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The $P2Y_2$ receptor mediates uptake of matrix-retained and aggregated low density lipoprotein in primary vascular smooth muscle cells

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Abstract

Background and aims—The internalization of aggregated low-density lipoproteins (agLDL) mediated by low-density lipoprotein receptor related protein (LRP1) may involve the actin cytoskeleton in ways that differ from the endocytosis of soluble LDL by the LDL receptor (LDLR). This study aims to define novel mechanisms of agLDL uptake through modulation of the actin cytoskeleton, to identify molecular targets involved in foam cell formation in vascular smooth muscle cells (VSMCs). The critical observation that formed the basis for these studies is that under pathophysiological conditions, nucleotide release from blood-derived and vascular cells activates SMC $P2Y_2$ receptors ($P2Y_2$Rs) leading to rearrangement of the actin cytoskeleton and cell motility. Therefore, we tested the hypothesis that $P2Y_2$R activation mediates agLDL uptake by VSMCs.

Methods—Primary VSMCs were isolated from aortas of wild type (WT) C57BL/6 and.*P2Y2R*−/− mice to investigate whether $P2Y_2$R activation modulates LRP1 expression. Cells were transiently transfected with cDNA encoding a hemagglutinin-tagged (HA-tagged) WT $P2Y_2$R, or a mutant $P2Y_2$R that unlike the WT $P2Y_2$R does not bind the cytoskeletal actin-binding protein filamin-A (FLN-A).

Results—$P2Y_2$R activation significantly increased agLDL uptake, and *LRP1* mRNA expression decreased in *P2Y2R*−/− VSMCs *versus* WT. SMCs, expressing $P2Y_2$R defective in FLN-A binding, exhibit 3-fold lower LDLR expression levels than SMCs expressing WT $P2Y_2$R, while

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Conflict of interest

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cells transfected with WT $P2Y_2R$ show greater agLDL uptake in both WT and *P2Y2R*−/− VSMCs *versus* cells transfected with the mutant $P2Y_2R$.

Conclusions—Together, these results show that both LRP1 and LDLR expression and agLDL uptake are regulated by $P2Y_2R$ in VSMCs, and that agLDL uptake due to $P2Y_2R$ activation is dependent upon cytoskeletal reorganization mediated by $P2Y_2R$ binding to FLN-A.

Keywords

LRP1; P2Y2R

1. Introduction

The foremost cause of mortality in men and women in the United States is atherosclerosis [1]. A major event in its development is the accumulation of lipids, mainly cholesterol esters (CEs), in smooth muscle cells (SMCs) of blood vessels, which leads to foam cell formation [2,3]. Although dietary and life-style factors are implicated in the development of atherosclerosis, a number of molecular targets have been shown to play significant roles in the pathological progression of atherosclerosis [4,5].

There is little knowledge as to how vascular SMCs (VSMCs) are transformed into foam cells. Lipoproteins that are retained in the extracellular matrix are taken up by VSMCs and macrophages, which subsequently results in CE accumulation and foam cell formation [6]. Macrophages become foam cells through the uptake of diversely modified LDLs [7], whereas the aggregation of LDLs seems to be a key condition for lipid accumulation in VSMCs [6,8]. Scavenger receptors on macrophages mediate their transformation to foam cells following uptake of modified and oxidized LDLs (oxLDLs). Compared to their relative abundance in macrophages, scavenger receptors are not observed in VSMCs from human atherosclerotic lesions. Uptake of aggregated LDL (agLDL) is a pre-requisite condition for lipid accumulation in VSMCs, and the LDL receptor related protein 1 (LRP1) is an important mediator in this uptake [6,9]. However, little is known about how LRP1 expression is regulated.

P2 receptors for extracellular purine and pyrimidine nucleotides are ubiquitously expressed in human tissues, including the blood vessel wall [10] Kanapuli. Under pathological conditions, nucleotide release from blood-derived and vascular cells activates the $P2Y_2$ receptor ($P2Y_2R$) in SMCs, leading to rearrangement of the actin cytoskeleton and cell motility [11,12]. The $P2Y_2R$ mediates these effects through its interaction with FLN-A, an actin-binding 280 kDa protein [13].

Several studies have demonstrated that in human VSMCs *in vitro*, LRP1 mediates the internalization of aggregated LDL (agLDL) [14,15]. We hypothesized that the $P2Y_2R$ may regulate foam cell formation in VSMCs. The $P2Y_2R$ plays an important role in stress or injury and the development of inflammation in the vasculature and arterial wall disease [16,17]. Under pathological conditions, extracellular nucleotides, including the equipotent $P2Y_2R$ agonists ATP and UTP, are released from various cellular sources to activate P2 nucleotide receptors. $P2Y_2R$ activation by ATP or UTP [18,19] has been associated with

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various cellular processes, including cytoskeletal remodeling, cell proliferation and migration of VSMCs [11,13,16,20]. It has also been shown that reorganization of the cytoskeleton plays a significant role in the uptake of aggregated lipoproteins by macrophages that is different than receptor-mediated endocytosis [21]. Specifically, Sakr et al. have shown that actin polymerization occurs in SMCs with matrix-retained LDL, and the activities of myosin ATPase, Rho family GTPases, and other signaling molecules are needed for the internalization of matrix-retained LDL and agLDL [21].

Extracellular nucleotides induce rapid dynamic reorganization of the actin network. As such, UTP-stimulated P2Y receptor activation was shown to induce reorganization of the actin cytoskeleton in rat VSMCs expressing $P2Y_2$ or $P2Y_4$ receptors [11], which, was coupled to activation of RhoA, a small GTPase that regulates actin cytoskeleton reorganization. UTP, an agonist of the $P2Y_2R$, was shown to significantly increase the spreading and migration of SMCs isolated from mouse aortas [13]. $P2Y_2R$/FLN-A interaction is necessary for $P2Y_2R$-mediated actin cytoskeleton reorganization, since UTP-induced spreading and migration was not observed when SMCs from *$P2Y_2R$* knockout mice were transfected with mutant *$P2Y_2R$* cDNA encoding a $P2Y_2R$ that does not bind to FLN-A [13]. In the present study, we investigated the role of $P2Y_2R$ in the internalization of matrix-bound agLDL in VSMCs, and we found that an interaction between FLN-A and $P2Y_2$ R was a necessary factor in the uptake of agLDL.

2. Materials and methods

2.1. Materials

Mouse aortas were surgically removed from 6 to 8 week old male *$P2Y_2R$* knockout mice and C57BL/6 control mice and enzymatically digested with 0.1% (w/v) collagenase II (Worthington Biochemical Corporation, Lakewood, NJ) in Medium Nutrient Mix 199 (M199) and suspended in Dulbecco's Modified Eagle's Medium Nutrient Mix F12 in a 1:1 ratio (DMEM F12), 100 μg/ml normocin, 5% (v/v) fetal bovine serum (FBS), 5% (v/v) 20X Smooth Muscle Growth Supplement (SMGS), 1% (v/v) 100X penicillin-streptomycin solution containing 10,000 IU units/ml penicillin and 10,000 μg/ml streptomycin and penicillin-streptomycin glutamine (Life Technologies, Carlsbad, CA). Uridine 5'-triphosphate trisodium salt dehydrate, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) and ribonucleic acid (RNA) synthesis inhibitor Actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine® 2000 Reagent, SOC medium and Electromax DH 10B™ competent cells used for cloning and transfection protocols were obtained through Life Technologies (Carlsbad, CA). Lipoproteins (unlabeled LDL or oxLDL labeled with 1,1'-dioctadecyl-3,3,3′,3'-tetramethyl-indocarbocyanine perchlorate (DiI-oxLDL) for LDL uptake studies) were purchased from Biomedical Technologies (Stoughton, MA). Alexa Fluor® 546 Monoclonal Antibody Labeling kit (Molecular Probes, Eugene OR) was used for labeling LDL. Primer sequences for *LRP1*, *GAPDH*, *$P2Y_1R$*, *$P2Y_2R$* and *$P2Y_4R$* were designed using the National Center for Biotechnology Information Local Alignment Search Tool (BLAST). The mRNA relative abundance was calculated using the delta-delta (ΔΔ) Ct method. *GAPDH* was used as a loading control gene.

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2.2. Cell preparation and culture

Primary VSMCs isolated from mouse aortas were grown in cell culture. The cells were obtained from enzymatically-digested aortas surgically removed from *P2Y2R*−/− and wild type (WT) C57BL/6 mice. The aortas were placed in a 0.5% (v/v) penicillinstreptomycin phosphate buffer solution (PBS) for 30 min to destroy surface contaminants. The aortas were then removed from the PBS and placed in enough M199 media to cover them. They were next cut open longitudinally and connective tissue and fat were removed. Aortas were placed in a culture dish with fresh PBS and 0.1% (w/v) collagenase, chopped into small pieces (2e4 mm) and transferred into a 50 ml tube. The tubes were incubated for 30 min in a water bath with gentle shaking. Following incubation, the tissue suspension was filtered through a 100 μm strainer and centrifuged for 5 min at 210g. The cell pellet was collected and resuspended in M199 medium supplemented with 20% (v/v) FBS and 1% (v/v) penicillin-streptomycin with 100 μg/ml normocin, 5% (v/v) SMGS. The cells were grown in M199 supplemented with 20% (v/v) FBS and 1% (v/v) 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_2. After 24 h, the media were aspirated, the cells rinsed with sterile PBS and fresh media were added. Thereafter, the cells were replenished with fresh media every 3 days. The cells were identified as VSMCs by their characteristic “hill-and-valley” growth pattern. Following the first passage at 70% confluence, cells were incubated in DMEM F12 culture medium supplemented with 15% (v/v) FBS, 5% (v/v) SMGS, 1% (v/v) penicillin-streptomycin with 5% (w/v) L-glutamine. Human 1321N1 astrocytoma cells were cultured in DMEM with 10% (v/v) fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM).

2.3. $P2Y_2$ receptor cDNA constructs

The open reading frame of wild type human $P2Y_2$ receptor cDNA was modified using PCR to incorporate the HA epitope (YPYDVP-DYA) from influenza virus at the N-terminus of the expressed protein, as described previously [22]. Three primers synthesized in the DNA Core Facility (University of Missouri, Columbia, MO) 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 $P2Y_2$ receptors; Primer 1: 5′ -AGGCTCGTACGCTTTGCCCGAGATGCCAAGGCTCGCCGCAGGCTGGGCCTGCGCAGATC-3’; Primer 2: 5′–ATCATGGATCCTTACT TGGCATCTCGGGC-3’; Primer 3: 5′-CACACCCTAACTGACAC -3’. The PCR products were resolved by agarose gel electrophoresis and the products were purified using the PCR Wizard kit from Amersham Biosciences (Piscataway, NY), digested with BsiWI and BamHI, and inserted into pLXSN. The mutant cDNAs were sequenced to verify that the mutations were incorporated correctly.

2.4. DH5α transformation, culture preparation and cell transfection

Plasmid DNA (100 ng/200 μl cells) encoding HA-tagged WT $P2Y_2R$ or a mutant $P2Y_2R$ that does not bind FLN-A, were added to DH5 competent cells and cells were heat-shocked at 42 °C for 2 min with gentle shaking, followed by addition of 4 vol of Lauria-Bertani (LB) media (400 μl) and incubation at 37 °C with gentle shaking for 50–60 min. The cells were pelleted at 5,000c for 10 min. Approximately 400 μl of the supernatant was removed and the

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pellet was re-suspended in the remaining 50 μl of supernatant. Then, cultures were prepared by inoculating cells in 1.5% (w/v) agar on petri dishes and incubating at 37 °C for 24 h. The colonies were collected and incubated with LB media with 1 μg/ml ampicillin in culture tubes at 37 °C with gentle shaking, until evidence of cloudy suspension appeared. The cultures were then centrifuged for 10 min at 10,000g and the pellet was collected followed by DNA isolation and purification using the Qiagen DNA Isolation kit with the manufacturer's protocol. VSMCs were transiently transfected with the plasmid DNA constructs using Lipofectamine® 2000 reagent from Life Technologies (Carlsbad, CA) following the manufacturer's instructions. The retroviral vector pLXSN was used to stably express the HA-tagged human $P2Y_2$ receptor in P2 receptor-null human *1321N1* astrocytoma cells, as previously described [23]. In brief, the recombinant $P2Y_2$R-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).

2.5. Protein abundance of LRP1 in mouse VSMCs

Serum-starved WT C57BL/6 cell cultures were incubated in the absence or presence of UTP (1, 10, 25, 50 or 100 μM). Cell lysates were prepared in 2X Laemmli sample buffer. Equivalent amounts of protein (100 μg) 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, pH 7.4) containing 0.1% (v/v) Tween-20 (TBST) and immunoblotted overnight at 4 °C in TBST containing 1% (w/v) BSA and anti-LRP1 antibody (1:3000 dilution; Abcam, Cambridge, MA), followed by horseradish peroxidase-conjugated donkey anti-mouse polyclonal antibody (1:10,000 dilution; Abcam, Cambridge, MA). For signal normalization, membranes were probed with rabbit polyclonal anti-mouse β-actin antibody (1:2000 dilution; Abcam, Cambridge, MA).

2.6. LRP1, LDLR, $P2Y_1$R, $P2Y_2$R and $P2Y_4$R mRNA expression in VSMCs

P2Y2R−/− and C57BL/6 control VSMCs were grown to 90% confluence in a monolayer in 12-well plates in DMEM F12 media supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin. Cells were serum starved by incubating for 24 h with DMEM F12 at 37 °C in a humidified atmosphere with 5% CO_2. After 24 h, cells for *LRP1* mRNA and *LDLR* mRNA expression were incubated with actinomycin D (2 mg/ml) in fresh media for 15 min to block transcriptional activity. Cells were then stimulated with UTP (10 μM) and incubated 18 h. Total RNA was isolated from VSMCs 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. An equal volume of 100% ethanol was added to the lysate, which was then 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 (10X RT Buffer, 25X dNTP mix, 10X Random Primers, and Multiscribe Reverse Transcriptase) (Invitrogen, Carlsbad CA). RT-PCR was conducted using Fast SYBR

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Green Master Mix. Two independent PCR reactions were carried out for each cDNA synthesis. The mRNA relative abundance was determined using the ΔΔCt method and *GAPDH* as a control gene. Oligonucleotide amplification primer sequences for *LRP1* were forward 5′-GCCAGCCAGATGTGCCCAAT-3′ and reverse 5′-TGGTGGGGCAGGCGCATTTA-3’. Primer sequences for *LDLR* were forward 5′-TGCCAATCGACTCACGGGTTCA-3′ and reverse 5′-AGTGTCGACTTCTCTAGGCTGTGT-3’. For $P2Y_1R$, $P2Y_2R$ and $P2Y_4R$ mRNA expression, cells were incubated with PPADS (2 mg/ml) in media for 15 min, then stimulated with UTP (10 μM) and incubated for 18 h. Sequences for $P2Y_1R$ were forward (NM_008772.4) 5′-CGGGAGCGCACTTGCAAACT-3′ and reverse 5′-TGAAGGACGTGCGGGCAACT-3’; sequences for $P2Y_2R$ were forward (NM_008773.3) 5′-GGTCGAGTCAGCGCCAAACA-3′ and reverse 5′-AGTTCATCAGCGCACCGGCA-3’; sequences for $P2Y_4R$ were forward (NM_020,621.4) 5′-TCGGCTCCGTTCTCTCCGCA-3′ and reverse 5′-CACCCGGCATTCGGCGTTCA-3’. Primer sequences were determined by BLAST (www.ncbi.nlm.nih.gov).

2.7. LDL labeling and aggregation

DiLDL was aggregated by incubation with bacterial sphingomyelinase (SMase), as previously described] [24]. In brief, 1 mg of LDL protein/ml of PBS was incubated with 50 milliunits/ml SMase in the presence of 5 mM $MgCl_2$, for 4 h, under argon, without shaking at 37 °C, followed by 10 mM EDTA to stop the reaction. The LDL was then diluted with binding buffer (10 mM Hepes, pH 7.4, 140 mM $NaCl_2$, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 3% (w/v) BSA). Unlabeled lipoprotein was fluorescently labeled by Alexa fluor® 546 monoclonal antibody labeling kit according to manufacturer's protocol, then aggregated by combining 500 ml with 500 ml PBS and centrifugation for 10 min at 10,000 × g, followed by collecting and resuspending the pellet in 700 μl PBS.

2.8. Matrix preparation, LDL binding and uptake

To produce SMC-derived matrix, 100 μl of the mouse SMC culture (1×10^6 cells/ml) was plated in 35-mm glass-bottom culture plates and incubated in DMEM with 10% (v/v) FBS. Cells were grown in a monolayer to 100% confluence. Following three washes with DMEM containing 0.2% (w/v) BSA, the SMC monolayer was air dried for 15 min and extracted twice with 3:2 (v/v) hexane:isopropanol for 30 min. The lipid extracts were removed and discarded, and the wells dried for 15 min at room temperature in a laminar flow hood. After washing three times with binding buffer, the matrix was incubated with binding buffer for 1 h at room temperature to block non-specific binding sites. In preparation for LDL-matrix binding, 1 μg of lipoprotein lipase (Sigma-Aldrich, St. Louis, MO) was added to the lipid-extracted SMC matrix for 1 h at room temperature, then 100 μl of SMase-treated LDL was added to the matrix and incubated for 18 h at 37 °C in a humidified atmosphere with 5% CO_2. The unbound LDL was removed by washing with DMEM and 0.2% (w/v) BSA.

2.9. UTP-stimulated uptake of agLDL in mouse VSMCs

To investigate the uptake of agLDL in the absence or presence of matrix, 100% (w/v) agLDL solution was vortexed for 4 min and then centrifuged at 10,000 × g for 10 min, and the pellet was recovered. VSMCs were incubated with 50 μg/ml agLDL for 2 h at 37 °C

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followed by 37 °C for 2 h in the presence or absence of 100 μM UTP. Cells were incubated with 200 μg/ml 1,1'dioctadecyl-3,3,3′,3'-tetramethylindo-carbocyanine (DiI) to stain lipid vacuoles. The nuclei were stained by incubating cells with 1 μg/ml of Hoechst solution (Thermo Scientific, Rockford, IL). Fluorescence microscopy experiments were carried out to visualize the internalization of agLDL by mouse VSMCs using a Zeiss LSM 510 laser scanning confocal microscope.

2.10. UTP-stimulated uptake of agLDL in VSMCs expressing WT or mutant $P2Y_2R$

To determine agLDL uptake by VSMCs, HA-tagged *WT* $P2Y_2R$- or *mutant* $P2Y_2R$-transfected VSMCs were incubated with 100 μM UTP for 4 h, then seeded on top of the matrix with 200 μg/ml DiI-oxLDL or Alexa fluor labeled LDL for 0, 1, 2 or 4 h. To visualize the internalization of fluorescently-labeled agLDL, the cells were viewed with a Zeiss LSM 700 confocal microscope. After brief rinsing in DMEM with 0.2% (w/v) BSA, cells were fixed with 3% (v/v) paraformaldehyde. The nucleus was identified using DAPI nuclear stain.

2.11. Statistical analysis

The treatment effects were analyzed using JMP (version 10.0, SAS Institute, Cary, NC), and data were evaluated by Student's *t*-test and expressed as means ± SEM with statistical significance defined as $p < 0.05$.

3. Results

3.1. UTP stimulates LRP1 expression in mouse VSMCs

LRP1 is highly expressed in VSMCs and has been shown to be upregulated *in vitro* by aggregated LDL (agLDL), and *in vivo* in a hypercholesterolemic porcine model [25,26]. Since agLDL is found in atherosclerotic lesions, our overall goal was to determine how agLDL uptake is regulated in foam cell formation. Therefore, we began our investigation by using western blot analysis to determine what effect UTP had on LRP1 expression in VSMCs from WT mice. Our results revealed that LRP1 expression was the greatest for cells stimulated with the highest concentration of UTP. In addition, there was a dose dependent response for LRP1 expression, with the highest UTP dose showing the greatest response versus the lowest doses with the least response (Fig. 1).

3.2. LRP1 mRNA expression is upregulated in mouse VSMCs with UTP stimulation

We next determined whether LRP1 was transcriptionally regulated in response to UTP stimulation of mouse VSMCs. For this reason, quantitative RT-PCR was carried out to assess LRP1 mRNA relative abundance in VSMCs. As shown in Fig. 2, UTP caused a two-fold increase in LRP1 mRNA expression, as compared to untreated mouse VSMCs expressing the wild type $P2Y_2R$. Addition of UTP to VSMCs from *P2Y2R*−/− mice showed no effect and was much lower than in WT cells with or without UTP ($p < 0.05$) (Fig. 2).

3.3. UTP stimulates uptake of aggregated LDL in mouse VSMCs

We investigated the uptake of agLDL in the presence and absence of SMC-derived matrix, prepared as mentioned above. Studies of PMA-activated macrophages seeded onto

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macrophage-derived matrix show significant increases in LDL binding and uptake [24,27], and our previous studies have established a role for the $P2Y_2R$ in actin stress fiber formation [16]. Since uptake of matrix-bound agLDL is associated with cytoskeleton reorganization, we hypothesized that stimulation of $P2Y_2R$ will increase agLDL uptake in VSMCs. Fluorescence microscopy studies indicate that UTP significantly increased the accumulation of DiL-labeled agLDL in cultured mouse VSMCs (Fig. 3).

3.4. $P2Y_2R$/FLN A interaction is required for UTP-mediated uptake of matrix-bound agLDL

We next hypothesized that $P2Y_2R$ interaction with filamin A was required for the uptake of DiL-labeled agLDL. For this purpose, VSMCs were isolated from *P2Y2R*−/− mice transduced with adenoviruses encoding either the full length $P2Y_2R$ or a mutant $P2Y_2R$ defective in FLN-A binding [16] [20]. As shown in Fig. 4, UTP significantly increases matrix-bound agLDL uptake in VSMCs expressing the full length WT $P2Y_2R$. In contrast, UTP failed to stimulate LDL uptake in VSMCs from mice expressing a mutant $P2Y_2R$ that does not bind FLN-A (Fig. 4).

3.5. UTP-stimulated LDLR mRNA expression in VSMCs is dependent upon $P2Y_2R$ interaction with FLN-A

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 $P2Y_2R$/FLN-A interaction. We hypothesized that the $P2Y_2R$ mediates the mRNA expression of the LDLR through interaction with FLN-A. To evaluate regulation of LDLR mRNA expression by the $P2Y_2R$, we transiently transfected *P2Y2R*−/− VSMCs with cDNA encoding a hemagglutinin-tagged WT $P2Y_2R$ or a mutant $P2Y_2R$ that does not bind FLN-A. VSMCs expressing the mutant $P2Y_2R$ exhibit 3-fold lower LDLR mRNA expression than VSMCs expressing the WT $P2Y_2R$ (Fig. 5). This shows that a full-length $P2Y_2R$ capable of FLN-A interaction is necessary for LDLR expression in mouse VSMCs.

3.6. UTP-stimulated $P2Y_1R$ and $P2Y_4R$ mRNA expression in VSMCs

The purpose of this study was to determine the efficiency of the $P2Y_2R$ knockout procedure, and whether other P2Y subtypes that respond to UTP are present in the VSMCs from the *P2Y2R*−/− mice. The results revealed UTP did not increase the abundance of $P2Y_1R$ mRNA in either WT or *P2Y2R*−/− VSMCs (Fig. 6). Although not significant, $P2Y_2R$ mRNA relative abundance showed a steady increase with UTP stimulation over time in both WT and *P2Y2R*−/− cells (Fig. 7), suggesting that the $P2Y_2R$ may not have been completely deleted in the *P2Y2R*−/− mice. The results also showed that UTP stimulation resulted in a significant increase ($p < 0.05$) in $P2Y_4R$ mRNA expression after 2 h in VSMCs from *P2Y2R* −/− mice *versus* WT cells, but mRNA expression declined within 4 h (Fig. 8).

4. Discussion

4.1. The $P2Y_2$ receptor regulates a protective mechanism during lipid accumulation

A substantial amount of immobilized lipoproteins are present in atherosclerotic lesions [28]. According to the response-to-retention hypothesis, lipoprotein retention in the arterial wall is

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an initiating event in atherogenesis that triggers an inflammatory response leading to lesion development [29]. Many studies have focused on roles played by activated endothelium and immune cells, such as macrophages and neutrophils, in the development of atherosclerosis. VSMCs and components within the cytoskeleton undoubtedly play a key role in disease progression. However, modifications of lipoproteins retained in the extracellular matrix of VSMCs necessitate a specific mechanism for aggregated lipoprotein uptake. LRP1 is a lipoprotein receptor expressed in arterial SMCs and other cells that is known to regulate the catabolism of lipoproteins [30], and modulates the activities of platelet-derived growth factor (PDGF) receptor-β and integrins [31,32]. The effects of integrins complexing with the $P2Y_2R$ was also investigated in a separate study, and it was demonstrated that the $P2Y_2R$, via a arginine-glycine-aspartic acid (RGD) binding domain in its first extracellular loop, is able to bind to specific integrins, which enables ATP and UTP to activate integrin signaling pathways [32]. $P2Y_2Rs$ for extracellular ATP and UTP have been suggested to play a significant role in inflammation, responses to injury and the development of atherosclerosis [16,33,34]. UTP, a relatively selective $P2Y_2R$ agonist, is shown here to increase LRP1 expression in murine VSMCs at both the mRNA and protein levels (Figs. 1 and 2). Furthermore, the activation of $P2Y_2Rs$ in VSMCs increases the uptake of agLDL (Figs. 3 and 4). We suggest that $P2Y_2R$ activation mediates LRP1 signaling as a protective mechanism in VSMCs to increase the uptake and degradation of cholesterol trapped within the extracellular matrix. LRP1 has been suggested to prevent the formation of atherosclerotic lesions via interaction with the PDGF-β and the transforming growth factor-β (TGFβ) signaling pathways, both of which have major roles in atherosclerosis development [30,35,36]. Deletion of LRP1 is associated with uncontrolled aortic SMC hyperplasia and increased PDGFRβ expression and phosphorylation, increased Smad 2 phosphorylation and alterations in TGFβ signaling [30]. However, apo-E binding to LRP1 inhibits PDGF-induced SMC migration within the arterial wall [37], suggesting that LRP1 protects the vasculature by suppressing PDGFRβ activation [31].

4.2. $P2Y_2R$ association with FLN-A is required for UTP-induced uptake of agLDL

The $P2Y_2R$ is associated with the actin cytoskeleton through a C-terminal interaction with FLN-A, and UTP-induced FLN-A phosphorylation is associated with actin polymerization [11]. UTP also induced FLN-A phosphorylation in human 1321N1 astrocytoma cells expressing the full length $P2Y_2R$, but not the mutant $P2Y_2R$ defective in FLN-A binding [13]. Thus, we propose that the $P2Y_2R$/ FLN-A interaction is required for UTP-induced agLDL uptake mediated by LRP1. As shown in Fig. 4, UTP significantly increased agLDL uptake in vascular smooth muscle cells expressing the full length WT $P2Y_2R$. In contrast, UTP failed to stimulate LDL uptake in VSMCs from mice expressing the mutant $P2Y_2R$ that does not bind FLN-A (Fig. 4). Phosphorylation of FLN-A at Ser/Thr regulates the association between FLN-A and the actin cytoskeleton for the stabilization of caveolae at the plasma membrane [38]. We postulate that FLN-A phosphorylation may be an important factor in UTP-induced LRP1 activation and cytoskeleton dynamics that promotes the uptake of agLDL in VSMCs. Dedieu et al. [39] observed a regulatory role for LRP1 in the architecture and dynamics of the cytoskeleton. In addition, adaptor proteins bind to the cytoplasmic domain of LRP1 to produce a variety of cellular responses. For example, Talin-like protein interacts with LRP1 to promote coupling to the actin cytoskeleton [40]. Some

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studies report that LRP1 is phosphorylated on both serine and tyrosine residues [41,42]. Li et al. [41] showed that LRP1 phosphorylation both *in vitro* and *in vivo* is mediated by cyclic AMP- (cAMP)-dependent protein kinase A (PKA), and used site-directed mutagenesis to identify serine 76 as the site of phosphorylation in the LRP1 cytoplasmic tail. Phosphorylation of tyrosine residues in LRP1 plays a role in association with Shc [42,43], a docking protein whose phosphorylation facilitates protein-protein interactions [42]. Tyrosine phosphorylated LRP1 specifically associates with the Src-homology 2 (SH2) binding domain on Shc [42]. SH2 binding domains mediate interactions between various intracellular signaling proteins, allowing these proteins to locate binding partners to regulate specific signaling pathways [44]. The cytoplasmic tail of the $P2Y_2R$ has two consensus proline rich (PXXP) SH3 binding domains that upon activation of the $P2Y_2R$ bind Src to promote the transactivation/ phosphorylation of growth factor receptors [45,46]. Both SH2 and SH3 may be involved in modulating interactions with the cytoskeleton, and receptor cross-talk between $P2Y_2R$ and LRP1 [42].

LRP1 signaling activity requires the direct interaction with the G stimulatory protein α-subunit ($G_{s\alpha}$) of a heterotrimeric G_s protein [47]. Thus, it is possible that cross-talk between LPR1-coupled $G_{s\alpha}$-dependent activation of PKA and $P2Y_2R$-coupled $G_{q\alpha}$-dependent activation of phospholipase C and protein kinase C [47] coordinately regulates agLDL uptake.

4.3. $P2Y_2R$ signaling plays a regulatory role in the uptake of agLDL

In conclusion, we have shown for the first time that $P2Y_2R$ activation increases LRP1 expression and the uptake of aggregated LDL by murine primary vascular smooth muscle cells. We suggest the existence of a regulatory element within the *LRP1* promoter that is responsive to activation of the $P2Y_2R$ signaling pathway (Fig. 9). We also propose that UTP-induced uptake of agLDL occurs via activation of the LRP1 signaling pathway and requires the presence of an FLN-A binding domain in the intracellular C-terminal domain of the $P2Y_2R$ and possibly FLN-A phosphorylation (Fig. 9). Overall, our novel findings show that increases in *LRP1* expression in VSMCs is UTP dose-dependent. Our findings showed that LRP1 expression was transcriptionally upregulated by UTP-induced $P2Y_2R$ activation, since a two-fold increase in LRP1 mRNA expression in response to UTP was observed in mouse VSMCs expressing the wild type $P2Y_2R$, as compared to untreated controls, whereas 10-fold lower *LRP1* mRNA expression was seen in UTP-treated VSMCs from *P2Y2R*−/− mice (Fig. 2). Information from this study could prove very useful by providing insights into future pharmacological targets in the treatment of hyperlipidemia and atherosclerosis.

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Abbreviations

LRP1	low-density lipoprotein receptor related protein
agLDL	aggregated low-density lipoproteins
P2Y2R	P2Y2 receptor

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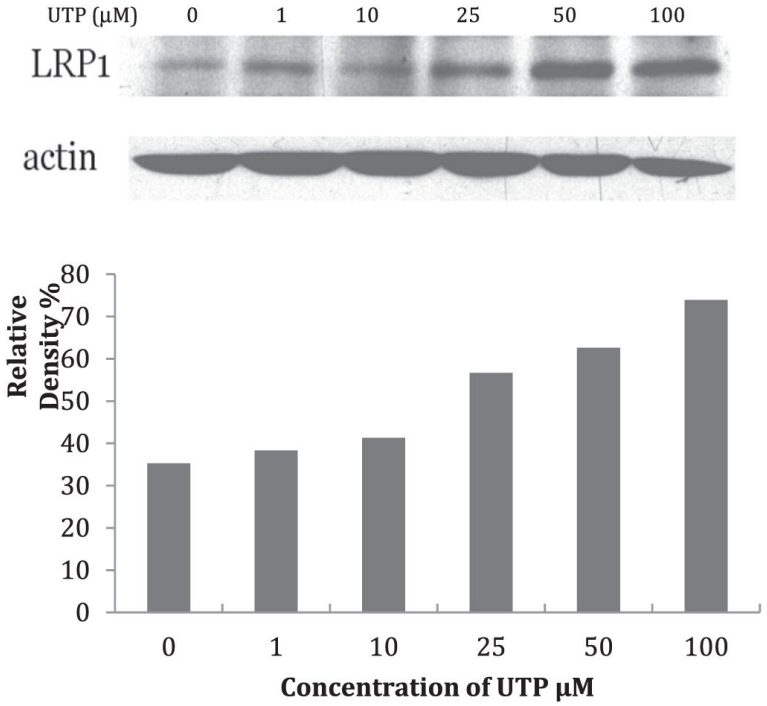

Fig. 1. Protein abundance of LRP1 expression in mouse VSMCs

Serum-starved VSMCs were incubated in the absence or presence of UTP (1, 10, 25, 50, 100 μM). (Upper panel) Detection of LRP1 was performed using mouse anti-LRP1 monoclonal antibody (1:3000 dilution; Abcam, Cambridge, MA) followed by horseradish peroxidase-conjugated donkey anti-mouse polyclonal antibody (1:10,000 dilution Abcam, Cambridge, MA). For signal normalization, membranes were probed with polyclonal anti-β-actin antibody (1:2000 dilution). (Lower panel) The results show that LRP1 expression is increased in a UTP dose-dependent manner.

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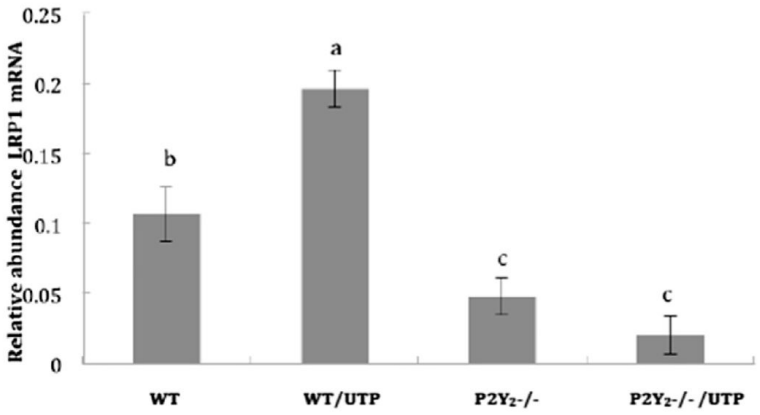

Fig. 2. *LRP1* mRNA expression in VSMCs stimulated with UTP

VSMCs isolated from WT and *P2Y2R*−/− 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 generate cDNA. The mRNA relative abundance was determined by RT-PCR using the ΔΔCt method. Data are shown as means ± SEM of results from 6 independent experiments where $p < 0.05$ represents a significant difference, as compared to untreated VSMCs from WT mice. GAPDH was used as a control gene to normalize the data.

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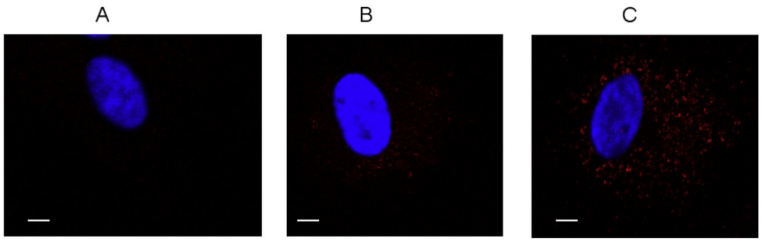

Fig. 3. UTP stimulates uptake of agLDL in mouse VSMCs

Confocal microscopy of mouse VSMCs incubated with 50 μg/ml agLDL for 2 h at 37 ° C. Lipids were stained red by incubating cells with 1,1′ dioctadecyl-3,3,3′ ,3’-tetramethyl-indocarbocyanine (Dil). VSMCs were incubated with (A) no LDL (B) LDL or (C) UTP (10 μM) þ LDL. Cells were washed and incubated with 5 μg/ml DiI for 30 min in the dark. The red granules indicate lipid uptake. The nuclei were stained by incubating cells with 1 μg/ml Hoechst dye (blue). The images are representative of results from at least 3 independent experiments. Cells were visualized using a Zeiss 510 laser scanning confocal microscope. Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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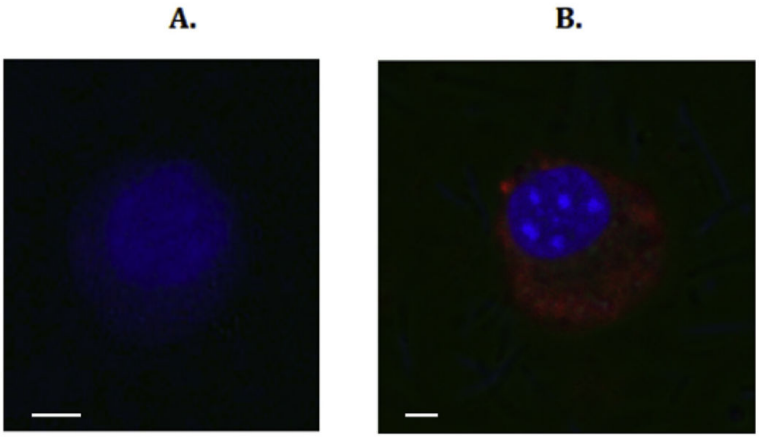

Fig. 4. UTP stimulates matrix-bound agLDL uptake in VSMCs expressing WT $P2Y_2R$ but not in VSMCs expressing a mutant $P2Y_2R$ that does not bind FLN-A

VSMCs from *P2Y2R*−/− mice were transfected with (A) a mutant $P2Y_2R$ that does not bind FLN-A or (B) WT $P2Y_2R$. Cells were grown on top of matrix, incubated with 50 μg/ml agLDL for 4 h at 37 ° C and then stimulated with 10 μM UTP. Lipids were stained by incubating cells with 1,1′ dioctadecyl-3,3,3′ ,3’-tetramethyl-indocarbocyanine (DiI; red) and cells were visualized using a Zeiss LSM 700 confocal microscope. The nuclei were stained by incubating cells with 1 μg/ml Hoeschst dye (blue). The images are representative of results from at least 3 independent experiments. Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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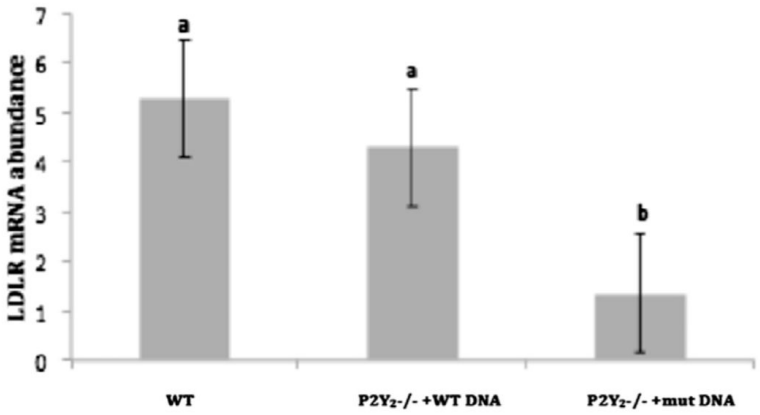

Fig. 5. LDL mRNA expression in VSMCs stimulated with UTP. VSMCs from *P2Y2R*−/− mice were transiently transfected with cDNA encoding a hemagglutinin-tagged WT $P2Y_2R$ or a mutant $P2Y_2R$ that does not bind FLN-A

Untransfected VSMCs showed the highest *LDLR* mRNA relative abundance compared to both transfected groups, although *LDLR* mRNA abundance was not significantly different between untransfected and WT $P2Y_2R^{-/-}$transfected VSMCs. Additionally, VSMCs expressing the mutant $P2Y_2R$ exhibited significantly lower LDLR mRNA relative abundance than VSMCs transfected with the WT $P2Y_2R$. Same letters represent no significant difference between the groups. Different letters represent significant differences between groups ($p < 0.05$). *GAPDH* was used as a control gene.

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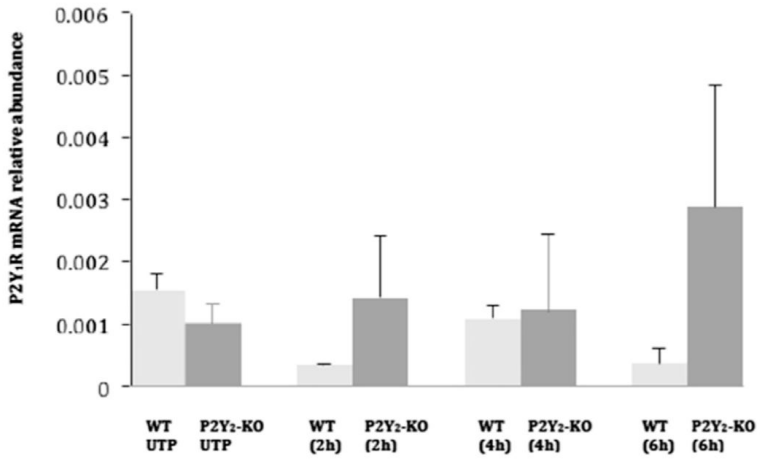

Fig. 6. *P2Y$_1$R* mRNA expression in VSMCs stimulated with UTP

VSMCs isolated from WT or *P2Y2R*−/− mice were cultured in 12-well plates and grown in DMEM/10% FBS 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$_1$R* forward and reverse primers and the ΔΔCt method to calculate fold increase in mRNA levels for results from 6 independent experiments. *P2Y$_1$R* expression is calculated relative to *GAPDH*. There were no significant differences in the levels of *P2Y$_1$R* mRNA detected over time.

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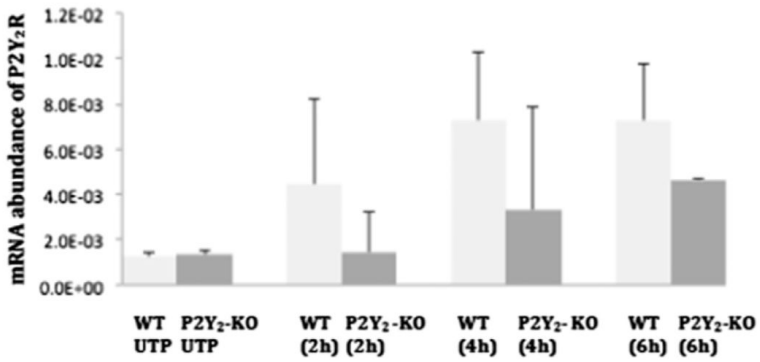

Fig. 7. P2Y$_2$R mRNA expression in VSMCs stimulated with UTP

VSMCs isolated from WT or *P2Y2R*−/− mice were cultured in 12-well plates and grown in DMEM/10% FBS 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$_2$R* forward and reverse primers and the ΔΔCt method to calculate fold increase in mRNA levels for results from 6 independent experiments. *P2Y$_2$R* expression is calculated relative to *GAPDH*. There were no significant differences in the levels of *P2Y$_2$R* mRNA detected over time.

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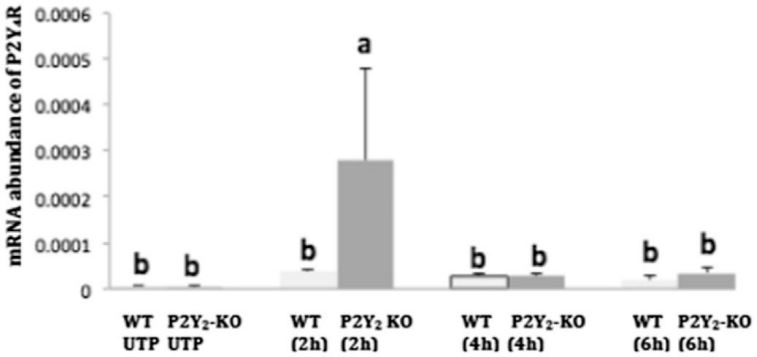

Fig. 8. *P2Y$_4$R* mRNA expression in VSMCs stimulated with UTP

VSMCs isolated from WT or *P2Y2R*−/− mice were cultured in 12-well plates and grown in DMEM/10% FBS 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$_4$R* forward and reverse primers and the ΔΔCt method to calculate fold increase in mRNA levels for results from 6 independent experiments. *P2Y$_4$R* expression is calculated relative to *GAPDH*. Different letters represent significant differences between groups ($p < 0.05$).

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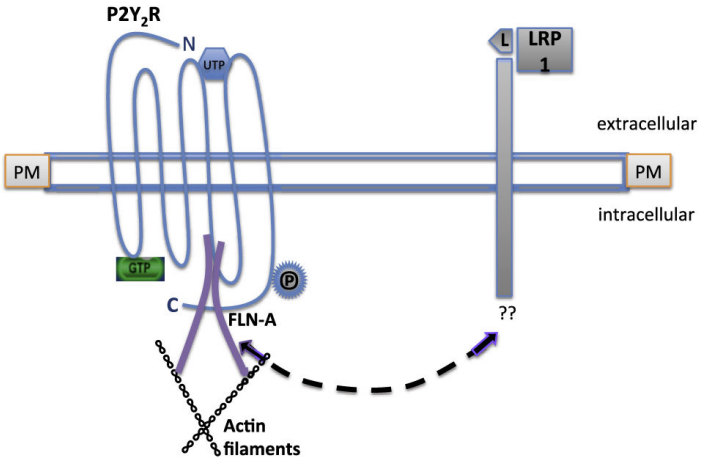

PM = plasma membrane
P = phosphate
L = ligand
UTP = uridine 5'-triphosphate
GTP = guanosine 5'-triphosphate

Fig. 9. Predicted pathway for $P2Y_2R$/LRP1 interaction in the regulation of agLDL uptake

Diagram represents a proposed mechanism for $P2Y_2R$-mediated uptake of agLDL in VSMCs and possible cross-talk between LRP1 and the $P2Y_2R$. The LRP1 extracellular binding domain indicates binding of apo-E ligand (L). *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 *LRP1* promoter that is responsive to $P2Y_2R$ activation. We propose that LRP1 associates with the $P2Y_2R$ (dashed line) possibly via a phosphorylated residue in the C-terminus of the $P2Y_2R$ or through binding of LRP1 to $P2Y_2R$-associated FLN-A and/or other cytoskeletal proteins. GTP indicates the activated state of the G protein-coupled $P2Y_2R$.