UTP stimulates the expression of pro-inflammatory vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells through activation of the P2Y2 nucleotide receptor P2Y2R. Here, we demonstrated that activation of the P2Y2R induced rapid tyrosine phosphorylation of vascular endothelial growth factor receptor (VEGFR)-2 in human coronary artery endothelial cells (HCAEC). RNA interference targeting VEGFR-2 or inhibition of VEGFR-2 tyrosine kinase activity abolishes P2Y2R-mediated VCAM-1 expression. Furthermore, VEGFR-2 and the P2Y2R co-localize upon UTP stimulation. Deletion or mutation of two Src homology-3-binding sites in the C-terminal tail of the P2Y2R or inhibition of Src kinase activity abolished the P2Y2R-mediated transactivation of VEGFR-2 and subsequently inhibited UTP-induced VCAM-1 expression. Moreover, activation of VEGFR-2 by UTP leads to the phosphorylation of Vav2, a guanine nucleotide exchange factor for Rho family GTPases. Using a binding assay to measure the activity of the small GTPases Rho, we found that stimulation of HCAEC by UTP increased the activity of RhoA and Rac1 (but not Cdc42). Significantly, a dominant negative form of RhoA inhibited P2Y2R-mediated VCAM-1 expression, whereas expression of dominant negative forms of Cdc42 and Rac1 had no effect. These data indicate a novel mechanism whereby a nucleotide receptor transactivates a receptor tyrosine kinase to generate an inflammatory response associated with atherosclerosis.

Extracellular nucleotides released by vascular cells act via P2 nucleotide receptors to regulate the vascular tone (1). There is increasing evidence to support a role for nucleotides in the development of vascular diseases such as atherosclerosis and restenosis after angioplasty. Indeed, UTP and ATP, equipotent agonists of the P2Y2R, induce proliferation and migration of vascular smooth muscle cells (2–4), two key processes involved in atherogenesis. Furthermore, our previous studies have shown that the development of intimal lesions in experimental models of atherosclerosis is closely associated with up-regulation of a functional P2Y2R (5, 6). The first direct evidence supporting a role for nucleotides in atherogenesis is the demonstration that local application of UTP to collared rabbit carotid arteries enhanced intimal hyperplasia and promoted infiltration of monocytes into the neointima (6). More recently, we reported that activation of the P2Y2R in vascular endothelial cells induces VCAM-1 expression and promotes the adherence of monocytes (7). Similarly, VEGF, a pleiotropic factor that regulates multiple biological phenomena, including endothelial cell survival, proliferation, and migration, also stimulates endothelial cell expression of adhesion molecules, including VCAM-1, ICAM-1 (intracellular adhesion molecule-1), and selectins (8). These multiple responses are mediated through the interaction of VEGF with two receptor tyrosine kinases, Flt-1 or VEGF receptor-1 and Flk-1/KDR or VEGF receptor-2 (8). It is widely accepted that VEGFR-2 mediates the growth and migration of endothelial cells, the permeability of blood cells, and the expression of VCAM-1 in human umbilical vascular endothelial cells (9). It is increasingly apparent that VEGFR-2 serves as a point of convergence of multiple signals arising from diverse stimuli. VEGFR-2 transactivation follows activation of G protein-coupled receptors, including the endothelial differentiation gene product and the bradykinin receptor (10, 11). We hypothesized that transactivation of VEGFR-2 may be involved in P2Y2R-mediated VCAM-1 expression in endothelial cells. Here, we show that P2Y2R activation by UTP induced tyrosine phosphorylation of VEGFR-2. Importantly, we demonstrated that inhibition of the tyrosine kinase activity of VEGFR-2 prevented UTP-induced VCAM-1 expression. Furthermore, we elucidated the structural determinants within the P2Y2R that are responsible for activating the signaling pathways involved in nucleotide-induced VCAM-1 expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—VEGF-185, rabbit anti-human Flk-1/VEGFR-2 polyclonal antibody, A/G PLUS-agarse, monoclonal anti-phosphotyrosine (4G10) antibody, and transfection grade eukaryotic expression vector pUSEamp (+) containing human dominant negative RhoA, Rac1, or Cdc42 were purchased from Upstate Biotechnology (Lake Placid, NY). A rabbit anti-VCAM-1 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The antibody against Vav2 (rabbit polyclonal) was generously provided by Dr. Keith Burridge (University of North Carolina at Chapel Hill). Anti-VEGF neutralization monoclonal antibody was from Chemicon (Temecula, CA). Specific inhibitors for VEGF-2 tyrosine phosphorylation (SU1498) and Src (PP2) were observed saline; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.
tained from Calbiochem (San Diego, CA). LipofectAMINE 2000 reagent was purchased from Invitrogen. The rat monoclonal anti-HA 3F10 affinity matrix was obtained from Roche Diagnostics. Dominant negative Src (K296R/Y528F) and its control vector (pUSE) were purchased from Upstate Biotechnology. All other reagents were from Sigma unless otherwise specified.

Cell Culture and Transfection—HCAEC were cultured in endothelial basal medium-2 (Clonetics, Walkerville, MD) at 37 °C in a humidified atmosphere of 5% CO₂. HCAEC were used between the third and sixth passages. The retroviral vector pLXSN was used for stable expression of the human P2Y₂R in human 1321N1 astrocytoma cells, as described previously (12). For transient transfections, plasmid constructs were delivered using LipofectAMINE 2000 reagent according to the manufacturer’s instructions.

**P2Y₂R cDNA Constructs**—The open reading frame of the wild type human P2Y₂R 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 (13). Three primers synthesized in the DNA Core Facility of the University of Missouri-Columbia (primer 1, 5’-AGGCTCGTACGCTTTGCCCGAGATGCCAAGGCTCGCGCAAGGCTGCCGTCGCGTCGCGTGTCCGGCAGTGC-3’; primer 2, 5’-ATCTGATCCCTTACGTGGGCTCCATCGGGCCTTGCAGATCT-3’; primer 3, 5’-CACACCTACTGACGACGTCGACGTTCCGGCAAGGCCTGA-3’) 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 P2Y₂ receptors. After verification of the size of the PCR products by agarose gel electrophoresis, the proteins were purified using the PCR Wizard kit (Amersham Biosciences), digested with BsiWI and BamHI, and inserted into pLXSN. The mutant cDNAs were sequenced to verify the mutations were incorporated correctly.

siRNAs Treatment—The sense and antisense strands of siRNAs were: VEGFR-2, beginning at nucleotide 3172, 5’-CCGGCGACTCAATCTACTAGCT-3’ (sense) and 5’-CCGGCGACTCAATCTACTAGCT-3’ (antisense). The control siRNAs were: scrambler, beginning at nucleotide 3172, 5’-CCGGCGACTCAATCTACTAGCT-3’ (antisense). The control siRNAs were: scrambler, beginning at nucleotide 3172, 5’-CCGGCGACTCAATCTACTAGCT-3’ (antisense). The control siRNAs were: scrambler, beginning at nucleotide 3172, 5’-CCGGCGACTCAATCTACTAGCT-3’ (antisense). Double-stranded siRNAs were generated in the pRNAT-U6.1 Neo vector (GenScript) that carries a green fluorescent protein marker used to track transfection efficiency. HCAEC were transfected with VEGFR-2 siRNA or control scrambler siRNA for 48 h in normal culture medium.

**Activity Assay for Rho, Rac, and Cdc24**—A Rho activation assay kit (Upstate Biotechnology) was used to assess Rho activity in HCAEC. Briefly, HCAEC were cultured in 150-mm tissue culture dishes and stimulated with UTP for the times indicated in the figure legends. The cells were then washed three times with ice-cold Tris-buffered saline and suspended in lysis buffer A containing 125 mM HEPES, pH 7.5, 7.5 mM NaCl, 5% (v/v) Igepal CA-630, 50 mM MgCl₂, 5 mM EDTA, and 10% (v/v) glycerol, and the lysates were transferred to 1.5-ml tubes. Fifty microliters of Rhotekin Rho-binding domain (RBD)-agarose were added to each tube for 45 min at 4 °C. The beads were pelleted by centrifugation (10 s, 14,000 × g, 4 °C) and washed three times with lysis buffer A. Finally, the beads were resuspended in 40 μl of 2× Laemmli buffer, and Western blot analysis was performed with anti-Rho antibody (Upstate Biotechnology). The assay for Rac1 and Cdc42 was done using an immobilized GST fusion construct of the Rac1-binding domain of p21Rac-binding domain that binds to Rac1-GTP but not to Rac1-GTP(P). Because Cdc42 also binds to the p21Rac-binding domain construct, the same assay was used to measure Cdc42 activity, except that the membranes were probed with an anti-Cdc42 antibody (Upstate Biotechnology).

**Immunoprecipitation and Immunoblotting**—HCAEC or 1321N1 cell transfectants were washed with PBS and suspended in lysis buffer B containing 0.5% (v/v) Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, and 0.1% (v/v) protease inhibitor mixture. Protein concentrations were measured using the Coomassie protein assay reagent following the manufacturer’s instructions (Pierce), and then 500 μg of the protein lysate were incubated overnight at 4 °C with anti-HA affinity matrix, anti-FliK1/VEGFR-2, anti-Vav2, or anti-phosphotyrosine antibodies. The immunoprecipitates were collected by incubation with protein A/G PLUS-agarose (Upstate Biotechnology) for 2 h when applicable and washed three times with lysis buffer B. Bound proteins were eluted by boiling in SDS-polyacrylamide gel electrophoresis sample buffer, and then immunoblotting was performed.

**Immunofluorescence**—Human 1321 astrocytoma cells stably expressing an N-terminal HA epitope-tagged human P2Y₂R were grown to 60% confluence on coverslips in 35-mm dishes. The cells were serum-starved overnight followed by incubation for 1 h at 37 °C with rabbit anti-HA (1:100) (Santa Cruz Biotehnologies) and mouse anti-VEGFR-2 (1:100) (Upstate Biotechnology) antibodies that recognize cell surface epitopes. The cells were washed three times with PBS and then incubated with a 1:200 dilution of goat anti-rabbit Alexa Fluor® 594- and goat anti-mouse Oregon Green® 488-conjugated IgG (Molecular Probes, Eugene, OR) in serum-free medium for 1 h at 37 °C. The cells were washed in PBS and re-equilibrated in serum-free medium for 1 h and then exposed to 100 μM UTP for 5 min at 37 °C, promptly washed in PBS, and immersed in PBS containing 4% (v/v) paraformaldehyde for 10 min. Coverslips were washed three times in PBS, rinsed in ddH₂O, and mounted on glass slides in ProLong® antifade reagent (Molecular Probes). Microscopy was performed on a Bio-Rad Radiance 2000 system coupled with an Olympus IX70 microscope using 60× water immersion lens (numerical aperture 1.3). Data were processed with Laser Sharp 2000 software. Co-localization experiments with Alexa Fluor® 594-conjugated IgG to detect VEGFR-2 and Oregon Green 488-conjugated IgG to detect the HA-tagged P2Y₂R were performed at 590 nm excitation/617 nm emission and 486 nm excitation/424 nm emission wavelengths for the respective antibodies.

**RESULTS**

**UTP Induces Tyrosine Phosphorylation of VEGFR-2**—We have shown previously that UTP induces VCAM-1 expression via P2Y₂-Rs in vascular endothelial cells (7). VEGF also stimulates the expression of endothelial cell adhesion molecules including VCAM-1 via activation of VEGFR-2 (9). Because ligand-independent activation of receptor tyrosine kinases is involved in many biological responses initiated by G protein-coupled receptors (11, 14), we hypothesized that activation of the P2Y₂R would increase VEGFR-2 phosphorylation in HCAEC. Indeed, we detected a rapid and intense tyrosine phosphorylation of VEGFR-2 in HCAEC as early as 1 min after UTP stimulation, which reached a peak within 5 min (Fig. 1A). Importantly, treatment of HCAEC with anti-VEGF antibody did not suppress UTP-induced VEGFR-2 phosphorylation, indicating that activation of VEGFR-2 did not occur secondarily to VEGF release (not shown). To determine whether the
P2Y2R could mediate VEGFR-2 phosphorylation, we utilized G protein-coupled P2 receptor-deficient 1321N1 cells transfected with the human P2Y2R. We first verified that VEGF could induce tyrosine phosphorylation of VEGFR-2 in 1321N1 cells prior to and after expression of the human P2Y2R (not shown). We then determined that UTP caused a time-dependent increase in VEGFR-2 tyrosine phosphorylation in 1321N1 cells expressing the P2Y2R (Fig. 1B) but not in untransfected 1321N1 cells (not shown). Thus, our data clearly indicate that the P2Y2R can mediate transactivation of the VEGFR-2.

RNA Interference Targeting of VEGFR-2 Inhibits P2Y2R-mediated VCAM-1 Expression—The rapid activation of VEGFR-2 by UTP prompted us to consider whether the VEGFR-2 signaling pathway was involved in UTP-induced P2Y2R-mediated VCAM-1 expression. To assess the role of VEGFR-2 in this process, siRNA was used to suppress the expression of the VEGFR-2 gene. Synthetic siRNAs of 21–23 nucleotides in length have been shown to silence cellular and viral gene expression in mammalian cells both in vitro and in vivo (15, 16). Results indicated that VEGFR-2 siRNA significantly decreased VEGFR-2 mRNA and protein levels 2 days after transfection (Fig. 2, A and B) without affecting UTP-induced mobilization of intracellular calcium (not shown). Treatment of HCAEC with VEGFR-2 siRNA completely inhibited UTP-induced VCAM-1 expression (Fig. 2C). Furthermore, SU1498, a specific inhibitor of VEGFR-2 tyrosine kinase activity, inhibited both UTP-induced phosphorylation of VEGFR-2 and VCAM-1 expression (Fig. 3), indicating that activation of VEGFR-2 is necessary for P2Y2R-mediated VCAM-1 expression in HCAEC.

UTP-dependent Co-localization and Interaction of VEGFR-2 with the P2Y2R—To determine whether the VEGFR-2 and the P2Y2R co-localize upon UTP stimulation, 1321N1 cells were transfected with the wild type P2Y2R in which a HA epitope tag was incorporated at the N terminus to facilitate immunodetection of the receptor. In the absence of UTP, confocal microscopy of dual labeled cells showed no co-localization between the VEGFR-2 (red) and the P2Y2R (green) in 1321N1 cell transfec-
Vegfr-2 for 6 h, and VCAM-1 expression was determined by Western blot analysis. The data are representative of results from four independent experiments.

**Fig. 3.** Inhibition of Vegfr-2 tyrosine phosphorylation prevents UTP-induced VCAM-1 expression. **A,** HCEAC were pretreated with the indicated concentrations of SU1498 for 30 min and then stimulated with 10 µM UTP for 5 min. Vegfr-2 was immunoprecipitated (IP) and tyrosine phosphorylation (PY) was detected by immunoblotting (IB) as described in the legend to Fig. 1. The blots were stripped and reprobed with anti-VEGFR-2 antibody. **B,** HCEAC were pretreated with the indicated concentrations of SU1498 for 30 min and stimulated with 10 µM UTP for 6 h, and VCAM-1 expression was determined by Western blot analysis. The data are representative of results from four independent experiments.

**Fig. 4.** The P2Y$_{2R}$ and VEGFR-2 co-localize and interact upon UTP stimulation. **A,** 1321N1 cells stably expressing the HA-tagged P2Y$_{2R}$ were incubated in the presence (+) or absence (−) of 100 µM UTP at 37°C for 5 min. Immunofluorescence of IgG-conjugated Oregon Green® (green) and IgG-conjugated Alexa Fluor® (red) was used to detect anti-HA-tagged P2Y$_{2R}$ and anti-VEGFR-2 antibodies, respectively, in intact cells as described under “Experimental Procedures.” Confocal micrographs are representative of results from four separate experiments. Magnification: ×60. **B,** 1321N1 cells stably expressing the HA-tagged P2Y$_{2R}$ were stimulated with 100 µM UTP for the indicated times, and cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody and immunoblotted (IB) with anti-VEGFR-2 antibody. Prior to immunoprecipitation, total VEGFR-2 was detected with anti-VEGFR-2 antibody using duplicate samples (lower blot). The results are representative of four independent experiments.

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**SH3-binding Sites in the C-terminal Domain of the P2Y$_{2R}$ and Src Kinase Activity Are Required for VEGFR-2 Tyrosine Phosphorylation**

It is unclear how the P2Y$_{2R}$, which lacks intrinsic tyrosine kinase activity, activates VEGFR-2. It has been reported previously that the angiotensin II-type receptor, which also lacks intrinsic tyrosine kinase activity, can activate the EGF receptor through binding with the SH2 domain containing tyrosine phosphatase 2 (14). There is also evidence that Src kinase activity is necessary for transactivation of the EGF receptor by the P2Y$_{2R}$ (17). Recently, it has been shown that P2Y$_{2R}$-mediated transactivation of the EGF receptor requires Src activity and two PXPP motifs in the C terminus of the P2Y$_{2R}$ that represent minimal consensus motifs for SH3 domain-binding proteins, including Src (18). We found that P2Y$_{2R}$-mediated tyr-88 phosphorylation of VEGFR-2 was dependent on Src kinase activity, as demonstrated by concentration-dependent sensitivity to PP2, the Src-specific tyrosine kinase inhibitor (Fig. 5A). Furthermore, expression of dominant negative Src abolished the physical interaction between VEGFR-2 and the P2Y$_{2R}$ (Fig. 5B). These results indicate that Src plays a critical role in mediating UTP-induced activation of VEGFR-2.

To test the role of the P2Y$_{2R}$ SH3-binding domains in UTP-
Nucleotide-induced Activation of VEGFR-2

Fig. 5. Src kinase activity and SH3-binding domains in the C-terminal tail of the P2Y<sub>2</sub>R are required for VEGFR-2 transactivation. A, PP2 inhibits UTP-induced VEGFR-2 phosphorylation. Human 1321N1 cells stably expressing the P2Y<sub>2</sub>R were incubated for 1 h with the indicated concentrations of the specific inhibitor of Src kinase PP2 and stimulated with 100 μM UTP for 5 min. VEGFR-2 phosphorylation was determined by immunoprecipitation (IP) with anti-VEGFR-2 antibody and immunoblotting (IB) with anti-phosphotyrosine (PY) antibody. The blots were stripped and reprobed with anti-VEGFR-2 antibody. B, dominant negative Src inhibits the interaction between VEGFR-2 and the P2Y<sub>2</sub>R. Human 1321N1 cells stably expressing the P2Y<sub>2</sub>R were transfected with dominant negative Src or control plasmid. Forty-eight h after transfection, the cells were stimulated with 100 μM UTP for 5 min, and the cell lysates were subjected to immunoprecipitation with anti-HA antibody and immunoblotting with anti VEGFR-2 antibody. Lane 1 (left to right), untransfected cells without UTP; lane 2, untransfected cells treated with UTP; lane 3, vector transfected cells with UTP; lane 4, dominant negative Src transfected cells with UTP. C, partial amino acid sequence of the C-terminal tail of the P2Y<sub>2</sub>R including the SH3-binding domains of the wild type receptor (wt) and mutant receptors in which prolines in the SH3-binding domains were mutated to alanines (4A) or deleted (del). D, VEGFR-2 phosphorylation in 1321N1 cells expressing the wt (upper panel), del (middle panel), or 4A mutant P2Y<sub>2</sub>R (lower panel). The cells were stimulated with 100 μM UTP for the indicated times or 10 ng/ml VEGF for 10 min, and the cell lysates were subjected to immunoprecipitation with anti-VEGFR-2 antibody and immunoblotting with anti-phosphotyrosine antibody. The blots were stripped and reprobed with anti-VEGFR-2 antibody. The results shown are representative of at least three independent experiments for each figure.

Induced transactivation of VEGFR-2, we stably expressed in 1321N1 cells P2Y<sub>2</sub>R cDNA encoding a mutant receptor (Fig. 5C) in which the PXXP sequences were deleted (del) or mutated to replace the prolines with alanines (4A). VEGF induced tyrosine phosphorylation of VEGFR-2 in 1321N1 cells expressing wild type or mutant P2Y<sub>2</sub>Rs (Fig. 5D). In contrast to cells expressing the wild type receptor, UTP failed to activate VEGFR-2 in cells expressing the del or 4A mutant P2Y<sub>2</sub>Rs (Fig. 5D). These data clearly indicate that the SH3-binding domains in the C terminus of the P2Y<sub>2</sub>R mediate UTP-induced transactivation of VEGFR-2. We also found that UTP did not induce VCA1 expression in cells expressing del or 4A mutant P2Y<sub>2</sub>Rs in contrast to cells expressing the wild type receptor (Fig. 6).

Activation of RhoA Downstream of VEGFR-2 Mediates VCAM-1 Expression in HCAEC—We next sought to identify the signaling mechanisms downstream of VEGFR-2 activation that are responsible for UTP-induced VCA1 expression. Activation of the P2Y<sub>2</sub>R and VEGFR-2 are linked to activation of the small GTPase RhoA (19, 20). Stimulation of the EGF receptor or the PDGF receptor leads to phosphorylation and activation of Vav2, a specific Rho exchange factor that activates RhoA (21). Therefore, we investigated whether Vav2 could be phosphorylated in response to P2Y<sub>2</sub>R-mediated VEGFR-2 transactivation. We found that UTP caused a strong time-dependent increase in the tyrosine phosphorylation of Vav2 in HCAEC (Fig. 7A). UTP-induced Vav2 tyrosine phosphorylation was blocked by pretreatment of cells with SU1498, an inhibitor of VEGFR-2 tyrosine phosphorylation (Fig. 7B). Because Vav2 can activate all three major Rho family GTPases, including RhoA, Rac, and Cdc42, we tested whether these G proteins were activated by UTP. Glutathione-Sepharose beads complexed to the GST constructs were used to affinity precipitate GTP-bound RhoA, Rac1, and Cdc42. As shown in Fig. 8A, RhoA was activated as early as 2 min after stimulation of HCAEC with UTP and remained activated through 10 min. We found a transient but significant UTP-induced increase in the activity of Rac1 in HCAEC, whereas Cdc42 was not activated (Fig. 8, B and C). To determine which of these G proteins was involved in UTP-induced VCA1 expression, we transiently expressed dominant negative forms of RhoA, Rac1, and Cdc42 in HCAEC. We found that expression of dominant negative forms of RhoA (but not Rac1 or Cdc42) blocked UTP-induced VCA1 expression (Fig. 9), indicating that RhoA (but not Rac1 or Cdc42) mediates VCA1 expression downstream of Vav2 activation.

Discussion

The P2Y<sub>2</sub>R is increasingly recognized as an important contributor to the development of atherosclerosis or restenosis after angioplasty (5, 22). We have reported previously that this G protein-coupled receptor mediates VCA1 expression and adhesion of leukocytes to vascular endothelial cells (7). The present study focuses on the mechanisms whereby P2Y<sub>2</sub>R activation increases the expression of VCA1. We demonstrated for the first time that activation of the P2Y<sub>2</sub>R in human coronary artery endothelial cells induces the rapid tyrosine phosphorylation of VEGFR-2 and the activation of Vav2, a Rho exchange factor, leading to the expression of VCA1. Two lines of evidence demonstrated the involvement of VEGFR-2 in
this process. First, SU1498, a specific VEGFR-2 tyrosine kinase inhibitor, diminished UTP-induced VCAM-1 expression (Fig. 3B). Second, selective inhibition of VEGFR-2 expression by siRNA inhibited the UTP-induced VCAM-1 expression (Fig. 2C). Ligand-independent activation of receptor tyrosine kinases is involved in many biological responses initiated by G protein-coupled receptors. It has been reported that activation of the endothelial differentiation gene in response to sphingosine 1-phosphate stimulates endothelial nitric-oxide synthase through activation of Flik1/KDR (10). EGF receptor transactivation by G protein-coupled receptor ligands may involve activation of matrix metalloproteinases, release of membrane heparin-bound EGF, and binding of heparin to the EGF receptor (23–25). At the structural level, two SH3-binding sites (PXXP motifs) have been identified in the intracellular C terminus of the P2Y<sub>2</sub>R and have been shown to interact with Src and regulate the activity of the EGF and PDGF receptors (18). In this study, we demonstrated that PP2, the Src kinase inhibitor, decreased P2Y<sub>2</sub>R-mediated VEGFR-2 tyrosine phosphorylation (Fig. 5A) consistent with the involvement of Src in EGF and PDGF receptor transactivation by P2Y<sub>2</sub>Rs (18). We also found that the P2Y<sub>2</sub>R and VEGFR-2 co-localized in cells upon UTP stimulation and demonstrated for the first time a direct interaction between these receptors in a UTP-dependent manner (Fig. 4). Deletion or mutation of the SH3-binding domains prevented UTP-induced expression of VCAM-1 (Fig. 6). Because transfection of dominant negative Src inhibited the

**Fig. 6.** Deletion or mutation of the SH3-binding sites in the P2Y<sub>2</sub>R prevents UTP-induced VCAM-1 expression in HCAEC. Wild type (WT) and mutant P2Y<sub>2</sub>Rs in which prolines in the SH3-binding domains were deleted (del) or mutated to alanines (4A) were stimulated with the indicated concentrations of UTP for 6 h or 10 ng/ml VEGF for 6 h, and then VCAM-1 expression was determined by Western analysis. The blots were stripped and reprobed with anti-VEGFR-2 antibody. The data are representative of results from three independent experiments.

**Fig. 7.** UTP-induced transactivation of VEGFR-2 causes tyrosine phosphorylation of Vav2 in HCAEC. A, HCAEC were treated with 10 μM UTP for the indicated times, and cell lysates were immunoprecipitated (IP) with polyclonal anti-Vav2 antibody and immunoblotted (IB) with anti-phosphotyrosine (PY) antibody. The blots were stripped and reprobed with anti-Vav2 antibody. Shown are bar graphs of densitometric quantifications of three independent experiments normalized to total Vav2 protein expression (n = 3, mean ± S.E.). B, serum-starved HCAEC were pretreated with the indicated concentrations of SU1498 for 30 min and stimulated with 10 μM UTP for 2 min. Phosphorylation of Vav2 was detected as described in A.

**Fig. 8.** UTP stimulates the activity of RhoA and Rac1. HCAEC were treated with 10 μM UTP for the indicated times. Immunoblots of RhoA (A), Rac1 (B), and Cdc42 (C) are shown. The top panels show immunoblots of protein from cell lysates precipitated by either GST-RBD beads (for Rho-GTP) or GST-p21-binding domain. beads (for Rac1-GTP or Cdc42-GTP). 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 or PAK-1 p21-binding domain. The lower panels show immunoblots of total RhoA, Rac1, or Cdc42 in total cell lysates.
P2Y2R-VEGFR-2 interaction, we concluded that Src binding to the SH3-binding domains in the P2Y2R mediates both the UTP-induced P2Y2R and VEGFR-2 interaction and the transactivation of VEGFR-2 involved in VCAM-1 expression.

VEGF-stimulated expression of VCAM-1 is mediated mainly through nuclear factor κB (NFκB) activation with phosphatidylinositol 3-kinase-mediated suppression (8). P2Y2Rs in endothelial cells activate a variety of signaling pathways including but not restricted to the mitogen- and stress-activated protein kinases ERK 1/2 and p38, and the small GTPase RhoA (7, 19). However, it is unclear whether activation of the small GTPase Rho is involved in UTP-induced VCAM-1 expression. Previous work has demonstrated that Vav and Vav2, two members of the Dbl family of proteins, act as guanine nucleotide exchange factors for Rho family proteins (26, 27). Rho family proteins function as GDP/GTP-regulated molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (28–30). Although Dbl family proteins stimulate GDP dissociation to promote the formation of the active GTP-bound protein, Rho family proteins are inactivated by GTPase-activating proteins that stimulate the intrinsic rate of GTP hydrolysis (31). Recent studies have demonstrated EGF- or PDGF-stimulated tyrosine phosphorylation of Vav2 in hematopoietic cells (21). We have found that UTP induced a rapid but transient phosphorylation of Vav2 (Fig. 7A), a Rho exchange factor that activates RhoA (21), and UTP-induced Vav2 phosphorylation was inhibited by SU1498 (Fig. 7B), a specific inhibitor of VEGFR-2 tyrosine kinase activity. In addition, UTP failed to activate Vav2 in HCAEC transfected with VEGFR-2 siRNA (data not shown). Therefore, we concluded that Vav2 is activated downstream of tyrosine phosphorylation of VEGFR-2.

Our results indicate that RhoA and Rac1 (but not Cdc42) activities were increased by UTP in HCAEC (Fig. 8). The tyrosine phosphorylation of Vav2 in response to UTP suggests that Vav2 contributes to RhoA and Rac1 activity. Although we cannot conclude that elevation of RhoA and Rac1 activity was due to direct activation by Vav2, preliminary experiments (not shown) indicated that over-expression of a constitutively active Vav2 in HCAEC activates Rho and Rac1 (but not Cdc42). Others studies have indicated that Vav2 overexpression elevates the activities of Rac1, RhoA, and Cdc42 (21). Although our data demonstrated that Rho and Rac1 were both activated downstream from VEGFR-2 tyrosine phosphorylation, only RhoA activation is required for VCAM-1 expression, because expression of dominant negative forms of RhoA (but not Rac1 or Cdc42) blocked UTP-induced VCAM-1 expression (Fig. 9).

In summary, results from the present study indicate that activation of the G protein-coupled P2Y2R promotes Src binding to SH3-binding motifs in the P2Y2R and facilitates the interaction and transactivation of VEGFR-2, resulting in the sequential activation of Vav2 and RhoA leading to VCAM-1 expression. The present study elucidated for the first time a novel mechanism whereby a nucleotide receptor interacts with a growth factor receptor to generate a pro-inflammatory response in vascular endothelial cells. Further delineation of the signaling pathway involved in this process may help prevent inflammation associated with atherosclerosis by limiting P2Y2R up-regulation and/or the consequences of P2Y2R activation.

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