

THE ROLE OF P2Y<sub>2</sub> NUCLEOTIDE RECEPTOR IN LIPOPROTEIN RECEPTOR-RELATED  
PROTEIN 1 EXPRESSION AND AGGREGATED LOW DENSITY LIPOPROTEIN UPTAKE IN  
VASCULAR SMOOTH MUSCLE CELLS

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition  
College of Human Ecology

KANSAS STATE UNIVERSITY  
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## Abstract

The internalization of aggregated low-density lipoprotein (agLDL) may involve the actin cytoskeleton in ways that differ from the endocytosis of soluble LDL. Based on previous findings the P2Y<sub>2</sub> receptor (P2Y<sub>2</sub>R) mediates these effects through interaction with filamin-A (FLN-A), an actin binding protein. Our findings also showed that uridine 5'-triphosphate (UTP), a preferential agonist of the P2Y<sub>2</sub>R, stimulates the uptake of agLDL, and increases expression of low-density lipoprotein receptor related protein 1 (LRP 1) in cultured mouse vascular smooth muscle cells (SMCs).

The strategy of this research was to define novel mechanisms of LDL uptake through the modulation of the actin cytoskeleton in order to identify molecular targets involved in foam cell formation in vascular SMCs. For this project, we isolated aortic SMCs from wild type (WT) and P2Y<sub>2</sub>R<sup>-/-</sup> mice to investigate whether UTP and the P2Y<sub>2</sub>R modulate expression of LRP 1 and low-density lipoprotein receptor (LDLR). We also investigated the effects of UTP on uptake of DiI-labeled agLDL in WT and P2Y<sub>2</sub>R<sup>-/-</sup> vascular SMCs. For LRP1 expression, cells were stimulated in the presence or absence of 10 μM UTP. To determine LDLR mRNA expression, and for agLDL uptake, cells were transiently transfected for 24 h with cDNA encoding hemagglutinin-tagged (HA-tagged) WT P2Y<sub>2</sub>R or a mutant P2Y<sub>2</sub>R that does not bind FLN-A, and afterwards treated with 10 μM UTP. Total RNA was isolated, reversed transcribed to cDNA, and mRNA relative abundance determined by RT-PCR using the delta-delta Ct method with *GAPDH* as control gene. Results show SMCs expressing the mutant P2Y<sub>2</sub>R that lacks the FLN-A binding domain exhibit 3-fold lower LDLR expression than SMCs expressing the WT P2Y<sub>2</sub>R. There was also decrease in LRP1 mRNA expression in response to UTP in P2Y<sub>2</sub>R<sup>-/-</sup> SMCs compared to WT. Actinomycin-D (20 μg/ml)

significantly reduced UTP-induced LRP1 mRNA expression in P2Y<sub>2</sub>R<sup>-/-</sup> SMCs ( $P < 0.05$ ).

Compared to cells transfected with mutant P2Y<sub>2</sub>R, cells transfected with WT P2Y<sub>2</sub>R showed greater agLDL uptake in both WT VSMCs and P2Y<sub>2</sub>R<sup>-/-</sup> cells. Together these results show that both LRP 1 and LDLR expressions are dependent on an intact P2Y<sub>2</sub>R, and P2Y<sub>2</sub>R/ FLN-A interaction is necessary for agLDL uptake.

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## CHAPTER 1 – Introduction

### A. Lipid Accumulation and Foam Cell Formation

The foremost cause of mortality in men and women in the United States is atherosclerosis. The major event in its development is the accumulation of lipids, mainly cholesterol esters (Fuster et al 1992), which leads to foam cell formation. Chronic inflammation, monocyte infiltration, matrix remodeling and endothelial dysfunction further characterize the progression of the disease.

Although dietary and life-style factors are implicated in the development of atherosclerosis, a number of molecular targets including G protein coupled receptors have been shown to play significant roles in its pathological progression. The G protein coupled P2 receptors for extracellular purine and pyrimidine nucleotides are ubiquitously expressed in human tissues, including the blood vessel wall. Under patho-physiological conditions, nucleotide release from blood-derived and vascular cells activates smooth muscle cell (SMC) P2Y<sub>2</sub> nucleotide receptors, leading to rearrangement of the actin cytoskeleton and cell motility. P2Y<sub>2</sub>R mediates these effects through its interaction with FLN-A, an actin-binding 280-kDa protein. To this end we hypothesize that P2Y<sub>2</sub>Rs can interact with the cytoskeletal proteins to promote LDL uptake by vascular SMCs.

Macrophages become foam cells through the uptake of diversely modified LDLs, whereas the aggregation of LDLs seems to be a key condition for lipid accumulation in VSMCs (Tertov et al 1989, Ismail et al 1994). Although many studies have examined macrophage foam-cell formation, there is still little knowledge as to how VSMCs are transformed into foam cells. Lipoproteins that are retained in the extra-cellular matrix are taken-up by VSMCs and macrophages, and subsequently result in cholesterol ester

accumulation and foam cell formation. Scavenger receptors on macrophages mediate their transformation to foam cells following modified and oxidized LDL. Compared to their relative abundance in macrophages, scavenger receptors are not observed in VSMCs from human atherosclerotic lesions. Studies show that uptake of aggregated LDL (agLDL) is a prerequisite condition for lipid accumulation in VSMCs, and that the LDL receptor related protein 1 (LRP 1) is an important mediator in this uptake. Based on these results, we believe that LRP 1 may play a significant role in atherosclerosis development, but little is known about how LRP 1 is upregulated.

Recent studies have demonstrated that in human VSMCs, LRP 1 mediates the internalization of aggregated LDL (agLDL) generated either by vortexing or sphingomyelinase treatment (Llorente-Cortes et al 2000, Xu et al 1995). Data presented in this dissertation show that UTP, a preferential agonist of the P2Y<sub>2</sub>R, stimulates the uptake of aggregated LDL in cultured VSMCs and increase LRP 1 expression in cultured mouse SMCs. Based on these observations, we hypothesized that the P2Y<sub>2</sub>R may regulate foam cell formation in VSMCs thereby contributing to the formation of atherosclerotic lesions. To test our hypothesis we have obtained wild type and P2Y<sub>2</sub>R<sup>-/-</sup> mice (Dr. Gary Weisman's Laboratory at the University of Missouri in Columbia Missouri) for evaluating the role of this receptor in the internalization of matrix-bound LDL in VSMCs.

## **B. Study Goals and Experimental Approaches**

The P2Y<sub>2</sub> receptor (P2Y<sub>2</sub>R) plays a very important role in stress or injury and the development of inflammation in the vasculature and in arterial wall disease (Seye et al 2002). Under pathophysiological conditions, extracellular nucleotides, including UTP the P2Y<sub>2</sub>R agonist, are released from various cellular sources to activate their respective

receptors. UTP was shown in our early data to stimulate the uptake of aggregated LDL in VSMCs and increase LRP 1 expression in cultured SMCs. UTP stimulation is also associated with various cellular processes including cytoskeleton remodeling, cell proliferation and migration of VSMCs (Yu et al 2008). The central research study goal of this dissertation is to investigate the role of P2Y<sub>2</sub>R in the internalization of matrix-bound agLDL in VSMCs.

Therefore the research aims to:

1. Determine the role of P2Y<sub>2</sub>R-mediated cytoskeleton rearrangement in the uptake of matrix-bound agLDL in cultured VSMCs obtained from wild type and P2Y<sub>2</sub>R<sup>-/-</sup> mice, by using confocal microscopy
2. Use cultured SMCs from P2Y<sub>2</sub>R<sup>-/-</sup> mice transduced with adenoviruses encoding a mutant P2Y<sub>2</sub>R defective in FLN-A binding to determine the effect of a loss of P2Y<sub>2</sub>R/FLN-A interactions in uptake of modified agLDL.
3. Use real-time PCR to determine whether P2Y<sub>2</sub>R agonists modulate LRP 1 expression at the transcriptional level in SMCs isolated from wild type and P2Y<sub>2</sub>R<sup>-/-</sup> mice.

## CHAPTER 2 - Literature Review

The general topics discussed will include 1) the P2 receptors and their subtypes, 2) factors that contribute to the development of atherosclerosis, 3) what we know about atherosclerosis from animal studies, 4) vascular physiology, and vascular smooth muscle cells in atherosclerosis, 5) the actin binding proteins of the cytoskeleton, and 6) LRP 1 structure and function in atherosclerosis development. The studies in this dissertation utilized vascular SMCs obtained from the P2Y<sub>2</sub>R knockout mouse model. These mice were genetically altered to produce a mutation in their *P2Y<sub>2</sub>R* gene, thereby allowing us to test our hypotheses of the involvement of the P2Y<sub>2</sub>R signaling in the uptake of agLDL by SMCs. The P2 class of receptors will be reviewed first in chapter 2, followed by a comprehensive summary of the P2Y<sub>2</sub> receptor subtype, because it is the mechanistic basis on which all of our investigations are carried out.

### A. P2 Nucleotide Receptors

P2 receptors for extracellular purine and pyrimidine nucleotides are ubiquitously expressed in human tissues, including the blood vessel wall. These nucleotide receptors play a very important physiological role in the vasculature with regard to hemostasis, platelet aggregation, vascular tone, muscle function, neurotransmission, and cardiac function. The primary ligands for activating P2 receptors in vivo are ATP, ADP, UTP, and UDP (Burnstock 2007). The P2 proteins are cell surface receptors that belong to two main classes of families, the P2X class and the P2Y class (Burnstock and Kennedy 1985). There are now seven human P2X receptor subunits (P2X<sub>1-7</sub>) (Garcia-Guzman et al 1997a; 1997b; Lee et al 1997; Lynch et al 1999; Rassendren et al 1997; Urano et al 1997; Valera et al

1994), and eight P2Y subunits (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, P2Y<sub>14</sub>) (Ayyanathan et al 1996; Chambers et al 2000; Communi et al 2001; 1997; 1996; 1995; Hollopeter et al 2001; Nguyen et al 1995; Parr et al 1994) that have been cloned and pharmacologically characterized (Table 1).

The P2X class of receptors are ligand-gated cation channels comprised of homo- or hetero-oligomers (Communi et al., 2001). These channels open up in response to the extracellular nucleotide adenosine 5' triphosphate (ATP), and facilitate the movement of cations (e.g. Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>) across the plasma membrane. This activity produces changes in the electrical potential of the cell followed by the propagation of a signal, as occurs with muscle contraction, and throughout the nervous system. The P2X<sub>1-7</sub> receptor family subunits display a topology that include intracellular N- and C-termini with protein kinase binding motifs and two transmembrane spanning regions, TM1 and TM2, that function in channel gating and ion pore lining, respectively, a large extracellular loop with disulfide bridges, and an ATP-binding site (Burnstock & Knight 2004). This dissertation research is based on select members of the P2Y class so the following discussion will only focus on those elements.

The P2Y receptors are seven-pass membrane spanning receptors coupled via the heterotrimeric G proteins (G<sub>q/11</sub> or G<sub>i/o</sub>) to phospholipase C (PLC) or adenylate cyclase (North et al., 2000; Cooper et al., 1979; Communi et al., 2001). PLC activation results in inositol 1,4,5-triphosphate (IP<sub>3</sub>), a mediator of Ca<sup>2+</sup> release from intracellular stores, and diacylglycerol, which is an activator of protein kinase C (PKC). Adenylate cyclase generates cyclic AMP, an activator of protein kinase A (PKA). The structure predicted for P2Y receptors (figure 1) include an extracellular N terminus that contain several N-linked



glycosylation sites, a seven transmembrane spanning ligand-binding domain, and an intracellular C terminus containing several consensus binding/phosphorylation sites for protein kinases (Erb et al 2006). P2Y receptors are activated by purine and pyrimidine nucleotides, and each P2Y receptor subtype is linked to one or more of the four subgroups of heterotrimeric G proteins,  $G_s$ ,  $G_{i/o}$ ,  $G_{q/11}$ , and  $G_{12/13}$  (Erb et al 2006). Following P2Y receptor stimulation, the heterotrimeric G-protein is activated and dissociates into its  $\alpha$  and  $\beta\gamma$  subunits which are then free to interact with various effector proteins. There is a 19-55% sequence identity among the eight cloned P2Y receptor subtypes (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, P2Y<sub>14</sub>) that have been identified and characterized at the level of protein. As a result they display significantly different pharmacological and functional profiles (Burnstock & Knight 2004). The subscripted numbers after P2Y represent their chronological order of cDNA cloning (Arrabacchio et al 2006). The P2Y receptor numbers that are not shown are those of either non-mammalian orthologs, or of receptors that have no responsiveness to nucleotides, but which share some sequence homology to P2Y receptors (Burnstock 2007). For example, the chicken ortholog of the P2Y<sub>6</sub> receptor is believed to be P2Y<sub>3</sub>, whereas the *Xenopus* and turkey orthologs of P2Y<sub>4</sub> could be P2Y<sub>8</sub> and tp2y, respectively (Li et al 1998).

The P2Y receptors can be further classified into two groups, the P2Y<sub>1,2,4,6,11</sub> and the P2Y<sub>12,13,14</sub> subgroups, which are based on phylogenetic and protein sequencing data (Costanzi et al 2004). Activation of P2Y<sub>1,2,4,6,11</sub> results in the stimulation of phospholipase C through  $G_q$  and activation of P2Y<sub>12,13,14</sub> results in the inhibition of adenylate cyclase through  $G_i$  protein (Jacobson et al 2012). With the exception of the P2Y<sub>11</sub> receptor gene, there are no introns in the DNA coding sequence of P2Y receptors. Also the amino acid

sequences, pharmacological profile, and signaling pathway of the P2Y receptors have significant differences. Regarding activation sites P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors were probed using site directed mutagenesis, and found to contain positively charged residues in the transmembrane helical domains (TM) 3, 6, and 7, which are critical binding sites for receptor activation by nucleotides (Jiang et al 1997). Activation of P2Y<sub>1,2,4,6</sub> receptors result in IP<sub>3</sub> release and calcium mobilization. Activation of P2Y<sub>11</sub> by ATP results in both an increase in cAMP and IP<sub>3</sub>, and only calcium mobilization when stimulated by UTP (Burnstock 2007). P2Y<sub>4</sub> has the greatest mRNA and protein expression in human and mouse intestine and other organs, and is activated by UTP and ATP. The P2Y<sub>2</sub> receptor antagonist suramin is often used to distinguish between P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors. P2Y<sub>6</sub> receptors are selective for UDP and are highly expressed in spleen, intestine liver brain and pituitary. Activations of P2Y<sub>6</sub> result in G<sub>q</sub>-dependent PLC activity and are notably co-expressed with P2Y<sub>14</sub> receptors in many tissues (Harden et al 2010). P2Y<sub>12</sub> receptor is expressed in neurons, brain cells, smooth muscle cells, glial cells, chromaffin cells, and in the megakaryocyte/platelet lineage (Burnstock 2007). P2Y<sub>12</sub> plays a major role in platelet aggregation and thrombosis, with ADP as the natural agonist. P2Y<sub>12</sub> knockout mice had increased bleeding time and impaired platelet adhesion to von Willebrand Factor, decreased platelet activity as observed with inhibition of fibrinogen-binding, resistance to thrombus formation, and thrombi instability in injured arteries (Andre et al 2003). P2Y<sub>13</sub> receptors are expressed mainly in spleen, placenta, liver, heart, bone marrow, monocytes, T cells, lung and brain. The P2Y<sub>14</sub> receptor has a wide tissue distribution, especially in epithelial and immune cells, and is believed to play a significant physiological role in immune system homeostasis, due to its high expression in leukocytes (Harden et al 2010).

It is found throughout the body, and couples to  $G_i$  upon activation by UDP, UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetyl glucosamine (Carter et al 2009; Harden et al 2010).

## **B. The P2Y<sub>2</sub> Receptor**

The P2Y<sub>2</sub> receptor (P2Y<sub>2</sub>R) has been cloned, and characterized using human, rat, mouse, canine, and porcine cells or tissues (Lustig et al 1993; Shen et al 2004). The P2Y<sub>2</sub> receptor is functionally responsive to its equipotent agonists UTP and ATP, but much weaker responses are elicited with ADP and UDP (Burnstock 2007). P2Y<sub>2</sub> receptor is upregulated during inflammation. For example, P2Y<sub>2</sub> mRNA was significantly increased in aortic smooth muscle cells by more than seven-fold, following stimulation from the inflammatory cytokine interleukin-1 $\beta$ . This effect was enhanced by the inflammatory cytokines interferon- $\gamma$ , and tumor necrosis factor- $\alpha$  (Hou et al 2000). P2Y<sub>2</sub> receptor mediates phospholipase C $\beta$  (PLC $\beta$ ) signaling when coupled to G proteins,  $G_{\alpha_q/11}$ , to release IP<sub>3</sub> and diacylglycerol, which are second messengers for intracellular calcium release, and activation of protein kinase C (PKC), respectively (Burnstock 2007).

Guanine nucleotide-binding proteins, commonly known as G proteins, function as molecular switches to transduce chemical signals and are activated by binding GTP and deactivated by hydrolysis of bound GTP to GDP. P2Y<sub>2</sub> receptors are upregulated during periods of stress and also in response to injury. For example, following balloon angioplasty, P2Y<sub>2</sub>R mRNA levels increased in blood vessels. Over-expression of the P2Y<sub>2</sub>R mRNA was observed at the neointimal thickening of rat aortic lumen indicating the participation of some P2Y<sub>2</sub>R agonist in atherogenesis (Seye et al 1997). This observation was later

confirmed in a stress-induced study involving placement of a vascular collar around rabbit carotid artery. In that study UTP was shown to contribute to the disease process through increased P2Y<sub>2</sub>R mRNA expression prior to neo-intima formation (Seye et al 2002). P2Y<sub>2</sub> receptor mRNA is observed in many tissues, including the cardiovascular system, and upon activation was shown to increase production of arachidonic acid, prostaglandins and nitric oxide (Burnstock 2007).

While these studies may indicate a causal involvement of P2Y<sub>2</sub> receptor in atherogenesis, they do not specify a mechanism whereby cholesterol accumulation and foam cell formation ensues. Therefore, the aim of this dissertation is to establish an association between agonist-induced P2Y<sub>2</sub> receptor activation and foam cell formation by using a P2Y<sub>2</sub> knock-out mouse model and examining LDL uptake. P2 receptors mediate the effects of extracellular nucleotides, and both ATP and UTP were shown to have equal effects on P2Y<sub>2</sub> receptor activation (Parr et al 1994). Seye et al 2000 reported that P2Y<sub>2</sub> receptor in vascular SMCs was rapidly up-regulated, following placement of a silicone collar on a rabbit carotid artery. In addition local UTP treatment to the arteries produced intimal thickening, and osteopontin expression. Osteopontin is a multifunctional cytokine molecule expressed in various cells including smooth muscle cells, and is a marker of inflammation. Recent clinical studies showed plasma concentrations of osteopontin as an important marker for inflammatory status and accelerated atherosclerosis in patients with coronary artery disease (Mazzone et al 2011).

### **C. Arterial Changes and Atherosclerosis**

Atherosclerosis is a major public health disease in the United States of America and

other westernized countries, and the leading cause of mortality in the United States among men and women  $\geq 65$  years old (Roger et al 2012). Cholesterol, while an important component of cell membranes, may initiate atherosclerosis (Goldstein et al. 1977; Guton and Klemp 1996). Atherosclerosis is a chronic low-grade inflammatory disease of the large arteries that is characterized as arterial wall build-up and subsequent hardening with plaque comprised of mainly cholesterol, calcium, fat, and other substances that are found in the blood (<http://www.nhlbi.nih.gov/health/health-topics/topics/atherosclerosis>). Over time, these substances accumulate inside the arteries causing hardening, loss of elasticity, and narrowing of the arteries, thus impeding normal flow of oxygen-rich blood throughout the body. As a result of this blockage, tissues are deprived of blood and oxygen, leading to tissue damage and tissue death, all factors implicated in heart attacks and stroke.

Lesions, intima media thickening and subsequently occlusive thrombi in the arterial walls also characterize atherosclerosis. Modified low-density lipoproteins (LDL) are taken up by macrophages to form foam cells, which later die and contribute to lesion formation (Lusis 2000). Other studies, using electron microscopy to study the aortas of young and middle-aged adults, suggest that the early lipid core forming the foam cells takes place in a manner that is independent of macrophages, and that there was a gradual accumulation of lipids in the extracellular matrix deep within the intima (Nakashima et al 2008; Guyton et al 1993). There is further support for LDL accumulation and oxidation in the intima of adult aortas and of human fetuses (Napoli et al 1997; Babaev et al 1993). Additional cell types such as endothelial cells, lymphocytes and smooth muscle cells play a role in the development of the disease. Monocyte-derived macrophages, T-lymphocytes and platelets interact with the endothelium and vascular smooth muscle cells to generate atherosclerotic

lesions and diminished arterial elasticity. These biochemical events leading to atherogenic pathology, represent the major cause of mortality in the United States, Europe and parts of Asia (WHO 1985).

The above events leading to atherosclerosis develop very slowly over many decades, and the rate of change varies from person to person, with lesion initiation and progression being virtually indistinguishable. These factors, along with the uncertainty of the precise origin of the extracellular lipids in the arterial wall, and the lack of an ideal animal model to study the early stages of the disease, have made research progress on early human atherosclerosis move at a very slow pace (Nakashima et al 2008).

Long before atherosclerosis develops early arterial changes such as a thickening of the intima are noted. There are two types of intimal thickenings, the eccentric or intimal cushion (intimal thickness at branch points and orifices), and diffuse intimal thickening or (DIT; musculoskeletal intimal thickening that spreads out around the circumference and along the length of non-branching segment of arteries). However, it is not clear whether these early changes are precursors for the more advanced lesion development. Some reports point to evidence that this type of smooth muscle cushioning seen in the eccentric intima is necessary but not sufficient to produce atherosclerotic lesions (Santerre et al 1972). The Council of American Heart Association has defined the DIT as a normal process in human arteries, with normal intact SMCs, elastin and proteoglycan composition (Stary et al 1997; Nakashima et al 2008). However, DIT can function as a repository for extracellular lipid accumulation in early stage atherosclerosis.

The apical and basolateral surfaces of vascular cells typically undergo morphological changes that result from blood flow and sequential rhythmical stretching

and retraction. These structural changes can subsequently alter the physiological function of the blood vessels and result in thrombosis or atherosclerosis. It is therefore important to understand the molecular mechanisms and processes within the cells that lead to the development of atherosclerosis in order to limit its progression and avert acute clinical events.

Under physiological conditions high-density lipoproteins (HDL) remove excess cholesterol from cells in the arterial wall, and shuttle it to the liver where it is excreted in bile, by a process known as “reverse cholesterol transport” (Francis 2010). In addition endothelial cells protect the vasculature by releasing nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS), thus preventing aggregation by adhesion molecules, inhibiting platelet activation, and by inducing vasodilation (Badimon et al 2011). In this literature review below, different aspects of the atherogenic process are detailed.

#### **D. Overview of the Structure and Function of Blood Vessels**

All blood vessel walls, except the capillaries, have three structurally distinct layers, referred to as tunica or tunics. As a functional unit, these tunics surround the blood vessel lumen, a central blood-containing space, to support the transport of blood by the vessels (Marieb 2001). The tunica intima is the innermost layer, and is so named due to its proximally intimate contact with the blood in the lumen. The intima contains the endothelium, which is comprised of simple squamous epithelium that forms the smooth surface necessary to minimize blood flow friction (Marieb 2001). In addition, large vessels like the aorta have an additional basement membrane, a subendothelial layer that supports

the endothelium. Minimizing friction is a critical factor in the prevention of increased peripheral resistance (opposition to flow in the peripheral circulation). When fatty debris and cholesterol accumulate in the arterial walls forming rough bulging areas, there is a narrowing of the vessel diameter, the blood flows closer to the vessel walls and flow is slower by reason of increased friction (Marieb 2001).

The tunica media forms the middle layer, and is made up of smooth muscle cells in a circular arrangement, and also elastin fibers. The tunica media plays a critical role in maintaining blood pressure and in the continuous flow of circulating blood. It accomplishes this through the vasoconstriction and vasodilation activities of the smooth muscles, which are regulated by vasomotor nerve fibers of the sympathetic portion of the autonomic nervous system (Marieb 2001).

The tunica adventitia is the outermost layer. This layer is equipped with tiny blood vessels called vasa vasorum, which provides nutrients to the external blood vessel wall. Collagen fibers that comprise the adventitia functions mainly as a protective layer around the blood vessel, while anchoring it to surrounding structures. Capillaries do not have an adventitia or media layer. They are very thin and are made up of only the tunica intima (Marieb 2001).

#### **E. General Role of Cholesterol and Lipoproteins in Atherogenesis**

Cholesterol is the primary sterol found in vertebrates, and it is the precursor for steroid hormones, bile acids and various steroids. Cholesterol homeostasis dictates that lipoproteins are first taken up, followed by hydrolysis of cholesterol ester, intracellular transport and esterification of cholesterol, then efflux of cholesterol from within the cells.



Apolipoprotein (apo)-A 1, high-density lipoproteins (HDL), scavenger receptors, and ATP-binding cassette (ABC) transporters play a major role in maintaining this balance between accumulation/influx, and removal/efflux of cholesterol, and the formation of foam cells. Atherogenic diets that are high in fat and cholesterol are reportedly major factors in foam cell formation and atherosclerosis development. The low-density lipoprotein receptor (LDLR) plays a significant role in taking up cholesterol from the plasma in order to maintain cholesterol homeostasis. As a cell surface receptor, it binds and internalizes LDL-cholesterol via the protein adaptin, in receptor-mediated endocytosis. In a study with LDLR deficient mice, extensive fatty streaks developed that are secondary to macrophage foam cell formation, following consumption of the atherogenic diet (Breslow 1996; Ishibashi et al 1994). LDLR has binding sites for the transport proteins apo B and apo E on LDL particles, thereby helping to facilitate cholesterol transport within the cells, and as a result their concentration levels are risk factor indicators for heart disease. Studies show that transgenic mice overexpressing the homologous apo B gene have elevated levels of plaque-forming LDL cholesterol, and lower levels of the more protective HDL cholesterol (McCormick et al 1996). Other studies show that in transgenic apo E deficient mice SMC infiltration is not evident until after 20 weeks (Niskashima et al 1994).

## **F. Endothelial Activation and Atherosclerosis**

The endothelium with its tight junctions is an anti-thrombogenic layer that acts as a permeability barrier, regulating the movement of nutrients and fluids between the plasma and the arterial wall. It is also involved in modifying or oxidizing lipoproteins as they are transported from the plasma into the arterial wall. The initial stages in the development of

atherosclerosis, known as atherogenesis, involve the internalization of lipids in the intima, and activation of the endothelium. Atherogenesis is initiated by endothelial dysfunction that is due to a number of factors including hypertension, diabetes, smoking, and high cholesterol. The process also involves adhesion of monocytes and lymphocytes to the endothelial surface, followed by monocyte migration and subsequent differentiation into macrophages in the sub-endothelial space.

The earliest endothelial change that is considered both necessary and sufficient for the central pathogenesis of atherogenesis is the retention of atherogenic lipoproteins in the subendothelial space (Williams & Tabas 1995). In accordance with the “response to retention hypothesis” other contributory processes that follow are merely an inflammatory response by normal healthy tissue to the presence of retained lipoproteins. The key evidence supporting the retention of lipoproteins point to the involvement of apolipoprotein B 100 (apo-B 100), the protein moiety of LDL, and the extracellular matrix, particularly proteoglycans. Using human recombinant LDL from human apo-B transgenic mice, and site directed mutagenesis studies to abolish binding activities, it was observed that the LDL receptor binding site B (amino acid residues 3359-3369) was also the main binding site for the proteoglycans (Boren et al 1998). This implies that atherogenesis may be impeded or averted in the presence of LDL defective in proteoglycan binding. Lipoprotein-proteoglycan complexes were shown *in vitro* to result in significantly faster uptake and degradation of rabbit apo-B lipoprotein (VLDL, IDL, LDL) by rabbit aortic SMCs, compared to lipoprotein only (Ismail et al 1994).

Early endothelial changes associated with atherogenesis produce leukocyte attachment and adherence due to activated intercellular adhesion molecule-1 (ICAM-1)

(Cybulsky 1991; Poston et al 1992) and vascular cell adhesion molecule-1 (VCAM-1) induced by P2Y<sub>2</sub> nucleotide receptor activation (Seye et al 2003). Prominent early changes that follow include endothelial permeability that allows increased amounts of lipoprotein to be oxidized, and transported into the subendothelial space.

Oxidized low-density lipoprotein (OxLDL) has the ability to alter gene expression in the endothelium (Nagel et al 1994). Constant injury to the endothelium can cause loss of endothelial cells due to inability to replicate, with accompanying loss of normal physiological properties which further results in increased thrombogenicity, increased leukocyte adhesion and increased lesion formation at sites of injury (Ross 1995). Endothelial dysfunction results in eNOS impairment, decrease in NO bioavailability, and subsequent decrease in the anti-atherogenicity of the endothelium (Badimon et al 2011; Vidal et al 1998).

## **G. Vascular Smooth Muscle Cells in Atherosclerosis**

### **1. Findings from Animal Studies**

The first notable animal study of experimental atherosclerosis was conducted in 1913 by Nikolaj Nikolajewitsch Anitschkow. Anitschkow was able to demonstrate that arterial lesions developed only when rabbits were fed a high cholesterol diet, and through this groundbreaking study showed that a causal link exists between dietary lipids and blood lipids. Anitschkow analyzed the development and histology of plaque, and he used immunohistochemistry to identify the cell types at the atherosclerotic site. He identified smooth muscle cells, macrophages and lymphocytes, and found that the amount dietary

cholesterol consumed was directly proportional to the size of the atherosclerotic lesion (Finking 1997).

Mouse models of atherosclerosis are often used to study the disease's development and progression, but there are notable differences between the morphological characteristics and processing of the intimal tissue that is found in mice and the intimal tissue that is found in humans (Nakashima et al 2008). For example, in mouse models the intima is very thin and there is no thickening of the intima in either the coronary artery or the aorta. Also, unlike in mice where lipids are deposited in a very narrowly defined subendothelial space with mostly extracellular matrix and very few SMCs, in humans there are greater amounts of both SMCs and extracellular matrix that have become reservoirs for excess lipid accumulation (Allahverdian et al 2010). Therefore, the SMCs in humans are proximally very close to the lipids that accumulate in the intima. Other small animals including rats and rabbits are sometimes used to study atherosclerosis. However, it has been noted that in rabbits there is intimal thickening only in the branches of the coronary arteries and also within the aortic arch (French 1966). Intimal thickenings were also detected at the celiac bifurcation of the aortas in both the atherosclerosis-prone White Carneau pigeon, and the atherosclerosis-resistant Show Racer breed, but only the White Carneau later developed atherosclerosis. Subsequent research showed differences between the two breeds of pigeons regarding the metabolic patterns of the vascular proteoglycans, major components of the extracellular matrix, which are involved in regulating molecules moving through the matrix. There are conflicting findings regarding the presence of intimal thickening in other mammals such as dogs, cows, sheep, horses, cats, and pigs (French 1966; Sims 1989). However, the intimal thickening in the coronary

artery of rhesus monkeys is similarly distributed as that in humans (Stacy et al 1976; Nakashima et al 2008).

There are clear differences in animal models compared to humans, and some disadvantages in using them for studying vascular disease affecting humans. However, genetic modifications and molecular cloning have made it possible to use them by way of *in vitro* and *in vivo* studies to investigate complex molecular systems and obtain valuable information that often leads to life-saving health solutions for a variety of human diseases.

## **2. Vascular Smooth Muscle Cell Physiology**

Smooth muscle cells comprise the major type of cells in early stage human atherosclerotic lesions, but are also found in other later stages of atherosclerosis. Although the precise origin of these cells is unknown, one study supports the notion of differentiation of bone marrow progenitor cells to intimal SMCs as the source for intimal SMCs that accumulate in atherosclerosis (Yu et al 2010). Intimal SMCs are also believed to be medial SMCs from the tunica media that matured and migrated into the intima following some atherogenic stimuli (Ross, 1995; Allahverdian and Francis 2010).

The tonus of the artery is provided by the SMCs, which are located in the tunica media. Due to proliferation and subsequent migration they are found in the intimal site of the arterial wall, an area characterized as the innermost layer within the arterial wall that is populated by endothelial cells (Nakashima et al 2008). The SMCs located in the tunica media are involved in maintaining the continuous and passive partial contraction of the muscle during diastole and systole. However, over time and with increasing endothelial damage, chemotactic factors and mitogens from the endothelium activate SMCs that migrate into the intima and proliferate.

In humans, most of the smooth muscle cells localize in the arterial wall, however a large amount is also located in the intima, referred to as intimal thickening that are either of a diffuse pattern or an eccentric pattern, although often indistinguishable. The eccentric intimal thickening or so called intimal cushion is a thickening around the branched areas and orifices, whereas the diffuse or musculoelastic intimal thickening is a thickening that spreads both linearly and around the circumference of the non-branching parts of the arteries.

Smooth muscle cells can exist in either a contractile or synthetic state, with the former state characterized by a greater amount of contractile myofilaments in the cytosol, and the latter characterized by proliferation and migration following activation (Ross 1995). In fact, vascular smooth muscle cells can be transformed from the contractile state to the highly proliferative phenotypic state following vascular injury, and the release of cytokines, growth factor and other mitogens (Zargham 2005).

### **3. Vascular Smooth Muscle Cell Migration**

Migration by cells is carried out in part by the coordinated activity of different cellular components that is powered by the actin cytoskeleton machinery. The actin filaments form various cell surface projections, which include lamellipodia and filopodia, to engage in dynamic activity and explore their surroundings. This complex process of cell locomotion involving lamellipodia refers to the forward planar extension of the encased actin network on the mobile edge of the cell. As the lamellipodia extends further and forms finger-like projections of the cell membrane they are defined as filipodia. The migratory aspect has been linked to the interaction between the actin cytoskeleton and microtubules,

possibly mediated through Rho GTPases, as activated RhoA has been shown to induce stress fibers formation, whereas activated forms of Cdc42 and Rac1 induced polymerization of filopodia and lamellipodia, respectively (Wehrle-Haller 2001).

#### **4. Vascular Smooth Muscle Cells and LDL Uptake**

Since the progression of atherosclerosis is associated with cell proliferation and migration, any mechanism that could disable this process would also interfere with the progression of this disease. That would mean that specific signaling pathways would be targeted without impeding the vital functioning of the cell. The main event in the atherogenic process is endothelial dysfunction, noted by lipid accumulation in the sub-endothelium.

Both in vitro (Frontini et al 2009; Mietus-Snyder et al 2000) and in vivo (Choi et al 2009; Stary et al 1994) studies show that SMCs can take up and form lipid-laden cells. SMCs were also transformed into foam cells when they were incubated with oxidized LDL (Yu et al 2010). In a separate study, mouse SMCs were reportedly transdifferentiated to macrophages following cholesterol loading and loss of SMC markers (Rong et al 2003). SMCs that expressed both smooth muscle  $\alpha$ -actin and a macrophage marker, CD 68, were shown to be linked with lipid accumulation and phagocytosis (Andreeva et al 1997). Foam cell formation in SMCs and the development of atherosclerosis depends on the efficiency with which this excess cholesterol is removed from the cell by apolipoprotein A1 (apoA-1) and HDL particles. If excess cholesterol is removed more rapidly by these particles, the rate of development of atherosclerosis decreases.

Although SMCs are the primary cell type that accumulate in the intima, the accumulation of excess lipids by macrophages are also a major contributing factor to the total content of foam cells that form lesions. Due to findings suggesting that some foam cells may actually be derived from SMCs instead of from monocyte-derived macrophages, it is a good idea to browse the contribution of macrophages to atherosclerosis. According to the current model that details the initiation of atherosclerosis, lipoproteins that contain apolipoprotein B diffuse from the circulation into the subendothelial space, are retained and subsequently modified. Following endothelial damage activated monocyte-derived macrophages take up lipids from these retained lipoproteins and become transformed into foam cells (Williams et al. 1995). Phorbol myristate acetate (PMA)-activated macrophages seeded onto macrophage-derived extracellular matrix (ECM) showed increased uptake of the ECM-bound lipoproteins (OxLDL or native LDL) by the macrophages, with increasing lipoprotein concentrations (10 to 100  $\mu\text{g protein/mL}$ ) (Kaplan, 2000). Furthermore, the binding capability of OxLDL to the ECM was dose dependent, and increased 3-fold compared to the native LDL. The ECM-bound OxLDL uptake by macrophage was 1.5-fold greater than uptake of ECM-bound native LDL (Kaplan, 2000).

There are several ways in which the cell is stimulated to accumulate OxLDL and other lipids. In one key finding, LDL receptor-related protein-1 (LRP1) in human VSMC was shown to be critical to the binding and internalization of aggregated LDL (agLDL) versus native LDL (nLDL). In LRP1-deficient VSMCs, uptake and accumulation of agLDL-CE was impaired (Llorente-Cortes et al 2000). Additionally, arterial wall secretory sphingomyelinase (SMase) was shown to be a contributory factor in subendothelial lipoprotein aggregation, a potent inducer of foam cell pathophysiology (Sakr et l. 2001)



(Wong ML, 2000). Sphingomyelinase is an enzyme that hydrolyzes sphingomyelin, a membrane sphingolipid, to its phosphoryl and ceramide components. Sphingolipids are important lipid mediators involved in various biological processes including signaling cascades related to apoptosis, inflammation, differentiation and proliferation. A deficiency in sphingomyelinase results in lipid storage disorders, in which lipids accumulate in various organs, and neurological damage.

There is some evidence that the extracellular matrix, especially proteoglycans, is also involved in the retention of atherogenic lipoproteins. In vitro studies show that lipoprotein lipase (LpL) further enhances the adherence of LDL to the matrix that is derived from normal smooth muscle and endothelial cells, and to proteoglycans (Williams et al 1995).

In a separate study, CD38, a scavenger receptor for OxLDL, showed increased expression during in vitro differentiation of blood monocytes into macrophages, with PMA activation (Yesner et al 1996). Multiple cell-surface molecules are likely involved in the macrophage uptake of aggregated lipoproteins. However, it has been shown that reorganization of the cytoskeleton plays a significant role in this process in a way that is different to receptor-mediated endocytosis (Sakr et al 2001). Specifically, Sakr et al 2001 have shown that actin polymerization of matrix-retained LDL, myosin ATPase activity, Rho family GTPases, and other signaling events are needed for the internalization of matrix-retained and aggregated LDL.

## **H. The Actin Cytoskeleton**

The main structural component of the cytoskeleton is actin, the globular protein, which exists as monomeric G-actin, or Filamentous F-actin. G-actin polymerizes to form the helical F-actin, in the presence of ATP,  $Mg^{2+}$  and  $K^+$ . F-actin can also depolymerize in weak ionic solutions to form G-actin, and this reversible process is the basis of many cell movements (Lechler 1997).

The stabilization of the actin network at the periphery of the cytoplasm and its attachment to the cellular membranes are conducted by binding proteins, such as the FLN family of actin-binding proteins, FLN-A, through protein-protein interactions (Cunningham et al 1992). In endothelial cells, FLN-A regulates how F-actin is distributed between the cortical actin band, a narrow area of dense filamentous actin and associated proteins just beneath the plasma membrane that are responsible for the cell surface growth, signaling and dynamic processes of the cytoskeleton, and actin stress fibers, non-muscle bundles of actomyosin that mediate cell contraction. These actin-binding proteins are necessary for cell migration, and their presence in tumor cells were shown, by light microscopy, to result in asymmetrical cell spreading and lamellae formation.

By contrast, cells lacking the actin-binding proteins also lacked the lamellae extensions, and their plasma membrane produced continuous, and extensive blebbing. This type of blebbing is a morphological change in the cell in which the actin protein dissociates from the plasma membrane and bulging results, possibly indicative of a protective response to injury (Babiychuk et al 2011). In addition, cells lacking actin fiber bundles and therefore deficient in actin-binding proteins, that were transfected with actin-binding protein cDNA in mammalian expression vectors, exhibited cell migration five times greater

than their untransfected counterparts (Cunningham et al 1992). This interaction was demonstrated between the cytoplasmic domain of the G protein coupled P2Y<sub>2</sub>R and FLN-A. At the University of Missouri in the Weisman Laboratory, we used a yeast 2-hybrid system screen with the C-terminal region of P2Y<sub>2</sub>R for bait, and identified FLN-A as an interacting protein for P2Y<sub>2</sub>R. In addition it was found that this interaction was critical for migration and spreading of aortic smooth muscle cells (Yu et al 2008).

Extracellular nucleotides induce rapid dynamic reorganization of the actin network to control various cellular processes including cell motility, intracellular transport, cell shape and polarity. As such, UTP-stimulated P2Y activation was shown to induce reorganization of the actin cytoskeleton in rat vascular SMCs expressing P2Y<sub>2</sub> or P2Y<sub>4</sub> receptor (Sauzeau et al 2000). This activation was also coupled to activation of RhoA, a small GTPase protein that regulates the actin cytoskeleton. UTP, an agonist for the full length P2Y<sub>2</sub>R, was shown to significantly increase the spreading of SMCs isolated from mouse aortas by a factor of 6.8 fold, and an increase in migration by 3.6 fold (Yu et al 2008). According to the research findings, P2Y<sub>2</sub>R/FLN-A interaction is necessary for P2Y<sub>2</sub>R-mediated actin cytoskeleton reorganization, since neither UTP-induced spreading nor migration was observed when SMCs from P2Y<sub>2</sub>R knockout mice were transfected with mutant P2Y<sub>2</sub>R that does not bind to FLN-A in a protein-protein interaction (Yu et al 2008). We conducted experiments to verify that there was a specific interaction between the P2Y<sub>2</sub>R and FLN-A in Dr. Gary Weisman's lab at the University of Missouri. Using human coronary artery endothelial cell lysate (HCAEC), we tested the ability of a Glutathione S-Transferase- (GST) fusion protein containing the C-terminus of P2Y<sub>2</sub>R (GST-P2Y<sub>2</sub>E-CTD) to interact with the endogenously expressed full-length FLN-A. A 280-kDa band that

immunoreacted with the anti-FLN-A antibody was detected, and the band was also detected when the lysates were incubated with GST-P2Y<sub>2</sub>R, but not with GST alone. We also verified the interaction, in human 1321N1 astrocytoma cells transfected with HA-tagged P2Y<sub>2</sub>R (HA-P2Y<sub>2</sub>R) (Figures 2-3).

## **I. LDL Receptor Related Protein 1 (LRP 1)**

LRP 1 is a member of the LDL receptor (LDLR) family and is highly expressed in VSMCs during excess LDL accumulation, but it is not regulated by intracellular cholesterol as occurs with the LDL receptor (LDLR). LRP 1, also referred to as cluster of differentiation 91 (CD 91), is a large 600 kilo-Dalton hepatic cell-surface receptor, that is structurally and biochemically similar to the LDLR (Herz et al 1988). It was first discovered as a cell surface receptor that functions in the binding and endocytosis of apo-E lipoproteins (Herz et al 1988; Beisiegel et al 1989; Kowal et al 1989; Kowal et al 1990). In addition, LRP 1 acts as a modulator and integrator in the signaling pathway and functions to maintain the integrity of the arterial wall in the vasculature (Boucher et al 2003).

One of the main events in the atherogenic processes is the accumulation of lipids, mainly cholesterol esters (CE) (Fuster 1992). The general mechanism that drives the disease development is the formation of macrophage- and VSMC- derived foam cells. Two different transformative processes distinguish macrophage-derived foam cells and VSMC-derived foam cells. In the first instance, macrophages are transformed to foam cells by uptake of diversely modified LDLs, whereas in the second instance VSMCs are ostensibly transformed by the uptake of lipids that accumulate in the intima. Notably, LDLR is regulated by normal intracellular cholesterol levels, and when there is excess LDL the LDLR

is down regulated in VSMCs to prevent lipid accumulation. Interestingly when there is excess LDL, the LRP 1 is most highly expressed in VSMCs. Furthermore, LDL that is vortexed to induce aggregation in vitro, or LDL that is aggregated through retention by extracellular matrix components such as proteoglycans, is structurally similar to the lipids found in the core of atherosclerotic lesions (Guyton et al 1991). It is believed that high LRP 1 expression in VSMCs may cause VSMCs to have an increased capacity to take up LDL from the intima through the regulation of cellular LRP 1 concentration (Llorente-Cortes et al 2005). In addition, other pathways that are engaged in atherothrombosis may be responsive to LRP1 upregulation, since LRP 1 has a role in dismantling complexes that are involved in thrombogenesis and fibrinolysis (Llorente-Cortes et al 2005).

LRP 1 is an endocytic receptor comprised of modular structures that include epidermal growth factor-like repeats (EGF), cysteine-rich complement-type repeats,  $\beta$ -propeller domains, a cytoplasmic domain, and a single-pass transmembrane domain. It is ubiquitously expressed in many tissues, but most highly expressed in liver, brain and lung in both humans and mice. Due to the various types of ligands that can be identified by LRP 1, it is tightly regulated by a receptor-associated protein (RAP), which is localized in the endoplasmic reticulum (ER). RAP has three domains, D1, D2, and D3, and forms a complex with LRP 1. This allows LRP 1 to be shuttled from the ER, where it is synthesized, to the Golgi. RAP binds very tightly to LRP 1 in the ER to prevent the premature binding of its many ligands. The side chains of D3 are protonated by histidine to lower the pH of the surface, which allows RAP to be released from LRP 1 once it arrives in the Golgi for modification and trafficking to other organelles (Lillis et al 2008).

LRP 1 is also believed to modulate VSMC proliferation and migration by binding to certain matrix metalloproteinase (MMP) to prevent the degradation of the extracellular matrix and inhibit cardiovascular remodeling. Studies show that LRP 1 was effective in modulating the levels of MMP-2, MMP-13 and MMP-9 in fibroblasts, thereby playing a role in regulating cell migration (Hahn-Dantona et al 2001). Other studies show that when agLDL was bound to LRP 1 the amount of cholesterol that accumulates in the blood vessel wall decreased. In a separate study using LRP 1<sup>-/-</sup> mice, there was an increase in platelet derived growth factor receptor beta (PDGFR $\beta$ ) expression, accelerated atherosclerotic lesion development, and significant cholesterol accumulation in VSMCs deficient in LRP 1 (Boucher 2003).

The cysteine-rich repeats (CR) known as ligand-binding repeats are located in the extracellular domain of LRP 1 in four clustered regions named clusters I, II, III, and IV, and they are the putative binding sites for most of its ligands (Herz et al 1988; Neels et al 1999). These cysteine rich domains are negatively charged, thereby allowing them to bind to ligands such as the positively-charged lipid transporters apolipoprotein B (apo-B) or apo-E. Apo-E is involved in triglyceride clearance from the liver, as it mediates the binding of lipoprotein particles to LRP 1 as well as to LDL receptors for this purpose.

The COOH-terminal EGF functions with the  $\beta$ -propeller domain in the uncoupling of the LRP 1 ligands at a low pH to promote their release in the endosomes (Lillis et al 2008). The transmembrane and cytoplasmic domains of LRP 1 are involved in cell signaling and trafficking through interactions with adaptor molecules such as Shc, disabled, and Fe65. *In vitro* studies suggest that the intracellular domain of the LRP 1 molecule (LRP 1-ICD) is involved in transcriptional modulation (Kinoshita et al 2003). Other adaptor proteins that

bind to the cytoplasmic domain of LRP 1 to produce a variety of cellular responses include Talin-like protein, PKC $\alpha$ , and GULP. For example Talin-like protein's interaction with LRP 1 promotes coupling to the actin cytoskeleton, whereas interaction of LRP 1 and PKC $\alpha$  promotes proliferation, apoptosis, differentiation and cellular motility, and GULP-LRP 1 interaction promotes phagocytosis (Lillis et al 2008).

LRP 1 mediates the endocytosis and intracellular degradation for a variety of ligands including lipoprotein lipase, chylomicron remnants, and very low-density lipoprotein remnants (VLDL). It has been shown in vitro to be upregulated by agLDL, and in vivo by a hypercholesterolemic porcine model (Llorente-Cortes et al 2002; 2006). LRP1 plays a role in the formation of foam cells originating from VSMCs and macrophages (Llorente-Cortes et al 2006; 2007), and is abundantly expressed in the arterial wall (Llorente-Cortes et al 2004; Hiltunen et al 1998). Based on our preliminary observations the P2Y<sub>2</sub>R agonist UTP stimulates LRP 1 protein expression in VSMCs, so this dissertation also investigated whether LRP 1 is also regulated at the transcriptional level.

## **J. Summary of Literature Review**

P2 receptor subtypes are expressed in a wide variety of tissues including the arterial walls. These receptors mediate multiple activities within cells, and are activated by extracellular nucleotides under pathophysiological conditions of inflammation, stress and injury such as atherosclerosis. UTP released from various sources, stimulates the P2Y<sub>2</sub> receptor subtype and causes cytoskeletal reorganization that influences migration of smooth muscle cells into the intima. Changes in the arterial walls that result from hyperlipidemic conditions initiate atherosclerosis, as the atherogenic lipoproteins become trapped and modified in the subendothelial space by matrix components. Vascular smooth

muscle cells take up these lipoproteins through LRP 1, an endocytic receptor of the LDL family. Therefore this purpose of this dissertation is to determine if the UTP-stimulated P2Y<sub>2</sub> receptor interacts with cytoskeleton proteins to mediate LRP 1 uptake of aggregated lipoproteins.



## CHAPTER 3 – Materials and Methods

All experimental procedures with cells were approved by the Kansas State University Institutional Review Board. Animal use at the University of Missouri Columbia was approved by the Institutional Animal Care and Use Committee.

### ***Materials***

Primary vascular smooth muscle cells were isolated from the aortas of P2Y<sub>2</sub>R knockout 6-8 week old male mice, and from C57 BL/6 control mice 6-8 week old. In this targeted P2Y<sub>2</sub>R mutation, a neomycin selection cassette replaced a genomic fragment corresponding to base pairs 552 - 1149 of the published cDNA (The Jackson Laboratory, [www.jax.org](http://www.jax.org)). All mice were obtained from Dr. Gary Weisman's Laboratory at the University of Missouri, Columbia Missouri. The aortas were surgically removed and enzymatically digested in 0.1% collagenase II purchased from Worthington Biochemical Corporation (Lakewood, NJ). Medium Nutrient Mix 199 (M199), Dulbecco's Modified Eagle Medium Nutrient Mix F12 (DMEM F-12), Normocin to prevent mycoplasma and fungal growth, fetal bovine serum (FBS), 20 X Smooth Muscle Growth Supplement (SMGS), 100 X penicillin-streptomycin solution containing 10,000 IU units/ml penicillin and 10000 µg/ml streptomycin, and penicillin-streptomycin glutamine were purchased from Life Technologies (Carlsbad, CA). Uridine 5'-triphosphate trisodium salt dihydrate used as agonist for P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> receptors was obtained from Sigma-Aldrich (Buchs, Switzerland). Pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) used as a P2 receptor antagonist, and ribonucleic acid (RNA) synthesis inhibitor Actinomycin D were purchased from Sigma-Aldrich (St Louis, MO).

Lipofectamine® 2000 Reagent, SOC medium, Electromax DH 10B™ competent cells used for cloning and transfection protocols were obtained through Life Technologies (Carlsbad, CA). Expression plasmid Prk5 vector was obtained from Upstate Biotechnology, Inc., and mutant P2Y<sub>2</sub>R defective in FLN binding inserted at the pCMVT19N promoter site was assembled at Dr. Weisman's Laboratory. Wild type P2Y<sub>2</sub>R was inserted at the pCMVT19N site and used as a negative control. Lipoproteins (unlabeled LDL or Oxidized LDL labeled with 1,1'-dioctadecyl-3,3',3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ox-LDL) for LDL uptake studies were purchased from Biomedical Technologies (Stoughton, MA). Alexa Fluor® 546 Monoclonal Antibody Labeling kit (Molecular Probes) was used for labeling LDL. Primer sequences for *LRP1*, *LDLR*, *GAPDH*, *P2Y<sub>1</sub>R*, *P2Y<sub>2</sub>R*, and *P2Y<sub>4</sub>R* were designed using the National Center for Biotechnology Information Local Alignment Search Tool (BLAST), and custom designed by Life Technologies (Carlsbad, CA). The mRNA relative abundance was calculated using delta-delta ( $\Delta\Delta$ ) Ct method. GAPDH was used as a control gene.

### ***Cell preparation and culture***

Primary VSMC were isolated from mice aorta and grown in cell culture. The cells were obtained from enzymatically-digested aortas, which were surgically removed from six to eight week old male wild type (WT) C57BL/6 and P2Y<sub>2</sub><sup>-/-</sup> mice. The aortas were placed in a 0.5% penn-strep phosphate buffer solution (PBS) upon removal for 30 min to destroy surface contaminants. The aortas were then removed from the PBS and placed in enough M199 media to cover. They were next cut open longitudinally, and tweezers were used to remove connective tissue and fat. The cleaned aortas were placed in a culture dish with

fresh media and 0.1% collagenase, chopped into small pieces (2-4 mm) and transferred into a 50-ml tube. The tubes were then incubated for 30 min in a water bath, with gentle shaking every 3 min. Following incubation, the tissue suspension was filtered through a 100- $\mu$ m strainer, and centrifuged for 5 min at 210 x g. The supernatant was removed by pipetting, and the cell pellet collected and re-suspended in M199 supplemented with 20% (v/v) FBS and 1% penicillin-streptomycin, plus normocin. Cells were seeded in 6-well plates and incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO<sub>2</sub>. After 24 h the media was aspirated, the cells rinsed with sterile PBS and fresh media was added. Thereafter, the cells were replenished with fresh media every 3 days. The cells were identified as vascular smooth muscle cells by their characteristic “hill-and-valley” growth pattern. Following the first passage at 70% confluence, DMEM F12 culture medium was supplemented with 15% FBS, 5% (v/v) SMGS, plus 1% penicillin-streptomycin with glutamine. Human 1321N1 astrocytoma cells were cultured in DMEM with 10% (v/v) fetal calf serum, penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml) and L-glutamine (2mM).

### ***P2Y<sub>2</sub> receptor cDNA constructs***

The cDNA constructs were produced at the Weisman laboratory at the University of Missouri (Columbia, MO). The open reading frame of the wild type human P2Y<sub>2</sub> receptor cDNA was modified using PCR to incorporate the HA epitope (YPYDVPDYA) from influenza virus at the N-terminus of the expressed protein, as described previously (Garrad et al 1998). Three primers synthesized in the DNA core Facility of the University of Missouri in Columbia were used in PCR to generate cDNA encoding ha-tagged deleted (del) (using

primer 1 and 3) or 4A (using primer 1 and 2) mutant P2Y<sub>2</sub> receptors. Primer 1: 5' -AGGCT CGTACGCTTTGCCCGAGATGCCAAGGCTCGCCGAGGCTGGGCCTGCGCAGATC-3'

Primer 2: 5' -ATCATGGATCCTTACTTGGCATCTCGGGC -3'; Primer 3: 5'-CACACCCTAACTG ACAC -3'. The sizes of the PCR products were resolved by agarose gel electrophoresis, and the products were purified using the PCR Wizard kit from Amersham Biosciences (Piscataway, NY), digestion with BsiWI and BamHI, and inserted into pLXSN. The mutant cDNAs were sequenced to verify that the mutants were incorporated correctly.

### ***Glutathione S-Transferase (GST) pull-down assay***

The GST-P2Y<sub>2</sub>R fusion protein pull-down assay was carried out according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Briefly, pGEX-5X-1-P2Y<sub>2</sub> C-Terminal Domain was transformed into *E. coli* strain BL21-Gold (Stratagene, La Jolla, CA). Fusion protein expression was immobilized by glutathione covalently linked to Sepharose. Lysates from mouse VSMC were incubated with fusion protein immobilized by glutathione-linked Sepharose, and bound proteins were eluted with soluble glutathione (100 mM). The eluted proteins were resolved by SDS-PAGE and analyzed by western analysis using a mouse monoclonal anti-FLN-A antibody (1:1000 dilution: Chemicon, Temecula, CA).

### ***Co-immunoprecipitation of FLN-A with HA-P2Y<sub>2</sub>R in 1321N1 cell transfections***

Mouse aortic SMC or 1321N1 astrocytoma cell transfectants expressing full length or truncated HA-tagged P2Y<sub>2</sub> receptors were washed with cold PBS and lysed in modified RIPA buffer at 4 °C for 15 min. The lysates were cleared by centrifugation at 10,000 x g at 4 °C for 15 min. The protein concentrations in the supernatant were determined according

to the Lowry assay and equal amounts of supernatant protein (200  $\mu$ g) were incubated with 50  $\mu$ l of HA-affinity matrix (Roche Applied Sciences, Indianapolis, IN) with gentle rocking at 4  $^{\circ}$ C for 2 h. The immunoprecipitate was sedimented by centrifugation, washed with RIPA buffer, resuspended in 50  $\mu$ l of 2X Laemmli's buffer and heated at 95  $^{\circ}$ C for 4 min. The solubilized proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat milk in TBST with gentle rocking at room temperature for 2 h. The membrane was then incubated at room temperature in TBST containing mouse monoclonal anti-FLN-A antibody (1/1000 dilution) (Chemicon, Temecula, CA), washed five times with TBST for 10 min each time, incubated with 1/2000 dilution of HRP-conjugated goat anti-mouse IgG for 1 h, and washed four times with TBST for 10 min each time. The proteins were visualized with Luminol reagent (Bio-Rad Laboratories, Hercules, CA)

### ***UTP induced FLN-A phosphorylation and actin stress fiber formation***

To assess FLN-A phosphorylation, human 1321N1 cells grown in culture were transfected with the HA-tagged P2Y<sub>2</sub>R or vector alone, then serum-starved and stimulated with 100  $\mu$ M UTP for 0, 5, and 15 min. The cells were lysed with RIPA buffer, and lysates were immunoprecipitated by anti-FLN antibody and analyzed by immunoblotting with anti-phospho-Ser or anti-phospho-Thr antibody. For assessing actin polymerization, human 1321N1 cells expressing the HA-tagged P2Y<sub>2</sub>R were grown to 60% confluence on coverslips in 35-mm dishes. The cells were serum-starved overnight and treated with 100  $\mu$ M UTP for 0, 5, and 30 min. The cells were washed 3 x in PBS and fixed in 3.7% formaldehyde then lysed with 0.1% triton X-100. The cells were then incubated with

Oregon Green™ 488-conjugated phalloidin (5 µg/ml) (Molecular Probes, Eugene Oregon) in serum-free medium for 45 min at room temperature and then washed with PBS. Texas Red-labeled DNase 1 was used to localize globular-actin. The coverslips were washed in cold PBS, and mounted on glass slides. The cells were visualized using microscopy at 40X magnification. Vector-transfected cells were used as controls.

### ***DH5α transformation, culture preparation and cell transfection***

Plasmid DNA (100 ng/200 µl cells) from hemagglutinin (HA)-tagged wild type (WT) P2Y<sub>2</sub>R or a mutant P2Y<sub>2</sub>R that does not bind filamin-A (FLN-A), were added to DH5 competent cells and heat-shocked at 42 °C for 2 min with gentle shaking, followed by addition of 4 volumes of LB (Luria-Bertani) media (400 µl) and incubation at 37 °C and gentle shaking for 50-60 min. The cells were spun at 5,000 x g for 10 min in a centrifuge to collect the pellet. Approximately 400 µl of the supernatant was removed and the pellet was re-suspended in the remaining 50 µl of supernatant. Then cultures were prepared by inoculating cells in 1.5% agar on petri dishes and incubating at 37 °C for 24 h. The colonies were collected and incubated with LB media (1 µg ampicillin/ml LB) in culture tubes at 37°C with gentle shaking, until evidence of cloudy growth appeared. The cultures were then centrifuged for 10 min at 10,000 x g and the pellet was collected, followed by DNA purification using Qiagen DNA isolation kit with the manufacturer's protocol. VSMC were transiently transfected with the plasmid DNA constructs delivered using Lipofectamine® 2000 reagent from Life Technologies (Carlsbad, CA), and following the manufacturer's instructions. The retroviral vector pLXSN was used to stably express the HA-tagged human P2Y<sub>2</sub> receptor in P2 receptor-null human 1321N1 astrocytoma cells, as previously

described (Erb et al 1995). In brief, the recombinant P2Y<sub>2</sub>-pLXSN construct or pLXSN (control) was used to transfect PA317 amphotropic packaging cells for production of the viral vectors. Then 1321N1 cells were infected with the viral vectors and cultured in DMEM plus 5%(v/v) FBS, 100 units/ml penicillin, and selected for neomycin resistance with 1 mg/ml G418 from Life Technologies (Carlsbad, CA).

### ***Western Blot Analysis of LRP 1 Expression in mouse VSMC***

Serum starved cultured WT C57 BL/6 cells were stimulated in the absence or presence of UTP (1, 10, 25, 50, and 100  $\mu$ M). Cell lysates were solubilized in 2X Lammeli sample buffer (120 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 1 mmol/L EDTA, 50 mmol/L dithiothreitol, 0.003% bromophenol blue). Equivalent amounts of protein (100  $\mu$ g) were subjected to 7.5% (w/v) SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked for 1 h with 5% (w/v) non-fat dry milk in tris-buffered saline (0.137 M NaCl, 0.025 M Tris (hydroxymethyl)-aminomethane, pH 7.4) containing 0.1% (v/v) Tween-20 (TBST) and immunoblotted overnight at 4 °C in TBST containing 1% BSA and anti-LRP 1 antibody (1:3000 dilution; Abcam, Cambridge MA). Detection of LRP 1 was performed using mouse anti-LRP 1 monoclonal antibody (1:3000 dilution; Abcam, Cambridge MA) followed by horseradish peroxidase-conjugated donkey anti-mouse polyclonal antibody (1:10000 dilution Abcam, Cambridge MA). For signal normalization, membranes were probed with polyclonal anti- $\beta$ -actin antibody (1:2000 dilution).

### ***LRP 1, LDLR, P2Y<sub>1</sub>R, P2Y<sub>2</sub>R, and P2Y<sub>4</sub>R, mRNA expression in VSMC***

P2Y<sub>2</sub>R<sup>-/-</sup> and C57BL/6 control VSMC were grown to 90% confluence in a monolayer in 12-well plates in DMEM F-12 media supplemented with 10% (v/v) FBS, 1% P/S. Cells were serum starved by incubating for 24 h with DMEM F-12 at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. After 24 h cells for LRP 1 mRNA and LDLR mRNA expression were incubated with actinomycin D (2mg/ml) in fresh media for 15 min to block transcriptional activity. Cells were then stimulated with UTP (10 μM) and incubated for 18 h. Total RNA was isolated from VSMC using the Qiagen RNeasy Mini Kit. Briefly, cells were washed in ice-cold PBS and collected by scraping following addition of fractionation buffer and lysis buffer. Equal volumes of 100% ethanol was added to the lysate and applied to a filter cartridge. Following several washes using different wash solutions with centrifugation, the RNA was eluted with heat-shocked elution buffer or RNase free water. First strand cDNA was synthesized from 1 μg total RNA in 20 μl reaction volume using random hexamers as primers. Synthesis of cDNA was performed using cDNA synthesis mix (10 X RT Buffer, 25 X dNTP mix, 10 X Random Primers, and Multiscribe Reverse Transcriptase) from Life Technologies (Carlsbad CA). RT-PCR was conducted using Fast SYBR Green Master Mix. Two independent PCR reactions were carried out for each cDNA synthesis. The mRNA relative abundance was determined using the  $\Delta\Delta C_t$  method and *GAPDH* as a control gene. Oligonucleotide amplification primer sequences for LRP 1 were forward 5'-GCCAGCCAGATGTGCCCAAT-3' and reverse 5'-TGGTGGGGCAGG CGCATTTA-3'. Primer sequences for LDLR were forward 5'-TGCCAATCGACT CACGGGTTCA-3' and reverse 5'-AGTGTGCGACTTCTCTAGGCTGTGT-3'. For *P2Y<sub>1</sub>R*, *P2Y<sub>2</sub>R*, and *P2Y<sub>4</sub>R* mRNA expression, cells were incubated with PPADS (2 mg/ml) in media for 15 min, then stimulated with UTP



(10  $\mu$ M) and incubated for 18 hours. Sequences for *P2Y<sub>1</sub>R* were forward (NM\_008772.4) 5'-CGGGAGCGCACTTGCAAAC-3' and reverse 5'-TGAAGGACGTGCGGGCAAAC-3'; sequences for *P2Y<sub>2</sub>R* were forward (NM\_008773.3) 5'-GGTCGAGTCAGCGCCAAACA-3' and reverse 5'-AGTTCATCAGCGCACCGGCA-3'; sequences for *P2Y<sub>4</sub>R* were forward (NM\_020621.4) 5'-TCGGCTCCGTTCTCTCCGCA-3' and reverse 5'-CACCCGGCATTCGGCGTTCA-3'. Primer sequences were determined by the Basic Local Alignment Search Tool (BLAST) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

### ***LDL labeling and aggregation***

DiI LDL was aggregated by incubation with bacterial sphingomyelinase (SMase) as previously described (Sakr 2001). In brief, 1 mg of LDL protein/ml of PBS was incubated with 50 milliunits/ml SMase in the presence of 5 mM MgCl<sub>2</sub> for 4 hours under argon without shaking at 37 °C, followed by 10 mM EDTA to stop the reaction. The LDL was then diluted with binding buffer. Unlabeled lipoprotein was fluorescently labeled by using Alexa fluor<sup>®</sup> 546 monoclonal antibody labeling kit according to manufacturer's protocol. Aggregation of the Alexa fluor labeled LDL was done as stated above.

### ***Matrix preparation, LDL binding and uptake***

To produce SMC-derived matrix, 100  $\mu$ l of the mouse SMC culture was plated in 35-mm glass-bottom culture plates, and incubated in DMEM, 10 % FBS. Cells were grown in a monolayer to 100 percent confluence. Following three washes with DMEM, 0.2% BSA, the SMC monolayer was air dried for 15 min and extracted twice with 3:2 hexane:isopropanol (v/v) for 30 min. The lipid extracts were removed and discarded, and the wells dried for

15 min at room temperature under a tissue hood. After washing three times with binding buffer (3% BSA, 140 mM NaCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub>, 10 mM Hepes, pH 7.4) the matrix was incubated with binding buffer for 1 h at room temperature to block non-specific sites. Lipoproteins were aggregated by incubation with bacterial sphingomyelinase (SMase) as previously described (Sakr 2001). In brief, 1 mg of LDL protein/ml of PBS was incubated with 50 milliunits/ml SMase in the presence of 5 mM MgCl<sub>2</sub> for 4 h under argon without shaking at 37 °C, followed by 10 mM EDTA to stop the reaction. In preparation for LDL-matrix binding, 1µg of lipoprotein lipase was added to the lipid-extracted SMC matrix for 1 hour at room temperature, then 100 µl SMase-treated LDL was added to the matrix and incubated for 18 h at 37 °C in a humidified atmosphere. The unbound LDL was removed by washing with DMEM and 0.2% BSA.

### ***UTP-stimulated uptake of aggregated LDL in mouse VSMC***

To obtain a fraction of 100% agLDL, the LDL solution was centrifuged at 10,000 x g for 10 min after vortexing for 4 min, and the pellet was recovered. VSMCs were incubated with agLDL 50 µg/ml for 2 h at 37 °C. VSMCs were incubated at 37 °C for 2 hours in the presence or absence of UTP. Lipids were stained by incubating cells with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine (DiI). Cells were subsequently incubated with DiI to stain lipid vacuoles. The nuclei were stained by incubating cells with 1 µg/ml of Hoeschst solution (Thermo Scientific, Rockford IL). Fluorescence microscopy experiments were carried to visualize the internalization of agLDL by mouse VSMC, using an LSM 510 laser scanning confocal microscope (Zeiss).

### ***UTP-stimulated uptake of agLDL in SMCs expressing WT or mutant P2Y<sub>2</sub> R***

To determine agLDL uptake for transfected SMC, HA-tagged WT- and mutant P2Y<sub>2</sub>R-transfected SMC were incubated with 100  $\mu$ M UTP for 4 h, then seeded on top of the matrix with the retained DiI LDL or Alexa fluor labeled LDL for different time points (0,1,2, and 4 hours). To visualize the internalization of fluorescently labeled agLDL the cells were viewed with an LSM 700 confocal microscope (Zeiss). Cells were fixed with 3% paraformaldehyde, after brief rinsing in DMEM, 0.2% BSA. The nucleus was identified using DAPI nuclear stain.

### ***Statistical analysis***

The treatment effects were analyzed using JMP (version 10.0, SAS Institute, Cary, NC), and individual treatment contrasts were evaluated by Student's t test. The residuals were normally distributed. Data are expressed as means  $\pm$  SEM with 95% confidence interval.

## CHAPTER 4 – Results

### *P2Y<sub>2</sub> receptor activation mediates FLN-A phosphorylation and formation of actin stress fibers*

As mentioned elsewhere in this dissertation, we previously determined that the P2Y<sub>2</sub>R specifically interacted with the cytoskeleton protein FLN-A in mouse cell lysate using the GST-P2Y<sub>2</sub>R fusion protein. The results show the presence of a 280-kDa protein in the cell lysate that immunoreacted with anti-FLN-A antibody (figure 2). The band was also detected in pull-down assays when SMCs were incubated with GST-P2Y<sub>2</sub>R, but not with GST alone (figure 2). We also verified the P2Y<sub>2</sub>R/FLN-A interaction in P2 receptor-null human 1321N1 astrocytoma cells transfected with HA-tagged P2Y<sub>2</sub>R (HA-P2Y<sub>2</sub>R), by immunoprecipitation using anti-HA antibody and immunodetection using anti-FLN-A antibody. As shown in figure 3, endogenous FLN-A interacts specifically with HA-P2Y<sub>2</sub>R. Since phosphorylation and dephosphorylation are believed to regulate FLN-A activation we hypothesized that UTP will activate P2Y<sub>2</sub>R and induce phosphorylation of FLN-A at serine/tyrosine residues. Our results show that UTP caused a rapid and sustained phosphorylation of FLN-A at serine/tyrosine residues in 1321N1 cells expressing the P2Y<sub>2</sub> receptor, but not in the vector-transfected control cells that do not express the P2Y<sub>2</sub> receptor (figure 4). In addition, UTP induced FLN-A phosphorylation in 1321N1 cells expressing the WT P2Y<sub>2</sub>R alone, but not the mutant P2Y<sub>2</sub>R (see Appendix A). Since phosphorylation of FLN-A mediates actin stress fiber formation, we next hypothesized that UTP-induced phosphorylation will cause actin cytoskeleton reorganization. As seen in figure 5, stimulation of P2Y<sub>2</sub>R in 1321N1 cells caused a rapid and sustained polymerization of actin filaments, whereas there was no such activity in the vector transfected controls.

Taken together, these two studies indicate that UTP-induced phosphorylation is also associated with reorganization of the actin cytoskeleton.

### ***UTP stimulates uptake of Aggregated LDL in mouse VSMCs***

Once we established the role of P2Y<sub>2</sub>R/FLN-A interaction in actin stress fiber formation, we speculated that there may be a regulatory role for the P2Y<sub>2</sub>R/FLN-A in the formation of foam cells in VSMCs. Since uptake of matrix-bound aggregated LDL is associated with cytoskeleton reorganization, we hypothesized that UTP will stimulate the uptake of aggregated LDL in VSMC. Fluorescence microscopy experiments were carried out to visualize the internalization of agLDL by mouse VSMCs. As seen in figure 6a, cells that were not treated with DiI labeled agLDL showed no evidence of LDL internalization. However LDL internalization was observed in the SMCs treated with DiI labeled agLDL as indicated by the red colored granules (figure 6b). The results also show that the addition of UTP resulted in a pronounced increase in the uptake of agLDL, again indicated by the red colored granules. As observed in figure 6c, UTP significantly increased the accumulation of agLDL in mouse VSMCs with an intact P2Y<sub>2</sub>R.

### ***UTP stimulates matrix-bound agLDL uptake in mouse VSMC expressing a mutant P2Y<sub>2</sub>R***

After establishing that the involvement of an intact P2Y<sub>2</sub>R was sufficient for the uptake of agLDL in VSMCs, we hypothesized that an intact P2Y<sub>2</sub>R was required for the uptake of agLDL. To determine the effect of P2Y<sub>2</sub>R agonist and the effect of the loss of P2Y<sub>2</sub>R/FLN-A interaction on the internalization of LDL, SMCs transduced with

adenoviruses encoding the mutant P2Y<sub>2</sub>R defective in FLN-A binding were seeded in poly-L-Lysine-coated dishes on top of the matrix with retained LDL and visualized with confocal microscopy. According to the results, there is no observed uptake of agLDL in P2Y<sub>2</sub>R<sup>-/-</sup> cells transfected with mutant P2Y<sub>2</sub>R that does not bind FLN-A (figure 7a). However there is noted agLDL uptake in P2Y<sub>2</sub>R<sup>-/-</sup> cells transfected with DNA from WT cells (figure 7b - 7c).

### ***UTP stimulates LRP 1 expression in mouse VSMCs***

LRP 1 is highly expressed in VSMCs and has been shown to be upregulated *in vitro* by aggregated LDL, and *in vivo* by a hyper-cholesterolemic porcine model (Llorente-Cortes et al 2002; 2006). Since aggregated LDL is found in atherosclerotic lesions, our overall goal was to determine how it is regulated in foam cell formation. Therefore we began our investigation by using western blot analysis to determine what effect UTP had on LRP 1 expression. Our results revealed that LRP 1 expression was the greatest for cells stimulated with the highest concentration of UTP. In addition, there was a dose dependent response for LRP 1, with the highest dose showing the greatest response versus the lowest doses with the least response (figure 8).

### ***LRP 1 mRNA expression is upregulated with UTP stimulation***

In order to determine whether LRP 1 was transcriptionally regulated, we used RT-PCR to assess LRP 1 mRNA relative abundance in UTP-stimulated SMCs from the P2Y<sub>2</sub>R<sup>-/-</sup> mice and C57BL6 controls (WT). Based on the results, there was a 2-fold significant increase in LRP1 mRNA relative abundance in response to UTP in WT cells compared to P2Y<sub>2</sub>R<sup>-/-</sup> cells ( $p < 0.05$ ) (figure 9). Additionally, an almost 10-fold lower difference is

observed in LRP 1 mRNA relative abundance in response to UTP in the P2Y<sub>2</sub>R<sup>-/-</sup> SMC compared to cells from the WT mice. We also found that actinomycin-D (20 µg/ml) significantly reduced UTP-induced LRP 1 mRNA expression in P2Y<sub>2</sub>R<sup>-/-</sup> SMC ( $P < 0.05$ ). There was not a significant difference between P2Y<sub>2</sub><sup>-/-</sup> and UTP-stimulated P2Y<sub>2</sub><sup>-/-</sup> cells.

#### ***UTP-stimulated LDLR mRNA expression in VSMC expressing mutant a P2Y<sub>2</sub>R***

It has already been established that the LDLR mediates the endocytosis of cholesterol rich LDL, but not the aggregated form of LDL. However, we wanted to see if the LDLR was transcriptionally regulated by the P2Y<sub>2</sub>R/FLN-A interaction. We hypothesized that the P2Y<sub>2</sub>R mediates the mRNA expression of the LDLR through interaction with FLN-A. To determine LDLR mRNA expression, we transiently transfected P2Y<sub>2</sub>R<sup>-/-</sup> VSMC with cDNA encoding a hemagglutinin-tagged WT P2Y<sub>2</sub>R or a mutant P2Y<sub>2</sub>R that does not bind FLN-A. Based on our results, SMC expressing the mutant P2Y<sub>2</sub>R that lacks the FLN-A binding domain exhibit 3-fold lower LDLR mRNA expression than SMC expressing the WT P2Y<sub>2</sub>R (figure 10). This shows that both an intact P2Y<sub>2</sub>R and FLN-A are necessary for LDLR expression.

#### ***UTP-stimulated P2Y<sub>1</sub>R, P2Y<sub>2</sub>R, P2Y<sub>4</sub>R mRNA expressions in VSMC***

The purpose of this study was to determine the efficiency of the P2Y<sub>2</sub>R knock-out procedure, and whether other P2Y subtypes that respond to UTP were present in the cells from the P2Y<sub>2</sub> knock-out mice. The results revealed UTP did not stimulate P2Y<sub>1</sub>R mRNA in either WT or P2Y<sub>2</sub>R<sup>-/-</sup> cells (figure 11). P2Y<sub>2</sub>R mRNA relative abundance showed a steady increase with UTP stimulation over time in both WT and P2Y<sub>2</sub>R<sup>-/-</sup> cells (figure 12).

However, this increase was not significant, indicating that P2Y<sub>2</sub>R may not have been completely knocked out. The results also showed that UTP stimulation resulted in a significant increase ( $p < 0.05$ ) in P2Y<sub>4</sub>R mRNA expression after 2 hours in the P2Y<sub>2</sub>R<sup>-/-</sup> cells versus WT cells, but then after 4 h there was a decline in expression (figure 13).



## CHAPTER 5 – Summary and Discussion

### A. Summary

Previous studies have shown that LRP 1 is highly expressed in vascular smooth muscle cells, and mediates the internalization of aggregated LDL in human vascular smooth muscle cells in a manner that is different from the internalization of soluble lipoproteins seen in the LDL receptor (Llorente-Cortes et al 2000). However, it was not known how LRP 1 was upregulated, so we investigated the role of P2Y<sub>2</sub> nucleotide receptors because of its high mRNA levels in vascular injury models, such as that present in medial SMCs following aortic balloon angioplasty (Seye et al 1997). The findings presented in this dissertation sought to highlight the importance of the agonist-induced P2Y<sub>2</sub> receptor in the uptake of aggregated LDL that is bound to SMC-derived matrix. Our central hypothesis stated that P2Y<sub>2</sub>Rs can interact with cytoskeletal proteins to promote LDL uptake by LRP 1 in VSMCs. We used the aortic SMCs from P2Y<sub>2</sub> knockout mice in order to define novel mechanisms by which UTP-induced P2Y<sub>2</sub> receptors may regulate foam cell formation in vascular smooth muscle cells, and contribute to the formation of advanced atherosclerotic lesions. We studied the involvement of cytoskeleton remodeling as a way in which the P2Y<sub>2</sub> receptor influences foam cell formation, based upon the role of the cytoskeleton in force generation, cellular motility, and filopodia extension (Lynch et al 2011). Extracellular nucleotides and P2Y<sub>2</sub> receptor activation are known to induce rapid dynamic organization of the actin cytoskeleton, leading to cell motility (Sauzeau et al 2000). We specifically tailored our investigation to include the FLN family of actin-binding proteins because of its apparent role in regulating the distribution of F-actin between the cortical band and actin stress

fibers in endothelial cells. To accomplish our objectives we prepared sphingomyelinase- or centrifuge-aggregated LDL plated on top of an SMC-derived matrix. We used confocal microscopy to study the internalization of the lipoproteins, and the role of the cytoskeleton in regulating this uptake. To this end we identified FLN-A as the cytoskeletal protein that interacts with the P2Y<sub>2</sub>R in a process that used the P2Y<sub>2</sub>R C-terminus as bait to screen a cDNA library generated from human aorta. Some of these studies were done in Dr. Gary Weisman's laboratory at the University of Missouri where this research project was initiated while I was a student there. We next verified that there was specific interaction between the P2Y<sub>2</sub> receptor and FLN-A in a pull-down assay using by a GST-fusion protein containing a fragment of the P2Y<sub>2</sub>R C-terminus, and the full length FLN-A protein in mouse VSMC lysate (figure 2). We further ascertained that this interaction occurs in mammalian cells by transfecting P2 receptor-null human 1321N1 astrocytoma cells with HA-tagged P2Y<sub>2</sub>R (HA-P2Y<sub>2</sub>R), then immunoprecipitated HA-P2Y<sub>2</sub>R from cell lysates using anti-HA antibody followed by immunodetection using anti-FLN-A antibody (figure 3). It was determined that the FLN-A binding site was localized between amino acids 322 and 333 of the P2Y<sub>2</sub> receptor, and the Weisman lab group found that deletion of the same region resulted in the loss of P2Y<sub>2</sub>R-induced tyrosine phosphorylation of FLN-A, as well as loss of P2Y<sub>2</sub>R/FLN-A interaction (Yu et al. 2008) Since phosphorylation of FLN-A mediates actin stress fiber formation (Gibbons and Dzau 1994), we then showed that UTP-induced FLN-A phosphorylation was also associated with the reorganization of the actin cytoskeleton. We showed by confocal microscopy how UTP-stimulated P2Y<sub>2</sub> receptors regulate actin polymerization in 1321N1 cells expressing the HA-tagged P2Y<sub>2</sub>R (figure 5).

To further assess the role of the P2Y<sub>2</sub> receptors in LRP 1 upregulation, we showed that, in the presence of UTP, the LRP 1 protein was expressed in mouse VSMC, and we found that UTP stimulated LRP 1 expression in a dose-dependent manner. In addition, we used quantitative RT-PCR and determined that LRP 1 was regulated at the transcriptional level. P2Y<sub>2</sub> receptors are expressed in vascular smooth muscle cells and modulate many cellular events, including the uptake of modified low-density lipoproteins. We demonstrated that UTP mediated many of the effects that were observed, including the interaction between P2Y<sub>2</sub>R and FLN-A, and that this interaction was necessary for the uptake of agLDL in VSMVs. In addition we showed that P2Y<sub>2</sub>R mediated the uptake of SMase-aggregated LDL in VSMCs that had intact P2Y<sub>2</sub>R/FLNA interactions, while no such uptake was observed in VSMCs that were transfected with the mutant P2Y<sub>2</sub>Rs that do not bind to FLN-A.

Four P2Y receptor subtypes, P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>, are coexpressed in VSMCs. We investigated P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> in the VSMCs from the P2Y<sub>2</sub><sup>-/-</sup> mice. P2Y<sub>1</sub> subtype was used as our negative control since it is not stimulated by UTP, and is hardly expressed by contractile VSMCs (Sauzeau et al 2000). We showed that UTP stimulation resulted in insignificant P2Y<sub>1</sub> mRNA relative abundance, but significant amount of P2Y<sub>4</sub> mRNA concentrations. However, a small insignificant amount of P2Y<sub>2</sub> mRNA was observed in our P2Y<sub>2</sub><sup>-/-</sup> SMCs, compared to wild type SMCs.

## **B. Discussion**

There is a substantial amount of immobilized lipoproteins that are present in atherosclerotic lesions (Smith et al 1976). According to the response-to-retention

hypothesis, lipoproteins that are retained in the arterial wall is the initiating event of atherogenesis that triggers an inflammatory response leading to lesion development (Williams and Tabas 1995). Many studies have focused on the roles that activated endothelium, or inflammatory cells such as macrophages or neutrophils, play in the development of the atherosclerosis. Vascular smooth muscle cells and components within the cytoskeleton undoubtedly play a key dynamic role in the progression of the disease. However, in the case of matrix-retained lipoproteins, a transformative process occurs that modifies these lipoproteins and necessitates a different mechanism for uptake.

Understanding the molecular mechanisms of factors that contribute to the development of atherosclerosis is critical to determining the types of strategies that need to be developed in order to engage targeted treatment options. LRP 1 is present in arterial wall SMCs, and has been shown to have a key role in multiple signaling pathways. It functions both as a receptor and a signaling molecule (Boucher and Herz 2011). The P2 receptors play a significant role in inflammation, injury and the development of atherosclerosis, through the modulating influence of extracellular nucleotides. UTP, the preferred agonist for the P2Y<sub>2</sub> receptor, was also shown here to mediate the LRP 1 expression at both the protein level and the transcriptional level. Since these studies were conducted with VSMCs from the P2Y<sub>2</sub> knockout mouse model, it appears reasonable to suggest there is some sort of co-regulation between LRP1 and the P2Y<sub>2</sub>R in the uptake of agLDL in VSMCs. The LRP 1 receptor is involved in catabolizing many ligands including lipoproteins, so it is conceivable that there is at least some cross-talk with the P2Y<sub>2</sub> receptor. LRP 1 signaling may act as a protective mechanism in SMCs that is upregulated by UTP during atherogenesis for the purpose of removal and degradation of cholesterol trapped within the extracellular matrix.

Indeed, LRP 1 is ubiquitously expressed in a wide variety of cells and is highly conserved among species, and it was noted that there is no apparent known functional coding mutation of the human LRP 1 gene (Boucher and Herz 2011). LRP 1 modulates SMC responses by interacting with other cellular receptors, including platelet-derived growth factor (PDGF) receptor  $\beta$  and integrins to protect the arterial structure (Boucher et al 2003; 2011). LRP 1 prevents the formation of atherosclerotic lesions by way of the PDGF- $\beta$  and the transforming growth factor- $\beta$  (TGF $\beta$ ) signaling pathways, both of which have major roles in atherosclerosis (Loukinova et al 2002; Boucher et al 2002; Boucher and Herz 2011). The LRP 1 receptor is involved in catabolizing many ligands including lipoproteins. Deletion of LRP 1 is associated with uncontrolled SMC hyperplasia of the aorta and increased PDGFR $\beta$  expression, increased PDGFR $\beta$  phosphorylation, and increased phosphorylation of Smad2, which mediates TGF $\beta$  signaling (Boucher and Herz 2002). On the other hand, apo-E binding to LRP 1 inhibits PDGF-associated SMC migration within the arterial wall (Swertfeger et al 2002). LRP 1 protects the vasculature against atherosclerosis by suppressing PDGFR $\beta$  activation (Boucher et al 2003).

As we have shown, the P2Y<sub>2</sub> receptor is associated with the actin cytoskeleton through interaction with filamin A, and UTP-induced FLN-A phosphorylation is associated with rapid and sustained polymerization of actin filaments. This suggests that P2Y<sub>2</sub>Rs can regulate cytoskeletal reorganization (figure 6). UTP induced FLN-A phosphorylation in 1321N1 cells expressing the full length P2Y<sub>2</sub>R, but failed to induce FLN-A phosphorylation in 1321N 1 cells expressing the mutant P2Y<sub>2</sub>R defective in FLN-A binding (figure 4). This shows there is a clear role for P2Y<sub>2</sub>R/FLN-A interaction in cytoskeleton reorganization. Phosphorylation of FLN-A on Ser2125 regulates the association between FLN-A and the

actin cytoskeleton for the stabilization of caveolae at the plasma membrane (Muriel et al 2011). Indeed, phosphorylation may be an important factor in the mechanism for response of UTP-induced LRP 1 activation. This could explain a co-regulatory role of the P2Y<sub>2</sub> receptor in the uptake of agLDL in VSMCs. Some studies report that LRP 1 is phosphorylated on both serine and tyrosine residues (Li et al 2001, Geer 2002). Li et al showed that LRP 1 phosphorylation both *in vitro* and *in vivo* is mediated by the cyclic AMP (cAMP)-dependent protein kinase A (PKA), and by using site-directed mutagenesis they identified a phosphorylation site at serine 76 on the LRP 1 cytoplasmic tail. Geer et al report that tyrosine residues on LRP 1 can be phosphorylated, and the tyrosine-phosphorylated LRP 1 associate with Shc, a docking protein (Barnes et al 2001, Geer et al 2002). Like other docking proteins, Shc does not have a catalytic domain, but it is phosphorylated on tyrosine residues in response to many types of stimuli and facilitate protein-protein interactions (Geer et al 2002). Tyrosine phosphorylated LRP 1 was specifically associated with the Src-homology 2 (SH2), a binding domain on the Shc protein (Geer 2002), which could offer a possible explanation for the involvement of the P2Y<sub>2</sub> receptor. These binding domains mediate interactions between intracellular signaling proteins, allowing the proteins to bind each other in various interlocking combinations and facilitate signaling (Alberts et al 2002). The cytoplasmic tail of the P2Y<sub>2</sub>R has two consensus proline rich (PXXP) SH3 binding domains that are involved in regulating the transactivation of growth factor receptors (Weisman et al 2005). Both SH2 and SH3 may be involved in modulating the interactions with the cytoskeleton, and co-receptor function between P2Y<sub>2</sub>R and LRP 1. Regarding the role of small G proteins, previous studies showed that Rho signaling in VSMCs was triggered by UTP-stimulated P2Y<sub>2</sub> receptor activation

(Sauzeau et al 2000), leading to RhoA translocation and cytoskeletal reorganization, and in endothelial cells the coupling of P2Y<sub>2</sub>R to Rho signaling was required for cell chemotaxis and cytoskeletal reorganization during vascular inflammation in endothelial cells (Zhongji et al 2007).

LRP 1 has multiple ligands, so it is within the realm of possibilities that the G-protein coupled P2Y<sub>2</sub> receptor could have an accessory role in its activation. In a somewhat related study, it was shown that LRP 1 associated directly with a G stimulatory subunit (G<sub>s</sub>α) of a heterotrimeric G-protein (Goretzki and Mueller 1998). The investigators showed that LRP 1 is coupled to a G-protein in a study using LRP 1 agonist lactoferrin with M21 human melanoma cell membrane supernatant to determine GTPase activity, in a reaction involving the use of  $\gamma$ -<sup>32</sup>P labeled GTP. They found that the GTPase activity was dose dependent as well as time dependent (Goretzki and Muller 1998). In addition they found that the LRP 1 and the G<sub>s</sub>α protein are physically associated in the cell membrane in a co-affinity precipitation study they conducted.

In conclusion, we have shown for the first time that the P2Y<sub>2</sub> receptor mediates LRP 1 expression and the uptake of aggregated LDL by primary vascular smooth muscle cells. Taken together, we suggest the existence of a regulatory element within the promoter region of the LRP 1 gene that is responsive to UTP induced P2Y<sub>2</sub>R activation. We propose that this activated region allows LRP 1 to associate with the P2Y<sub>2</sub> receptor at its C-terminus, through possible phosphorylation, and other events involving FLN-A and/or other cytoskeletal proteins (figure 14). LRP 1 can be phosphorylated on serine-tyrosine residues within its cytoplasmic domain, and with the help of adapter molecules or scaffold proteins can engage in protein-protein interactions. Interestingly, one study found that the

LRP 1 cytoplasmic domain was subjected to proteolytic processing by a protease with  $\gamma$ -secretase like properties (May et al 2002). The  $\gamma$ -secretase proteases are integral membrane proteins that cleave single-pass transmembrane proteins, like the LRP 1, at residues within the transmembrane region. The study found that proteolytic processing within the LRP 1 cytoplasmic domain resulted in the release and translocation of the cytoplasmic tail to the nucleus (May et al 2002). There are numerous binding sites on the LRP 1 intracellular domain for adaptor proteins and scaffold proteins that can be used as docking sites for biologically active regulatory proteins, such as mitogen-activated protein kinases (MAPK), JNK, or Src. The resulting complex that forms can be then cleaved and translocated to subcellular target sites to modulate signaling events, based on the regulatory protein that is bound.



## CHAPTER 6 – Future Directions

Lipoproteins are the primary transporters of lipids in the blood, and are largely viewed as carriers of cholesterol, triglycerides, and phospholipids. However with recent research, including this current dissertation, it is becoming more evident that lipoproteins and their receptors are involved in intracellular signaling that produce downstream effects. It is clear that this study has shown for the first time a mechanistic role for the P2Y<sub>2</sub> receptor in mediating LRP 1 uptake of aggregated LDL by VSMCs. However, it is not known how the P2Y<sub>2</sub> receptor interacts with the LRP 1 to mediate these effects. LRP 1 has multiple binding sites for a wide variety of ligands, including UTP. Indeed, assumptions were made about the involvement of LRP 1 without the use of the 39-kDa receptor activating protein (RAP), its regulatory protein. Like G-protein coupled P2Y receptor signaling, LRP 1 signaling may require hetero-dimerization leading to the phosphorylation of its cytoplasmic tail. Future studies should be conducted to identify the regulatory site on the promoter region of the LRP 1 gene to determine potential transcription factor binding sites. Since RAP prevents all ligands from binding to LRP 1, an immunosorbent assay could be performed using labeled LRP 1. Future research should use study designs that include the use of RAP. Studies that delete or mutate the potential sites and determine which site is involved in UTP-induced LRP 1 expression would be an appropriate step in our understanding. A supershift assay using antibodies against known transcription factors should be conducted to determine which transcription factors mediate the site activity. In summary, future research directions should address the following questions: 1) Is there a direct interaction between LRP 1 and the P2Y<sub>2</sub> receptor? 2) Is there a regulatory element in the LRP 1 promoter that responds to P2Y<sub>2</sub>R agonists that allow ligand binding and

transduction of molecular signals? 3) What phosphorylation events are involved in the interaction between LRP 1 and the P2Y<sub>2</sub> receptor? 4) Is there involvement of a specific regulatory pathway coupled to both the P2Y<sub>2</sub>R and LRP 1 that allow for these protein-protein interactions in vascular smooth muscle cells, and 5) Does LRP 1 involvement in agLDL uptake by SMCs hinge on a G-protein dependent pathway? In seeking to answer these questions experimentally, researchers may better understand the molecular processes involved in LRP 1 activation and foam cell formation in the vasculature, and help move the field forward in atherosclerosis research.

The goal of atherosclerosis research is ultimately to improve the heart health status of all individuals. Since dietary cholesterol and lifestyle factors play a significant role in atherosclerosis, reducing overall cholesterol levels is a first step in preventing the development of heart disease. According to the American Heart Association's recommendations, a balanced caloric diet and physical activity are the best preventive approach to fighting heart disease. Eating fruits and vegetables that are high in vitamins, minerals and fiber are highly recommended in order to maintain a healthy weight and lower the risk of death from coronary heart disease. In addition, lean meats, poultry and fish contribute to a healthy lifestyle, and consumption of fish rich in omega-3 fatty acids such as salmon, trout, and herring at least two times weekly are highly recommended to help maintain healthy blood lipid profiles.

**Table 1. Tissue localizations of P2Y receptor subtypes and G-protein coupling<sup>1</sup>**

Receptor	Tissue distribution	Agonists (human*)	G protein	Transduction Mechanism
P2Y <sub>1</sub>	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	ADP* 2MeSADP ATP	G <sub>q/11</sub>	PLC (+) Ca <sup>2+</sup> release
P2Y <sub>2</sub>	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP*=ATP* MRS2698 UTPγS Up <sub>4</sub> -phenyl ester, Ap <sub>4</sub> A MRS2768	G <sub>q/11</sub> G <sub>o</sub>  G <sub>12</sub>	PLC (+) Ca <sup>2+</sup> release PLC (+) Ca <sup>2+</sup> release Rac (+) RhoA (+)
P2Y <sub>4</sub>	Endothelial cells	UTP>ATP Ap <sub>4</sub> A, UP <sub>4</sub> U	G <sub>q/11</sub> G <sub>i/o</sub>	PLC (+) Ca <sup>2+</sup> release
P2Y <sub>6</sub>	T cells, thymus placenta, epithelial cells	UDP*>UTP* >>ATP* MRS2693 Up <sub>3</sub> U, UDPβS	G <sub>q/11</sub>	PLC (+) Ca <sup>2+</sup> release
P2Y <sub>11</sub>	Spleen, intestine, granulocytes	ATP*, ATPγS BzATP, NF546 NAD <sup>+</sup> , NAADP <sup>+</sup>	G <sub>q/11</sub> G <sub>s</sub>	PLC (+) Ca <sup>2+</sup> release
P2Y <sub>12</sub>	Platelets, glial cells	ADP*, ADP-β-S 2MeSADP, ATP	G <sub>i/o</sub>	AC (-), ↓cAMP
P2Y <sub>13</sub>	Spleen, brain, lymph nodes, bone marrow	ADP*, 2MeSADP 2MeSATP, ATP	G <sub>i/o</sub>	AC (-), ↓cAMP
P2Y <sub>14</sub>	Placenta, adipose tissue, stomach, intestine, brain	UDP-Glucose* MRS2690, UDP UDP-galactose UDP-glucosamine	G <sub>i/o</sub>	AC (-) ↓cAMP

PLC = phospholipase C  
AC = adenylate cyclase

<sup>1</sup>Adapted from Burnstock (2007) and Erb et al. (2006).

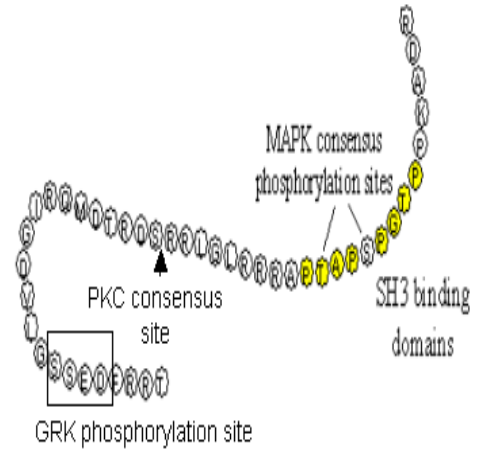
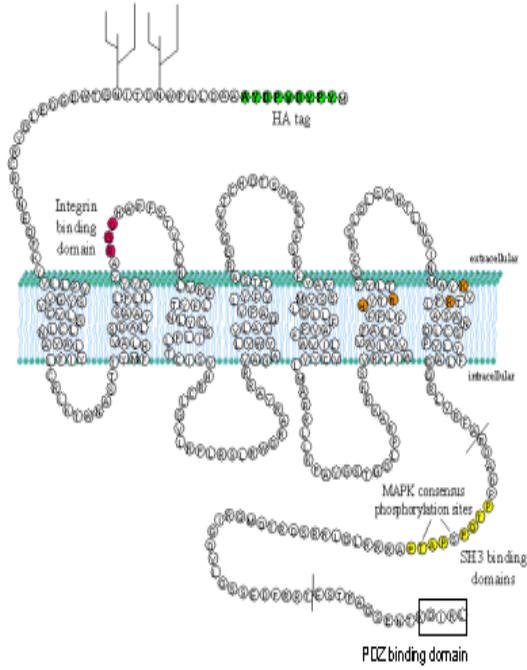
**Table 2. Primer sequences for *GAPDH*, *LRP 1*, *P2Y<sub>1</sub>R*, *P2Y<sub>2</sub>R*, *P2Y<sub>4</sub>R*, *LDLR***

<b>Primers (Mus Musculus)</b>	<b>Sequences</b>
<i>LRP 1</i> (NM_008512.2) Forward Reverse	5'-GCCAGCCAGATGTGCCCAAT-3' 5'-TGGTGGGGCAGG CGCATTTA-3'
<i>GAPDH</i> (NM_008084.2) Forward Reverse	5'-TTGTGCAGTGCCAGCCTCGT-3' 5'-AGGCGCCCAATACGGCCAAA-3'
<i>P2Y<sub>1</sub>R</i> (NM_008772.4) Forward Reverse	5'-CGGGAGCGCACTTGCAAAC-3' 5'-TGAAGGACGTGCGGGCAAAC-3'
<i>P2Y<sub>2</sub>R</i> (NM_008773.3) Forward Reverse	5'-GGTCGAGTCAGCGCCAAACA-3' 5'-AGTTCATCAGCGCACCGGCA-3'
<i>P2Y<sub>4</sub>R</i> (NM_020621.4) Forward Reverse	5'-TCGGCTCCGTTCTCTCCGCA-3' 5'-CACCCGGCATTCCGGCGTTCA-3'
<i>LDLR</i> (NM_001252658.1) Forward Reverse	5'-TGCCAATCGACT CACGGGTTCA-3' 5'-AGTGTGCGACTTCTCTAGGCTGTGT-3'

**Figure 1. Structural organization of the P2Y<sub>2</sub>R.**

A fragment of the P2Y<sub>2</sub>R C-terminus consisting of amino acids 317-361 was expressed in yeast for two-hybrid library screening. This fragment contains two SH3 binding sites, a PDZ binding domain, mitogen-activated protein kinase (MAPK) consensus phosphorylation sites, a protein kinase C (PKC) consensus target site, and a G protein-coupled receptor kinase (GRK) phosphorylation site. It also includes an incorporated hemagglutinin (HA) tag used for immunofluorescence and immunoprecipitation of diverse P2Y<sub>2</sub>R constructs. To identify the cytoskeletal proteins that interact with P2Y<sub>2</sub>R the Weisman group used the intracellular C-terminus of the P2Y<sub>2</sub>R as bait to screen a cDNA library generated from human aorta. Several P2Y<sub>2</sub>R-interacting clones containing an ~1.2 kb cDNA insert encoding the C-terminal region of FLN-A were isolated.

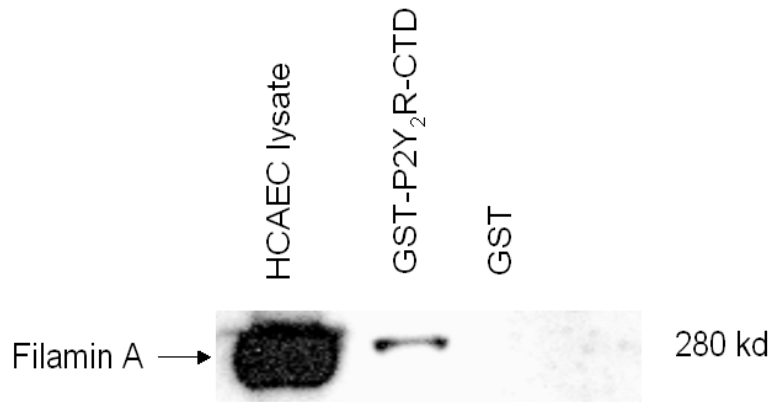
## N-Terminus



## C-terminus

**Figure 2. GST-P2Y2R fusion protein interacts with FLN-A.**

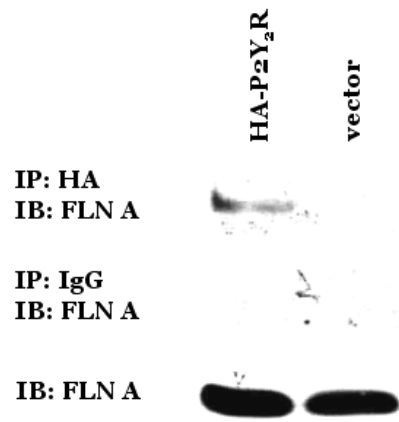
Protein expression was induced by 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside in *E. coli* BL21 transformed with GST or GST- P2Y<sub>2</sub>R C-terminal construct. Bacterial lysates were incubated with immobilized glutathione for 4 h at 4°C with gentle rocking. After extensive washing, mouse VSMCs lysates were added for overnight incubation at 4°C with gentle rocking. The protein complexes were eluted by 100 mM glutathione. Proteins in the elutant were resolved by SDS-PAGE and gels were probed with anti-FLN-A monoclonal antibodies. Results shown are representative of three independent experiments.





**Figure 3. Co-immunoprecipitation of FLN-A with HA-P2Y<sub>2</sub>R in 1321N1 cell transfectants.**

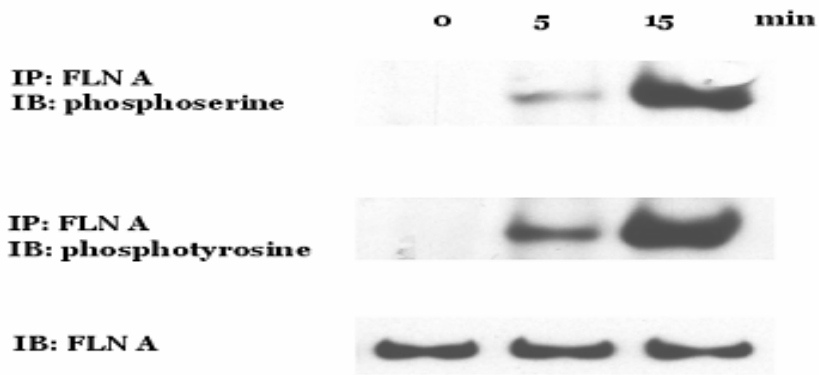
Human 1321N1 cells transfected with cDNA encoding the HA-tagged P2Y<sub>2</sub>R in the pLXSN vector were lysed and lysates were immunoprecipitated (IP) with anti-HA antibody or normal IgG, and analyzed by immunoblotting (IB) with anti-FLN-A antibody (upper panel). FLN-A levels in total cell lysates were analyzed for normalization (lower panel). IgG was used as negative control. Results shown are representative of three independent experiments (Yu et al 2008).



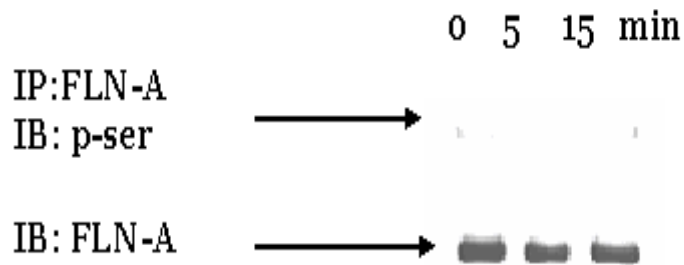
**Figure 4. UTP induces FLN-A phosphorylation on Ser/Tyr residues in 1321N1 cells transfected with the P2Y<sub>2</sub>R.**

Human 1321N1 cells transfected with the HA-tagged P2Y<sub>2</sub>R or pLXSN vector alone, were serum-starved and stimulated with 100  $\mu$ M UTP for the indicated time. The cells were then lysed and lysates were immunoprecipitated by anti-FLN-A antibody and analyzed by immunoblotting with anti-phospho-Serine or anti-phospho-tyrosine antibody (top panel). FLN-A levels in total cell lysates were analyzed for normalization. Results shown are representative of three independent experiments. The empty vector transfected into 1321N1 cells was used as a control (Vector-transfected).

### P<sub>2</sub>Y<sub>2</sub>R-transfected

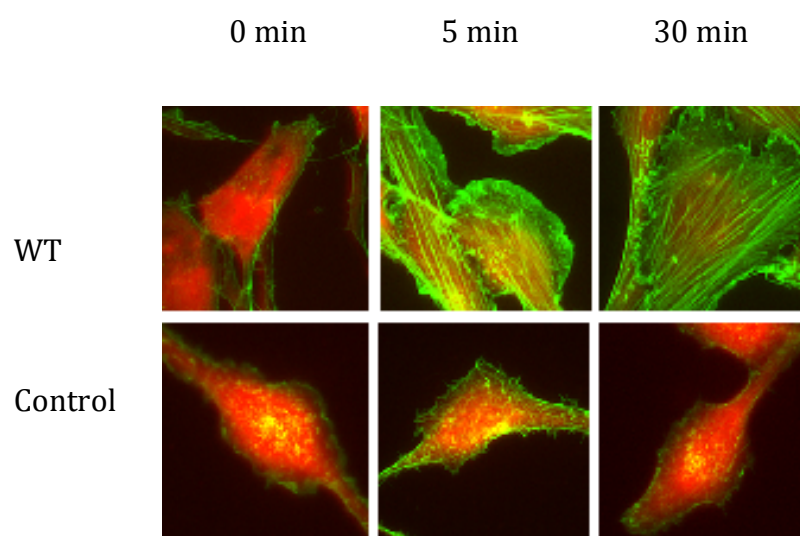


### Vector-transfected



**Figure 5. UTP stimulates actin polymerization in 1321N1 cells expressing the HA-tagged P2Y<sub>2</sub>R.**

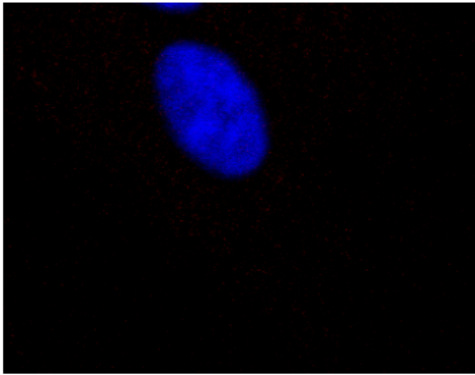
Serum-starved cells were treated with 100  $\mu$ M UTP for the indicated time, fixed in 3.7% formaldehyde and lysed with 0.1% triton X-100. Then, the cells were incubated with Oregon Green™488-conjugated phalloidin (5  $\mu$ g/ml, Molecular Probes) for 45 min at room temperature and then washed with PBS. Texas Red-labeled DNase I was used to localize globular-actin (shown in red). The cells were visualized using microscopy at 40X magnification. Vector-transfected cells were used as controls, and 1321N1 cells transfected with the HA-P2Y<sub>2</sub>R are referred to as wild type (WT).



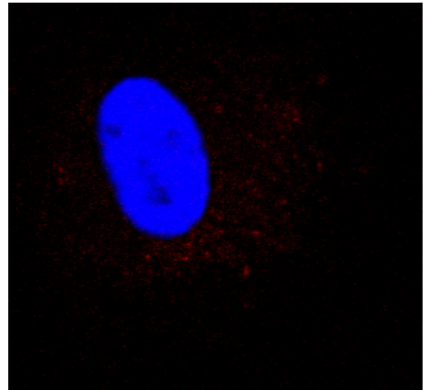
**Figure 6. UTP stimulates uptake of Aggregated LDL in mouse VSMCs**

Confocal microscopy of mouse VSMCs incubated with aggregated LDL 50 $\mu$ g/ml for 2 h at 37 $^{\circ}$ C. Lipids were stained red by incubating cells with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI). (A) no LDL (B) LDL (C) UTP 10  $\mu$ M + LDL. Cells were afterwards washed and incubated with 5  $\mu$ g/ml of DiI for 30 minutes in the dark. The red granules indicate lipid uptake. The nuclei (blue color) were stained by incubating cells with 1  $\mu$ g/ml of Hoeschst dye. The images are representative samples. Cells visualized using Zeiss 510 laser scanning confocal microscope.

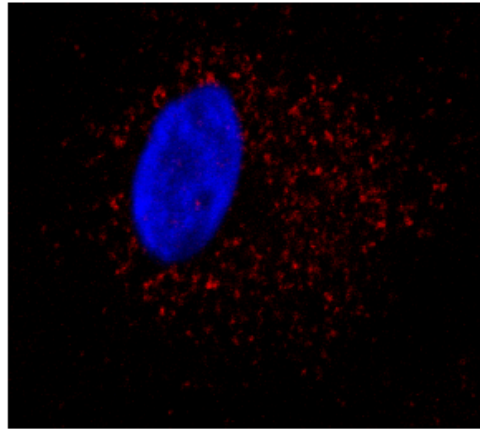
A



B



C





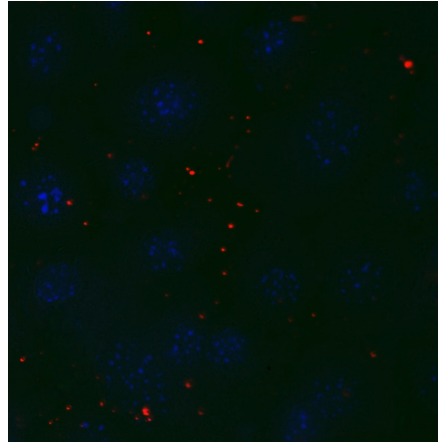
**Figure 7. UTP stimulated and matrix-bound agLDL uptake in VSMC expressing WT P2Y<sub>2</sub>R or mutant P2Y<sub>2</sub>R that does not bind FLN-A**

Confocal microscopy of mouse VSMCs incubated with aggregated LDL 50 µg/ml for 4 hours at 37°C. Lipids were stained by incubating cells with 1,1'-diiodo-3,3',3',3'-tetramethylindocarbocyanine (DiI). Cells were transfected with DNA from WT or mutant P2Y<sub>2</sub>R constructs, then stimulated with 10 µM UTP and grown on top of a matrix with retained agLDL. (A) no agLDL uptake in cells transfected with P2Y<sub>2</sub>R mutants, (B) agLDL uptake in P2Y<sub>2</sub>R<sup>-/-</sup> cells transfected with DNA from WT P2Y<sub>2</sub>R. Cells visualized using Zeiss LSM 700 confocal microscope.

A.

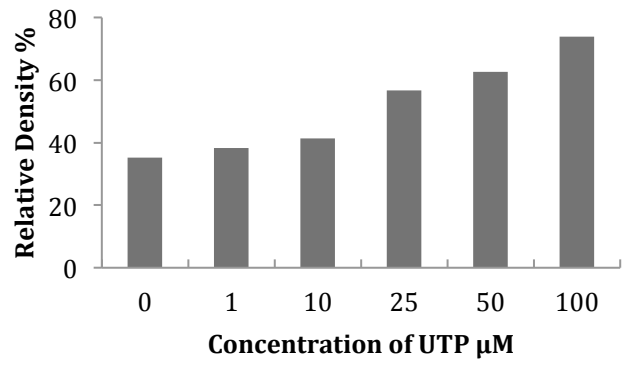
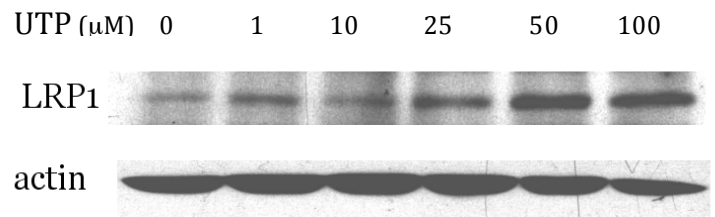


B.



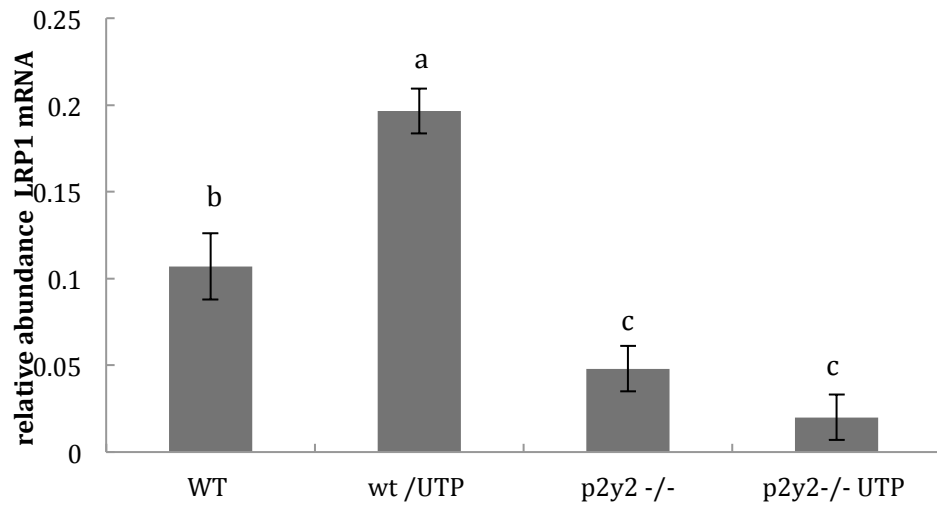
**Figure 8. Western Blot Analysis of LRP 1 expression in mouse VSMCs.**

Serum starved cells were stimulated in the absence (lane1) or presence of UTP (1, 10, 25, 50, 100  $\mu$ M) (lanes 2-6). Detection of LRP 1 was performed using mouse anti-LRP 1 monoclonal antibody (1:3000 dilution; Abcam, Cambridge MA) followed by horseradish peroxidase-conjugated donkey anti-mouse polyclonal antibody (1:10000 dilution Abcam, Cambridge MA). For signal normalization, membranes were probed with polyclonal anti- $\beta$ -actin antibody (1:2000 dilution). The results show LRP 1 is expressed in a dose-dependent manner and expression increasing with increased UTP stimulation.



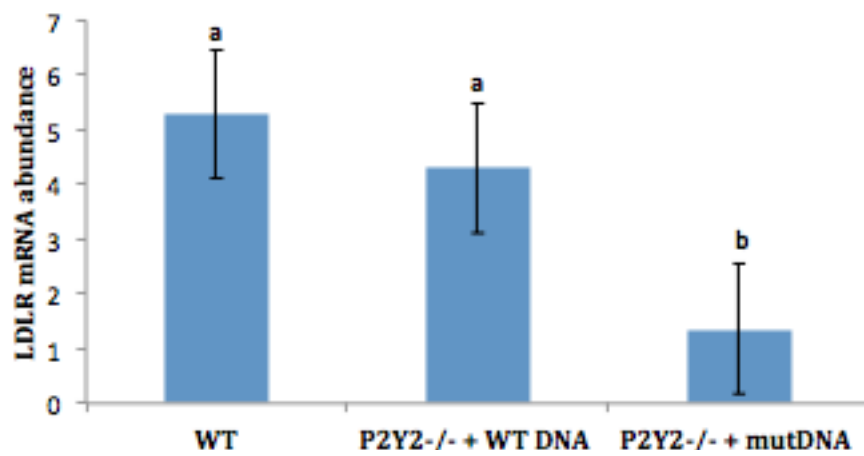
**Figure 9. LRP 1 mRNA expression in VSMC stimulated with UTP.**

Cells isolated from WT and P2Y<sub>2</sub> mice were cultured in 12-well plates and grown to at least 80% confluence. The cells were serum-starved overnight followed by treatment with 10 μM UTP. Total RNA was isolated, and reverse transcribed to cDNA. The mRNA relative abundance was determined by RT-PCR using the  $\Delta\Delta C_t$  method to calculate fold increase in mRNA levels expressed at 95% confidence intervals of results from 6 independent experiments. The letters (a) and (b) indicate a significant difference in mRNA levels in response to UTP in WT VSMCs ( $p < 0.05$ ), whereas (c) represents no significant difference in cells from the p2y<sub>2</sub>R<sup>-/-</sup> mice. *GAPDH* was used as a control gene.



**Figure 10. LDLR mRNA expression in VSMC stimulated with UTP.**

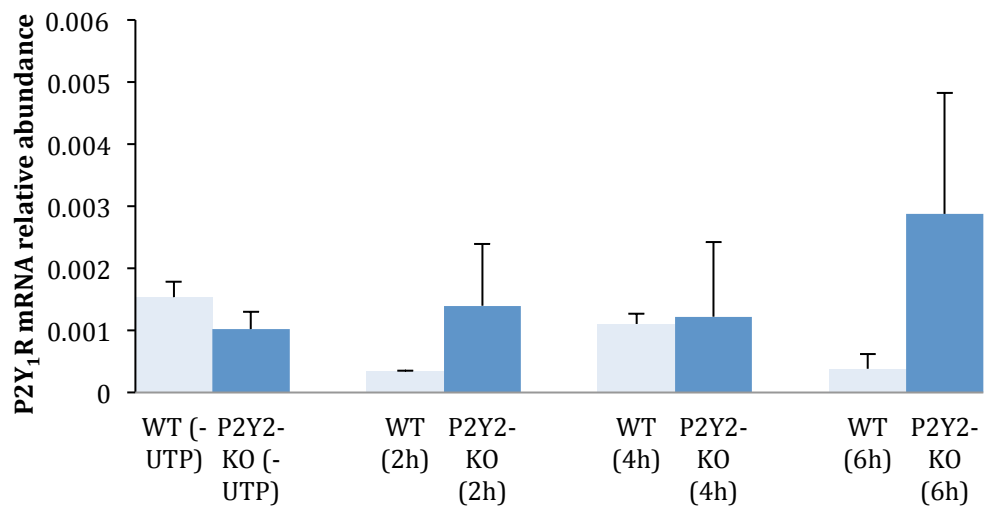
Cells were transiently transfected with cDNA encoding a hemagglutinin-tagged WT P2Y<sub>2</sub>R or a mutant P2Y<sub>2</sub>R that does not bind FLN-A. Based on our results, the wild type untransfected SMCs showed the highest LDLR mRNA relative abundance compared to both transfected groups. Additionally, the P2Y<sub>2</sub>R mutant SMCs transfected with the mutant P2Y<sub>2</sub>R DNA exhibited significantly lower LDLR mRNA relative abundance, than the P2Y<sub>2</sub>R mutant SMCs transfected with the WT P2Y<sub>2</sub>R. However, the high LDLR expression in the untransfected wild type SMCs was not significantly different from the P2Y<sub>2</sub>R mutant transfected with WT DNA. These results may indicate that both an intact P2Y<sub>2</sub>R and FLN-A may be critical for LDLR expression. Same letters represent no difference between the groups. Different letters mean there is a significant difference from the other groups. *GAPDH* was used as a control gene.





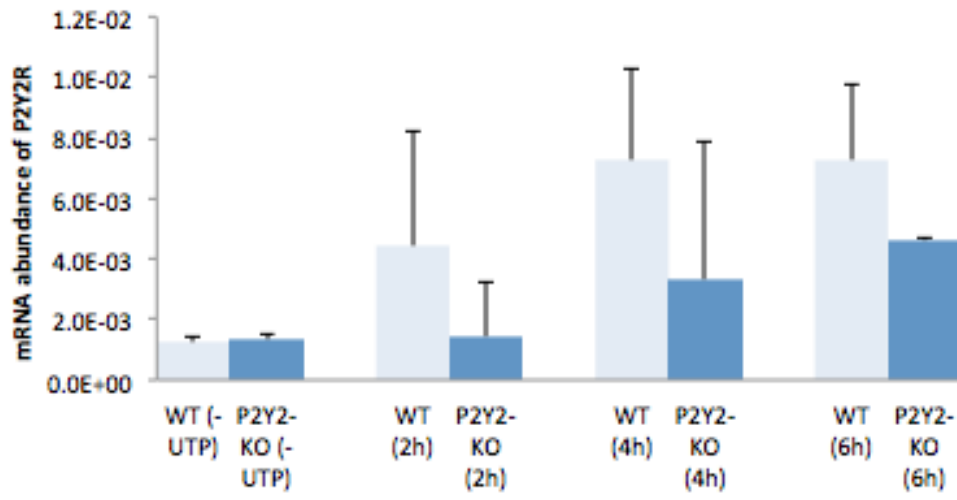
**Figure 11. P2Y<sub>1</sub>R mRNA expression in VSMC stimulated with UTP.**

Cells isolated from WT and P2Y<sub>2</sub> mice were cultured in 12-well plates and grown to at least 80% confluence. Cells were serum starved overnight, followed by stimulation with 10 μM UTP. Total RNA was isolated, and reverse transcribed to cDNA using multiscribe reverse transcriptase and random primers. The mRNA relative abundance was determined by RT-PCR using P2Y<sub>1</sub>R forward and reverse primers and the  $\Delta\Delta C_t$  method to calculate fold increase in mRNA levels expressed at 95% confidence intervals of results from 6 independent experiments. *GAPDH* was used as a control gene. There was no significant difference in the levels of P2Y<sub>1</sub>R mRNA over time. *GAPDH* was used as a control gene.



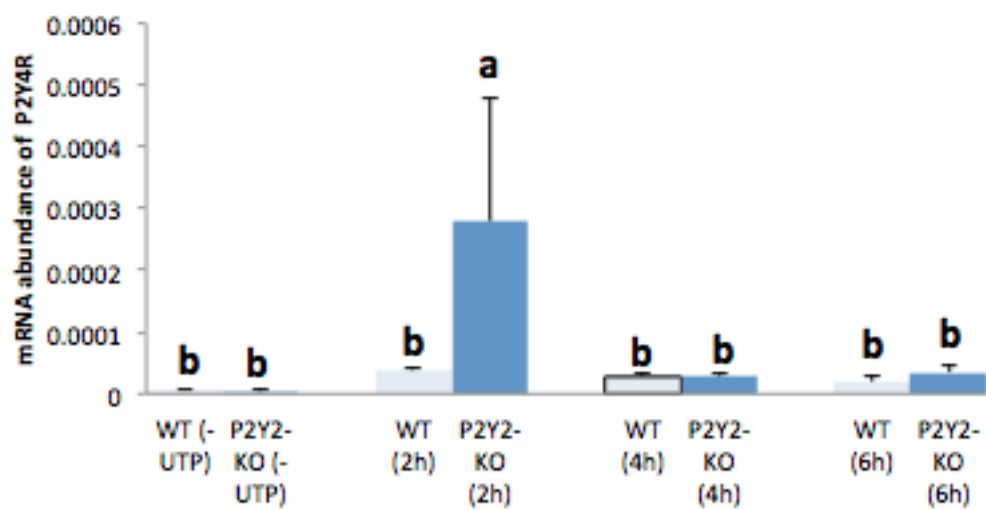
**Figure 12. P2Y<sub>2</sub>R mRNA expression in VSMC stimulated with UTP.**

Cells isolated from WT and P2Y<sub>2</sub><sup>-/-</sup> mice were cultured in 12-well plates and grown to at least 80% confluence. Cells were serum starved overnight, followed by stimulation with 10 μM UTP. Total RNA was isolated, and reverse transcribed to cDNA using multiscribe reverse transcriptase and random primers. The mRNA relative abundance was determined by RT-PCR using P2Y<sub>2</sub>R forward and reverse primers and the  $\Delta\Delta C_t$  method to calculate fold increase in mRNA levels expressed at 95% confidence intervals of results from 6 independent experiments. *GAPDH* was used as a control gene. No significant levels of P2Y<sub>2</sub>R mRNA were observed in the samples.



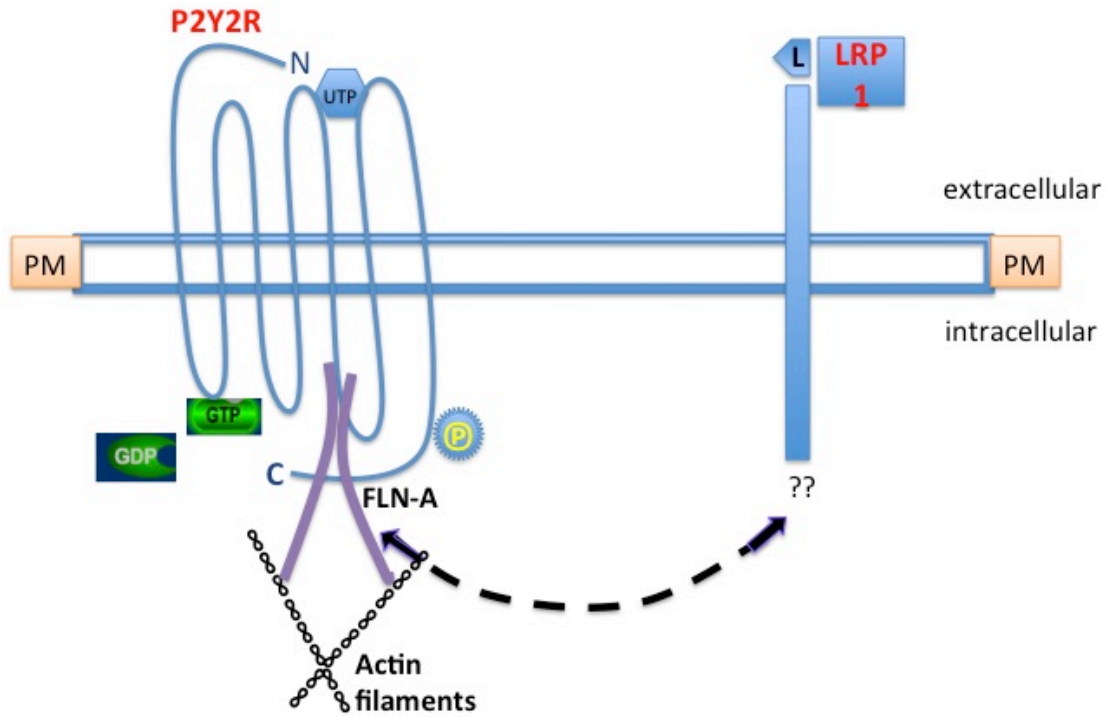
**Figure 13. P2Y<sub>4</sub>R mRNA expression in VSMC stimulated with UTP.**

Cells isolated from WT and P2Y<sub>2</sub><sup>-/-</sup> mice were cultured in 12-well plates and grown to at least 80% confluence. Cells were serum starved overnight, followed by stimulation with 10 μM UTP. Total RNA was isolated, and reverse transcribed to cDNA using multiscribe reverse transcriptase and random primers. The relative mRNA abundance was determined by RT-PCR using P2Y<sub>4</sub>R forward and reverse primers and the ΔΔCt method to calculate fold increase in mRNA levels expressed at 95% confidence intervals of results from 6 independent experiments. The mRNA levels are expressed at 95% confidence intervals of results from 6 independent experiments. The letters indicate a significant difference in mRNA levels in response to UTP by P2Y<sub>2</sub><sup>-/-</sup> VSMCs ( $p < 0.05$ ) at 2 h, whereas (b) represents no significant difference in cells from the p2y<sub>2</sub>R<sup>-/-</sup> mice. *GAPDH* was used as a control gene.



**Figure 14. Predicted pathway for P2Y<sub>2</sub>R-LRP 1 interaction for agLDL uptake by SMC**

This diagram represents a proposed mechanism for P2Y<sub>2</sub>R mediated uptake of agLDL in VSMC, and the possible cross-talk between LRP 1 and P2Y<sub>2</sub>R. Ligand binding on the LRP 1 extracellular binding domain represent binding of the apo-E molecules. *In vitro* studies show that Apo E binding inhibits platelet-derived growth factor induced migration and proliferation in smooth muscle cells (Boucher et al 2003). We predict the existence of a regulatory element within the promoter region of the LRP 1 gene that is responsive to UTP induced P2Y<sub>2</sub>R activation. We propose that this activated region allows LRP 1 to associate with the P2Y<sub>2</sub> receptor at the C-terminus, through possible phosphorylation, and other events involving FLN-A and/or other cytoskeletal proteins. P2Y<sub>2</sub>R is a G-protein coupled receptor. The bound GTP represents its activated state. The dashed line represents the predicted interaction between LRP 1 and the P2Y<sub>2</sub>R.



PM = plasma membrane

P = phosphate

L = ligand

UTP = uridine triphosphate

GTP = guanosine triphosphate

GDP = guanosine diphosphate



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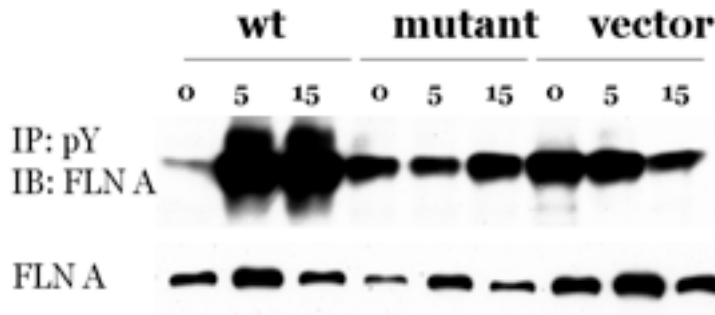
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## Appendix A

**UTP induced phosphorylation of FLN-A in 1321 N1 cells expressing the WT P2Y<sub>2</sub>R, but not in the cells expressing the mutant P2Y<sub>2</sub>R.**



Cells were stimulated with UTP (100  $\mu$ M) for 0, 5, and 15 min. Immunoprecipitation was done using a phosphotyrosine antibody followed by western blotting with FLN-A antibody.

Data are representative of three independent experiments.