

P2Y₂ Receptor-mediated Lymphotoxin- α Secretion Regulates Intercellular Cell Adhesion Molecule-1 Expression in Vascular Smooth Muscle Cells*

Received for publication, October 13, 2011, and in revised form, January 22, 2012. Published, JBC Papers in Press, February 1, 2012, DOI 10.1074/jbc.M111.313189

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Background: Lymphotoxin- α (LTA) is involved in the pathogenesis of atherosclerosis.

Results: The P2Y₂ nucleotide receptor (P2Y₂R) recruits filamin A to activate Rho signaling upstream of LTA secretion leading to intercellular cell adhesion molecule-1 expression.

Conclusion: P2Y₂R regulates LTA gene activation and secretion in vascular smooth muscle cells.

Significance: This suggests that P2Y₂R is an important regulator of vascular inflammation.

The proinflammatory cytokine lymphotoxin- α (LTA) is thought to contribute to the pathogenesis of atherosclerosis. However, the mechanisms that regulate its expression in vascular smooth muscle cells (VSMC) are poorly understood. The ability of exogenous nucleotides to stimulate LTA production was evaluated in VSMC by ELISA. The P2Y₂ nucleotide receptor (P2Y₂R) agonist UTP stimulates a strong and sustained release of LTA from WT but not P2Y₂R^{-/-} SMC. Assessment of LTA gene transcription by LTA promoter-luciferase construct indicated that LTA levels are controlled at the level of transcription. We show using RNAi techniques that knockdown of the actin-binding protein filamin-A (FLNa) severely impaired nucleotide-induced Rho activation and consequent Rho-mediated LTA secretion. Reintroduction of FLNa in FLNa RNAi SMC rescued UTP-induced LTA expression. In addition, we found that UTP-stimulated LTA secretion is not sensitive to brefeldin A, which blocks the formation of vesicles involved in protein transport from the endoplasmic reticulum to the Golgi apparatus, suggesting that P2Y₂R/filamin-mediated secretion of LTA is independent of the endoplasmic reticulum/Golgi secretory vesicle route. Furthermore, UTP selectively induces ICAM-1 expression in WT but not SMC expressing a truncated P2Y₂R deficient in LTA secretion. These data suggest that P2Y₂R recruits FLNa to provide a cytoskeletal scaffold necessary for Rho signaling pathway upstream of LTA release and subsequent stimulation of ICAM-1 expression on vascular smooth muscle cells.

The ability of nucleotides to modulate the release of cytokines has generated increasing interest in regards to the development of novel anti-inflammatory therapeutics targeted to specific nucleotide receptors (1–3). Release of nucleotides has been proposed to occur by exocytosis of ATP/UTP-containing

vesicles, facilitated diffusion by putative ABC transporters, cytoplasmic leakage, and electrodiffusional movements through ATP/nucleotide channels (4). Under pathological conditions, it is clear that extracellular nucleotides are released from injured or stressed cells and tissues in response to oxidative stress, ischemia, hypoxia, or mechanical stretch (5–10), whereupon they activate a variety of cellular responses in addition to cell adhesion and inflammation, including neurotransmission, apoptosis, and cell growth (11–15). Two families of plasma membrane receptors mediate the actions of extracellular nucleotides, the metabotropic G protein-coupled P2Y purinergic receptors, and the ionotropic P2X purinergic receptor ion channels (2, 17). Stimulation of P2 receptors is coupled to the release of the proinflammatory cytokines IL-1 β , IL-1 α , IL-6, IL-8, and TNF- α that are of obvious relevance to inflammation in atherosclerosis (18–23). Lymphotoxin- α (LTA)² is another member of the TNF ligand family that is synthesized predominantly by activated T- and B-lymphocytes (24, 25). The signaling network in which lymphotoxins act is complex, comprising unique and shared ligand receptor systems. TNF- α and LTA elicit responses through two receptors termed p55 (TNFR1) and p75 (TNFR2), of which p55 activates the majority of biological responses (25). Studies in mice have indicated that loss of LA but not TNF- α reduced the size of lesions, suggesting a preeminent role for LTA in atherosclerosis (26). A case-control association study has shown that a single nucleotide polymorphism in the galectin-2 (Gal-2) gene is associated with susceptibility to myocardial infarction (27). This genetic substitution affects the transcriptional levels of Gal-2 *in vitro*, potentially leading to altered secretion of LTA in VSMC. Polymorphisms in the LTA gene have been reported to cause a 2-fold increase in induction of cell adhesion molecules in VSMC of human coronary arteries (28). LTA has also been shown to modulate adhe-

* This work was supported by a grant from the Showalter Foundation (to C. I. S.).

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² The abbreviations used are: LTA, lymphotoxin- α ; P2Y₂R, P2Y₂ nucleotide receptor; ICAM-1, intercellular cell adhesion molecule-1; FLNa, filamin-A; VSMC, vascular smooth muscle cell(s); ER, endoplasmic reticulum; Gal-2, galectin-2.

sion molecule expression in endothelial cells, including ICAM-1 (29, 30).

The P2Y₂ nucleotide receptor (P2Y₂R) is a G_{q11}-coupled protein that is activated equipotently by extracellular ATP or UTP, leading to G_{αq}-dependent activation of phospholipase C, which increases [Ca²⁺]_i via calcium release from intracellular stores (31, 32). The P2Y₂R is up-regulated in a variety of tissues in response to injury or stress, including the vasculature (33). We have shown that stress-activated up-regulation of P2Y₂Rs in vascular endothelium *in vivo* increases the expression of adhesion molecules, monocyte adherence, and transendothelial migration (34, 35), suggesting that P2Y₂Rs for cytokine-like nucleotides play a role in endothelial-dependent inflammation.

In the current study, we examined the physiological consequences of P2Y₂R activation in the regulation of LTA secretion in VSMC. Our data show that activation of P2Y₂R selectively triggers LTA gene activation and secretion in VSMC. P2Y₂R interaction with the actin-binding protein FLNa results in Rho activation and consequent Rho-dependent secretion of LTA that occurs in a nonclassical Golgi-independent pathway. In addition, we found that secreted LTA positively regulates ICAM-1 expression in SMC. We propose that C-terminal P2Y₂R recruits FLNa to provide a cytoskeleton scaffold necessary for Rho signaling pathway upstream of LTA release and subsequent stimulation of adhesion molecule expression.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal rat anti-LTA (clone 168717) was purchased from R & D Systems. Rabbit anti-mouse ICAM-1 antibody was purchased from Zymed Laboratories Inc.. Secondary antibodies conjugated to Alexa Fluor used for immunostaining experiments were purchased from Invitrogen. EGFP polyclonal antibody was from Thermo Scientific. Transfection grade eukaryotic expression vector pUSEamp (+) containing human dominant negative RhoA, Rac1, or Cdc42 were purchased from Millipore. Galectin-2 antibody (polyclonal sheep IgG) was obtained from R & D Systems. All other reagents were purchased from Sigma unless otherwise specified.

Cell Culture—Mouse SMC were prepared from aortas of 4-month-old WT and P2Y₂R^{-/-} C57BL/6J mice strains (Jackson laboratory) and cultured as previously described (36). SMC were used at the second passage. Quiescent SMC were obtained by incubation in serum-free DMEM for 48 h.

ELISA—The concentrations of LTA in cultured supernatant were determined with ELISA using a kit (TSZ ELISA; catalogue number M7779). The detection limit of the assays was 3 ng/ml.

Activity Assay for Small GTPase Rho-A—Rho activation assay kit (Millipore) was used to assess Rho activity in mouse SMC according to the instructions of the manufacturer.

FLNa siRNA Constructs—A 21-bp siRNA target sequence for FLNa was chosen by the siRNA Target Finder program (Ambion). A pair of complimentary oligonucleotides for each target sequence was synthesized by custom primers (Invitrogen). The sense and antisense sequences are separated by a 9-bp loop region (ttcaagaga), and each oligonucleotide terminates with restriction endonuclease half-sites (BamHI and HindIII) for further cloning into the expression vector. Complimentary

oligonucleotides were annealed and cloned into pSilencer 2.1-U6 hygro (Ambion). The FLNa siRNA target sequence was aagattgtgagtcctcaggt, and the hairpin sequences generated were as follows: antisense 5'-gatccgattgtgagtcctcaggttcaaggaaccaggaggactcacaatcttttggaaa-3' and sense 5'-agcttttccaaa-aagagattgtgagtcctcaggttcttcttggaaaacctgaggaggactcacaatcg-3'. To generate the rescue construct, six nucleotides of siRNA target sequence in FLNa were mutated by PCR. The resulting FLNa cDNA was subcloned into the retroviral EGFP fusion vector, pLEGFP-C1 (BD Science) for retroviral infection. The rescue construct encodes the same amino acid as endogenous FLNa but contains a different nucleotide sequence in the FLNa siRNA target region. The endogenous FLNa sequence was 5'-aagattgtgagtcctcaggtT-3', and the rescue FLNa sequence was 5'-aagatcgtaagcccatccggt-3'. Mutated nucleotides are shown in bold type.

Cell Transfection and Viral Infection—FLNa protein knockdown was performed using the siRNA construct pSilencer 2.1-U6 hygro-FLNa. Mouse aortic SMC (1 × 10⁶) were transfected with 0.8 μg of FLNa siRNA construct using the Nucleofector electroporator and a basic Nucleofector kit (primary smooth muscle cells) (Amaya Inc, Gaithersburg, MD) according to the instructions of the manufacturer. After selection with 200 μg/ml of hygromycin B (Invitrogen) for 2 weeks, clones were isolated to generate stable cell lines. For retroviral infection, the EGFP-FLNa cDNA subcloned into retroviral EGFP fusion vector, pLEGFP-C1 (BD Science) was transfected in amphotropic packaging cell line, Phoenix A cells, as described above. After 48 h, virus-containing supernatants were collected and filtered through a 0.45-μm membrane to remove cell debris. SMC were infected by virus with 5 μg/ml polybrene. At 48 h after infection, virus was removed, and the medium was replaced by selection medium with 400 μg/ml of G418 (Invitrogen). Dominant negative RhoA, Rac1, or Cdc42 were transfected into SMC using Nucleofector electroporator as described above. Galectin-2 protein knockdown was performed using shRNA lentiviral particles according to the manufacturer's instructions (Santa Cruz Biotechnology).

Reporter Assays—Mouse SMC were co-transfected with pGL₂ basic vectors (Promega) containing mouse LTA promoter sequence obtained from amplification of mouse genomic DNA (0.6 μg) and by pHook-LacZ (Invitrogen) (0.6 μg). The cells were then cultured in serum-free DMEM for 48 h and stimulated with graded concentrations of UTP for 6 h. A luciferase assay system (Promega) was used to measure luciferase activities. For transfection efficiency, luciferase activity was normalized using β-galactosidase activities determined by a spectrophotometric method using ortho-nitrophenyl-β-D-galactopyranoside as substrate.

Real Time PCR—Total RNA was extracted from SMC lysed in TRIzol reagent (Invitrogen), using an RNeasy mini kit (Qiagen). High quality RNA extracted (5 μg/sample) was used for synthesis of single-strand cDNA with Superscript II (Invitrogen). Real time PCR was conducted with 45 ng of cDNA using an ABI Prism 7900HT PCR machine (Applied Biosystems). PCR primers for LTA were as follows: sense 5'-cccatggcatcctgaac-3' and antisense 5'-ggaggcctggaatcaat-3'. Primers for galectin-2 were as follows: forward 5'-aacaatgaaccaggat-

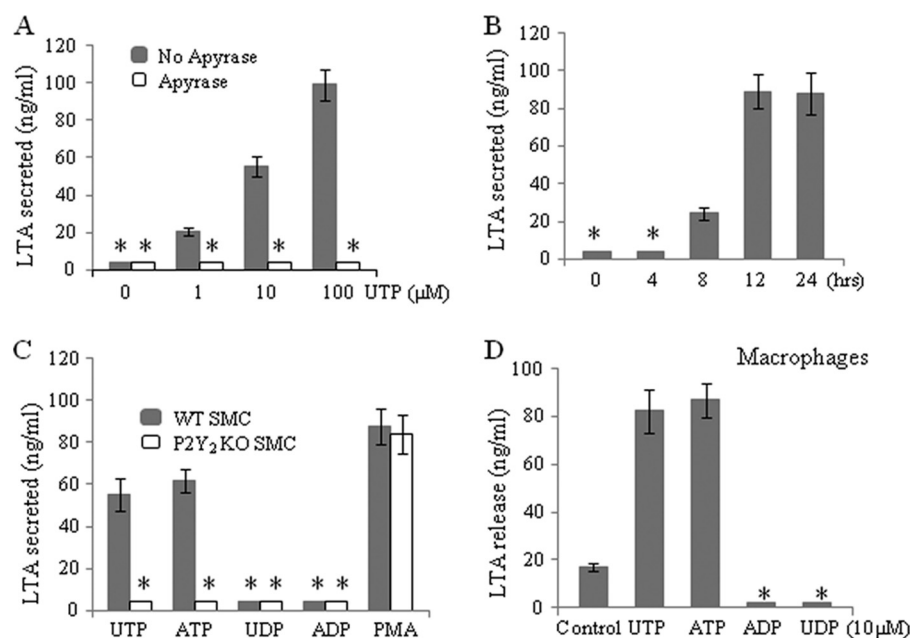


FIGURE 1. P2Y₂R activation mediates LTA secretion. A, SMC from WT mice were treated with graded concentrations of UTP in the presence or absence of the enzyme potato apyrase grade III (2 units/ml) for 12 h, and the amount of LTA secreted into the extracellular medium was measured by ELISA. B, SMC were stimulated with an effective dose of UTP (10 μM), and LTA secretion was measured for the indicated periods of time. C, WT and P2Y₂R^{-/-} SMC were stimulated with ATP, UTP, ADP, UDP (10 μM), or phorbol myristate acetate (PMA, 10 ng/ml) for 12 h, and the secreted LTA was analyzed by ELISA. D, LTA secretion was analyzed in mouse peritoneal macrophages treated with nucleotides as described above. The data represent the means ± S.E. of results from three independent experiments. *, under detection limit (3 ng/ml).

gtcc-3' and reverse 5'-cgagggttaaatgcaggttgag-3'. The reaction mixes included 10× PCR buffer, 20 mM magnesium chloride, 0.2 mM deoxy-NTP, and 10 nmol of TaqMan probe with a pair of 10-nmol LTA primers, 10-nmol GAPDH primers, and probe of each molecule. Real time PCR was performed in a Rotor Gene 3000 Detector (PerkinElmer Life Sciences). The thermal cycling program consisted of 3 min at 94 °C, followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle, C_t). Each reaction was performed in quadruplicate, and the average C_t value was used in all analyses. To account for variability in total RNA input, the expressions of the transcriptions were normalized to GAPDH in the samples.

Immunofluorescence Staining—Mouse SMC were grown to 80% confluence on coverslips in 35-mm dishes. The cells were made quiescent by incubation in serum-free DMEM for 48 h at 37 °C before exposure to UTP. After treatment with UTP (10 μM), the cells were fixed in acetone for 15 min and treated with 0.2% (v/v) Triton X-100 in PBS at room temperature. After washing in PBS, ICAM-1 expression was detected in cells by incubation for 2 h at room temperature with a rabbit polyclonal anti-ICAM-1 antibody (1:200 dilution in PBS containing 5% (v/v) normal goat serum), followed by Alexa Fluor-labeled goat anti-rabbit IgG antibody (1:200 dilution in PBS). Then the cells were incubated in PBS containing 2 μM Hoechst 33258 for nuclear counterstaining.

Statistical Analysis—Paired Student's *t* test was used for statistical analysis. The results are expressed as the means ± S.E., and significance was inferred at $p < 0.05$.

RESULTS

P2Y₂R Agonists Stimulate LTA Secretion from Vascular SMC—The ability of exogenous nucleotides to stimulate LTA release was evaluated in WT and P2Y₂R^{-/-} SMC by ELISA. When added directly into WT SMC cultures, UTP dose-dependently increased LTA secretion in the supernatant (Fig. 1A). LTA release was detected as early as 8 h following UTP stimulation, reached a peak at 12 h, and remained elevated at 24 h (Fig. 1B). Incubation of SMC with the enzyme potato apyrase grade III (2 units/ml) that degrades nucleoside di- and triphosphates inhibited UTP-mediated LTA secretion (Fig. 1A). Similar effects on LTA secretion were observed when cells were stimulated with ATP (data not shown), suggesting the involvement of P2Y₂Rs at which UTP and ATP are equipotent. Consistent with these data, UTP failed to stimulate LTA secretion from P2Y₂R^{-/-} SMC (Fig. 1B). The addition of 10 ng/ml phorbol myristate acetate, a known inducer of LTA in human T cell (37), similarly increased LTA release from WT and P2Y₂R^{-/-} SMC, indicating that P2Y₂R activation selectively mediates LTA secretion. The P2Y₂R is coupled to downstream signaling pathways via G_{q/11} proteins (31). In addition to the presence of P2Y₂Rs, VSMC also express the G_{q/11}-coupled P2Y₁ and P2Y₆Rs (38, 39), which are activated by ADP and UDP, respectively. We next investigated whether activation of these G_{q/11}-coupled receptors triggers LTA gene activation expression and secretion in VSMC. The addition of either ADP or UDP failed to activate LTA secretion in SMC from WT or P2Y₂R-KO SMC (Fig. 1C), suggesting that other specific properties of P2Y₂R are required for LTA secretion.

We next determined whether P2Y₂R triggers similar induction of LTA gene expression/secretion in a non-VSMC cell lin-

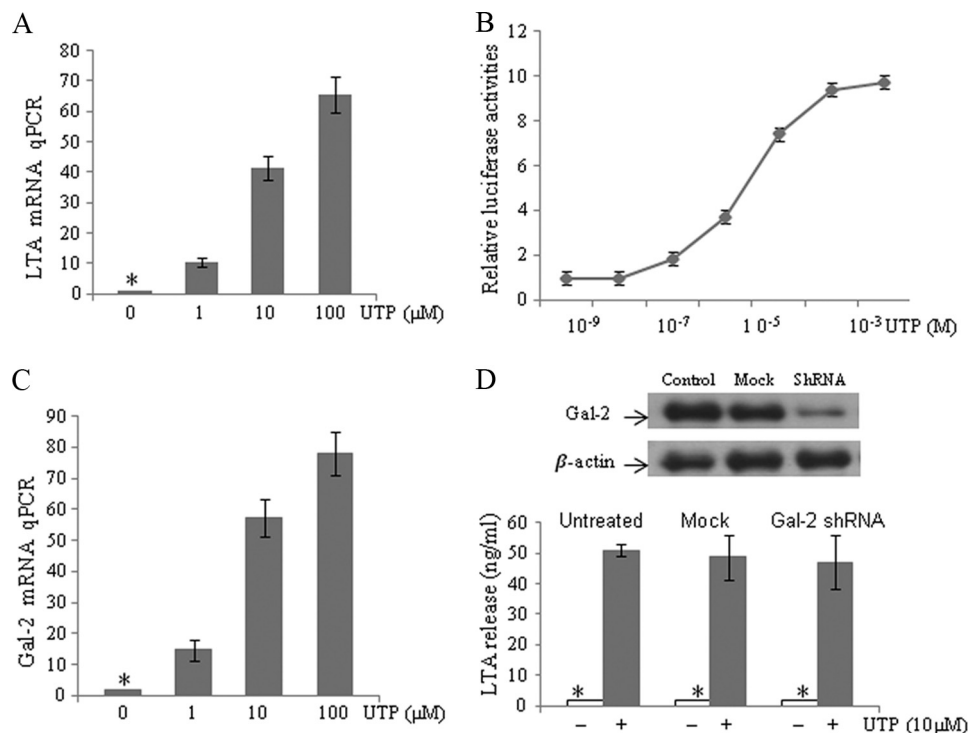


FIGURE 2. P2Y₂R regulates transcriptional activation of LTA and galectin-2 genes, but LTA release is not controlled by galectin-2. *A*, SMC were treated with UTP (0–100 μM) for 4 h, and LTA transcripts were measured by real time PCR. The data (means ± S.E.) are presented as the ratio of LTA to GAPDH mRNA, and the experiments were tripled under the same conditions. *B*, WT SMC were co-transfected with the pGL2 basic vector containing the mouse LTA promoter and pRLTK. After 24 h of incubation in serum-free medium, SMC were stimulated for 6 h with UTP (0.1–1000 μM). Luciferase activities measured in quiescent cells are given the value 1. Each condition was performed in triplicate for $n = 3$ experiments. *C*, real time PCR quantification of Gal-2 mRNA in SMC treated with UTP as described for *A*. The data (means ± S.E.) were presented as the ratio of LTA to GAPDH mRNA, and the experiments were tripled under the same condition. *D*, SMC expressing the full-length P2Y₂R were transfected either with shRNA lentiviral particles targeting galectin-2 or control lentiviral particles (mock). The cells were incubated with UTP (μM), and then cell lysates were prepared and immunoblotted with anti-galectin-2 antibody. LTA secretion into medium prior to lysate preparation was measured by ELISA. The data represent the means ± S.E. of results from three independent experiments.

age using mouse peritoneal macrophages. UTP (10 μM) stimulation of these cells triggered a significant release of LTA in a time-dependent manner. As expected, LTA secretion was prevented by knockdown of P2Y₂R in macrophages (Fig. 1D). These data rule out the possibility that LTA secretion requires the intersection of proximal P2Y₂R signals with distal signaling pathways/proteins predominantly expressed in the VSMC lineage.

P2Y₂R Activation Coordinates Up-regulation of Galectin-2 and LTA Gene Expression, but Galectin-2 Is Not Required for LTA Secretion—To study the mechanism leading to increase secretion of LTA by SMC, we analyzed the effects of UTP on LTA mRNA expression using real time PCR. As shown in Fig. 2A, UTP dose-dependently increased LTA mRNA content. We next studied the effect of UTP on the transcriptional activity of the LTA gene using a reporter gene strategy. UTP stimulation of WT SMC expressing a construct containing the luciferase reporter gene downstream of the mouse LTA promoter increased luciferase activity in a dose-dependent manner (Fig. 2B). These data indicate that P2Y₂R activation regulates synthesis and secretion of LTA by SMC. Gal-2 is presumed to play a regulatory role in the intracellular trafficking of the proinflammatory cytokine LTA. A case control association study showed that a single nucleotide polymorphism in Gal-2 is associated with susceptibility to myocardial infarction (27). This genetic substitution affects the transcriptional level of Gal-2 *in vitro*, potentially leading to altered secretion of LTA. Therefore,

we considered whether activation of P2Y₂R triggers Gal-2 up-regulation in VSMC. Treatment of VSMC with UTP (10 μM) stimulates the expression of galectin-2 mRNA in a dose-dependent manner (Fig. 2C). We next investigated whether UTP-induced increases in Gal-2 expression affect LTA secretion by using shRNA lentiviral particles to knockdown Gal-2 in VSMC. UTP-induced LTA secretion was unaffected by successful galectin-2 knockdown, indicating that galectin-2 is not required for P2Y₂R-mediated release of LTA in SMC (Fig. 2D).

Actin-binding Protein FLNa Is Required for P2Y₂R-mediated LTA Secretion—Activation of P2Y₂R induces stress fiber formation by causing the phosphorylation of myosin cofilin, an actin-depolymerizing protein (40). We explored the role of the actin cytoskeleton in UTP-induced LTA secretion by using cytochalasin D, an inhibitor of actin polymerization. Graded concentrations of cytochalasin D (0.001–10 ng/ml) induced a dose-dependent inhibition of LTA when WT SMC were stimulated with an effective dose (10 μM) of UTP (Fig. 3A). We have shown that the actin-binding protein FLNa links P2Y₂R to the actin cytoskeleton (2). Therefore, we further examined the role of FLNa in nucleotide-induced LTA secretion by knocking down FLNa expression. Using RNAi techniques, we successfully developed FLNa knockdown SMC in which the expression of FLNa was drastically reduced (Fig. 3B). We found that UTP-induced LTA secretion was significantly impaired in FLNa RNAi SMC (Fig. 3C). To confirm that the defect in LTA secretion was due to a loss of FLNa expression, we used the EGFP

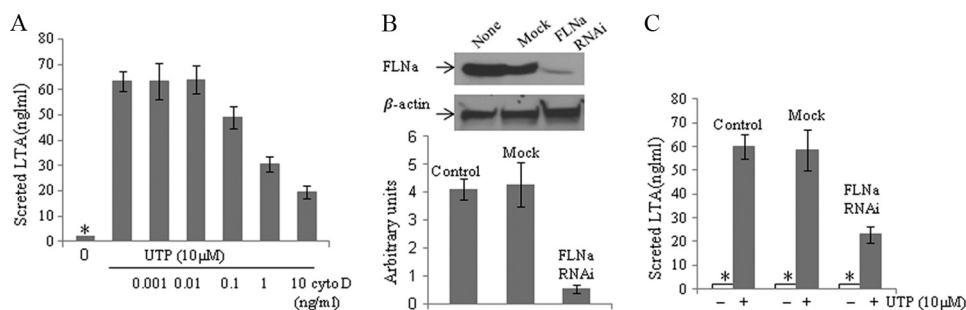


FIGURE 3. FLNa RNA interference inhibits UTP-stimulated LTA secretion. A, SMC were incubated with graded concentrations of cytochalasin D (cytochalasin D, 0.001–10 ng/ml) in the presence of UTP (10 μM). The results are expressed as the means ± S.E. of the percentage of LTA secreted in the incubation medium over 12 h ($n = 3$). B, lysates derived from control and FLNa RNAi expressing SMC were resolved by SDS-PAGE, followed by Western blot analysis for FLNa. Blotting for β-actin was used as a loading control. C, control and FLNa RNAi SMC were subjected to UTP stimulations for 12 h to assess the secretion of LTA by ELISA. The data represent the means ± S.E. of results from three independent experiments. *, under detection limit (3 ng/ml).

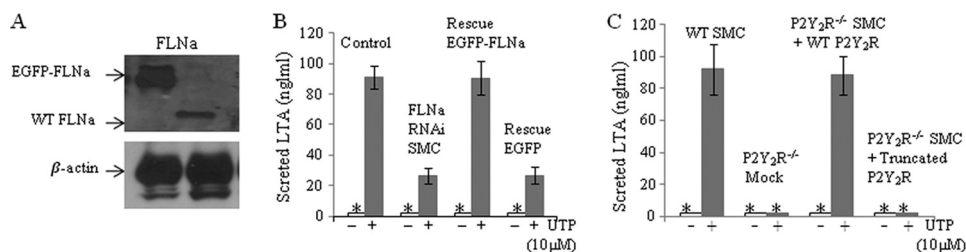


FIGURE 4. Expression of EGFP-FLNa in FLNa RNAi SMC restores UTP-induced LTA secretion. A, lysates were prepared from control (first lane) and EGFP-FLNa rescue FLNa RNAi SMC (second lane). Specific antibodies were used for Western blot analysis to detect endogenous FLNa and EGFP-FLNa. B, EGFP and EGFP-FLNa rescue FLNa RNAi SMC were stimulated with UTP for 12 h, and LTA secreted into the medium was measured by ELISA. C, P2Y₂R^{-/-} SMC expressing WT or a truncated P2Y₂R were stimulated with UTP for 12 h, and secreted LTA was measured by ELISA. The data represent the means ± S.E. of results from three independent experiments. *, under detection limit (3 ng/ml).

fusion vector, pLEGFP-C1 retroviral system for efficient introduction of exogenous FLNa in FLNa-RNAi SMC. The rescue construct encodes the same amino acid as endogenous FLNa but contains a different nucleotide sequence in FLNa RNAi target region to protect it from degradation. As shown in Fig. 4A, EGFP-FLNa rescued cells displayed expression of EGFP-FLNa but not endogenous FLNa. Expression of EGFP alone failed to restore LTA secretion in FLNa RNAi cells (Fig. 4B). In contrast, the expression of EGFP-FLNa fully restored nucleotide-induced LTA secretion (Fig. 4B). We next evaluated the importance of P2Y₂R/FLNa interaction in LTA secretion by transfecting P2Y₂R^{-/-} SMC with WT cDNAs encoding WT or a truncated P2Y₂R deficient in FLNa binding (36). We first determined that the P2Y₂R agonist UTP stimulates LTA mRNA expression in P2Y₂R^{-/-} SMC expressing either WT or truncated P2Y₂R cDNAs (data not shown), suggesting that P2Y₂R/FLNa interaction is not important for LTA transcriptional activation. Remarkably, expression of WT P2Y₂R cDNA in P2Y₂R^{-/-} SMC restored UTP-induced LTA in these cells as compared with the truncated P2Y₂R (Fig. 4C), indicating that P2Y₂R binding to FLNa is essential for LTA release.

P2Y₂R/FLNa Interaction Is Required for Rho Activation and Consequent Rho-dependent Secretion of LTA—Studies have shown that filamin interaction with the C terminus of calcium sensing receptor is involved Rho activation Rho and subsequent Rho-mediated signaling pathway (41). We next examined whether P2Y₂R/FLNa interaction is required for Rho activation and consequent Rho-dependent secretion of LTA. We first analyzed Rho GTPase activation in SMC in response to P2Y₂R activation with the use of a GST-Rhotekin pull down assay. As shown in Fig. 5A, RhoA was activated in SMC expressing WT

P2Y₂R but not in cells expressing the truncated P2Y₂R that does not interact with FLNa-A. UTP stimulations failed to increase Rac-1 or Cdc42 activity in these cells (data not shown). Notably, expression of the dominant negative form of RhoA (but not Rac1 or Cdc42) blocked UTP-induced LTA secretion (Fig. 5C), indicating that LTA secretion is selectively mediated through RhoA activation. Remarkably, expression of dominant negative RhoA does not prevent LTA gene activation (Fig. 5B), suggesting that the P2Y₂/RhoA signaling cascade is used exclusively for the secretion of LTA and is not involved in stimulating LTA gene expression.

Filamin-mediated LTA Secretion Is Independent of the ER/Golgi Secretory Pathway—Mammalian cells export most proteins by the endoplasmic reticulum/Golgi-dependent pathway. However, cytokines may also be released through alternative pathways in nonclassical secretion. We tested whether UTP-stimulated LTA secretion is sensitive to the antiviral antibiotic brefeldin A, which rapidly blocks the formation of vesicles involved in protein transport from the ER to the *cis*-Golgi cisternae (42). As shown in Fig. 6A, treatment of SMC with brefeldin A (1–5 μg/ml) had no effect on LTA secretion. This drug, however, strongly inhibited phorbol myristate acetate-induced LTA in a dose-dependent manner (Fig. 6B). These data suggest that P2Y₂R/filamin-mediated secretion of LTA is independent of the ER/Golgi secretory vesicle route.

LTA Regulates ICAM-1 Expression on VSMC—Although the presence of LTA has been documented in vascular SMC of human coronary artery lesions (27), the role of this cytokine in SMC function is not known. We next examined whether signaling via P2Y₂R modulates adhesion molecule expression on SMC. Using immunofluorescence staining, we found that qui-

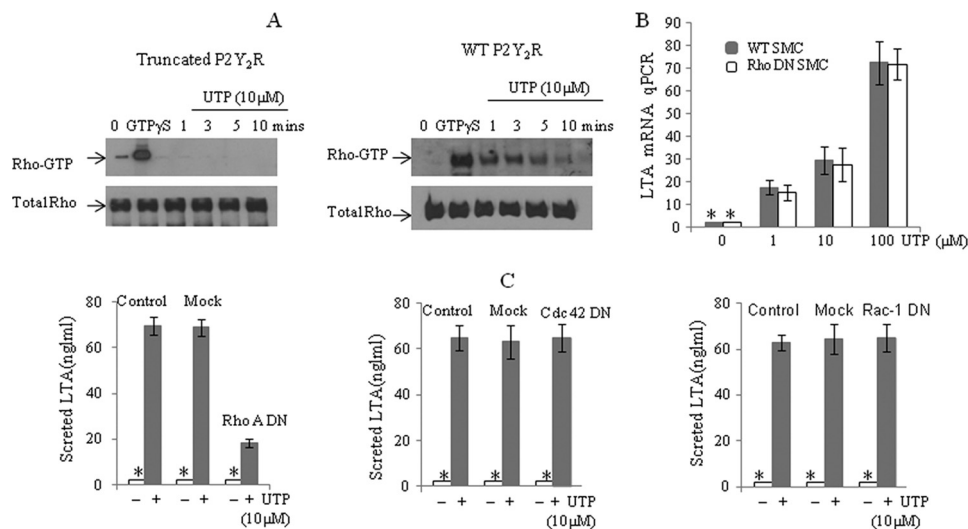


FIGURE 5. P2Y₂R/FLNa interaction mediates Rho activation and consequent Rho-dependent secretion of LTA. A, SMC expressing WT or truncated P2Y₂R cDNA were treated with UTP (10 μM) for the indicated times. The left panel shows immunoblot of protein from cell lysates precipitated by GST-RBD beads. The signal from the stimulated cell lysates was compared with signals from lysates loaded with guanosine 5'-O-(3-thiotriphosphate) (GTPγS) before precipitation with GST-RBD. The right panel shows immunoblot of total RhoA. B, real time PCR quantification of LTA mRNA in WT and SMC expressing RhoA dominant negative and treated with UTP (10 μM). The data (means ± S.E.) were presented as ratios of LTA to GAPDH mRNA, and the experiments were tripled under the same condition. C, dominant negative FLNa inhibits LTA secretion. SMC were transfected with vector alone (mock) or dominant negative (DN) RhoA (A), Rac1 (B), or Cdc42 or Cdc42 (C). After stimulation with UTP (10 μM) for 12 h, LTA was analyzed by ELISA. The data are representative of results from three independent experiments. *, under detection limit (3 ng/ml).

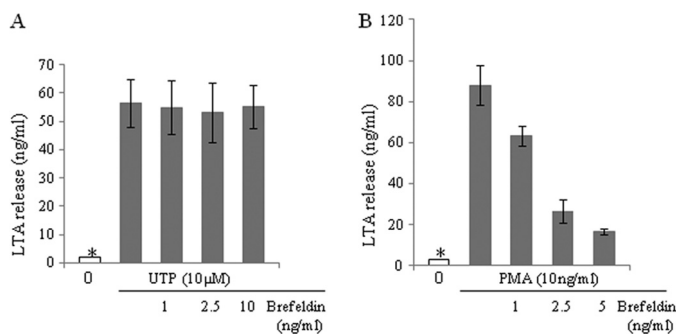


FIGURE 6. UTP-mediated LTA secretion is independent of the ER/Golgi secretory pathway. SMC were preincubated with graded concentrations of brefeldin for 30 min, and the cells were stimulated with UTP (A) or phorbol myristate acetate (PMA, B) for 12 h. LTA secreted in the medium was then measured by ELISA as described above. The results are expressed as the means ± S.E. of the percentage of LTA secreted in the incubation medium over 12 h (n = 3).

escent untreated SMC expressed a low basal level of ICAM-1 (Fig. 7A). The addition of UTP (10 μM) increased ICAM-1 staining over the entire cell surface (Fig. 7A). Western blot analysis showed that UTP caused a dose-dependent increase in ICAM-1 (Fig. 7B). However, this effect was absent in P2Y₂R^{-/-} SMC (Fig. 7B). In contrast to ICAM-1 expression, vascular cell adhesion molecule-1 was not detectable in these VSMC (data not shown). To explore the role of LTA in this process, we analyzed ICAM-1 expression in P2Y₂R^{-/-} SMC transduced with WT P2Y₂R or the truncated P2Y₂R deficient in LTA secretion (Fig. 4C). Reintroduction of WT P2Y₂R cDNA into P2Y₂R^{-/-} SMC restored ICAM-1 expression in response to UTP as compared with P2Y₂R^{-/-} SMC expressing a truncated P2Y₂R (Fig. 8A). Similarly, LTA (but not TNF-α) neutralizing antibody selectively inhibited UTP-induced ICAM-1 expression (Fig. 8B). Furthermore, the addition of exogenous LTA in cultured SMC dose-dependently stimulates ICAM-1 expres-

sion. In conclusion, these data demonstrate that secreted LTA plays an essential autocrine role in the regulation of ICAM-1 expression.

DISCUSSION

More detailed studies of the specific steps involved in the secretion of cytokines in VSMC may lead to a better understanding of the molecular basis of the mechanisms involved in vascular inflammation. In this study, we describe a new pathway whereby the P2Y₂ nucleotide receptor (P2Y₂R) regulates LTA secretion in vascular SMC. We first show that exogenous nucleotides increase transcriptional activation of the LTA gene leading to increased production and secretion by SMC. These effects are selectively mediated through activation of P2Y₂Rs because both UTP-induced LTA mRNA and an increase in protein secretion were absent from P2Y₂R^{-/-} SMC. Similar to VSMC, P2Y₂R also triggers induction of LTA secretion in mouse peritoneal macrophages, thus ruling out the possibility that LTA secretion requires the intersection of proximal P2Y₂R signals with distal signaling pathways/proteins predominantly expressed in the VSMC lineage. The P2Y₂R is coupled to downstream signaling pathways via G_{q/11} proteins (31). Our data indicate that selective activation of the G_{q/11}-coupled P2Y₁ and P2Y₆Rs expressed in these cells failed to trigger LTA secretion, indicating that other specific properties of P2Y₂R are required for LTA secretion.

We have previously reported that P2Y₂R selectively interacts with the actin-binding protein FLNa to modulate SMC migration (36). Using RNAi approaches, we show that disruption of the interaction between P2Y₂R and FLNa prevented nucleotide-mediated LTA release from VSMC. To our knowledge, this is the first study that directly implicates FLNa in the secretion of LTA. Although previous studies (27) have suggested a role for the actin cytoskeleton and microtubules in cytokine

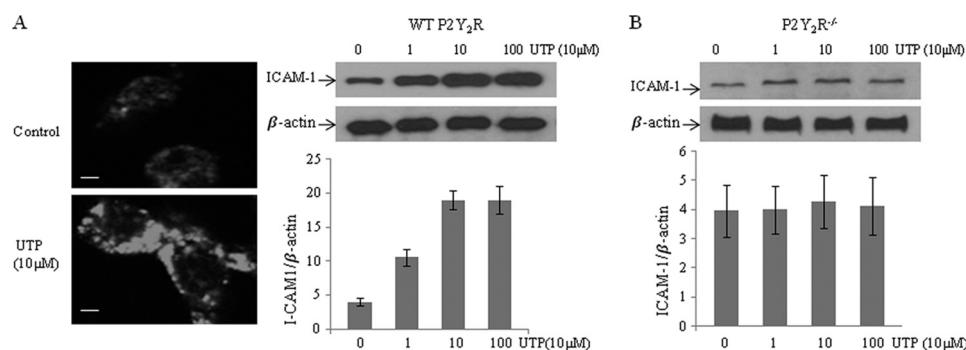


FIGURE 7. **UTP increases ICAM-1 expression on SMC.** WT and P2Y₂R^{-/-} SMC were stimulated with UTP (0–100 μM) for 12 h, and ICAM-1 expression was analyzed by immunofluorescence staining (A) or Western blot analysis (B) followed by quantitative analysis (mean values ± S.E., *n* = 3). Scale bar = 20 μm.

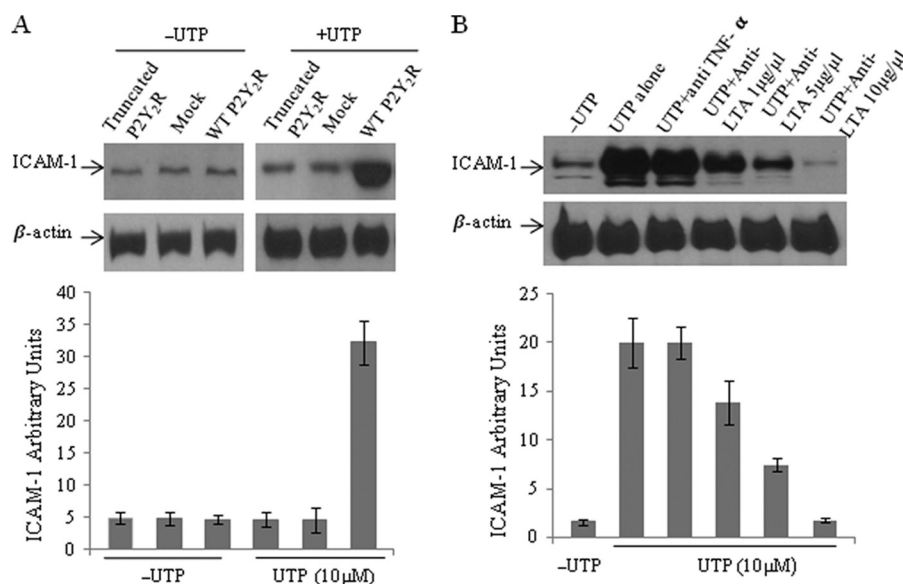


FIGURE 8. **LTA mediates autocrine regulation of ICAM-1 in SMC.** A, levels of ICAM-1 were measured in P2Y₂R^{-/-} SMC transduced with either WT or a truncated P2Y₂R and stimulated with UTP for 12 h (mean values ± S.E., *n* = 4). B, SMC from WT mice were stimulated with UTP (12 h) in the presence of anti-LTA neutralizing antibody or a nonspecific IgG, and ICAM-1 was then analyzed by Western blot followed by quantitative analysis (mean values ± S.E., *n* = 3).

secretion, little is known regarding the specific intracellular regulatory molecules involved in cytokine trafficking and release in vascular cells.

Small GTPases Rac1, Rac2, and Cdc42 have been implicated in granule exocytosis in mast cells and neutrophils through modulation of the actin cytoskeleton (43, 44) and have been associated with cytokine secretion in other cells, such as IL-8 from polarized epithelial cells (45). However, no specific role for Rho GTPases in cytokine secretion from SMC has yet been established. To identify the downstream effector of P2Y₂R/FLNa-mediated LTA secretion in VSMC, we examined whether the C terminus of P2Y₂R, which has recently been reported to bind to filamin (36), is involved in activation of Rho and consequent Rho-dependent secretion of LTA. We compared the ability of the truncated P2Y₂R to stimulate small Rho GTPases activity in vascular SMC. UTP caused a strong increase in RhoA activity in SMC expressing the full-length P2Y₂R, a response that was abolished in cells expressing a truncated P2Y₂R deficient in FLN-A binding. We found that P2Y₂R activation of FLNa results in a Rho-dependent stimulation of LTA secretion that is disrupted by expression of a dominant negative RhoA construct.

Galectin-2 is presumed to play a regulatory role in the intracellular trafficking of the proinflammatory cytokine LTA. A case control association study showed that a single nucleotide polymorphism in galectin-2 is associated with susceptibility to myocardial infarction (27). This genetic substitution affects the transcriptional level of galectin-2 *in vitro*, potentially leading to altered secretion of LTA. We found that P2Y₂R activation triggers both LTA and galectin-2 gene activation. However, UTP-induced LTA secretion was unaffected by successful galectin-2 knockdown, indicating that galectin-2 is not required for P2Y₂R-mediated release of LTA in SMC.

The secretion of cytokines from cells is a fundamental response to injury. Most of our knowledge regarding the distal steps of cytokine secretion from the Golgi complex, which occurs through membrane-bound organelles for classical secretion involving membrane fusion and exocytosis, is coming to light from innate immune cells. However, the distal mechanisms of cytokine release in nonimmune cells are poorly understood. Cytokines may also be released through alternative pathways, such as molecular transporters, in nonclassical secretion (46). By using the antiviral antibiotic brefeldin A, which rapidly blocks the formation of vesicles involved in protein transport

from the ER to the *cis*-Golgi cisternae (42), we provided convincing evidence suggesting that P2Y₂R/filamin-mediated secretion of LTA is independent of the ER/Golgi secretory vesicle route. There is a large body of evidence that supports the role of nucleotide receptors in the release of cytokines via ER-Golgi-independent mechanisms. Extracellular ATP acting via P2X₇Rs is a very potent stimulus for IL-1 β processing and release (47–49). It has been shown that P2X₇R activation in THP-1 macrophages triggers shedding of plasma membrane-derived IL-1 β -loaded microvesicles (50). Other studies have shown that astrocyte-derived ATP induces vesicle shedding and release from microglia (51). Furthermore, stimulation of P2 receptors causes release of IL-1 β -loaded microvesicles from human dendritic cells. In the present study, prolonged UTP stimulations did not result in SMC cell death (data not shown), thus excluding the possibility that LTA may be released as a secondary mechanism of apoptosis or necrosis. However, further investigations are needed to determine whether P2Y₂R activation triggers LTA release via shedding of plasma membrane-loaded vesicles.

It is unclear why LTA, a signal peptide-containing protein, bypasses the Golgi apparatus when induced by nucleotides. Previous studies have suggested that T cells are able to differentially direct cytokines through two molecularly distinct export pathways, thus ensuring that some only reach the immunological synapse, whereas others are released in multiple directions to induce an inflammatory response and establish chemokine gradients (52). Thus, further studies are warranted to determine whether ER/Golgi-independent transport of LTA represents a novel regulatory mechanism used by VSMC to selectively trigger inflammatory responses in nearby SMC.

Although it was shown that LTA is expressed in human atherosclerotic lesions (27), the role of this cytokine in SMC function is poorly understood. Our findings indicate that P2Y₂R activation leads to increased ICAM-1 expression on SMC. We demonstrated that neutralization of secreted LTA inhibited nucleotide-induced ICAM-1 up-regulation in SMC. Furthermore, blockade of LTA secretion by disrupting the interaction between P2Y₂R and FLNa prevented ICAM-1 up-regulation on SMC. Previous reports demonstrated that large amounts of nucleotides are released into the extracellular milieu in response to vascular stress conditions including ischemia/oxidative stress and mechanical stretch (7, 16, 53). Although the metabolism of extracellular nucleotides by ecto-nucleotidases plays a role in the regulation of purinergic signaling, pathological conditions, especially those associated with endothelial cell activation, can decrease ATP-diphosphorylase activity (54), suggesting that pathological conditions affecting blood vessels contribute to local elevations in nucleotide concentrations in the vessel wall. Thus, P2Y₂R could play an essential autocrine regulatory role in ICAM-1 expression in the early steps of vascular injury leading to the recruitment of blood-derived cells into the neointima.

In conclusion, our data show that signaling by nucleotides via P2Y₂Rs mediate FLNa-dependent activation of RhoA and subsequent release of LTA from VSMC. The mounting evidence suggesting a role for P2Y₂Rs in the regulation of vascular inflammation warrants additional studies to identify potential

P2Y₂R inhibitory molecules for the treatment of chronic vascular diseases.

Acknowledgment—We thank Shaomin Qian for help in preparation of the manuscript.

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