The P2Y2 Nucleotide Receptor Mediates UTP-induced Vascular Cell Adhesion Molecule-1 Expression in Coronary Artery Endothelial Cells

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P2Y2 receptor up-regulation and activation induces intimal hyperplasia and monocyte/macrophage infiltration in the collared rabbit carotid artery model of vascular injury, suggesting a potential role for P2Y2 receptors in monocyte recruitment by vascular endothelium. In this study, we addressed the hypothesis that activation of P2Y2 receptors by extracellular nucleotides modulates the expression of adhesion molecules on vascular endothelial cells that are important for monocyte recruitment. Results indicated that the equipotent P2Y2 receptor agonists UTP or ATP (1–100 μM) stimulated the expression of vascular cell adhesion molecule-1 (VCAM-1) in human coronary artery endothelial cells (HCAEC) in a time- and dose-dependent manner. P2Y2 antisense oligonucleotides inhibited VCAM-1 expression induced by UTP but not by tumor necrosis factor-α. Furthermore, UTP induced VCAM-1 expression in human 1321N1 astrocytoma cell transfectants expressing the recombinant P2Y2 receptor, whereas vector-transfected control cells did not respond to UTP. The effect of UTP on VCAM-1 expression in HCAEC was prevented by depletion of intracellular calcium stores with thapsigargin or by inhibition of p38 mitogen-activated protein kinase or Rho kinase, but was not affected by inhibitors of the mitogen-activated protein/extracellular signal-regulated kinase pathway (i.e. MEK1/2). Consistent with a role for VCAM-1 in the recruitment of monocytes, UTP or ATP increased the adherence of monocytes U937 cells to HCAEC, an effect that was inhibited by anti-VCAM-1 antibodies. These findings suggest a novel role for the P2Y2 receptor in the p38- and Rho kinase-dependent expression of VCAM-1 that mediates the recruitment of monocytes by vascular endothelium associated with the development of atherosclerosis.

Extracellular nucleotides cause a wide range of cellular responses and appear to play a role in the regulation of many vascular functions (1, 2). Vascular cells release nucleotides when exposed to stimuli such as ischemia, hypoxia, and chemical or mechanical stress (3, 4). It also is becoming apparent that extracellular nucleotides can promote the development of a variety of pathologies including disorders of the immune system, and neurodegenerative and vascular diseases (1). Indeed, ATP or UTP induces proliferation and migration of vascular smooth muscle cells, two processes involved in the development of intimal lesions found in atherosclerosis and post-angioplasty restenosis. The biological effects of extracellular nucleotides are mediated through activation of P1 and P2 purinergic receptors. P1 receptors are responsive to adenosine, whereas P2 receptors are activated by a variety of nucleotides including ATP and UTP (5, 6). The P2 receptors are subdivided into two distinct categories, the metabotropic G-protein-coupled (P2Y) receptors and the ionotropic ligand-gated channel (P2X) receptors (6, 7). Vascular cells have been shown to express metabotropic P2Y and ionotropic P2X receptors (5, 8). It has been reported that P2Y2 receptors are up-regulated in cells of rat intimal lesions following balloon angioplasty (9). Our recent studies showed that placement of a silicone collar around the rabbit carotid artery promoted up-regulation of P2Y2 receptors in vascular smooth muscle cells and endothelium (10). Subsequently, local infusion of UTP was shown to stimulate intimal hyperplasia and increase intimal monocyte infiltration (10), suggesting a role for P2Y2 receptors in the recruitment of blood monocytes leading to inflammation in atherosclerosis.

Monocyte recruitment into the vessel wall is a complex process that includes cell rolling, firm attachment, and directed migration. It is now becoming evident that adhesion molecules such as VCAM-1 play an important role in leukocyte adherence to vascular endothelial cells (11, 12). VCAM-1 expression is induced or up-regulated by proinflammatory cytokines such as TNF-α and interleukin 1-β on cellular components of the arterial wall including endothelial cells, smooth muscle cells, and fibroblasts (13–15). ATP and UTP have been shown to induce cell-cell adhesion in a monocyte/macrophage lineage and neutrophil adherence to an endothelial cell monolayer (16, 17), raising the possibility that released ATP and UTP could induce endothelial cell activation by an autocrine/paracrine mechanism to regulate leukocyte adherence.

The purpose of this study was to test the hypothesis that...
extracellular nucleotides stimulate the expression of adhesion molecules on vascular endothelial cells and promote monocyte adherence. Results showed that the P2Y2 receptor agonists UTP or ATP induced the expression of VCAM-1 in human coronary artery endothelial cells (HCAEC). The ability of P2Y2 antisense oligonucleotides to inhibit UTP-induced VCAM-1 expression strongly suggests that this response is mediated via P2Y2 receptors. The effects of UTP were dependent upon release of calcium from intracellular stores and activation of the p38 kinase pathway. Finally, results indicated that nucleotide-induced VCAM-1 expression increased monocyte binding to endothelial cells, supporting the hypothesis that nucleotides released during vascular injury contribute to leukocyte/endothelial cell interactions and promote inflammatory responses involved in the development of atherosclerosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—HCAEC and endothelial cell basal medium-2 (EBM-2) were purchased from Clonetics (Walkerville, MD). U937 cells were obtained from American Type Cell Collection (ATCC), and RPMI 1640 medium was purchased from Invitrogen. Anti-VCAM-1 (P3C4), anti-ICAM-1 (P2A4), and anti-E-selectin (P2F3) antibodies used in blocking studies were purchased from Chemicon International (Temecula, CA). Anti-ICAM-1 (clone G-5), anti-β-actin (rabbit polyclonal), anti-VCAM-1 (c-19), and anti-VCAM-1 (HAE-2z) antibodies were used for Western analysis and fluorescence, respectively, and obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Oregon green-labeled goat anti-rabbit IgG antibody and Hoechst 33258 were purchased from Molecular Probes (Eugene, OR). The polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody was obtained from Cell Signaling (Beverly, MA). The p38 inhibitor SB203580, MEK1/2 inhibitor U0126, and both were purchased from Calbiochem (Indianapolis, IN). DOTAP liposomal reagent was purchased from Roche Diagnostics. Phosphorothioate-modified oligonucleotides to human P2Y2 were synthesized and purified by Integrated DNA Technologies. All other reagents including nucleotides and the PKH2 green fluorescent cell linker were obtained from Sigma, unless otherwise specified.

**Cell Cultures**—HCAEC were cultured in EBM-2 supplemented with vascular endothelial growth factor, human fibroblast growth factor, epidermal growth factor, hydrocortisone, ascorbic acid, insulin-like growth factor, GA 1000, and 5% bovine fetal serum at 37 °C in a humidified atmosphere of 5% CO2. HCAEC were used between the third and fifth passages. Monocyte U937 cells were purchased from RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum. The retroviral vector pLXSN was used for stable expression of the human P2Y2 receptor in human 1321N1 astrocytoma cells, as described previously (18). Briefly, the recombinant P2Y2-pLXSN construct or pLXSN (control) was used to transfect PA317 amphotrophic packaging cells for production of the viral vectors. Then, 1321N1 cells were infected with the viral vectors and cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 1 mg/ml G418 (Invitrogen) for neomycin resistance selection.

**P2Y2 Antisense S-oligonucleotides**—Sequences including translation initiation site were as follows: sense, 5'-GGCGAATGGCACAGACCTGCGCCCTGCGA-3'; antisense, 5'-TCCAGGGGCGACGTCTCGGCGATCGCCGC-3'. The sequences were chosen for uniqueness using the National Center for Biotechnology Information’s Local Alignment Search tool (BLAST). Briefly, HCAEC were incubated with 2 μM P2Y2 sense or antisense S-oligonucleotides for 6 h at 37 °C in EBM-2 medium containing 1.4% (v/v) DOTAP liposomal reagent. Then, fresh EBM-2 medium was added and cells were cultured for an additional 20 h, as previously reported (19). Fluorescein isothiocyanate-conjugated P2Y2 antisense oligonucleotides were used to monitor cellular oligonucleotide uptake by HCAEC.

**Immunofluorescence Staining**—HCAEC were made quiescent by incubation in EBM-2 for 24 h at 37 °C before exposure to UTP. After treatment with UTP (100 μM) or TFN (10 ng/ml) for 8 h at 37 °C, 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 phosphate-buffered saline (PBS), VCAM-1 expression was detected in cells by incubation for 2 h at room temperature with a rabbit polyclonal anti-VCAM-1 (HAE-2z) antibody (1:200 dilution in PBS containing 5% (v/v) normal goat serum), followed by Oregon green-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.

**Binding of U937 Cells to Human Endothelial Cells**—HCAEC were grown to confluence in 24-well plates in EBM-2 supplemented with 5% (v/v) fetal bovine serum, and then the cells were incubated for 24 h at 37 °C in EBM-2 followed by stimulation with 100 μM ADP, ATP, UTP, adenosine, or 2MeS-ATP for an additional 8 h at 37 °C. In some experiments, HCAEC were preincubated with BAPTA/AM (10 μM) for 30 min before the addition of nucleotides. U937 cells were labeled with the green fluorescent dye PKH2, as described previously (20). Labeled U937 cells (106 cells/well) were added to confluent cultures of HCAEC for 1 h at 37 °C. The cells were washed several times in PBS, and adherent U937 cell numbers were determined by microscopy using fluorescin isothiocyanate illumination. In experiments designed to investigate the effect of blocking antibodies on binding of U937 cells, HCAEC were treated with UTP for 8 h at 37 °C and then 10 μg/ml anti-VCAM-1, anti-ICAM-1, or anti-E-selectin antibody was added to HCAEC for 45 min before addition of U937 cells. For each condition, cell numbers were determined in seven different fields of view in three wells.

**Western Blot Analysis**—Equivalent amounts of protein (100 μg) in extracts of HCAEC were subjected to 7.5% (w/v) SDS-PAGE and transferred to nitrocellulose membranes for immunoblottting. Detection of VCAM-1 and ICAM-1 was performed using goat anti-mouse VCAM-1 or goat anti-mouse ICAM-1 monoclonal antibodies (1:1000 dilution), followed by horseradish peroxidase-conjugated anti-goat IgG antibody (1:2000 dilution). For signal normalization, membranes were probed with polyclonal anti-β-actin antibody (1:2000 dilution). A mouse monoclonal p-ERK (E-4) antibody and a rabbit polyclonal anti-p38 MAP kinase antibody were used to analyze ERK1/2 and p38 phosphorylation.

**Statistical Analysis**—Results are represented as mean ± S.E. Statistical analyses were performed by analysis of variance and unpaired Student’s t test (significance, p < 0.05).

**RESULTS**

**ATP and UTP Induce VCAM-1 Expression in Vascular Endothelial Cells**—Incubation of serum-starved HCAEC with UTP or ATP for 8 h caused a dose-dependent increase in the expression of VCAM-1 (Fig. 1, A and B). In contrast, UDP, ADP, 2MeS-ATP, or adenosine had no significant effect even at concentrations as high as 100 μM (not shown). VCAM-1 expression was induced within 2 h of UTP or ATP addition, reached a maximum level within 6 h, and remained elevated through 16 h (Fig. 1, C and D). A significant increase in VCAM-1 expression occurred even when HCAEC were exposed to 100 μM UTP for as little as 30–60 min before the nucleotide agonist was removed (Fig. 1E). Incubation of HCAEC with the enzyme potato acrylamide grade III (2 units/ml) that degrades nucleoside di- and triphosphates inhibited UTP or ATP-induced VCAM-1 expression (not shown). In contrast to VCAM-1 expression, ATP or UTP did not significantly increase ICAM-1 expression in quiescent HCAEC (Fig. 2). These data suggest a selective effect of UTP and ATP on VCAM-1 expression in human endothelial cells. Immunofluorescence staining of quiescent untreated HCAEC detected a low basal level of VCAM-1 expression with a punctate staining (Fig. 3, panel A). In cells treated with UTP (100 μM), there was diffuse VCAM-1 staining over the entire cell surface (Fig. 3, panel B). A similar VCAM-1 staining pattern was observed when cells were treated with 10 ng/ml TNF-α (Fig. 3, panel C).

**Calcium Release from Internal Stores Is Required For UTP-Induced VCAM-1 Expression**—UTP caused an increase in the intracellular free calcium concentration, [Ca2+], in HCAEC, a response that was inhibited by the intracellular calcium chelator BAPTA/AM (not shown). VCAM-1 expression induced by UTP was also inhibited in HCEAC that were preincubated with 10 μM BAPTA/AM for 30 min at 37 °C (Fig. 4). Furthermore, thapsigargin (2 μg/ml), an agent that depletes intracellular calcium stores, also inhibited UTP-induced VCAM-1 expression (Fig. 4), further demonstrating a role for UTP-induced increases in [Ca2+], in the expression of VCAM-1. Thapsigargin treatment had no apparent toxic effect on HCEAC as measured by trypan blue exclusion (not shown).
UTP-induced VCAM-1 Expression Is Mediated by P2Y2 Receptors—Pertussis toxin (100 ng/ml), an inhibitor of Gαi/o proteins, attenuated the effects of UTP on VCAM-1 expression in HCAEC (Fig. 5A). Because the P2Y2 receptor subtype has been shown to be partially sensitive to pertussis toxin (21), we evaluated the effect on UTP-stimulated VCAM-1 expression in HCAEC for inhibition of P2Y2 receptor activity with P2Y2 antisense S-oligonucleotides. Treatment of HCAEC with P2Y2 antisense S-oligonucleotides (2 μM), complementary to the P2Y2 translation initiation region, inhibited UTP-induced VCAM-1 expression by ~50% (Fig. 5, B and C; p < 0.05) compared with cells treated with P2Y2 sense oligonucleotides. In contrast, P2Y2 antisense S-oligonucleotides had no effect on TNF-α-induced VCAM-1 expression in HCAEC (Fig. 5, B and C). To confirm the involvement of P2Y2 receptors in UTP-induced VCAM-1 expression in HCAEC, we stably expressed P2Y2 receptor cDNA in 1321N1 cells that lack endogenous P2 receptors (22). Cells transfected with P2Y2 receptor cDNA and treated with UTP for 8 h at 37 °C expressed VCAM-1, whereas vector-transfected control cells failed to respond to UTP (Fig. 6). Taken together, these results demonstrate that P2Y2 receptors mediate UTP-induced VCAM-1 expression.

Rho Kinase Activation and p38 Phosphorylation Mediate UTP-induced VCAM-1 Expression—The UTP-induced increase in [Ca2+]i mediates mitogen-activated protein kinase phosphorylation in vascular cell types including endothelial cells (23, 24). In HCAEC, UTP (100 μM) caused the rapid phosphorylation of ERK1/2 and p38 that was inhibited by the MEK1/2 inhibitor U0126 (5 μM), whereas the MEK1/2 inhibitor U0126 (5 μM) had no effect on p38 phosphorylation (Fig. 7). Preincubation of HCAEC with 10 μM Y27632 prevented UTP-induced VCAM-1 expression in HCAEC, whereas the MEK1/2 inhibitor U0126 (5 μM) did not inhibit phosphorylation of Rho kinase and p38 (Fig. 7). The rapid phosphorylation of Rho kinase and p38, but not ERK1/2, is involved in the intracellular signaling pathways leading to VCAM-1 expression induced by UTP.

VCAM-1 Expression Correlates with Increased Monocytic Cell Adherence to Endothelial Cells—Stimulation of HCAEC with UTP or ATP (100 μM) for 8 h increased the binding of U937 monocytes to HCAEC by ~4- and ~3.6-fold, respectively, compared with HCAEC incubated in the absence of nucleotides (Fig. 8). In contrast, treatment of HCAEC with UDP, adenosine, or 2MeS-ATP (Fig. 8) or ADP (not shown) did not significantly increase U937 cell adherence to HCAEC. Introduction of BAPTA/AM (10 μM) into HCAEC prior to treatment with
UTP attenuated U937 cell binding to endothelial cells (Fig. 8). Incubation of HCAEC with anti-VCAM-1 antibody, but not anti-ICAM-1 or anti-E-selectin antibody, significantly decreased U937 cell binding to UTP-treated HCAEC (Fig. 8). These antibodies did not affect U937 cell adherence to HCAEC in the absence of UTP (not shown).

**DISCUSSION**

The present study demonstrates that exogenous UTP or ATP stimulate VCAM-1 expression via P2Y2 receptors in HCAEC. It also indicates that the P2Y2 receptor-mediated increase in VCAM-1 expression promotes the adherence of U937 monocytes to HCAEC. These results, for the first time, link P2Y purinergic receptor signaling to the mechanisms controlling the expression of an endothelial cell adhesion molecule involved in monocyte recruitment in early atherosclerosis.

Vascular diseases such as atherosclerosis and post-angioplasty restenosis are initiated by vascular injury. Previous reports demonstrated that large amounts of nucleotides are released into the extracellular milieu in response to vascular stress conditions including ischemia/oxidative stress, flow, and mechanical stretch (3, 4). In addition, high concentrations of extracellular ATP and UTP generated in vivo by platelet aggregation or cell damage that occurs during transmural angioplasty should increase the concentration of nucleotides in the vessel wall. Although the metabolism of extracellular nucleotides by ectonucleotidases plays a role in the regulation of purinergic signaling, recent studies indicate that chronic hypoxic exposure of pulmonary artery endothelial cells can decrease the rate of ecto-nucleotidase activity (26). In addition, it also has been reported that certain pathological conditions, especially those associated with endothelial cell activation, can decrease ATP-diphosphorylase activity (27), suggesting that pathological conditions affecting blood vessels contribute to local elevations in nucleotide concentrations in the vessel wall.

The P2Y2 receptor is up-regulated in activated thymocytes.
(28) and in epithelial cells in response to ligation of the salivary gland duct (29). It is now becoming apparent that P2Y2 receptor up-regulation occurs in various models of vascular injury including balloon denudation of the rat aorta (9) and placement of a silicone collar around the rabbit carotid artery (10). In the latter model, we showed that activation of the P2Y2 receptor by UTP stimulated intimal hyperplasia and induced monocyte/macrophage infiltration into the vessel wall, suggesting a potential role for this receptor in monocyte recruitment by vascular endothelium. The relative equipotency and equiefficacy of UTP and ATP for induction of VCAM-1 expression in HCAEC (Fig. 1) strongly suggests the involvement of P2Y2 receptors. Nonetheless, the ability of antisense, but not sense, P2Y2 oligonucleotides to inhibit UTP-induced VCAM-1 expression (Fig. 5) unambiguously demonstrates the role of P2Y2 receptors.

Stimulation of the P2Y2 receptor in endothelial cells is known to activate a variety of signaling molecules including the mitogen- and stress-activated protein kinases ERK1/2, p38, and c-Jun NH2-terminal kinase, the small GTPase RhoA, and the release of the second messenger Ca2+/H+ into the cytoplasm (23–25). In this study, UTP-induced VCAM-1 expression in HCAEC was prevented by the calcium chelator BAPTA or by depletion of intracellular calcium stores with thapsigargin (Fig. 4), indicating a role for P2Y2 receptor-mediated increases in 

\[ \text{Ca}^{2+}/\text{H}^+ \]i in VCAM-1 expression. UTP-induced VCAM-1 expression also was suppressed by inhibitors of p38 and Rho kinase but not by inhibition of ERK1/2 (Fig. 7), suggesting that p38 and RhoA are involved in the increased expression of VCAM-1 mediated by P2Y2 receptors.

Vascular endothelial growth factor induces VCAM-1, ICAM-1,
and E-selectin expression in endothelial cells through a pathway involving the MEK/ERK-independent activation of NF-κB (30). Activation of the P2Y2 receptor has been reported to increase prostacyclin and nitric oxide release from endothelial cells (31, 32). Studies have shown that nitric oxide modulates the level of VCAM-1 protein expression in endothelial cells (33). Although nitric oxide did not play a role in vascular endothelial growth factor-induced adhesion molecule expression (30), it could potentially modulate the effects of UTP on VCAM-1 expression in endothelial cells. Further studies are needed to identify the factors involved in nucleotide-induced VCAM-1 expression both at the transcripational and translational levels.

Consistent with earlier reports showing that ATP and UTP stimulate cell-cell adhesion in a monocyte/macrophage lineage and enhance neutrophil adherence to endothelial cell monolayers (16), our data demonstrate that ATP and UTP stimulate U937 monocyte adherence to HCAEC (Fig. 8). The P2Y2 receptor contains the integrin binding domain arginine-glycine-aspartic acid (RGD) in its first extracellular loop (21). The RGD motif has been shown to be the core recognition sequence for many integrins including αβ3 and αβ5 (34). Recent studies have indicated that the RGD sequence in the P2Y2 receptor mediates its interaction with αβ3/β5 integrins, and is required for Gαs-mediated signal transduction (21). It has been reported previously that the αβ3 integrin on monocytes mediates their adherence to endothelial and epithelial cells, an early event in the acute inflammatory response (35). Thus, protein complex formation between αβ3/β5 integrins and P2Y2 receptors also may be involved in the regulation of monocyte binding to endothelial cells mediated by P2Y2 receptors.

In summary, this study is the first to demonstrate that extracellular nucleotides and P2Y2 receptor activation can induce VCAM-1 expression in endothelial cells and promote the adherence of monocytes. In combination with our previous studies, these results indicate that P2Y2 receptor up-regulation in vascular cells mediates the nucleotide-stimulated proliferation of smooth muscle cells and the adherence of monocytes to endothelium, responses associated with intimal hyperplasia and inflammation seen in atherosclerosis. These results encourage the development of therapeutic strategies to limit P2Y2 receptor up-regulation and activation as a means to prevent arterial disease.

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