrast, phosphorylation of JNK and p-38 pathways were not different between $P2Y2^{-/-}$ and WT mice (**Fig. 3.7B**). These results substantiate the above evidence that 2'-O-ME UTP truly acts through the P2Y2R, and indicates that signaling bias also exists on P2Y2R in primary peritoneal macrophage from murine species. In bone marrow derived macrophages, P2Y2R mRNA expression can be detected from Day 3 to Day 5 when treated with GM-CSF (20ng/ml) (**Fig. 3.8A**). 2'-O-Me UTP can activate ERK1/2 in a concentration- dependent manner on derived macrophages on Day5 (**Fig. 3.8B**), and 2'-O-ME UTP itself did not induce tissue factor mRNA expression, but repressed UTP treatment-induced tissue factor increase to basal level (**Fig. 3.8C**). This suggests 2'-

O-ME UTP is novel biased P2Y2R agonist leading to functional selectivity and thus leads to suppression of inflammation mediator expression on multiple cell types.

Figure and legends

Figure 3.1

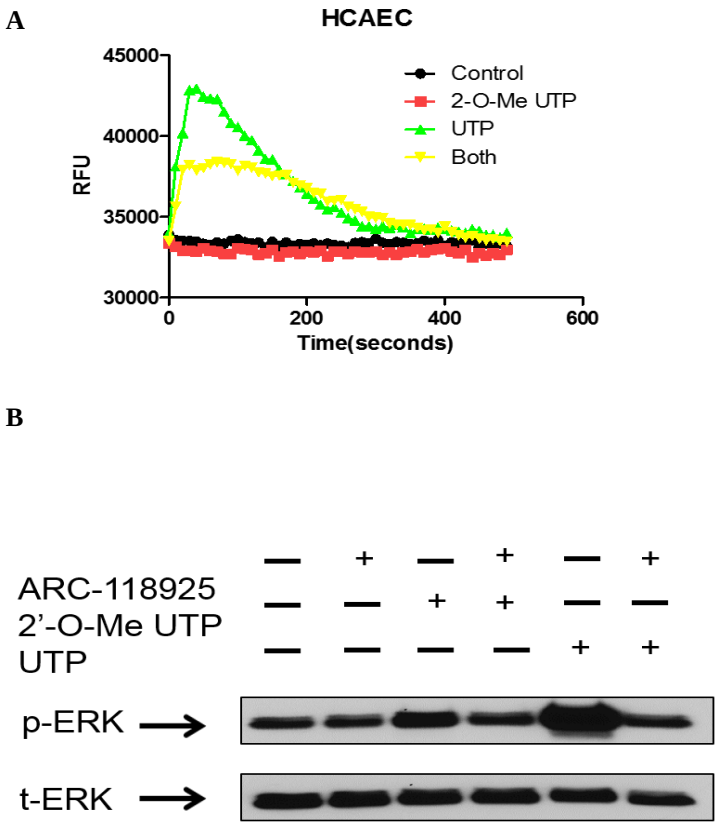


Figure 3.1. 2'-O-Me-UTP actions through P2Y2R and mediates MAPK-ERK1/2 signaling. UTP- induced [Ca^{2+}] increase was determined after HCAECs were pretreated with or without 2'-O-Me-UTP treatment (**A**). Effect of ARC-118925 (3μM) on 2'-O-Me-UTP- and UTP-induced ERK1/2 phosphorylation (**B**). N=4.

Figure 3.2

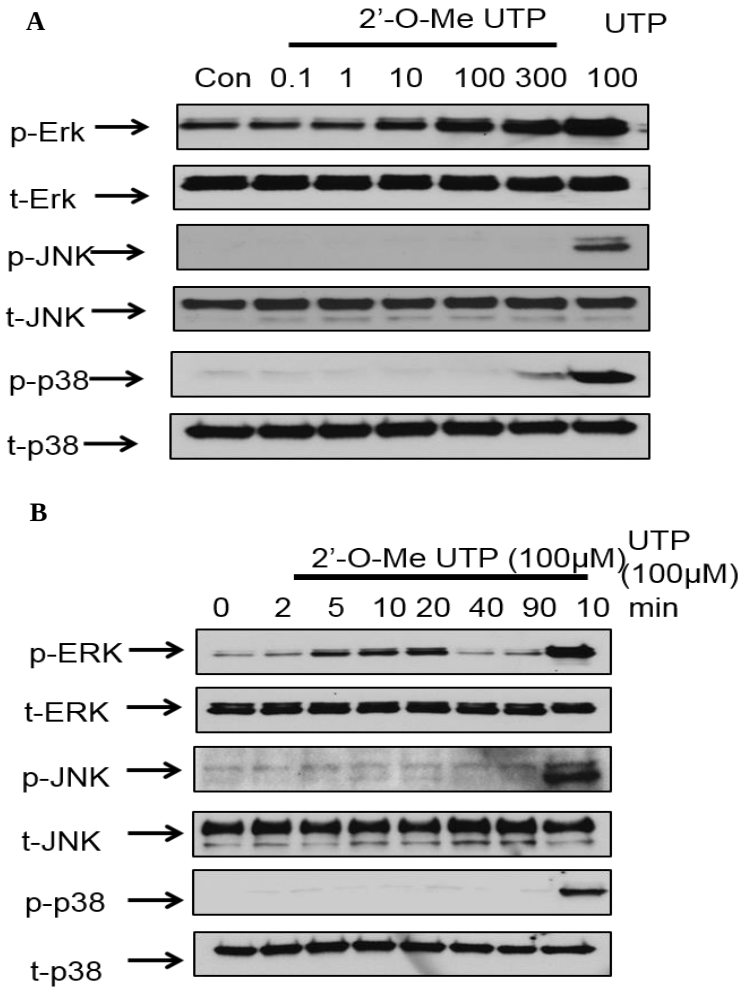
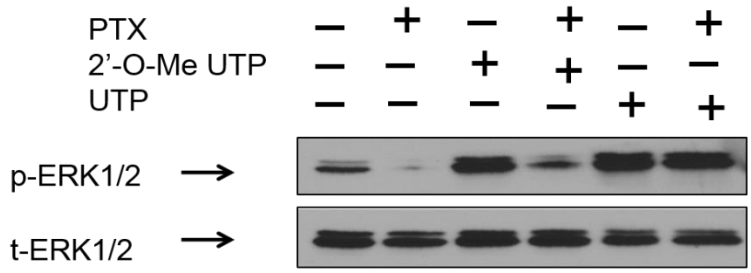


Figure 3.2. Differential effects of 2'-O-Me-UTP and UTP on MAPKs signaling in HCAEC. Time-course of ERK1/2, p38, and JNK phosphorylation after HCAEC were stimulated by 2'-O-Me-UTP (100 μM) for indicated times. UTP as a control (**A**). Phosphorylation levels of ERK1/2, p38, JNK, AKT and AMPK after HCAEC were stimulated by 2'-O-Me-UTP (0.1-300μM) for 10 min. As a biased ligand, 2'-O-Me-UTP activate ERK1/2 pathway with a minimal or no effect on the JNK and p38 pathways (**B**).

Figure 3.3

A



B

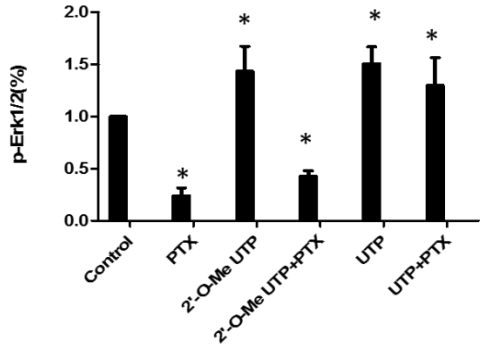


Figure 3.3. Biased agonism of 2'-O-Me-UTP-mainly coupled to P2Y2R-G*αi*/o subunit.

UTP or 2'-O-Me-UTP-induced ERK phosphorylation was determined after HCAECs were pretreated with or without PTX(100ng/ml) (**A**). Densitometry of above western blotting bands were quantified (n=3) (**B**).

Figure 3.4

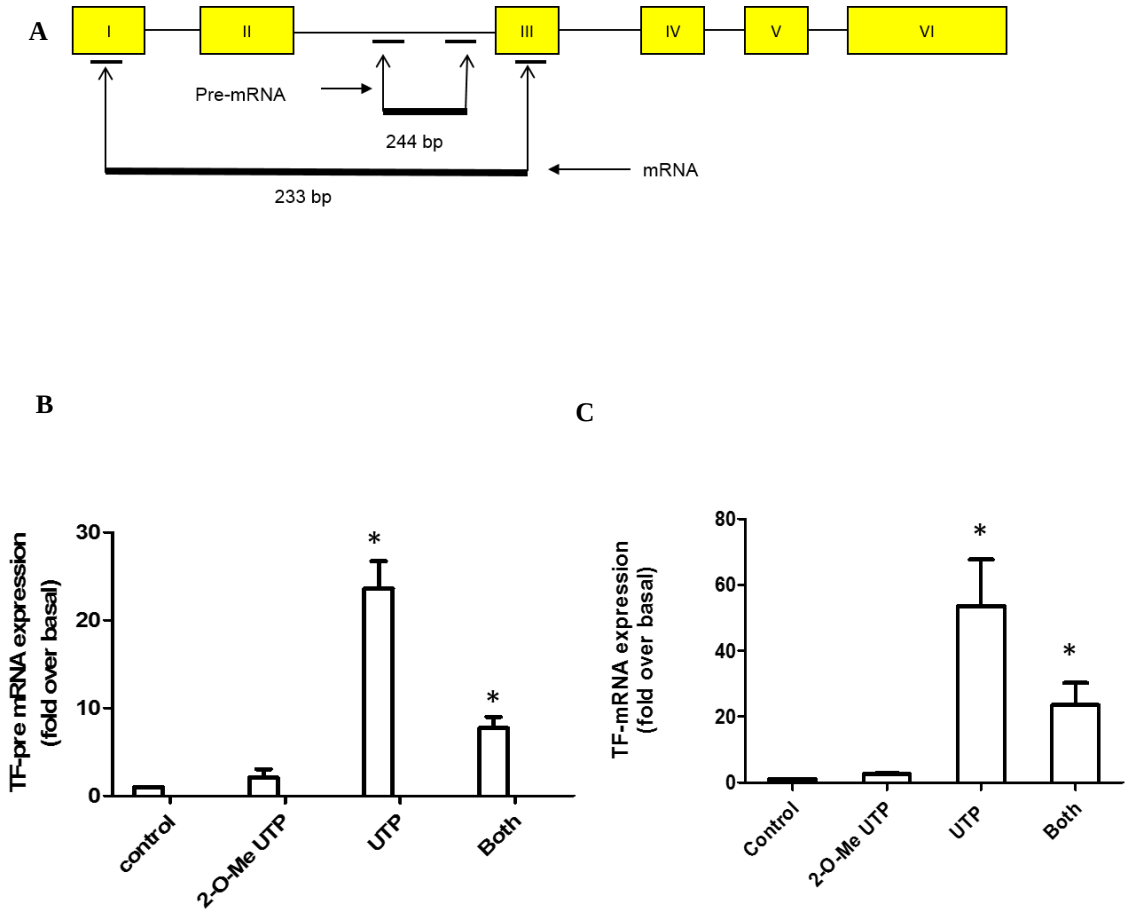


Figure 3.4 2'-O-Me-UTP inhibits TF gene transcription induced by UTP in HCAEC.

Two pairs of primers were designed to quantify human TF pre-mRNA (hnRNA) and TF mature mRNA by Real-time RT-PCR respectively. Relative position of primers of the TF gene is depicted in (**A**). Effect of 2'-O-Me-UTP (10μM), UTP (10μM) or their co-treatment on TF pre-mRNA and mature mRNA expression in HCAEC were determined by Real-time RT-PCR. (n=5, *, $p<0.05$) (**B & C**).

Figure 3.5

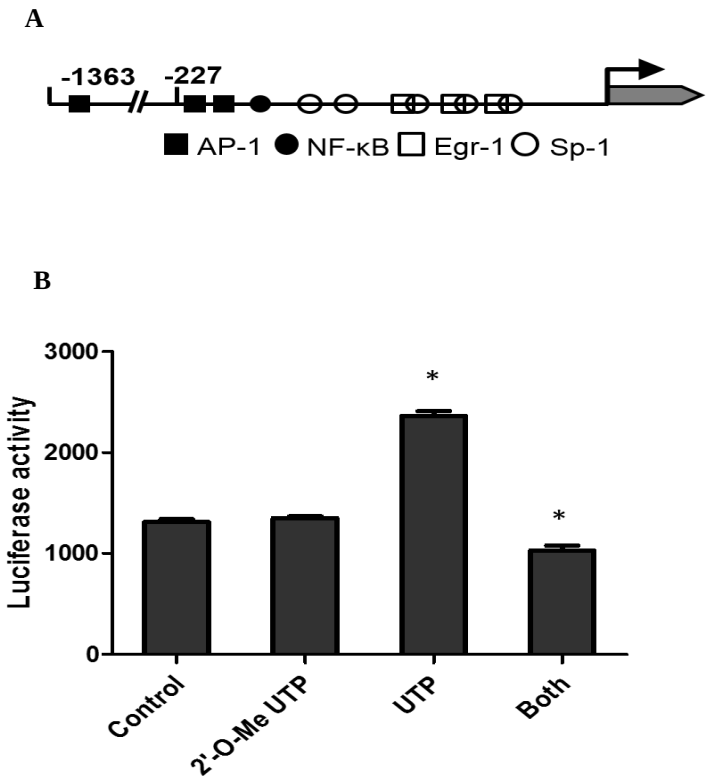
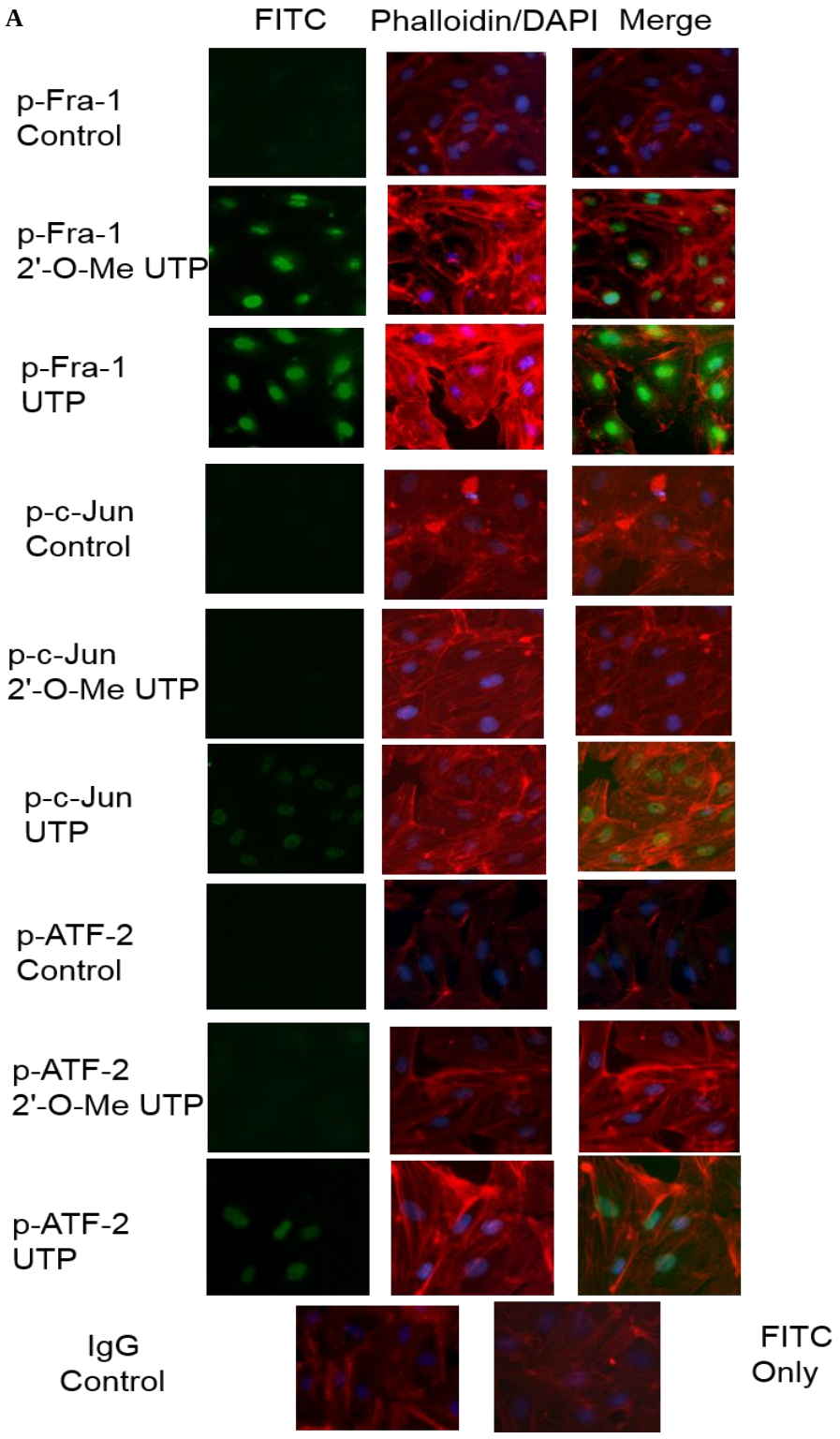


Figure3.5 Differential effect of 2'-O-Me-UTP and UTP on TF promoter activity in HCAEC. Schematic description of human TF gene promoter and relevant transcription factor binding sites. A new distal AP-1 binding site at -1363bp in TF promoter was identified in our previous study (**A**). HCACE were transfected with luciferase plasmid constructs and stimulated with UTP (10μM), PSB1114 (10μM) or both for 40min, after which levels of luciferase activity relative to non-stimulated control were determined and summarized (**B**). (n=4, *,$p<0.05$).

Figure 3.6


A
FITC
Phalloidin/DAPI
Merge
p-Fra-1
Control
p-Fra-1
2'-O-Me UTP
p-Fra-1
UTP
p-c-Jun
Control
p-c-Jun
2'-O-Me UTP
p-c-Jun
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UTP
IgG
Control
FITC
Only

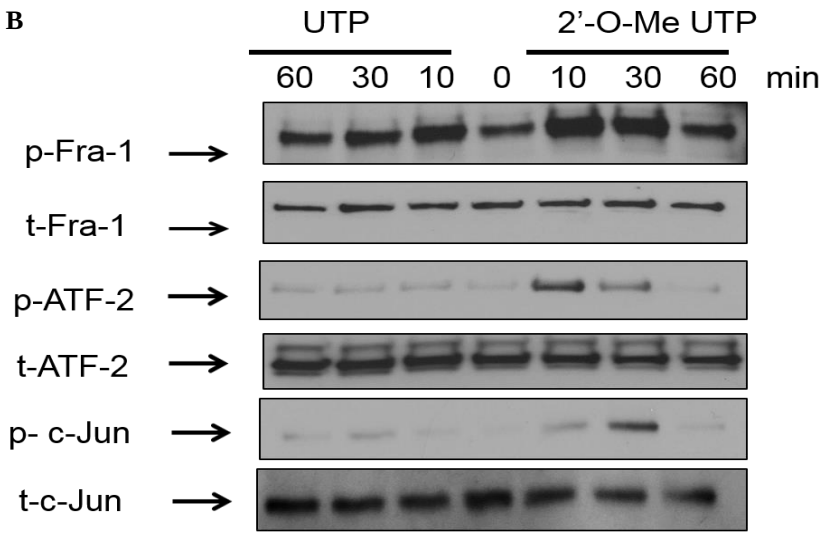

Figure 3.6 2'-O-Me UTP selectively activates TF promoter repressor Fra-1 in HCAEC.

HCAEC were stimulated with vehicle (control) or 2'-O-Me UTP (100μM) or UTP (100μM) for 30 min before fixed for stained immunofluorescence assays. Locations of p-Fra-1, p-c-Jun, and p-ATF-2 were indicated by FITC (green). Cytoskeleton was stained with phalloidin (red) and cell nuclei were counterstained with DAPI (blue). Isotype-matched primary antibody or FITC-conjugated secondary antibody were used for negative controls. Scale bar=20μm (**A**). The time course of AP-1 subunits phosphorylation in response to UTP (10μM) or PSB1114 (10μM) were determined by Western blotting (**B**).

Figure 3.7

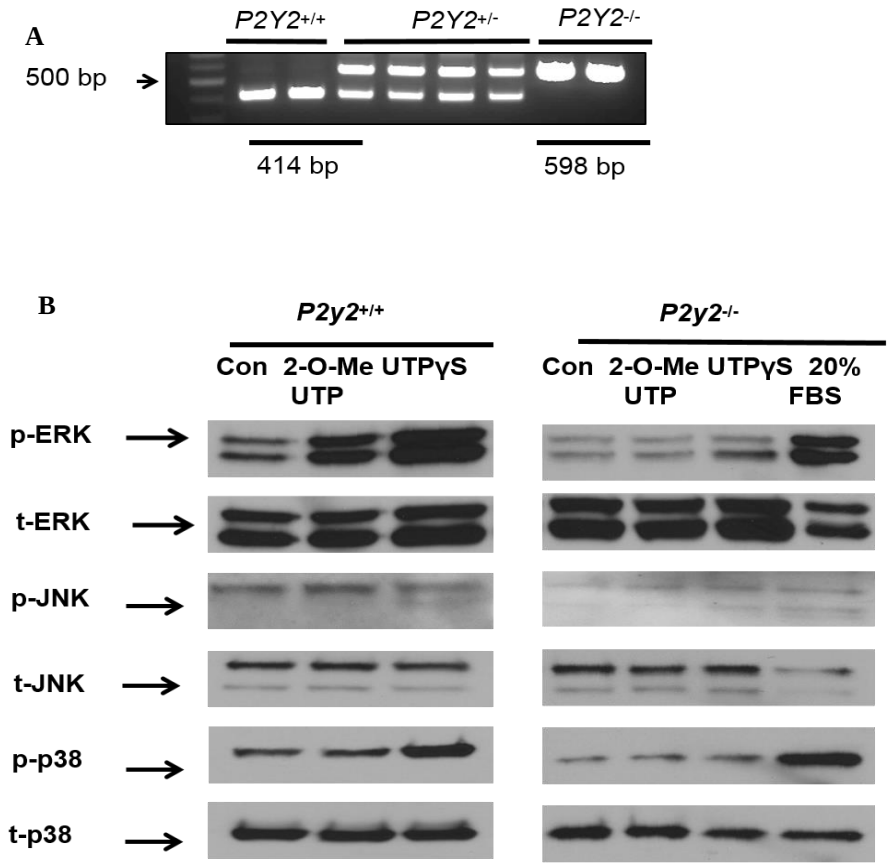

Figure 3.7. 2'-O-Me UTP mediated biased signaling at P2Y2R in macrophages from WT and P2Y2-/- mice.

The genotypes of wide-type, heterozygous and P2Y2-null mice were determined by PCR as shown in image (**A**). Peritoneal macrophages were isolated from WT and P2Y2-null mice respectively. Phosphorylation levels of ERK1/2, p38, and JNK were determined by Western blotting after the P2Y2-positive macrophages were stimulated by 2'-O-Me UTP (100 μM) for 5 min. UTP (100 μM) was used as a positive control **(B)**. Phosphorylation levels of MAPKs were determined by Western blotting after P2Y2-null macrophages were stimulated by PSB1114 (0.1-100 μM) or UTP (100 μM) for 5 min. 20% FBS was used as a positive control.

Figure 3.8

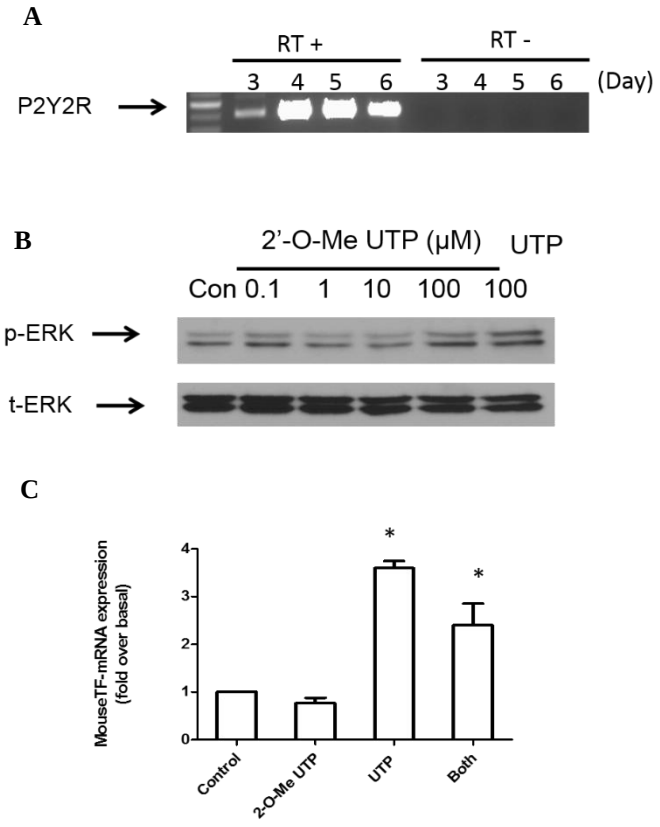

Figure 3.8. 2'-O-Me UTP mediated TF expression at P2Y2R in bone-marrow derived macrophages from WT mice.

P2Y2R mRNA from bone-marrow derived macrophages from wide-type mice on Day 3 to Day 5 were determined by PCR as shown in image (**A**). Phosphorylation levels of ERK1/2 after the P2Y2-positive macrophages were stimulated by 2'-O-Me UTP (0.1-100 μM) for 5 min. UTP (100 μM) was used as a positive control **(B)**. Effect of 2'-O-Me UTP (10μM), UTP (10μM) or their co-treatment on mouse TF mature mRNA expression in HCAEC were determined by Real-time RT-PCR. (n=5, *, $p<0.05$) (**C**).

Figure 3.9

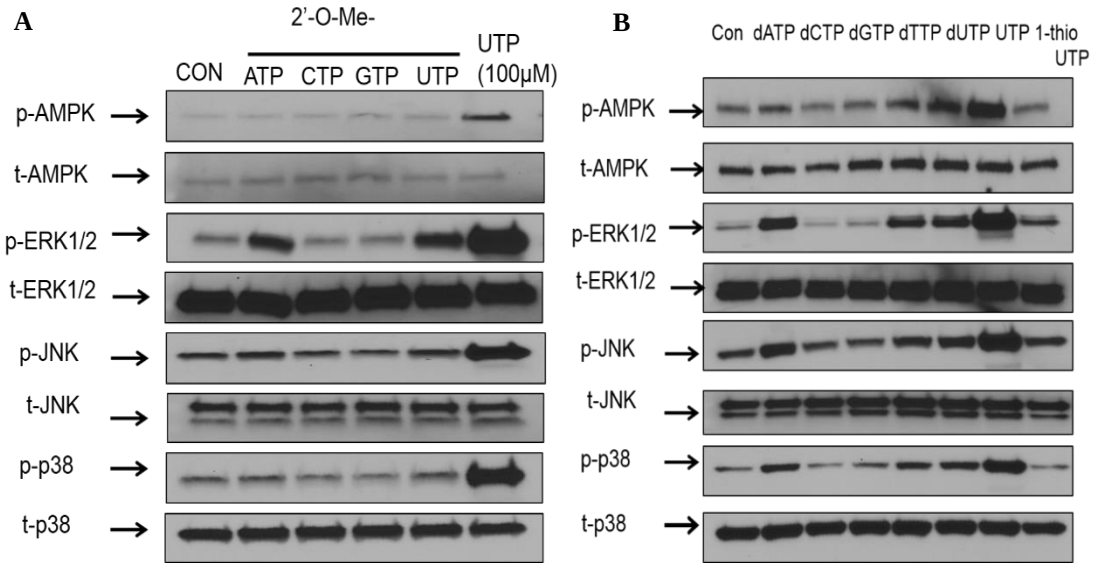

Figure 3.9. 2'-O-Me modified nucleotide phosphates in MAPKs signaling in HCAECs.

AMPK, ERK1/2, p38, and JNK phosphorylation after HCAEC were stimulated by 2'-O-Me– (ATP, CTP, GTP or UTP) (100μM) for indicated times. UTP as a control was determined by Western blot (**A**). Phosphorylation levels of ERK1/2, p38, and AMPK after HCAEC were stimulated by 2',3'-Dideoxyl-ATP, CTP, GTP or UTP for 5 mins was determined by Western blot (**B**).

Figure 3.10

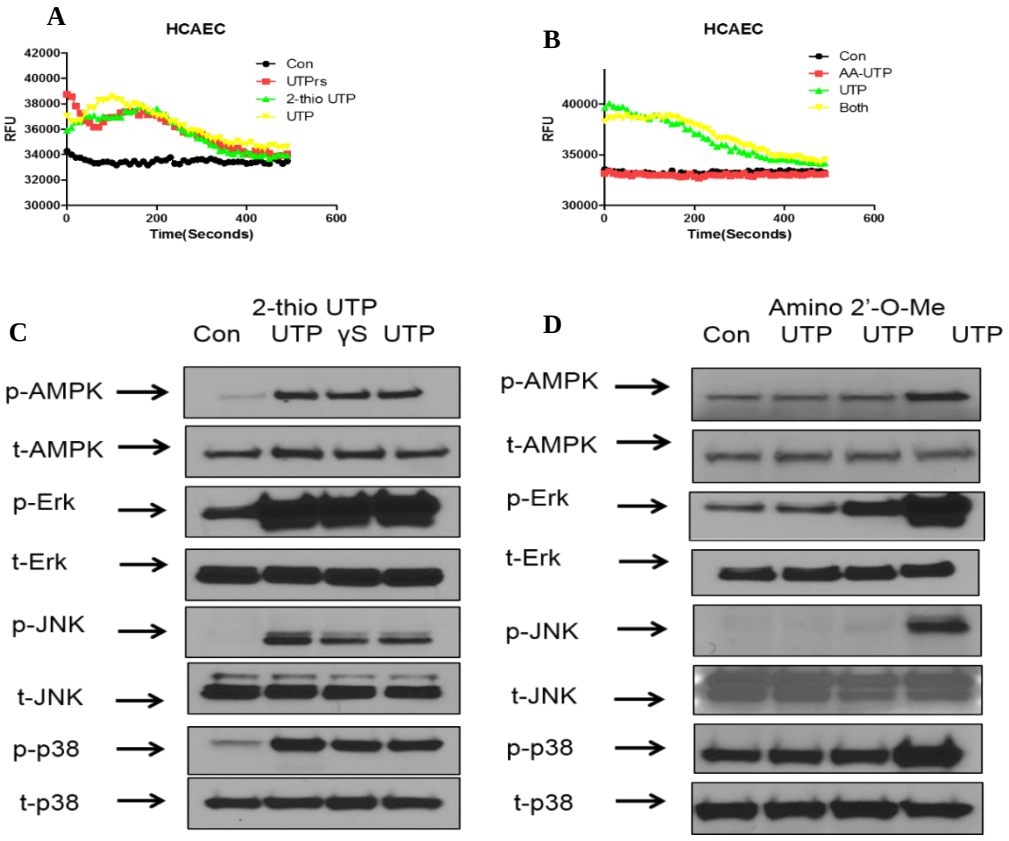

Figure 3.10. Different UTP analogs induced AMPK and MAPKs signaling in HCAECs.

P2Y2R agonist UTPγS (100μM)- or 2-thio UTP (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in HCAECs (**A**). UTP (100μM) waignss used as positive control. Amino-UTP or UTP or both -induced [Ca^{2+}] signaling in HCAECs (**B**). Measurement of [Ca^{2+}] signal was performed by a fluorometer plate reader with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU). N=3. ERK1/2, p38, and JNK phosphorylation after HCAEC were stimulated by 2-thio UTP, UTPγS (100μM) for indicated times. UTP as a control was determined by Western blot (**C**). Phosphorylation levels of ERK1/2, p38, and AMPK after HCAEC were stimulated by Amino-UTP, 2'-O-Me UTP or UTP for 5 mins was determined by Western blot (**D**).

3.4 Discussion

Activation of the P2Y2R by UTP in HCAEC stimulates various intracellular signaling pathways, including Ca^{2+} mobilization, AMPK, and MAPKs, etc. Among MAPKs, p38 and JNK have been shown to positively regulate TF expression upon P2Y2R activation (Ding, Ma et al. 2011). However, our recent follow-up study has found the P2Y2R-ERK1/2-Fra-1 pathway being a negative feedback mechanism, fine-tuning TF transcription (Liu, Zhang et al. 2016). In addition, in the previous chapter, we showed that PSB1114 can preferentially activate P2Y2R-AMPK and the P2Y2R-ERK1/2 pathways, with an efficacy comparable to the endogenous agonist UTP, but PSB1114 has a negligible or no effect on JNK and p38 activation. Furthermore, for the first time, we found 2'-O-Me-UTP as a new P2Y2R biased ligand, though it is unable to activate Gαq-mediated Ca^{2+} signaling, it retains the ability to activate the ERK1/2 by Gαi/o, but not JNK and p38 pathways through P2Y2R. Thus, 2'-O-Me-UTP may be another good parental lead compound to study the role of P2Y2R-ERK1/2 signaling pathway in vascular inflammation.

We performed quantitative Real-time PCR and Western blot/ELISA, immunofluorescence microscopy assay to determine TF mRNA and protein expression, localization of transcription factors (c-Jun, ATF-2, and Fra-1) in the presence of 2'-O-Me-UTP alone or its combination with other inflammation mediators in HCAEC. UTP was used as a positive control. The TF promoter activity was measured after the plasmid construct is transfected into HCAEC followed by stimulation of the cells with 2'-O-Me-UTP or UTP or both. 2'-O-Me-UTP exclusively activates the ERK1/2 pathway in HCAEC. In addition, we found 2'-O-Me-UTP suppress TF gene transcription induced by UTP through a mechanism that may involve different G protein coupling.

Biased ligands for some GPCRs are suggested to promote the stabilization of distinct receptor activation states, and preferentially signal through different G proteins- or β-arrestin-mediated pathway. Therefore, for mechanistic study, we ddetermine whether UTP, and 2'-O-Me-UTP would

activate different kinds of G proteins (Gq vs. Gi) or β-arrestin after binding to the P2Y2R (Steen, Larsen et al. 2014). To determine the role of Gαi protein, we treated HCAEC with PTX 100ng/ml overnight and then assess the potential differential impact of the 2'-O-Me-UTP on cellular signaling. Our results show that 2'-O-Me-UTP coupled to Gαi/o and exclusively activates ERK1/2-Fra-1 pathway can repress TF expression in HCAECs and macrophages from mice.

We also test other 2'-O-Me modified NTPs, and other UTP analogs such as amino-UTP, UTP γS or 2',3'-Didexoy-NTPs signaling behaviors on HCAECs (**Figure 3.9 & 3.10**). For the first time, we found 2'-O-Me-UTP/ATP as a new P2Y2R biased ligand, 2'-O-Me-UTP may be another good parental lead compound to study P2Y2R signaling bias in control of inflammatory gene expression. We can design prospect pathway-specific agonists for P2Y2R based on the parental compound such as 2'-O-Me-UTP to avoid on-target side effects. In the future, we will further explore the conformational change for the P2Y2R in response to 2'-O-Me-UTP, PSB1114 or UTP. In addition, we will also determine if 2'-O-Me-UTP could be detected human body, because it was shown that it is a component of piRNA (Mei, Clark et al. 2013, Weick and Miska 2014).

Chapter 4. 2-MeS ATP as a tissue/cell-specific bias ligand for P2Y2 receptor to control of inflammation in sepsis (preliminary study)

4.1 Abstract

The P2Y receptor ligand 2-MeS-ATP was reported to protect mice from endotoxic death, although the exact receptor mechanism(s) remains unclear. We previously demonstrated that HCAEC only express P2Y2R and P2Y11R mRNA, with no detectable mRNAs for the other six subtype P2Y receptors (Ding, Ma et al. 2011). P2Y2R is a subset of Gαq-coupled receptor, the activation of which induces an increase in IP3 and intracellular calcium mobilization. To determine the activity of 2-MeS-ATP on P2Y2R, we transfected mouse or human P2Y2R cDNA into 1321N1 human astrocytoma cell line which does not express any endogenous P2Y receptors. P2Y2R activity was analyzed by measuring calcium mobilization after 2-MeS-ATP treatment alone or in the presence of P2Y2R selective antagonist ARC-118925. We also isolated bone marrow-derived or peritoneal macrophages from WT and $P2Y2R^{-/-}$ mice to assess potential differential effects of 2-MeS-ATP on Ca^{2+} mobilization and post-receptor kinase activation, including MAPKs, AKT, and AMPK. Our study found that P2Y2R is expressed in different primary mouse macrophages. 2-MeS-ATP can induce a similar peak of Ca^{2+} response as UTP in both human and mouse P2Y2R-transfected 1321N1 cell line, but 2-MeS-ATP has a negligible or no agonistic activity in HCAEC, but functions as a partial agonist on macrophages. ATP analog, 2-Mes ATP exerts cell-type specific biased manner via P2Y2 receptor, which shed light on its unexplained anti-sepsis mechanism. Our findings also reveal that 2-Mes ATP can achieve anti-inflammatory effects via cell-type specific bias to repress pro-inflammatory pathway induced by classical agonist ATP, which could be a promising lead compound for synthesis or high-throughput drug screen in the future sepsis research.

4.2 Introduction

Vascular inflammation is commonly observed in sepsis, which results in high morbidity and mortality worldwide (Aird 2003, Hotchkiss and Karl 2003, Treacher and Brown 2009). Current medication for sepsis mainly depends on antibiotics with limited options. The death rate for sepsis is as high as 30%, with severe sepsis being ~50% and septic shock being ~80% (Schoenberg, Weiss et al. 1998). The influence of various risk factors, including LPS, leads to alteration in inflammatory and thrombotic gene expression in the endothelium (Schoenberg, Weiss et al. 1998, Remick 2007, Fink 2014). One of the potential new therapeutic strategies for sepsis is to target endothelial receptors to regulate the expression of inflammatory mediators, adhesion molecules and coagulant factors, etc. Our previous study showed that the principal P2Y nucleotide receptor subtype in human coronary artery endothelial cells (HCAEC) is the P2Y2 receptor (P2Y2R) (Ding, Ma et al. 2011). P2Y2R has been shown to induce key inflammatory and thrombotic gene expression including VCAM-1 and tissue factor (TF), suggesting that unveiling the molecular mechanism of P2Y2R-mediated inflammatory response may provide new target for sepsis treatment (Seye, Yu et al. 2004, Ding, Ma et al. 2011). Also, there has been accumulating lines of evidence suggests that P2Y2 receptor can also elicit anti-inflammatory effects. Nevertheless, the underlying mechanism for its protective role in inflammation is not fully investigated. Indeed, nucleotide analog, 2-methylthiol ATP (2-MeS-ATP) was reported to rescue mice from endotoxin shock and death nearly 100%, but the exact mechanism remains unclear (Proctor, Denlinger et al. 1994). Our preliminary study found that 2-MeS-ATP activates AMPK and MAPKs pathways in naïve cells transfected with human or mouse P2Y2R, but it has no agonistic effect on the endothelial P2Y2R. Thus, 2-MeS-ATP may be a biased P2Y2R ligand, signaling in a tissue/cell-specific manner. Understanding how 2-MeS-ATP affects P2Y2R signaling in relevant cells is worthwhile to investigate, we believe this could lead to illustration of its protective role in sepsis. Although GPCR biased signaling in general has been known for a decade, as of this point there is no report showing such a biased signaling case for any known P2Y receptors. Thus, our preliminary study could be the first to reveal

a biased signaling by 2-MeS-ATP mediated P2Y2R activation, which may also well explain its protective role in sepsis.

4.3 Materials and methods

4.3.1Cell culture

4.3.1.1 Human coronary artery endothelial cells

HCAEC were cultured in EBM-2 medium supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA1000 (Lonza), and 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. HCAEC were used between the third and eighth passages. Before stimulation, cells were seeded to grow for 24 h and starved overnight. Where inhibitor or antagonist was used, cells were pretreated with the inhibitor/antagonist for 40 minutes before cell stimulation.

4.3.1.2 Human and mouse transfected -1321N1 cells

Wild-type and human P2Y2 receptor 1321N1 cell lines were kindly offered by Dr. Gary Weisman (The University of Missouri) and selection of receptor-expressing cells accomplished by treatment with 0.5mg/ml G418. Wild-type and transfected 1321N1 cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. All experiments on transfected receptors are run in parallel with WT controls.

4.3.1.3 Raw264.7cells

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 μg/L streptomycin, and 100 IU/mL penicillin at 37 °C in a 5% CO2 atmosphere.

4.3.2 Intracellular Ca^{2+} mobilization assay

Cells were seeded at a density of $4x10^4$ cells per well into 96-well culture plates and cultured for one day. On day two, the original medium was removed and the assay medium from FluoForte™ kit (Enzo Life Sciences) containing the Ca^{2+} dye was added and receptor-mediated Ca^{2+} mobilization was determined as previously described (Ding, Ma et al. 2011). Fluorescence was determined immediately after adding of different reagents, with an excitation wavelength set to 485 nm and an emission wavelength set to 525 nm, and readings were taken every 1s for 500s. For antagonist inhibition experiment, cells were pre-incubated with the antagonist for 30 min before agonist addition. Measurement of Ca^{2+} signal was performed with the fluorometer plate reader (BMG FLUOstar) with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU).

4.3.3 General RT-PCR analysis

Total RNA and DNA were extracted from HCAEC and macrophages using the RNeasy and DNeasy kits, respectively (Qiagen). For the synthesis of the first strand of cDNA, 1 μg of total RNA after DNase (Ambion) treatment was reverse transcribed using a cDNA synthesis kit (Applied Biosystems). The cDNA samples were then amplified by PCR using 2.5 units of TaqDNA polymerase (Qiagen). Forward: 5'-AGC CAC CCG GCG GGC ATA AC-3'; common Reeverse: 5'-GAG GGG GAC GAA CTG GGA TAC-3'.

4.3.4 Western Blotting Assay.

After stimulation, cells were lysed, and standard Western blotting assay was performed as previously described (Ding, Ma et al. 2011). The individual primary antibodies used were anti-p-AMPKα, anti-p-ERK1/2, anti-p-p38, anti-p-SAPK/JNK, anti-AMPKα, anti-ERK1/2, anti-p38, and anti-SAPK/JNK (Cell Signaling Technology). Equal protein loading was verified by stripping off the original antibodies and re-probing the membranes with a different primary antibody such as anti-β-actin (Cell Signaling Technology).

4.4. Results

Evidence of 2-MeS-ATP mediated Ca2+ signaling through the P2Y2R.

2-MeS-ATP has been shown to rescue sepsis-induced death in mice (Proctor, Denlinger et al. 1994); however, the underlying mechanism is not clear. To test whether it affects P2Y2R signaling, we stimulated 1321N1 human astrocytoma cells transfected with the mouse P2Y2R gene. Figure 4.1A & B show that 2-MeS-ATP induced intracellular Ca^{2+} signaling in mouse transfected cells, but not in wild-type cells. Although 2-MeS-ATP has a comparable efficacy with UTP or ATP, it is about 100-fold less potent than ATP/UTP. The EC_{50} of 2-MeS-ATP on mouse-transfected 1321N1 is 3μM as shown in dose-response curve, also its effect was abolished by ARC-118925, a P2Y2R-selective antagonist (**Fig. 4.1C**).

2-MeS-ATP does not activate AMPK and p38, ERK1/2 and JNK- MAPKs activation on WT-1321N1 cells which lack of P2Y2R at any time point (**Fig 4.2A**). On mouse P2Y2R transfected 1321N1 cells, 2-MeS-ATP mediated full activation of AMPK and p38, ERK1/2 and JNK-MAPKs as compared to UTP treatment at different time points (**Fig. 4.2B**) and this activation is also in a dose-dependent manner at 2 or 20 minutes treatment (**Fig. 4.2C & D**).

To determine whether 2-MeS-ATP mediated P2Y2R signaling in a species bias manner, we found 2-MeS-ATP (100μM) can induce intracellular Ca^{2+} mobilization and AMPK and MAPKs activation in human-transfected 1321N1 cells with similar patterns in mouse P2Y2R transfected 1321N1 cells (**Fig. 4.3A & B**).

Evidence of 2-MeS-ATP cell type/tissue- biased signaling on P2Y2R

We compared the signaling difference between 2-MeS-ATP and UTP in addition to the Ca^{2+} response, which revealed more signaling difference in various tissues or cell types through the seemingly same P2Y2R. Specifically, we studied the difference between 2-MeS-ATP and UTP-

activated P2Y2R in terms of its signaling efficacy to the MAPKs, and AMPK pathways in HCAEC, mouse macrophages. Interestingly, we found that in HCAEC, even at a maximal dose of 100μM, 2-MeS-ATP elicited very little Ca^{2+} signaling, whereas the effect of ATP/UTP is robust, as shown in (**Fig. 4.4A**). 2-MeS ATP does not activate AMPK or MAPKs on HCAEC from 1 to 20 mins (**Fig. 4.4B**). 2-MeS-ATP can antagonize ATP induce Ca^{2+} signaling nearly to basal level and partially antagonize UTP induce Ca^{2+} signaling (**Fig. 4.4C & D**). In line with Ca2+ signaling studies, 2-MeS ATP inhibits ATP-induced p38 signaling, but 2-MesATP (1-100μM) has no effects on UTP- induced AMPK activity (**Fig. 4.4E & F**).

The macrophages are an important player in sepsis; we also tested the role of 2-MeS-ATP in Raw cells, which expresses P2Y2R (**Fig. 4.5A**). 2-MeS ATP-induced a calcium influx with the peak 1/3 as UTP but still higher than the peak induced by ATP (**Fig. 4.5B & C**). On Raw 264.7 cells, 2-MeS-ATP mediated partial activation of AMPK and p38, ERK1/2, and JNK-MAPKs as compared to UTP treatment at different time points, we used different time points with no treatment as negative control to avoid the activation of signaling pathways due to sheer stress (**Fig. 4.6A & B**). The activation is also in a dose-dependent manner with 0.1-100μM treatment (**Fig. 4.6C**). These data suggest that 2-MeS-ATP may be a biased ligand for P2Y2R signaling in a species or tissue/cell-specific manner. 2-MeS-ATP can induce a similar peak of Ca^{2+} response as UTP in both human and mouse P2Y2R-transfected 1321N1 cell lines, but 2-MeS-ATP has a negligible or no agonistic activity and can antagonize ATP activity in HCAEC and functions as a partial agonist on macrophages.

Figures and legends

Figure 4.1

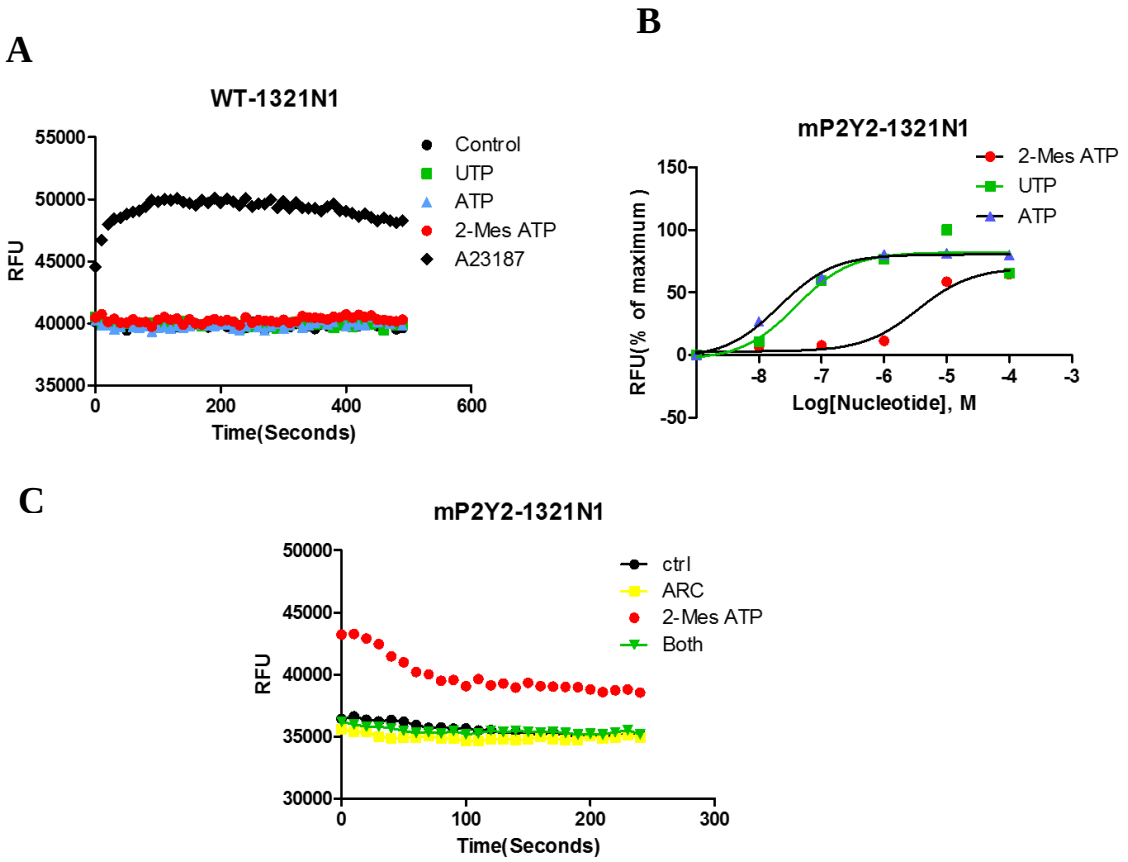

Figure4.1. Evidence of 2-MeS-ATP mediated Ca^{2+} signaling through mouse P2Y2R transfected 1321N1 cells.

UTP, ATP, 2-MeS-ATP (100µM)-induced intracellular [Ca^{2+}] mobilization was determined in wild-type 1321N1 cells which lack of any P2Y receptors. A23187 (1µM) as a positive control (**A**). Dose response curves for 2-MeS-ATP, UTP, and ATP in mouse-transfected 1321N1 cells (**B**). 2-MeS-ATP- induced [Ca^{2+}] increase was inhibited by ARC-118925 (3 µM) (**C**).

Figure 4.2

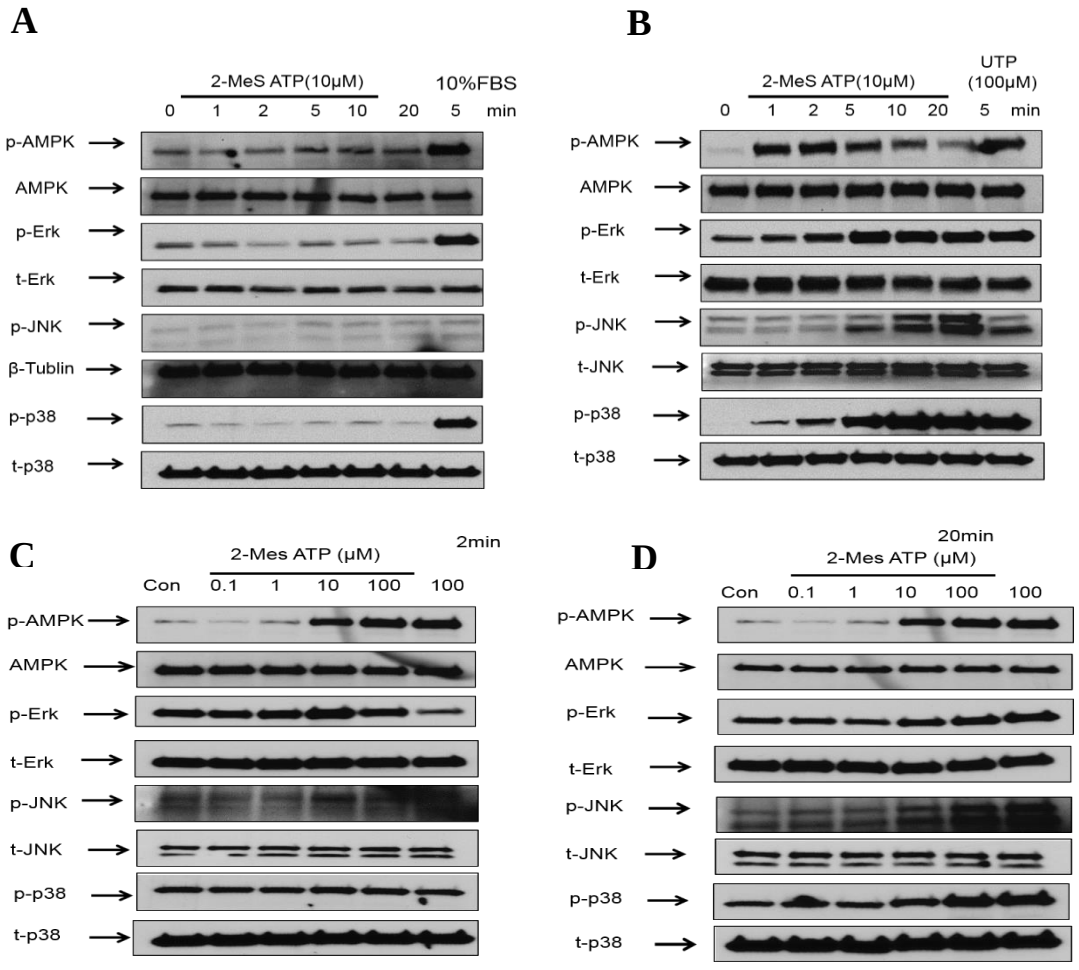

Figure 4.2 Evidence of 2-MeS-ATP signaling through mouse P2Y2R-1321N1 cells.

Phosphorylation levels of ERK1/2, p38, and JNK after WT-1321N1 cells were stimulated by 2-MeS ATP (10μM) at the indicated times (**A**). Time course (0-20min) for ERK1/2, p38, and JNK phosphorylation after mouse P2Y2R were stimulated by 2- MeS ATP (10μM) for 1-20 min and compared to the effect induced by UTP (100 μM) treatment for 5 min (**B**). ERK1/2, p38 and JNK phosphorylation after 2- MeS ATP (10μM) treatment on mouse P2Y2R for 2 min or 20 min were detected and compared to that induced by classical agonist UTP (**C&D**) N=3.

Figure 4.3

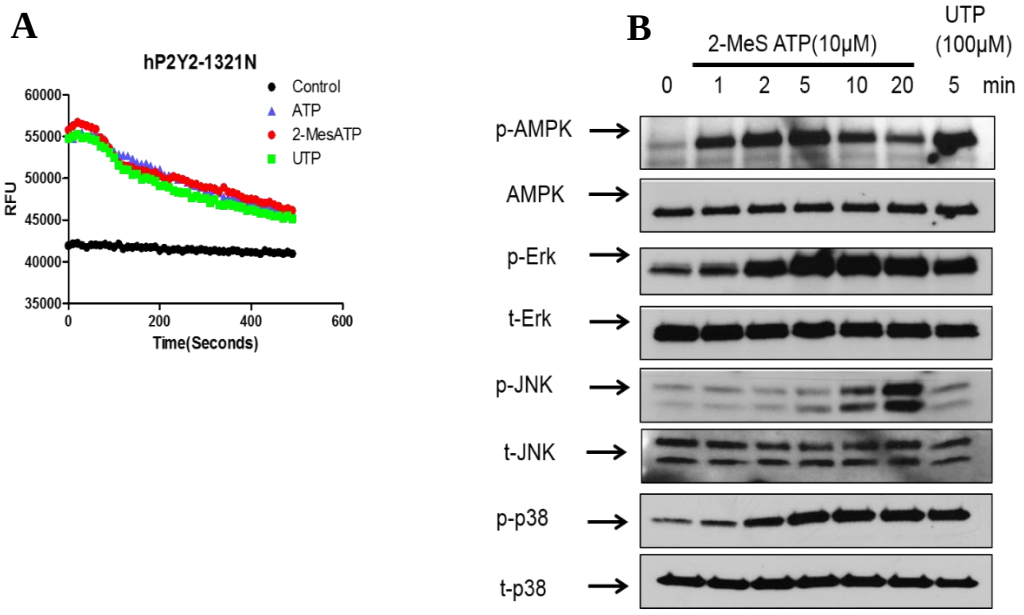

Figure 4.3 Evidence of 2-MeS-ATP signaling through human P2Y2R-1321N1 cells.

UTP, ATP, 2-MeS-ATP (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in wild-type human P2Y2R transfected 1321N1 cells. A23187 (1μM) as a positive control (**A**). Time course (0-40min) for ERK1/2, p38, and JNK phosphorylation after human P2Y2R were stimulated by 2- MeS ATP (10μM) for 1-20 min and compared to the effect induced by UTP (100 μM) treatment for 5 min (**B**).

Figure 4.4

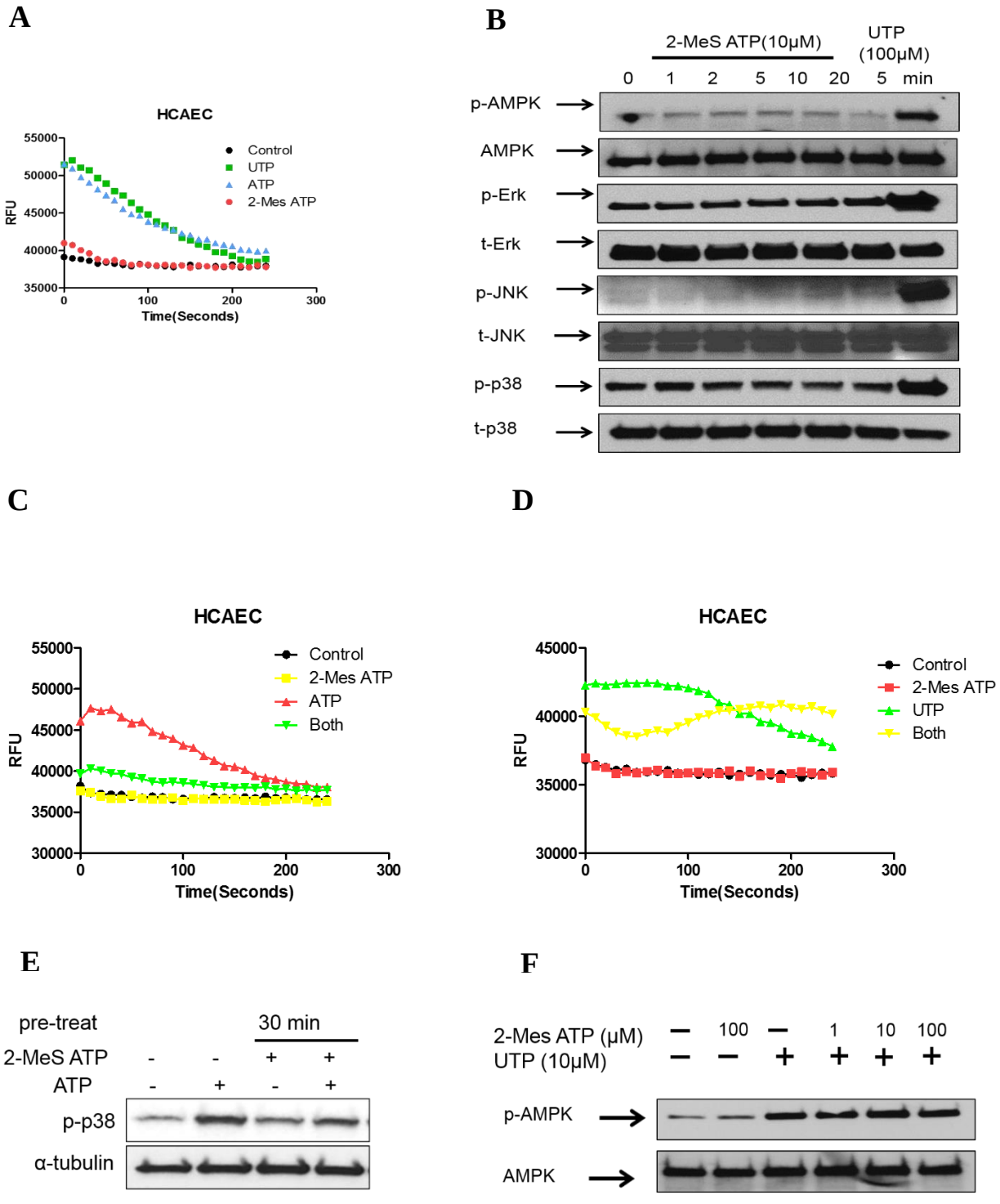
A
HCAEC
RFU
Time(Seconds)
Control
UTP
ATP
2-Mes ATP
B
2-MeS ATP(10μM)
UTP (100μM)
0 1 2 5 10 20 5 min
p-AMPK
AMPK
p-Erk
t-Erk
p-JNK
t-JNK
p-p38
t-p38
C
HCAEC
RFU
Time(Seconds)
Control
2-Mes ATP
ATP
Both
D
HCAEC
RFU
Time(Seconds)
Control
2-Mes ATP
UTP
Both
E
pre-treat
30 min
2-MeS ATP
ATP
p-p38
α-tubulin
F
2-Mes ATP (μM)
UTP (10μM)
p-AMPK
AMPK

Figure 4.4 Evidence of 2-MeS-ATP is antagonist of P2Y2R on HCAEC.

UTP, ATP, 2-MeS-ATP (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in HCAECs. A23187 (1μM) as a positive control (**A**). Time course (0-20min) for ERK1/2, p38, and JNK phosphorylation after HCAECs were stimulated by 2-MeS ATP (10μM) for 1-20 min and compared to the effect induced by UTP (100 μM) treatment for 5 min (**B**) ATP- induced [Ca^{2+}] increase was inhibited by 2-MeSATP (100μM) to basal level (**C**). UTP- induced [Ca^{2+}] increase was inhibited by 2-MeSATP (100μM) from 0-50s and [Ca^{2+}] influx remained increasing from 50-300s (**D**). 2-MeS-ATP (100μM) pretreatment for 30 min before add ATP/UTP treatment for 5 mins on HCAEC, p38 or AMPK phosphorylation was determined by western blot (**E&F**). N=3.

Figure 4.5

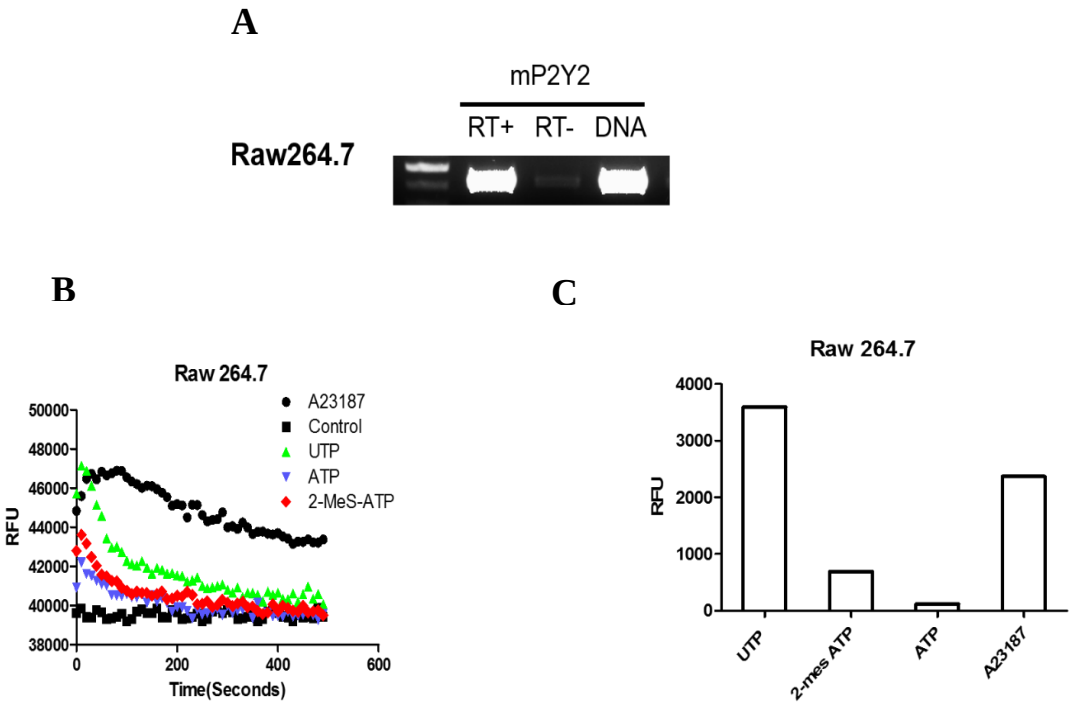

Figure 4.5 Evidence of 2-MeS-ATP is partial agonist of P2Y2R in Raw264.7 cells.

The P2Y2R mRNA expression on Raw 264.7 cells were determined by PCR as shown in image (**A**). UTP, ATP, 2-MeS-ATP (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in Raw cells which expresses P2Y2R..A23187 (1μM) as a positive control (**B**). Average values of UTP, ATP, and 2-MeS-ATP relative fluorescence intensity induced by calcium influx (**C**).

Figure 4.6

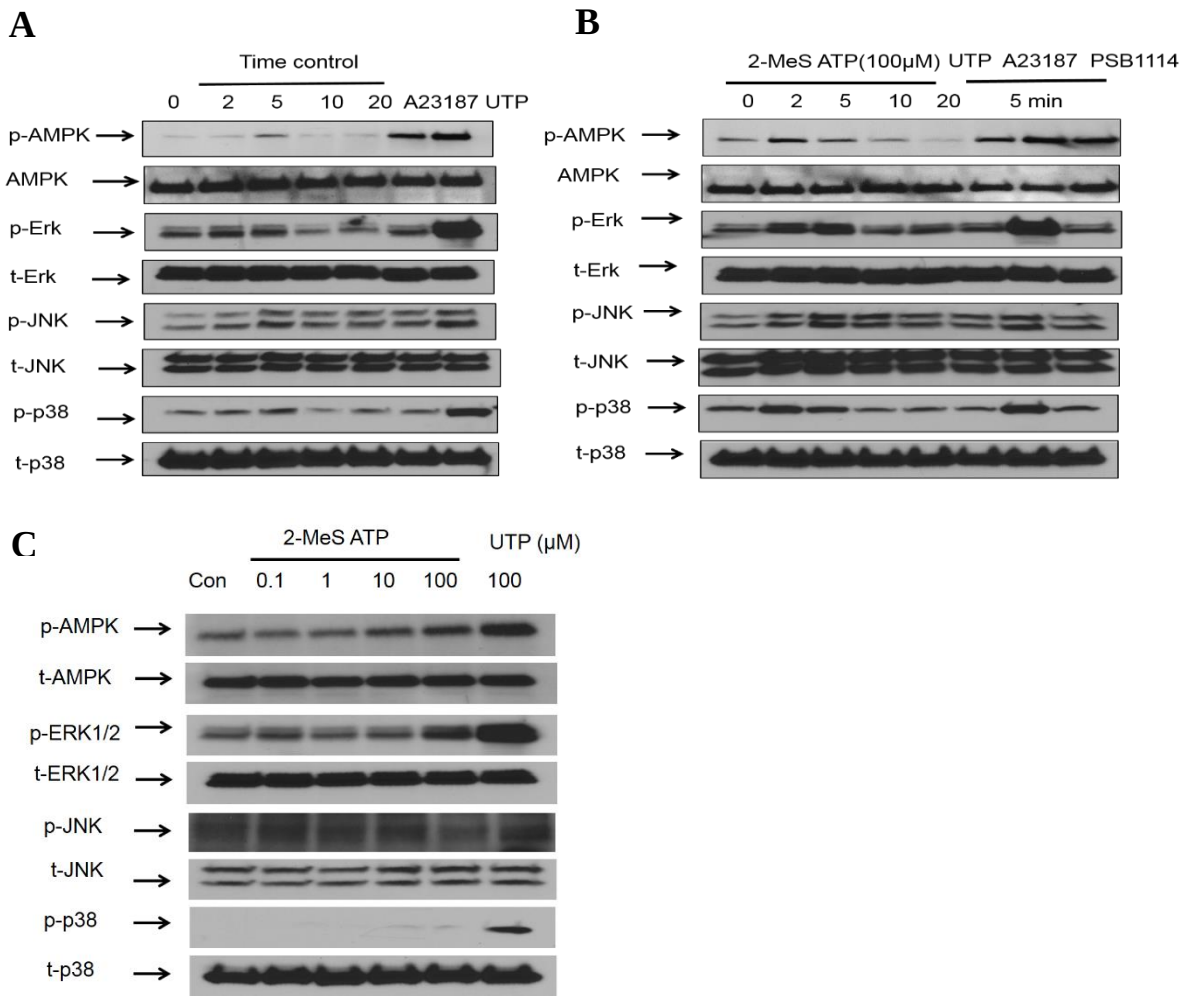

Figure 4.6 Evidence of 2-MeS-ATP is a partial agonist of P2Y2R in Raw264.7 cells.

Time point control of AMPK and MAPKs after 2-MeS ATP stimulation of P2Y2R in HCAEC were determined by Western blotting (**A**). Time-course of AMPK and MAPKs after 2-MeS ATP stimulation of P2Y2R on Raw cells were determined by Western blotting (**B**). Dose-dependent of AMPK and MAPKs phosphorylation after 2-MeS ATP stimulation of P2Y2R in HCAEC were determined by Western blotting and compared to classical agonist UTP, selective agonist PSB1114 or calcium ionophore A23187 (**C**). Total AMPK or MAPKs was used as loading controls. N=4.

Figure 4.7

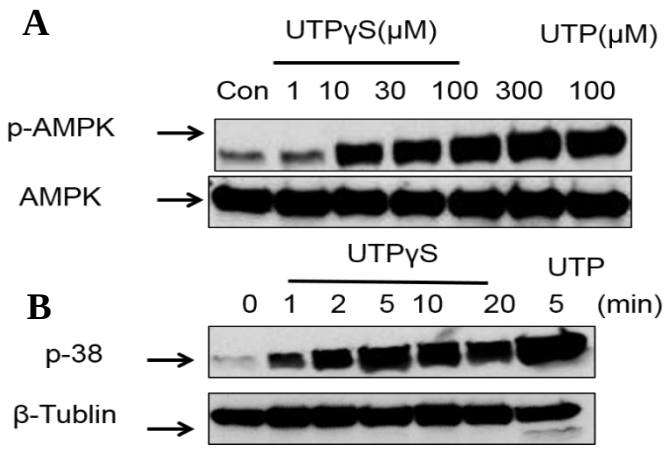

Figure 4.7 Effects of UTP and UTPrS on AMPK and p38-MAPK signaling in HCAEC.

Time-course for AMPK and p38 phosphorylation after HCAEC were stimulated by UTPγS or UTP (100μM) (**A**). Dose-dependent increase of AMPK and p38 phosphorylation after HCAEC was stimulated by UTPγS (**B**). N=3.

4.5 Discussion

For in vitro study, we observed that 2-MeS-ATP can significantly induce Ca^{2+} mobilization in P2Y2R-transfected 1321N1 cells, but not in control 1321N1 cells. Blockade of P2Y2R by the selective antagonist ARC-118925 abolished the response, demonstrating that 2-MeS-ATP can be a full agonist on P2Y2R, at least in 1321N1-transfected cells. We compared the signaling difference between 2-MeS-ATP and UTP in addition to the Ca^{2+} response, revealing more signaling difference in various tissues or cell types through the seemingly same P2Y2R. Specifically, we will study the difference between 2-MeS-ATP and UTP-activated P2Y2R regarding its signaling efficacy to the MAPKs and AMPK pathways in HCAEC, raw cells and macrophages from mice. Therefore, we discover 2-MeS-ATP can not be simply defined as agonist or antagonist, but as a cell type/tissue-biased ligand for G-protein coupled P2Y2R. Enhanced tissue selectivity has the potential to greatly improve drug safety in the context of on-target adverse effects. Furthermore, as P2Y2R is expressed in diverse tissues it is necessary to ensure that the observed response is identical in other tissues or cell types.

In summary, 2-MeS ATP is a full agonist in both human and mouse P2Y2 receptor transfected into 1321N1 cell line which does not have any endogenous P2 receptors. Secondly, 2-MeS ATP has no agonistic activity in human coronary artery endothelial cells but appears to have some antagonistic activity on P2Y2 receptor. In addition, 2-MeS ATP functions as a weak partial agonist on mouse macrophages. We can make a preliminary conclusion that 2-MeS ATP is a biased P2Y2 receptor ligand, signaling in a tissue or cell specific manner.

Chapter 5. Identify a putative new post-P2Y2R signaling molecule p25 on human coronary artery endothelial cells

5.1 Introduction

Endothelial dysfunction is one of the earliest events in the pathogenesis of acute inflammation, sepsis, or chronic inflammation such as atherosclerosis and thrombosis (van Hinsbergh 2012, Rajendran, Rengarajan et al. 2013). Endothelial cells lining the vessel lumen are constantly subjected to conditions of varying blood flow and shear stress. Under the stimulation of inflammatory mediators, nucleotide (ATP/UTP) will release from endothelium as an extracellular signaling molecule and modulate a wide range of pathophysiological responses mainly via activation of cell surface purinergic P2Y2 receptor (Milner, Bodin et al. 1990). When endothelial cells stimulated by ATP/UTP or other agonists, we unexpectedly found a prominent band with a molecular weight in a diffuse region of Mw 25,000-28,000, which can be detected with polyclonal p-Acetyl-CoA carboxylase antibody. Although it is now widely accepted that ATP/UTP plays a major role in vascular tone and inflammation, however, the role of p25 in the function of endothelial cells is still not well understood. One way to address the contributions of the p25 is to characterize the biochemical properties of p25 and to establish whether p25 can be induced by more than one ligand or exists in different cell types and exerts various actions in biological function. The p25 described here is a phosphorylated protein in the cytosol of endothelial cells and can be induced by various stimuli, but whether it induces inflammatory or anti-inflammatory response has yet to be determined. To date, such studies have not been possible because p25 has not been isolated and characterized and the intracellular signaling mechanism for p25 through P2Y2R activation has not been identified. In fact, only a few cell types have been found which express p25 and could lead to a biological response upon P2Y2R activation. Associations between p25 and upstream or

downstream components in the endothelial cells must be characterized. While the physiologic importance of p25 remains uncertain, a role in hemostasis of endothelium is a possibility.

5.2 Material and Reagents

5.2.1 Chemicals

Table 5.1 Chemicals

Items	Catalog Number	Company
Uridine 5'- triphosphate tris salt	U 6875	Sigma-Aldrich
TritonX-100	T8787	Sigma-Aldrich
ATP	3245	Tocris
Adenosine	3624	Tocris
A23187	100106	Calbiochem
SDF-1	300-28A	Peprotech
Human VEGF	293-VE	R&D systems
Thrombin	P00734	R&D systems
2-thioUTP tetrasodium salt	3280	Tocris
UTPγS trisodium salt	3279	Tocris
H-9 dihydrochloride	0396	Tocris
Geneistein	1110	Tocris
PKC 412	2992	Tocris
Recombinant human TNF-α	210-TA	R&D Systems
p-RhoA (Ser 188) Rabbit Ab	sc-32954	Santa Cruz
Rho A (67B9) Rabbit mAb	2117	Cell signaling
Cdc42(11A11) Rabbit mAb	2466	Cell signaling
p-Rac1/cdc42(Ser71) Rabbit mAb	2461	Cell signaling

HaltTM protease inhibitor cocktail	78430	Thermo Scientific

5.2.2 Cell Culture and Drug Treatment

HCAEC were cultured in EBM2 supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA 1000 (Lonza), and 5% FBS at 37 ºC in a humidified atmosphere of 5% CO2. HCAEC were seeded at 105 cells/well in a six-well plate or 4X106 and grown for 24h, reaching~ 80-90% confluence. Then the cells were starved overnight and treated with different agonists at the indicated time and concentrations.

The human myelomonocytic cell line THP-1, acute monocytic leukemia cells, was purchased form American Type Culture Collection(ATCC). THP-1 cells were cultured in suspension with RPMI-1640 (Hyclone, Thermo) supplemented with 10% fetal bovine serum.100U/ml Penicillin, and 100μg/ml streptomycin (Lonza) at 37 ºC in a humidified atmosphere of 5% CO2. THP-1 can be further differentiated into macrophage-like cells after serum starvation.

Hela cells culture were kindly offered by Dr. Richard Bird (Veterinary school, Auburn, AL). Hela cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO2. Hela cells were seeded in 10 cm dish and expanded to the appropriate density. Then the cells were starved overnight and treated with 10% FBS at the indicated time and concentrations.

5.2.3 Protein extraction

Treat cells by adding desired treatment for desired time. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS. Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer (Cell Signaling) to each plate (10 cm) and incubate the plates on ice for 5 minutes. Scrape cells off the plates and sonicate samples on ice three times for 5 seconds each.

Microcentrifuge samples for 10 minutes at 14,000g, 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at –80°C.

5.2.4 Determination of protein concentration

Protein concentration was determined by the lowery method with bovine serum albumin (BSA) as a standard, measuring the absorbance at 595nm (BCA assay, Themro Scientific).

5.2.5 Polyacrylamide gel electrophoresis and Western Blot Analysis

The molecular weight of p25 was roughly determined by SDS-PAGE under reducing denaturing condition and p25 was visualized by immunoblot with the polyclonal p-ACC antibody. Protein analysis and molecular weight determination were carried out through SDS-PAGE using polyacrylamide gel electrophoresis (12%) was performed according to laemmli. Protein bands were identified by Imperial Coomassie Blue Stain. Gels were stained with Imperial Protein Stain (Thermo Scientific) or silver stain (Thermo Scientific). After stimulation, cells were lysed, and standard Western blotting was performed as previously described (Ding, Ma et al. 2011). The primary antibodies used were anti-p-ACC (1:1000). Equal protein loading was verified by stripping off the original antibodies and re-probing the membranes with the primary antibody β-Tublin (1:2000). Molecular weights were estimated by comparison with the position of molecular weight standards purchased from Bio-Rad Laboratories.

5.2.6. Subcellular fractionation analysis

HCAEC cells were seeded in the 10-cm culture dish after overnight starvation then add UTP (100μM) treatment. Subcellular protein fractionation kit (Millipore) was used to extract soluble compartment (S1 and S2), the nuclear compartment (N), and cytoskeletal compartment (C) with respective extraction buffer; cellular, nuclear extraction buffer and cytoskeleton solubilization buffer which add both the protease inhibitor cocktail and sodium orthovanadate in each buffer. All

incubation was performed at 4 ºC except cytoskeleton was solubilize at 37 ºC. Extracts obtained from different compartments were quantified and subjected to western blotting.

5.2.7 Determine of molecular weight and isoelectric point

Hela cells were seeded in the 10-cm dish after overnight starvation, 8μM urea as solubilizing reagent with phosphor-protein inhibitor was added to the cells, and the proteins were extracted by freezing and thawing procedure. The sample was loaded with loading buffer with a ratio of 1:1 and then was loaded in Isoelectric focusing (IEF) gels (7.5% polyacrylamide with 3% cross-linkage) containing carrier ampholytes purchased from Bio-Rad (Ready Precast IEF Gels, Bio-Rad) for detection under native conditions. The gel is composed by 2% ampholyte, pH 3-10 Anode buffer: 7mM phosphoric acid Cathode Buffer: 20mM Lysine/20mM Arginine. Isoelectric focusing was carried out using the PowerPac high voltage power supply (Bio-Rad) with the following run settings: 1h at 100V, 1h at 250 V, 30 min at 500V. During the run, the tank was placed in a shallow tray containing ice at room temperature, and a magnetic stir bar was added to aid heat dispersal.

After IEF focusing gel, the focused gel was rinsed in water for 5 min and stained with Imperial Protein Stain or was transferred to nitrocellulose membrane with 0.7% acetic acid. After the secondary antibodies visualization, the nitrocellulose membrane was stained with Coomassie G-250 Simply Blue Safe Stain (Invitrogen, #LC6060) to ensure the separation of proteins and compared to the standards. The IEF standards (Broad Range, Bio-Rad) were: β-Lactoglobulin B, pI 5.1; Bovine Carbonic anhydrase , pI 6.0; Human carbonic anhydrase, pI 6.5 ; Equine myoglobin, pI 6.8, 7.0; Human hemoglobin A, pI7.1; Human hemoglobin C, pI 7.5; Lectin, pI 7.80, 8.00, 8.20; Cytochrome C, pI 9.6.

5.2.8 Immunoprecipitation

HCAECs were starved in serum-free medium overnight before treatment with or without UTP (100μM) for 5mins, then rinsed with cold PBS and scraped from the 10-cm dish in cell lysis buffer (Cell Signaling) with protease inhibitors. The lysis buffer was composed of 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2 EDTA, 1mM EGTA, 1% Triton, 2.5mM Sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml Leupeptin and 1mM PMSF immediately before use. After sonication, the cell lysates was ncentrifuged for 10 minutes at 14,000g, 4 ºC, and the concentration of the supernatant was measured and kept under non-denaturing conditions. Immunoprecipitation was performed with cell lysates and anti-p-ACC Briefly, immunoprecipitate was collected after lysates was pre-cleared and then incubation with protein A-agarose beads for 3h when applicable and washed five times with lysis buffer. Bound protein was eluted and boiled for 5 minutes in the DTT sample buffer before electrophoresis. SDS-PAGE was performed at room temperature using 5% stacking and 12% resolving polyacrylamide gel and transfer to PVDF membrane and analyzed by immunoblot with the anti-p-ACC antibody as primary antibody and conformation-specific secondary antibodies (Cell signaling). The molecular weight of the p25 was roughly determined by comparing the standard ladders.

5.2.9 Phosphoprotein Enrichment and Determination

HCAECs were starved in serum-free medium overnight before treatment with or without UTP (100μM) for 5mins, then rinsed with cold PBS and the proteins were collected by scraping the cells in the 10-cm dish after freezing and thawing. Buffer A (Clontech) as extraction buffer with EDTA-free phosphatase inhibitor cocktail (Cell Signaling). The cellular extract was loaded on TALON PMAC Phosphor-protein enrichment kit (Clontech), and elution with detergent-free buffer (buffer B). The total cell extract, flowthrough, wash, and elution fraction were collected, after quantification extracted was subjected to western blot analysis with p25 antibodies. All the procedure was performed on ice and as quickly as possible.

5.2.10 De-phosphorylation treatment

After overnight starvation, HCAEC cells were treated with 100μM UTP for 5 minutes, and then cells were homogenized in a buffer containing 20mM Tris-HCl, pH7.4, 150mM NaCl, 0.5% Triton X-100, protease inhibitor PMSF and then add phosphatase (Biolabs) treatment of interest. Harvest cell lysates were treated with PP1 phosphate (50Unit) and also supplemented with 1mM MnCl2 in 1X NEB buffer (50mM HEPES ,100mN NaCl, 2mM DTT ,0.01% Brij 35, pH7.5). PP1 phosphatase treatments where carried out 30°C, while Alkaline phosphatase CIP (300 Unit) treatment was performed in 1XNEB buffer 3 (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$,1mM DTT pH 7.9) at 37°C, as recommended by the manufacturer. Control samples were treated identically and simultaneously, but without any enzyme or phosphatase deactivated heat at 65 ºC for one hour.

For phosphorylation inhibitor assay, each inhibitor was used at 30μM in the serum-deprived EBM-2 medium for 30 minutes treatment before adding UTP (100μM) treatment for 5 mins on HCAECs. All the inhibitors treatment alone on HCAEC were used as negative controls.

5.2.11 Ion-exchange Chromatography

HCAECs were starved in serum-free medium overnight before treatment with or without UTP (100μM) for 5mins, then rinsed with cold PBS and scraped from the multiple 10-cm dishes in cell lysis buffer (Cell Signaling) with protease inhibitors. The extracts from the cell lysates were quantified and diluted 1:4 with binding buffer (20mM Tris, pH 8.3) for AEX column and binding buffer (20mM sodium acetate, pH5.0) for CEX column. The diluted materials were loaded onto AEX/CEX column respectively (Aurum Ion exchange, Bio-Rad) and waited until equilibration. The columns were then washed with Elution buffer (Binding buffer + 1.0 M NaCl). The elution fraction was concentrated with 3KD cut-off concentrator (Nanosep, Pall Corporation), quantified and equally loaded on SDS-PAGE and followed with Western Blotting Assay.

5.2.12 Protein Sequence Analysis

Precipitate colllected from immunoprecipitation from above procedure and loaded on SDS-PAGE gel, protein bands were stained with Coomassie Blue, and protein spots of interest (around 25KD) was excised, destained and samples not immediately used were stored at -20°C. The sample was digested with trypsin and extracted peptides were examined by LC-MS on an LTQ Orbitrap mass spectrometry (University of Missouri). Database search of NCBI limited to humans was conducted using Sorcerer-Sequest.

5.3 Results

This report describes the identification, partial characterization, and purification of a putative novel cytosolic protein from endothelial cells. This phosphorylated protein has an apparent size of 25kD as determined by reduced sodium dodecyl sulfate SDS-PAGE gel electrophoresis. P2Y2 receptor activation not only induced the phosphorylation of the anticipated substrate ACC but also significantly induced phosphorylation of a 25kD unknown protein that we termed p25. First, we found p25 is not well focused on SDS-PAGE gels and migrates as a smear between Mw 25,000-28,000 (**Fig. 5.1A**). In addition, p25 is activated in a timely pattern when stimulated by UTP, p25 quickly increased from 1 to 10mins, decreased after 20mins. p25 is also up-regulated by UTP in a dose-dependent manner from 1 to 300 μM (**Fig. 5.1B**). Also, immunoprecipitation of p25 from HCAECs cell lysates by p-ACC antibodies and then detected with western blot, the results show UTP treatment can increase p25 expression from p-ACC immunoprecipitate compared to control group. To ensure the antigen effectively released from the antibody, the immunoprecipitant is eluted by boiling in SDS-containing buffer, which disrupts the protein A–immunoglobulin (Ig) interaction. The inherent complication of this method is the co-elution of IgG, which will mask the blot with high background. To avoid the appearance of very high background signals, arisen from the heavy chain (HC, 55kDa) and especially light chain (LC, 25kDa), dithiothreitol as reducing agent was used to break the disulfide bonds and detection with the conformation-specific secondary

antibody which yields a relatively clean and specific band (**Fig. 5.1C**). To further characterize the receptor subtype, we use P2Y2R selective agonist PSB1114 to stimulate HCAECs at different time points, as shown in (**Fig. 5.1D**); p25 can be induced by PSB1114 (100μM), further indicating that p25 can be regulated through the P2Y2R.

The following study showed phosphorylation of p25 by UTP can be mimicked by other receptor agonists including thrombin, histamine, and VEGF, but not by TNFα, SDF-1, and adenosine (**Fig. 5.2A**). The P2Y2Rs belongs to a distinct subset of G-protein coupled receptors, that act through the Gαq-coupled signaling cascade by stimulating PLC and increasing Ca^{2+} from intracellular stores, and thus triggering downstream Ca^{2+} signaling events in HCAECs. For this purpose, cell permeable and receptor-independent calcium ionophore A23187 served as the positive control. A23187 can prominently up-regulate p25, suggesting the up-regulation of p25 is a calcium-related event. Impressively, any ligand can induce the release of Ca^{2+}, this well-known second messenger, which plays as important in the regulation of cell functions, can also induce p25. Directly activation of AMPK or ACC by AICAR can not induce p25 expression in HCAEC, suggesting p25 is not upstream of downstream effector of AMPK or ACC (Fig. 2B). Other studies demonstrated that P2Y2R had shown the activation of this receptor transiently increases the Rho family of small ATPases (RhoA, Rac1, and Cdc42), which has a similar molecular weight around 25kd and regulates cytoskeletal organization(Seye, Yu et al. 2004, Liao, Cao et al. 2014). Many any inflammatory mediators, such as vascular endothelial growth factor (VEGF), lipopolysaccharide (LPS), thrombin, tumor necrosis factor α (TNFα), histamine, modulate endothelial function by altering the activities of Rho GTPase (Wójciak-Stothard, Entwistle et al. 1998, Soga, Namba et al. 2001, Woo and Kim 2002).

To rule out the possibility that p25 is one of already known signaling molecules, we probed the cell lysates with p-Rac/cdc (28KDa) or RhoA antibodies, but saw no visible band. We also examined

another G-protein receptor downstream signaling molecule RhoA (28KDa) and p-Rho (23KDa), and no band was shown (**Fig. 5.7**). Above data indicates p25 is not a degradation product of p-ACC or structurally related, and p25 does not relate to any of the previously described G-protein downstream effectors reported before. As far as we know, there is no other post-receptor signaling proteins that have a molecular weight around 25 kDa.

The subcellular localization of p25 is also of interest and can be characterized by biochemical separation of subcellular fractions, and then followed by immunoblotting. To this end, subcellular fractionation of HCAECs was carried out according to the manual of commercial kit. Different subcellular compartments of endothelial cells were separated by extraction buffers, collected, and then detected by immunoblot with the anti-p-ACC antibody. The resulting immunoblot revealed that most of the p25 was found in the soluble extract, indicating that 25kD was primarily present in the cytoplasm. The distribution of p25 was similar to that of p-ACC, except that some of the p-ACC appeared in the nuclear extract (**Fig 5.3**). The cellular location of p25 suggested a possible functional role of p25 in regulating signaling transduction.

As phosphorylation is a common post-translational modification of protein involved in transmitting intracellular signals(Hunter 1995), it is of our interest to test whether p25 is a phosphorylated protein or not. Post-translational modification of phosphorylated p25 can be enriched up to 100-fold by an ion-metal affinity chromatography (TMAC). From Fig. 4A, the majority of p25 binds to affinity column and appears in the elution portion, suggesting that p25 is a phosphorylated protein. Whether p25 is a phosphorylated protein was assessed by other methods including phosphatase or kinase inhibitors treatment. Two of these phosphatases, protein phosphatase 1 (PP1) and alkaline phosphatase (CIP) were used to antagonize the phosphorylation of p25. As shown in Fig. 4B, PP1 and CIP catalyze the dephosphorylation of p25, both phosphatases significantly decreased p25 phosphorylation. To confirm above study, cultured endothelial cells were pretreated with different

phosphorylation inhibitors for 45 minutes before UTP treatment. Our results show that PKC and H9 (30μM), but not Genistein (30μM) significantly reduced the basal level or UTP-induced phosphorylation of p25 (**Fig 5.4**). All lines of evidence suggest that p25 is a phosphorylated protein which is a vital property for cell signal transduction. Therefore, the existence of p25 as a phosphorylated protein is not surprising to be assumed as a post-P2Y2R signaling component.

The pI value of p25 was determined by the Isoelectric focusing (IEF), as such a technique separates p25 according to its isoelectric point (pI). IEF gel together with immunoblot is an effective method for visualizing the property of p25. As p25 is a phosphorylated protein, only occupy 10% in total protein from primary human cell lines; therefore, it seemed desirable to use Hela cells presents a similar p25 expression pattern as HCAECs with or without treatment as the source of starting material for characterization of p25 (**Fig. 5.5A**). Compared to the standards, p25 has a pI value around 7.8 in the physiological range, whereas the pI value of p-ACC is around 5.89. Subsequent Coomassie Blue staining revealed bands for proteins, indicating that the electrophoresis conditions successfully separate proteins (**Fig. 5.5B**).

We also used anion and cation exchange columns to further characterize p25; cells were homogenized and diluted in loading buffer, and the homogenates with the same amount of protein were passed through the columns respectively. To increase the recovery percentage, the elution buffer was composed of loading buffer with NaCl in it. Next, we compared the separation efficiency of two columns by detection of elution portion with western blotting. The maximal of p25 recovery was attained in the elution from the anion exchange resin, and p25 can also be collected from cation exchange resin. The pH of buffer for ion exchange chromatography is critical, as the ideal loading buffer pH is 0.5-1.5 pH units greater than or lesser than the pI of the protein of interest. For anion exchanger, the elution condition with pH value 8.1 is within the ideal buffer range for p25, as the wash efficiency within the range of the observation of pI value of p25. Our current results suggested

a potential functional role of p25 by demonstrating its presence in different cell lines; p25 appears in endothelial cells, Hela cells but not in THP-1 cells (Fig 7). Above observation raised an interesting question about the functional significance of p25. However, this putative new signaling protein p25 is not fully identified and has apparently escaped appreciation due to its poor abundance.

Figures and legends
Figure5.1

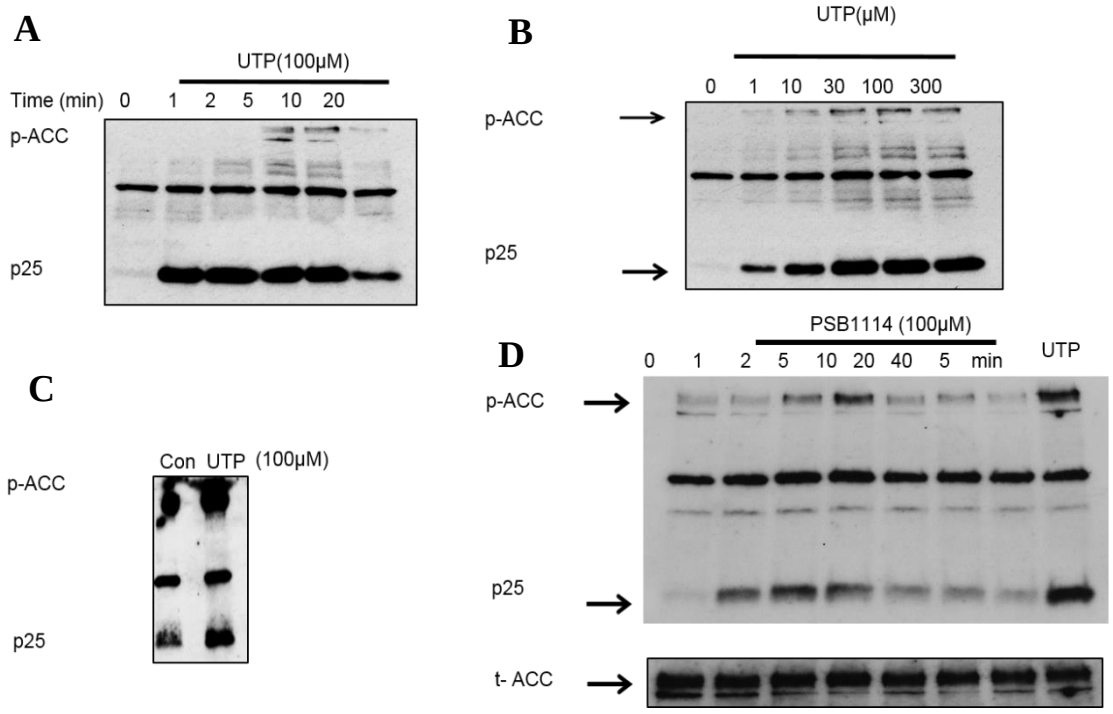

Figure 5.1. UTP induction of p25 expression in HCAEC.

HCAEC were stimulated by UTP (100μM) for the indicated times (**A**), or by indicated concentrations of UTP for 5 min (**B**). The anticipated p-ACC protein and an unknown protein p25 in total cell lysates were detected with an anti-p-ACC (280 kDa) polyclonal antibody by standard Western blotting (**A** & **B**). Cell lysates with or without UTP treatment were immune-precipitated using the anti-p-ACC antibody, after which p25 was detected by Western blotting with the same anti-p-ACC antibody (**C**). N=3. HCAEC were stimulated by PSB1114 (100μM) for the indicated times, the anticipated p-ACC protein and an unknown protein p25 in total cell lysates were detected with an anti-p-ACC (280 kDa) polyclonal antibody by standard Western blotting (**D**).

Figure5.2

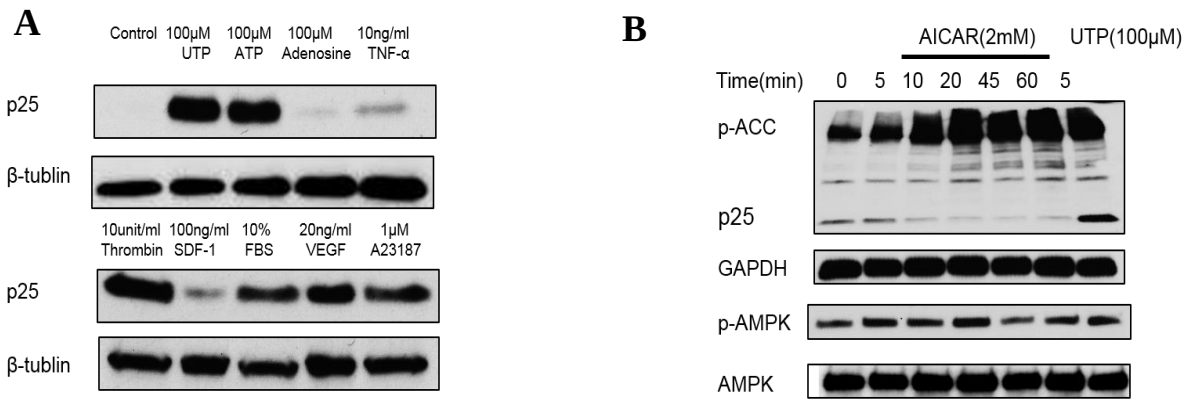

Figure 5.2 Multiple receptor agonists induction of p25 expression in HCAEC.

The effect of other stimuli such as ATP (100μM), adenosine (100μM), TNF-α (10 ng/ml), Thrombin (10 unit/ml), SDF-1(100 ng/ml), 10%FBS, and VEGF (20 ng/ml) on p25 expression was also determined by Western blotting (**A**). HCAEC were stimulated by 2mM AICAR for the indicated times were detected by Western blotting with the anti-AMPK or anti-p-ACC antibody (**B**).

Figure5.3

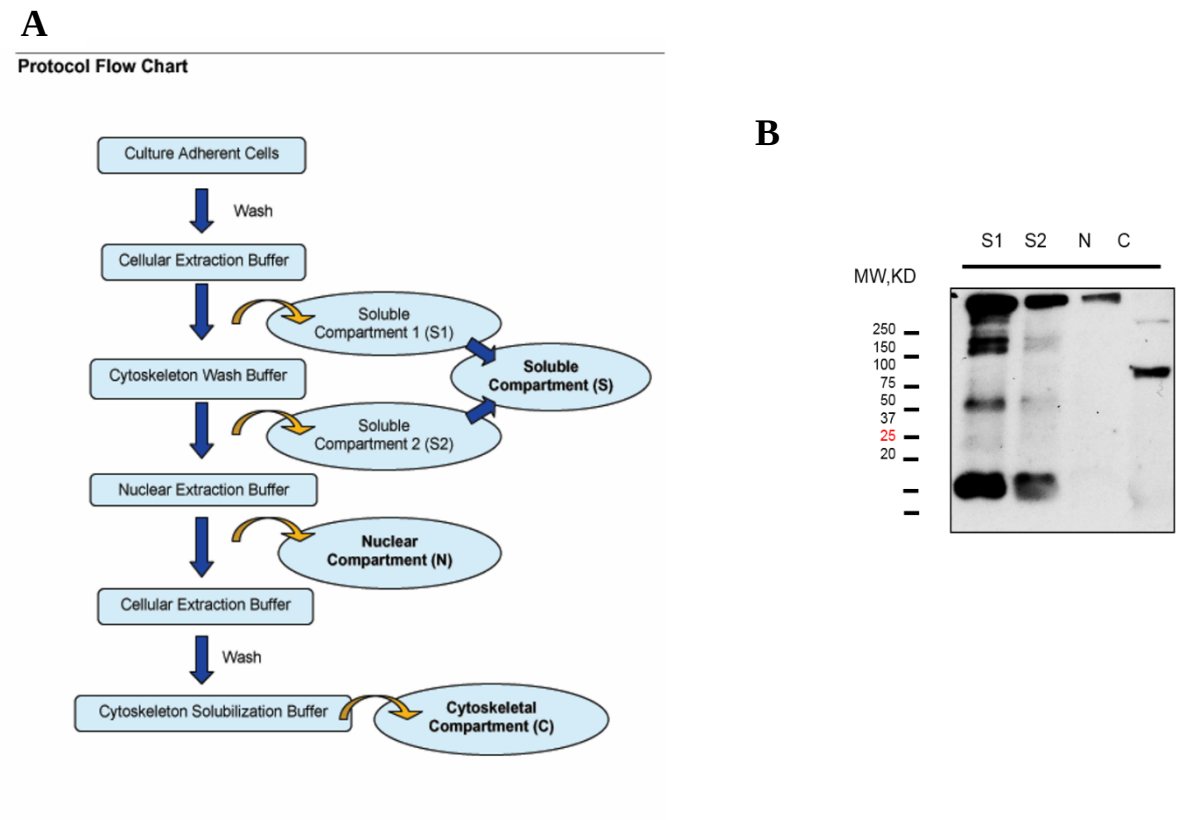

Figure 5.3 Cellular location of p25

Different subcellular components of HCAEC were seperated into soluble compartment (S1 and S2), the nuclear compartment (N), and cytoskeletal compartment (C), after quantification, equal amount protein was loaded on SDS-PAGE gel and then p25 expression was determined by Western blotting using p-ACC antibody.

Figure5.4

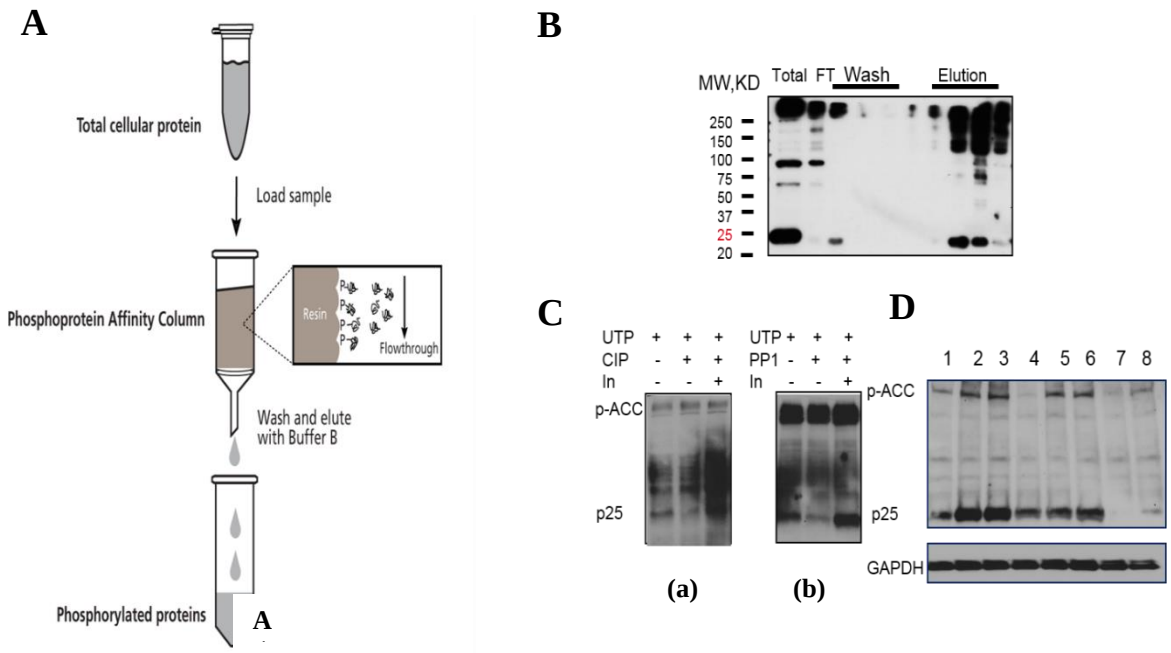

Figure 5.4 Phosphor properties of p25.

The cellular extract was loaded on TALON PMAC Phosphor-protein enrichment kit, and elution with detergent free buffer. The total cell extract, flowthrough, wash, and elution fraction were collected and subjected to western blot analysis with p25 antibodies (**B**). The homogenate was incubated in 30 °C with or without CIP or heat-deactivated CIP. (**C.a**) The homogenate was incubated in 30 °C without or with PP1 or heat-deactivated PP1 (**C.b**). Lane 1, control group; lane 2, 10% FBS treatment on HCAECs for 5 minutes; lane 3,4,and 5, pretreat HCAEC with 30μM Genistein, PKC412 or H9 for 1h and then treated with 10% FBS for 5 minutes; lane 6,7,and 8, treatment HCAEC with 30μM Genistein, PKC412 or H9 alone. The lysates were subjected to western blotting (**D**).

Figure5.5

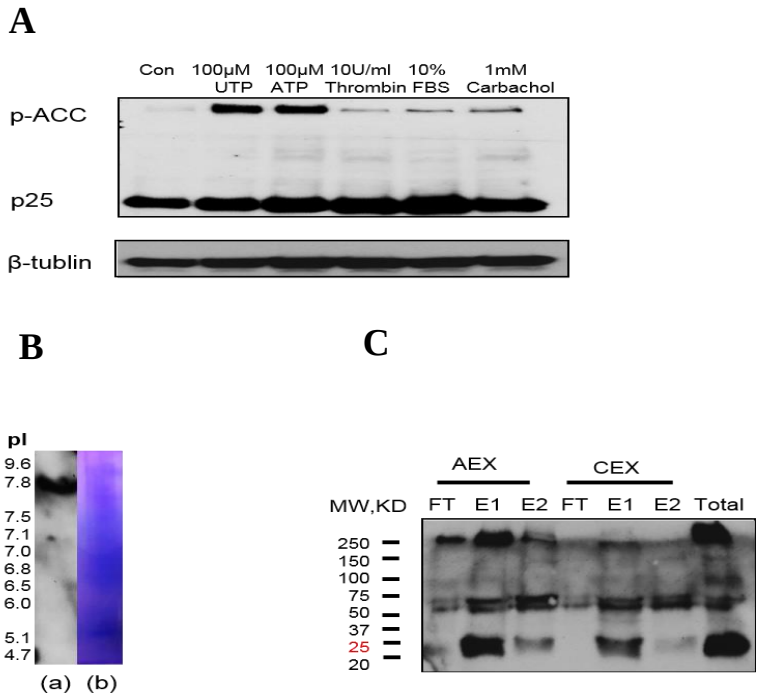

Figure 5.5 Isoelectric focusing (IEF)

The Hela cells were treated with 100μM UTP, ATP, 10unit/ml thrombin, 10% FBS, or 1mM carbachol for 5 minutes. p25 expression were subjected to western blot analyses with p-ACC polyclonal antibodies (**A**). Isoelectric focusing (IEF) gels detection under native conditions was perforemed. 8M urea as solubilizing agent was added to the Hela cells, and the proteins were extracted by freezing and thawing procedure. Protein native extract was loaded on Ready Precast IEF Gels from Bio-Rad and detected with p-ACC antibodies by Millipore SNAP i.d.® 2.0 Protein Detection System (a).Proteins on the nitrocellular membrane was detected by Coomassie blue stain (b) (**B**). Chromatograph of p25 on anion exchange column. Crude cell extract was equilibrated with 20mM Tris, pH 8.3 and then the column was eluted with 20mM Tris, pH8.3 and 1.0M NaCl. The total cell extract, flowthrough, wash and elution fraction were collected and subjected to western blot analysis with p-ACC antibodies (**C**).

Figure5.6

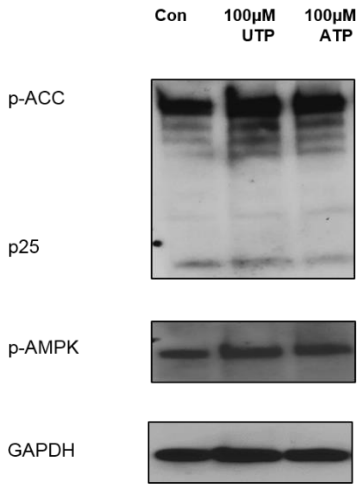

Figure 5.6 p25 expression pattern in THP-1 cell line.

THP-1 cells were starved and treated with 100μM UTP or ATP for 5 minutes.The corresponding total cell lysates were prepared and subjected to Western blot with polyclonal p-ACC or p-AMPK antibodies. GAPDH was used as loading control to ensure equally loading.

Figure5.7

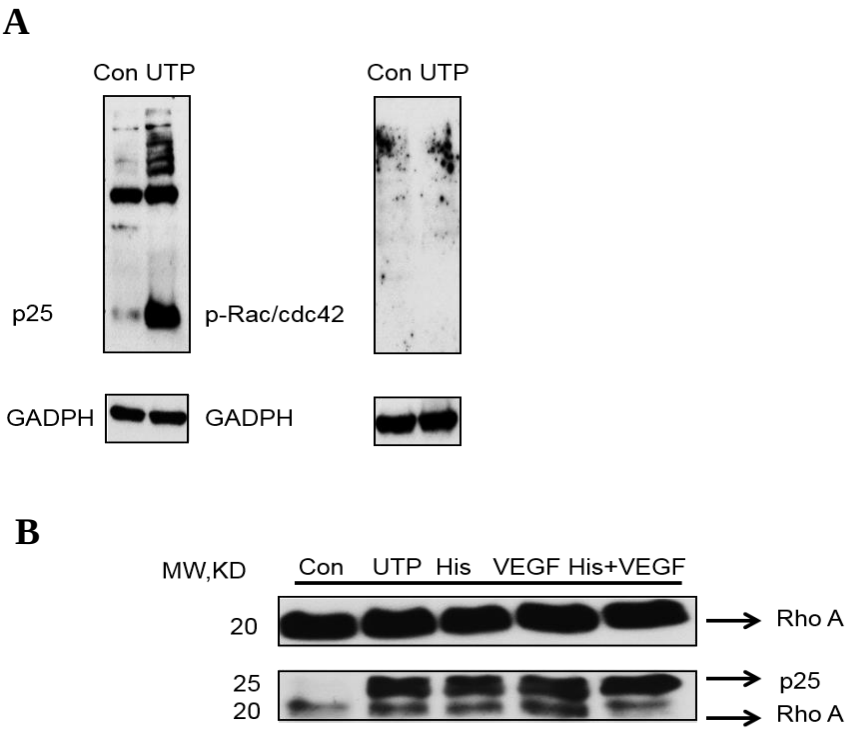

Figure 5.7 p25 is not an already known protein

The same cell lysates from Figure 1 were probed with p-Rac/cdc (28KDa) (**A**) or RhoA (28KDa) antibody (B), after stripping, they were reprobed with p-ACC antibody and visulized by western blotting.

Figure5.8

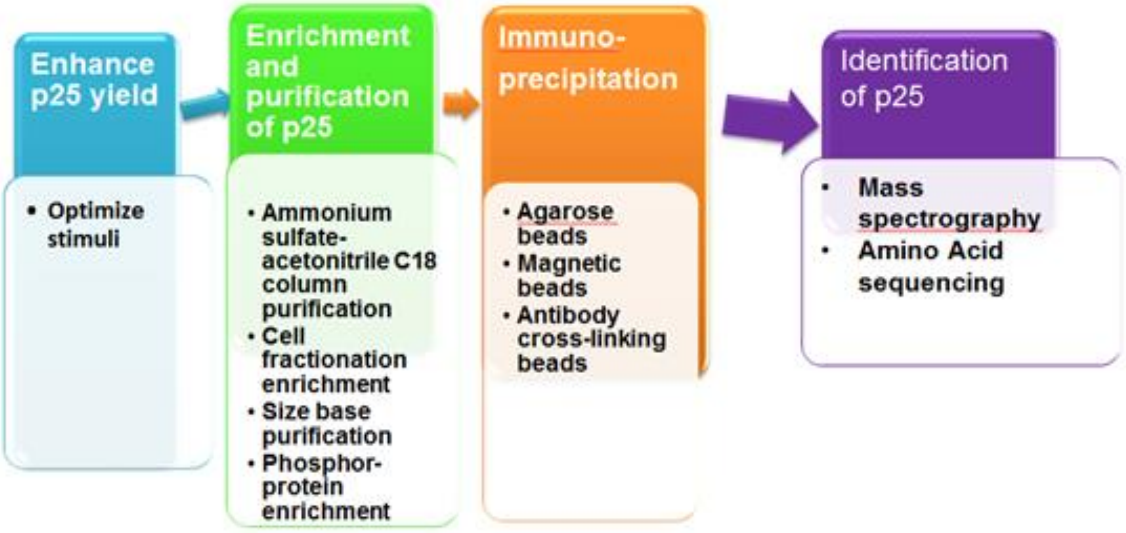

Figure5.8 Proposed purification and identification approaches for p25.

5.4 Discussion

Here, we first found that this unknown protein p25 could be induced by UTP or multiple other stimuli. The up-regulation of p25 expression in endothelial cells is rapid but not accumulated, indicating p25 is not a degradation product of p-ACC. Also, the expression of p25 is Ca^{2+} dependent as it can be induced by calcium ionophores such as A23187 or the activation of other receptor agonists, such as ATP, UTP, or thrombin which increase intracellular Ca^{2+} concentration. Based on above results, we speculated that P2Y2R might modulate the function of endothelium by affecting calcium signaling. Additionally, p25 cannot be induced by direct AMPK activator AICAR, suggests it has no direct relation with p-AMPK or p-ACC. The localization of p25 is an important dimension of knowledge. Cell fractionation and immunoblotting can provide the readout from HCAECs fractionation, thus providing another layer of information that the expression of p25 was mainly found in the cytoplasm. Substantial evidence indicates that post-translational modification of a protein such as phosphorylation, plays a significant role in mediation or modulation of post P2Y2R signaling transduction. The pI value of p25 is around 7.8 which is in the physiological range, and the phosphorylation of p25 is sustained for less than 20min. All lines of evidence indicate that p25 might have an ability to induce post-P2Y2R signaling transduction. To date, little is known about the role of p25 in the regulation of physiological or pathological processes in the endothelial cells or cell lines, as indicated in above data, p25 was also induced by multiple stimuli in Hela cells but not in THP-1 cells, indicating that p25 may play a role in cell adhesion. It was also tempting to hypothesize p25 may be involved in the modulation of essential pathways to regulate cellular function. Further investigation into the expression patterns and regulatory mechanisms governing p25 activity in vascular endothelial cells or cancer cell line will help reveal novel signaling pathways that may participate in the regulation of inflammatory factor activation or therapeutic intervention. However, the final proof of the role of p25 in the signal transduction of P2Y2R receptor must await the determination of sequence of the second prominent protein detected by poly-clonal antibody and thus reconstitution of a functional system containing the overexpress p25

or genetic analysis of p25. In the current study, due to the complexity of sample, the sequencing results still cannot give us a clear answer. To achieve this goal, we design a workflow to purify p25 to near homogeneity as shown in Fig 7. It is well known that purifying an unknown protein is a very challenging work, but we think this is a high risk and high rewarding project that worth the efforts. Many of the alternative approaches are detailed above. At the very worst scenario that all the proposed strategies are not successful in p25 purification, we will consider expression cloning by transfecting the cDNA plasmid library combined with Western blotting detection of p25. Once we know the cDNA coding sequence for p25, we will over-express or employ siRNA approach to knockdown p25 and further explore its cellular function and/or disease relevance.

Chapter 6. Commercial antibodies against P2Y2 receptor lacks specificity

6.1 Introduction

The purinergic signaling system is an attractive therapeutic target for in vascular system such as regulation of tone modulation, inflammation, and coagulation. The purinergic receptor P2 nucleotide receptors are further divided into a P2Y family of G-protein-coupled receptors (P2Y1, 2, 4, 6, 11, 12, 13 and 14) and a ligand gated ion channel family (P2X1-7) (Kaebisch, Schipper et al. 2015). P2Y2 receptor is widely distributed in immune cells, epithelail cells and endotheial cells and neuron cells (von Kugelgen and Hoffmann 2016). Several previous studies aimed at investigating accurate tissue and cellular distribution of P2Y2R and unveil its functional role by using commerial available antibodies from various sources.Carefully evaluation of the specificity of antibodies against P2Y2 receptor is crucially important before we can reach any conclusion. However, concerns remain regarding the specificity of antibodies against P2Y2R (Hamdani and van der Velden 2009, Michel, Wieland et al. 2009, Pradidarcheep, Stallen et al. 2009). Many antibodies raised against receptor are not sepcific for us in protein-targeted technique such as immuunoassays(Hamdani and van der Velden 2009, Beermann, Seifert et al. 2012, Cecyre, Thomas et al. 2014, Talmont and Mouledous 2014). Therefore, the purpose of this study was to evaluate the specificity of several commerial available P2Y2R antiboides. For the detection of endogneous receptor expression, we use HCAEC, as our previous study has demonstrated that the P2Y2R is the dominant P2Y subtype receptor expressed in human coronary artery endothelial cells (HCAEC) (Ding, Ma et al. 2011). Moreover, we expressed recombinant mouse/human P2Y2R proteins in 1321N1 cells, which are genetically modified with an epitope-tag. Thus, in this study, we investigated the specificity and quality of several commerial available P2Y2R antibodies on multiple cells lines and primary macrophages obtianed from wild-type and $P2Y2^{-/-}$ mice by Western blot. Several pubulished studies using those antibodies, (McGivern, Patitucci et al. 2013,

Gailly, Szutkowska et al. 2014) (Choi, Chu et al. 2013) but unfortunately, our results show that all of the tested P2Y2R antibodies bind to above cells in an unspecific fashion at designed size, around 40-60kDa and recognized several protein bands at different molecular wieghts. However, we found one P2Y2R antibody from Abclonal (Catalog A5779) has a specific band shown around 260kDa, which suggests this antibody can detect the P2Y2R aggregate.Next, we use different methods and systems including cells have endogenous receptor and expression of tagged receptors in 1321N1. Apart from Western blot, immunofluorescence was used to evaluate Abclonal (A5779). We found this antibody would be suited to investiate the expression of P2Y2R, the subcellular localiztion, and P2Y2R receptor trafficking.

6.2 Material and Methods

6.2.1 Materials

Dulbecco's Modified Eagle medium (DMEM) and EBM-2 were purchased from Lonza. FluoForte™ Kit was purchased from Enzo Life Sciences. Fetal Bovine Serum (FBS) was purchased from Thermo Fisher Scientific. DNA primers were purchased from Integrated DNA Technologies. P2Y2R antibodies were purchased from various sources as listed in Table 6.1.

Table 6.1 Sources of antibodies for P2Y2R

Antibody	Catalog	Sequence	predicted size
Abclonal	A5779	Recombinant protein of human P2Y2R	55kDa
Abcam	ab10270	CRRTESTPAGSENTKDIRL DVLGSSEDSR RTESTPAGSE	42kDa
	ab46537	DVLGSSEDSRRTESTP	42kDa
	ab125474	KPPTGPSPAT PARRRLGLRR SDRTDMQRIE DVLGSSEDSR RTESTPAGSE	42kDa
	ab168535	Full-length protein	42kDa
	ab168984	Full-length protein corresponding to human P2Y2 aa 1-37	42kDa
Alomone Labs	APR-010	KPAY GTTGL PRAKR KSVR	60kDa
Santa Cruz	sc-15209	Peptide mapping near the C-terminus of P2Y2 of human	42kDa

6.2.2 Mice

Wild-type (C57BL/6) and P2Y2R KO (P2Y2R-/-) on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor) and bred at the animal facility at Auburn University. DNA extraction from mouse tail and PCR genotyping were routinely performed according to the instruction of the animal supplier. Mouse P2Y2R genotyping primers are the following: wild-type forward: 5'-AGC CAC CCG GCG GGC ATA AC-3'; mutant forward: 5'- AAA TGC CTG CTC TTT ACT GAA GG-3'; common reverse: 5'-GAG GGG GAC GAA CTG GGA TAC-3'. Mice were housed in Auburn University's Biological Research Facility in a controlled and pathogen-free environment (25 °C; 12:12-h light-dark cycle) with free access to water and standard chow diet.

6.2.3 Cell culture

HCAEC were cultured in EBM-2 medium supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA1000 (Lonza), and 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. HCAEC were

used between the third and eighth passages. Before stimulation, cells were seeded to grow for 24 h and starved overnight. Wild-type and P2Y2R-transfected 1321N1 cell lines were kindly offered by Dr. Gary Weisman (The University of Missouri-Columbia). Wild-type and P2Y2R-transfected 1321N1 cells were cultured in DMEM supplemented with 10% FBS with or without G418 (0.5 mg/mL).

6.2.4 Intracellular Ca^{2+} mobilization assay

Cells were seeded at a density of $4x10^4$ cells per well into 96-well culture plates and cultured for one day. On day two, the original medium was removed, and the assay medium from FluoForte™ kit (Enzo Life Sciences) containing the Ca^{2+} dye was added, and receptor-mediated Ca^{2+} mobilization was determined as previously described (Ding, Ma et al. 2011). Fluorescence was determined immediately after adding of different reagents, with an excitation wavelength set to 485 nm and an emission wavelength set to 525 nm, and readings were taken every 1s for 500s. For antagonist inhibition experiment, cells were pre-incubated with the antagonist for 30 min before agonist addition. Measurement of Ca^{2+} signal was performed with the fluorometer plate reader (BMG FLUOstar) with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU).

6.2.5 Generation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages were obtained according to the literature (Manzanero 2012). Briefly, bone marrow plugs were flushed from femurs and tibiae of mice with serum-free DMEM medium, after filtration and centrifugation, the harvested cells were resuspended in DMEM medium containing 10% FBS, penicillin (50 U/ml), streptomycin (50 μg/ml), and M-CSF (10 ng/ml). Bone marrow cells (2×10^6) were seeded in 6-well plate with 2ml DMEM medium and incubated at 37°C. On day 3, medium was removed and replaced with fresh DMEM medium plus

new M-CSF. On day 5, the cells were starved overnight in serum-free DMEM medium, after which the macrophages were used for further experiments.

6.2.6 Western Blotting Assay.

After stimulation, cells were lysed, and standard Western blotting assay was performed as previously described, and use bovine serum albumin % as blocking agent (Ding, Ma et al. 2011). The individual primary antibodies used as in Table 6.1. According the information provided by the supplier, each antibody supposed to detect epitope or full-length protein sequence. The supplier provided detail sequences were also listed in Table 6.1.The molecular wight of the detected protein bands was estimated by the molecular weight marker(Precision Plus Protein Standards Dual Color).

6.2.7 Immunofluorescence

HCAEC or macrophages from mice were seeded into 8-chamber glass slides (Nunc), starved overnight, and then treated with vehicle, PSB1114 or UTP. After the 30-minute treatment, the medium was aspirated, and cells were then fixed with 4% paraformaldehyde for 15 minutes and washed with PBS for three times and blocked with 5% horse serum for 1h at room temperature. Then the cells were incubated with rabbit P2Y2R antibody (Abclonal, A5779), overnight at 4°C followed by incubation with FITC-conjugated anti-rabbit IgG (1:200) (KPL) for 90 minutes at room temperature in darkness. For negative controls, cells were incubated with non-immune rabbit IgG in place of specific primary antibody. Finally, mounting medium containing DAPI was added to seal the slides. Images with fluorescent signals in random fields were acquired and captured using an AMG EVOS digital inverted multi-functional microscope (AMG).

6.3. Results and Disscussion

The most common strategy to generate antibodies against GPCR consist of 10 to 40 amino acid from pepetide sequences in GPCR domains (Cecyre, Thomas et al. 2014). Several antibodies from Abcam in use are rabbit polyclonal from peptide antigen, a portion of epitopes of P2Y2R or the full length of P2Y2R protein. We attempted to test those P2Y2 receptor antibodies from Abcam on endogenous receptor expressed HCAEC or transfected P2Y2R in 1321N1 cells. Firstly, we performed intracellular Ca^{2+} mobilization assay to show the presence of human/mouse P2Y2R in transfected 1321N1 cells or HCAEC (**Figure 6.1**). Significant calcium influx was observed on transfected cells and endothelial cells. Next, we tested several anti-P2Y2R antibodies from Abcam with different epitopes as Table 6.1. The selectivity of above antibodies was tested by Western Blot assays. However, WT and transfected P2Y2R cells all show similar patterns of the non-specific bands on designated size at 42kDa for antibodies from Abcam. Unfortunately, none of other antibodies raised against the peptide protein from Abcam exhibits selectivity on human or mouse P2Y2 receptor (**Figure 6.2**). Therefore, we have raised doubts about the immunoassays and the conclusion drawn by the previous articles (McGivern, Patitucci et al. 2013, Gailly, Szutkowska et al. 2014) (Choi, Chu et al. 2013). For above articles, the distinction between WT and P2Y2 KO mice group is very slight. The conclusion is debatable due to the selectivity of those antibodies.We cannot rule out the possibility the observed discrepancy may simply due to the parameters setup or lack of proper controls. Of course, the authors should not take the blame. Since the antibodies are commercially available for profits, the providers should test and present antibodies specificity data with caution. Besides, the other question is even though we can trust the interpretation given by providers, the experiment reproducibility, and lot-to-lot variability remains in concern.

To find an effective antibody for P2Y2R, we also tested several antibodies from other companies. We found P2Y2R antibody (Abclonal, A5779) raised against the recombinant protein of human P2Y2R could be an effective one. Though it didn't show a specific band at predicted 55kDa, we

observed a specific band of molecular weight around 250kDa, which suggests this antibody can detect aggregate of P2Y2R on primary macrophages from WT and P2Y2$^{-/-}$ mice, transfected 1321N1 cells by Western blots. Also, we tested this antibody on macrophages isolated from mice and HCAEC by immunofluorescence and use IgG as the control (**Figure 6.4**).

Currently, the best way for the obtaining the specific P2Y2R antibodies has raised the antibody against protein rather than the peptide. While peptide antigens are attractive due to their cost and speed of generation, it is important to consider their limitations. Where protein antigens are capable of eliciting antibodies against conformational epitopes, antibodies raised against peptide antigens only recognize linear epitopes. Their value is therefore primarily found in the context of PTM detection, although peptide generated antibodies may work in other applications. For example, anti-peptide antibodies may also perform in Western blots and ELISAs. However, the linearized protein must recapitulate the epitopes of the inherently linear peptide antigen used for immunization to assure positive results.

In summary , current commerical available antibodies against P2Y2R are questionable for immunoassay. Abclonal, A5779 use the recombinant protein of human P2Y2R as antigen appears as a useful antibody for downstream detection. Therefore, there is need to invest in the production of Ab from protein antigen, which could be specific for P2Y2R.

Figures and legends

Figure 6.1

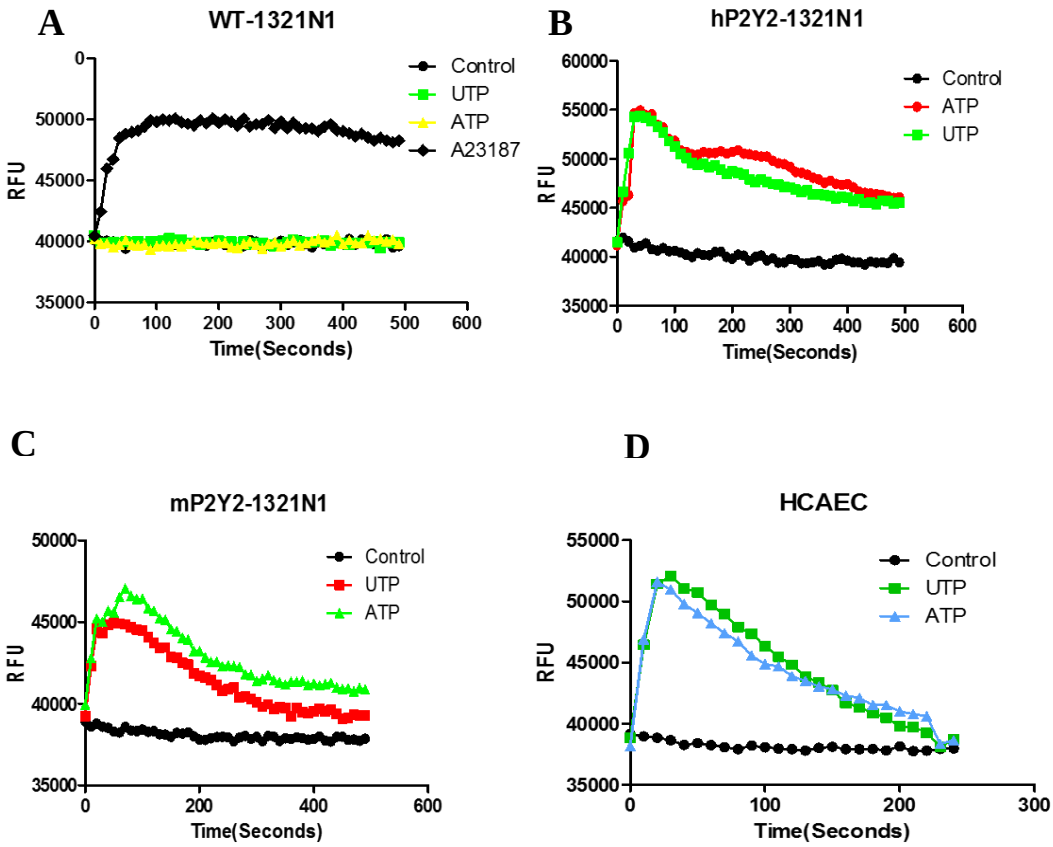

Figure 6.1 UTP/ATP activation of P2Y2R mediated Ca^{2+} signaling in different cell lines.

P2Y2R agonist UTP (100μM)- or ATP (100μM)-induced intracellular [Ca^{2+}] mobilization was determined in wildtype 1321N1 cells which lack of any P2Y receptors. Calcium ionophore A23187 (1 μM) was used as positive control (**A**). UTP- or ATP-induced [Ca^{2+}] increase in 1321N1 cells transfected with human P2Y2R gene (**B**). UTP- or ATP-induced [Ca^{2+}] increase in 1321N1 cells transfected with mouse P2Y2R gene (**C**). UTP- or ATP-induced [Ca^{2+}] increase in HCAEC(**D**). Measurement of [Ca^{2+}] signal was performed by a fluorometer plate reader with a 490/525nm bandpass filter, the results of which was shown as relative fluorescence units (RFU). N=3.

Figure 6.2

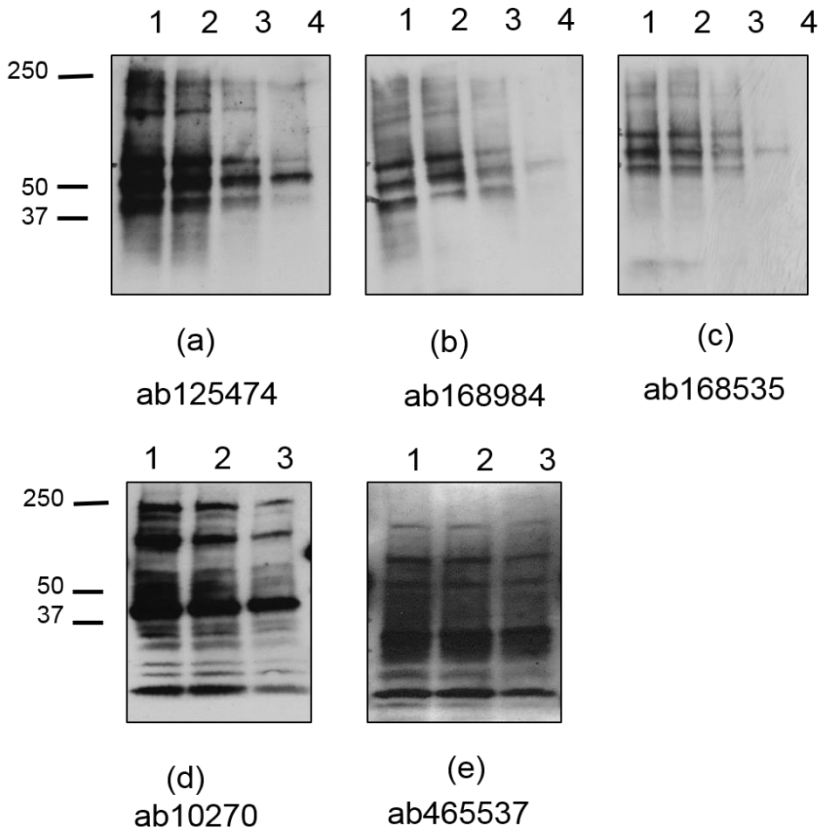

Figure 6.2 Western blot staining HCAEC samples by commercial P2Y2R antibodies.

Protein bands detected by several different antibodies against P2Y2R isotypes from Abcam. Molecular weight of the protein band was calculated on the basis of the molecular weight marker.

Figure 6.3

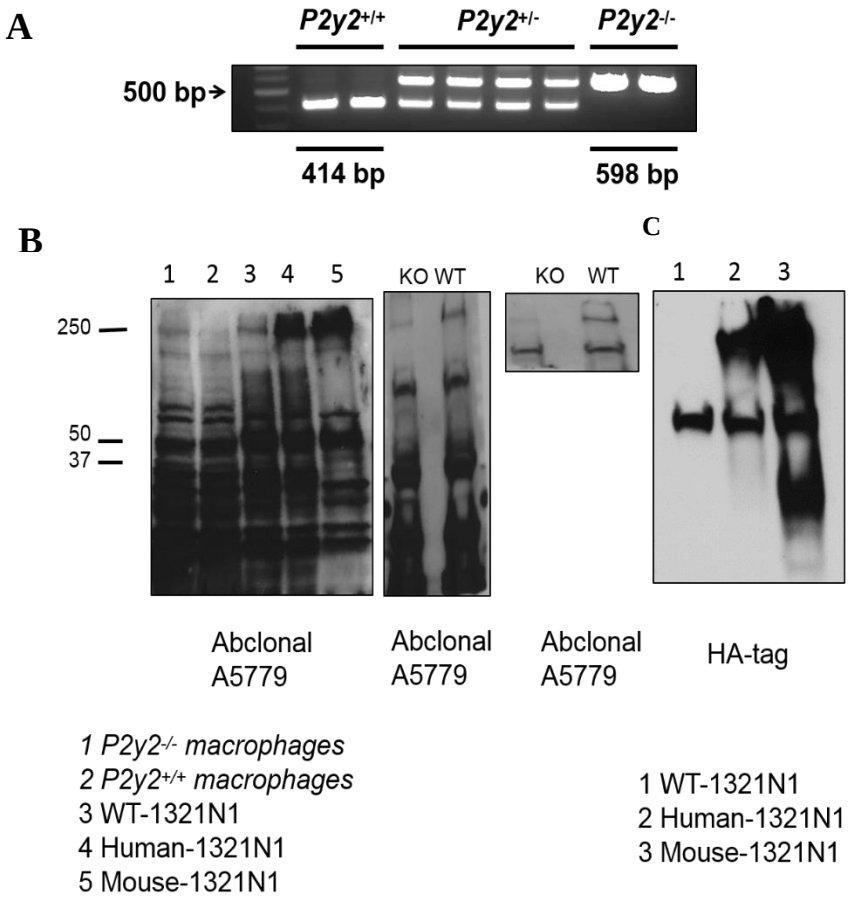

Figure 6.3 Specificity of P2Y2R antibody (Abclonal A5779)

The genotypes of wide-type, heterozygous and P2Y2-null mice were determined by PCR as shown in image (**A**). Bone-marrow derived macrophages were isolated from WT and P2Y2-null mice respectively. Expression of P2Y2R were determined by Western blotting after incubation of P2Y2R (Abclonal A5779) (**B**). Expression of P2Y2R were determined by Western blotting was detected by HA-tag antibody.

Figure 6.4

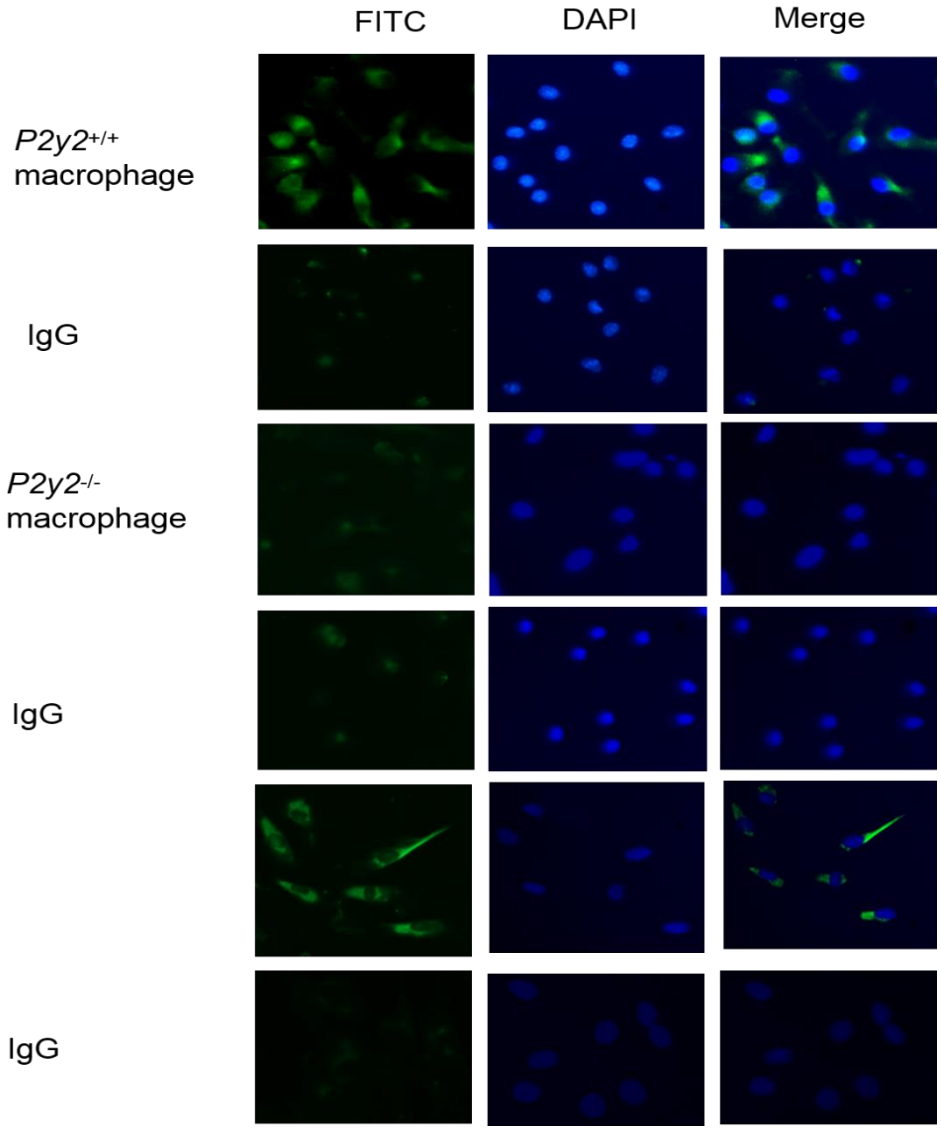

Figure6.4 Abclonal(A1557) specifically binds to P2Y2R in HCAEC and bone-marrow derived macrophages.

HCAEC or macrophages were incubated with Abclonal(A1557) or IgG isotype control for 30 min before fixed for stained immunofluorescence assays. Locations of P2Y2Rwere indicated by FITC (green). Cell nuclei were counterstained with DAPI (blue). Isotype-matched primary antibody or FITC-conjugated secondary antibody were used for negative controls. Scale bar=20μm.

Figure 6.5

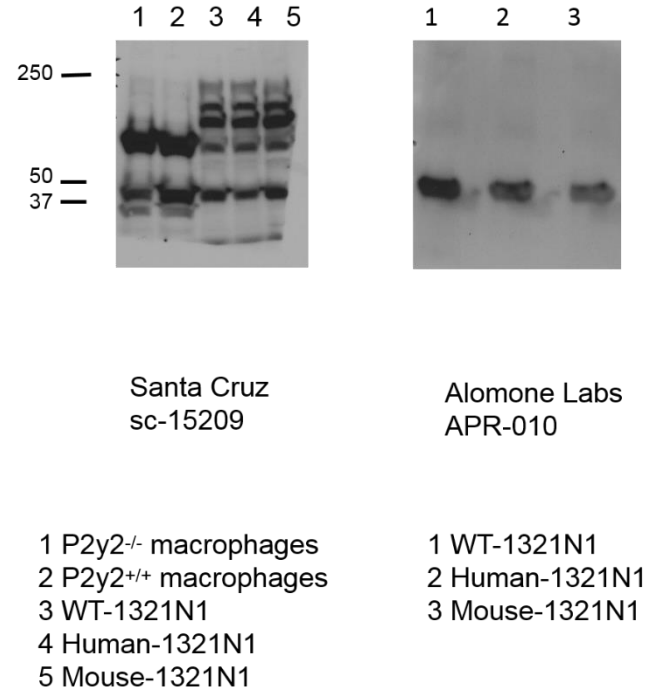

Figure 6.5 Specificity of other commercial P2Y2R antibodies.

Protein bands detected by several different antibodies against P2Y2R isotypes from other companies. Molecular weight of the protein band was calculated on the basis of the molecular weight marker.

Chapter 7. Scope of study and future direction

We indicate that extracellular nucleotide UTP rapidly induces mRNA expression of GM-CSF in HCAEC. However, GM-CSF protein expression is indeed significantly reduced (**Fig. 2.11A & B**). Importantly, this unexpected translational suppression of GM-CSF is also observed when the ECs were treated by both UTP and TNF-α (**Fig. 2.12A & B**). This is consistent with the aforementioned description that P2Y2R can mediate anti-inflammatory effects, but obviously through post-transcriptional mechanism. To unveil the underlying mechanism, we proposed that it may be due to the possible involvement of AMP-activated protein kinase (AMPK), which has been shown to suppress protein translation (Chan, Soltys et al. 2004, da Silva, Jarzyna et al. 2006, Wang, Song et al. 2012). In line with an earlier study in umbilical vein ECs (Hess, Kou et al. 2009), our data indicate that UTP can activate P2Y2R and induce significant activation of the AMPK pathway in HCAEC (**Fig. 2.13A**). To date, P2Y receptor regulation of AMPK has not been fully studied, especially in the context of vascular inflammation evoked by P2Y2R. As P2Y2 receptor is a G protein coupled receptor, IP3 induction of calcium mobilization is observed, P2Y2-CaMKK-AMPK axis in inflammatory gene translational regulation in vitro and in vivo. Therefore, we hypothesize that the P2Y2R/AMPK axis is a previously unappreciated anti-inflammatory pathway that leads to translational suppression of a set of key inflammatory proteins. The major objective of future study is to determine P2Y2R biased signaling in control of pro-inflammatory and pro-thrombotic gene expression through translational mechanisms.

During our study, we found that unlike the endogenous P2Y2R agonist ATP or UTP, P2Y2R-selective agonist PSB1114 significantly activates AMPK and ERK1/2 pathways, but not the pro-inflammatory p38 or JNK pathway (**Fig. 2.13B**). This original observation intrigued us to propose

that P2Y2R may induce signaling bias in a ligand-dependent manner. The growing understanding of complexity of GPCR signaling inspires us to study P2Y2R biased ligands and their functional selectivity. Thus, one of the future direction is to investigate that P2Y2R biased signaling to the AMPK and ERK1/2 pathways leads to suppression of key pro-inflammatory genes by both transcriptional and translational mechanisms (**Fig. 2.14**).

In chapter 2, we observed that PSB1114 can preferentially activate the anti-inflammatory signaling pathways, with no or negligible effect on the pro-inflammatory signaling pathways such as JNK and p38. Moreover, applying PSB1114 on HCAEC may lessen the pro-inflammatory effect induced by endogenous ligand UTP by keeping the anti-inflammatory pathway effect to transcriptional repress TF mRNA. Our data also showed that endothelial P2Y2R signaling can mediate translational suppression for some key inflammatory genes such as GM-CSF, despite a significant induction for GM-CSF mRNA. These findings suggest that the P2Y2R can be manipulated by different ligands to induce a preferred signaling profile that suppresses inflammatory response. Our preliminary data also indicate that extracellular nucleotide UTP rapidly, PSB1114 as a selective P2Y2R biased agonist can translationally suppress a set of pro-inflammatory genes such as GM-CSF expression.

In addition, our future work will examine the functional consequence and in vivo relevance of P2Y2R biased signaling. WT and P2Y2R-null mice will be injected with LPS followed by injection of different doses of 2'-O-Me-UTP or PSB1114, after which mice survival rate and serum levels of various cytokines or chemokine will be determined. Histology study in major organs will also be performed. To compare the efficacy between traditional agonism versus biased agonism in combating inflammatory response in vivo, the P2Y2R agonist UTPγS will be also injected after LPS challenge and the data will be compared with those from the groups of 2'-O-Me-UTP or PSB1114. We anticipate that biased ligand 2'-O-Me-UTP or PSB1114 treatment in WT groups will

show a higher survival rate than the traditional agonist UTPγS-treated group and a lower level of inflammatory markers expression. We expect that the proposed biased ligands are equally or even more efficacious than the reported effect of 2-MeS-ATP in rescue of mouse endotoxic shock and death. The challenge of this study is the stability of PSB1114, but at least 2'-O-Me-UTP is resistant to hydrolysis. Dose titration and injection times will be considered to achieve optimal blood level of the compounds. Finally, to determine the existence of endogenous 2-O-Me-UTP in HCAEC and preliminary detect its physiological role, we performed LC-MS analysis for the quantification of 2-O-Methyl modified UTP. These findings reveal that 2'-O-methyl UTP could be a promising lead compound for synthesis or high-throughput drug screen in the future thrombosis research.

We determined the agonistic or antagonistic effect of 2-MeS-ATP in EC or macrophages; we will further study whether 2-MeS-ATP affects inflammatory response of EC and macrophages isolated from WT and P2Y2R-null mice. We anticipate that this agonistic effect of 2-MeS-ATP can be observed in HCAEC and in primary mouse EC or macrophages, but not in cells isolated from P2Y2R-null mice. Secondly, it also should be noted that 2-Mes ATP functions differently in endothelial cells and transfect human or mice P2Y2R-1321N1 may be ascribed to the overexpression of receptor in cellular hosts and yield altered signaling. For in vivo study, we will generate the endotoxic shock model. Although human cells such as HCAEC have P2Y11 receptor in addition to P2Y2R, a P2Y11 ortholog gene is not found in the murine genome. Thus, we can rule out the involvement of P2Y11 receptor upon 2-MeS-ATP treatment in mice (Abbracchio, Burnstock et al. 2006). UTPγS is against hydrolyzation and has similar signaling profile as UTP as shown in Figure 2.2.7. UTPγS is an agonist for P2Y4 receptor in addition to P2Y2R, thus more P2Y2R-selective ligand such as PSB1114 or P2Y4 receptor-selective agonist may be used. We expect that the survival rate may be different in 2-MeS-ATP-treated versus UTPγS-treated mice due to a potential tissue/cell-specific action of these two nucleotides on the P2Y2R We are aware that the proposed anti-inflammatory effect of 2-MeS-ATP may not be related to its agonistic

property on P2Y2R; instead, it may be due to its partial agonist property in some cells, which will be handled in the future. Of course, we may not be able to rule out P2Y2R-independent mechanism for 2-MeS-ATP, which could be our future study. Future work should focus on synthesis or screening of more effective compounds that build upon the knowledge learned from 2-MeS-ATP, PSB1114, and 2'-O-Me-UTP.

Finally, it is well known that purifying an unknown protein is a very challenging work, but we think this is a high risk and high rewarding project that worth the efforts. Many of the alternative approaches are detailed above. At the very worst scenario that all the proposed strategies are not successful in p25 purification, we will consider expression cloning by transfecting the cDNA plasmid library combined with Western blotting detection of p25. Once we know the cDNA coding sequence for p25, we will over-express or employ siRNA approach to knockdown p25 and further explore its cellular function and/or disease relevance.

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