Functional P2Y$_2$ Nucleotide Receptors Mediate Uridine 5’-Triphosphate–Induced Intimal Hyperplasia in Collared Rabbit Carotid Arteries

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Background—Extracellular uridine 5’-triphosphate (UTP) induces mitogenic activation of smooth muscle cells (SMCs) through binding to P2Y$_2$ nucleotide receptors. P2Y$_2$ receptor mRNA is upregulated in intimal lesions of rat aorta, but it is unclear how this G-protein–coupled receptor contributes to development of intimal hyperplasia.

Methods and Results—This study used a silicone collar placed around rabbit carotid arteries to induce vascular injury and intimal thickening. Collar placement caused rapid upregulation of P2Y$_2$ receptor mRNA in medial SMCs before appearance of neointima. Fura-2 digital imaging of single SMCs was used to measure changes in myoplasmic calcium concentration ($Ca_{m}$) in response to P2Y receptor agonists. In contrast to UDP, activation by UTP or adenosine 5’-triphosphate (ATP) greatly increased $Ca_{m}$, which indicates upregulation of functional P2Y$_2$ receptors at which UTP and ATP are equipotent agonists. The number of responsive cells was significantly greater for freshly dispersed SMCs from collared arteries than for controls. Perivascular infusion of UTP (100 $\mu$mol/L) within the collar significantly enhanced neointimal development. Intimas that resulted from UTP exposure were infiltrated by macrophages. Moreover, increased expression of osteopontin occurred in response to in situ application of UTP. ATP or UTP also stimulated osteopontin expression in cultured SMCs in a dose-dependent manner. Furthermore, P2Y$_2$ antisense oligonucleotide inhibited osteopontin expression induced by UTP.

Conclusions—These findings indicate for the first time a role for the UTP/ATP receptor, P2Y$_2$, in development of intimal hyperplasia associated with atherosclerosis and restenosis. (Circulation. 2002;106:2720-2726.)

Key Words: nucleotide ▪ carotid arteries ▪ receptors ▪ restenosis ▪ calcium

Smooth muscle cell (SMC) proliferation and migration play key roles in the development of intimal thickening that accompanies atherosclerosis and restenosis. Many factors contribute to the development of intimal hyperplasia, although the role of extracellular nucleotides remains largely unknown. Studies indicate that extracellular adenosine 5’-triphosphate (ATP) and uridine 5’-triphosphate (UTP) mediate SMC activation and proliferation. Recently, it was shown that UTP induces migration of aortic SMCs via osteopontin expression. Effects of extracellular nucleotides are mediated by P2 receptors: ligand-gated ion channels (P2X) and G-protein–coupled receptors (P2Y). P2Y$_2$ receptors (P2Y$_2$R) are activated equipotently by ATP and UTP, and P2Y$_2$ mRNA is overexpressed in rat intimal thickenings induced by balloon angioplasty, which suggests a role for P2Y$_2$R in arterial intimal lesion development. Organ culture of arteries also elicits functional expression of a UTP receptor. In vitro studies showed that extracellular ATP and UTP are released during platelet aggregation and from vascular or blood cells under mechanical or chemical stresses. Alterations in extracellular nucleotide concentrations in response to in vivo injury have been reported. Because trauma to vascular endothelium in angioplasty should induce focal release of intracellular nucleotides, it is hypothesized that extracellular nucleotides and P2Y$_2$R promote intimal thickening of arteries, although in vivo studies have not addressed this possibility. The aim of this study was to determine whether activation of P2Y$_2$R promotes intimal hyperplasia under conditions that maintain the vascular endothelium. To directly test this hypothesis, a silicone collar was placed around rabbit carotid arteries to enable local delivery of UTP. Results indicated that collar placement caused rapid upregulation of P2Y$_2$R in SMCs. UTP application to collared carotid arteries induced intimal thickening, expression of osteopontin...
(OPN), and an inflammatory-proliferative response characteristic of human atherosclerotic lesions. These studies demonstrate a novel role for P2Y2R in development of arterial intimal lesions.

**Methods**

**Cell Culture**

Rabbit SMCs obtained from normal carotid arteries by enzymatic dissociation were maintained in culture as described for rat aortic SMCs\(^8\) and used from passages 4 to 6. Phosphorothioate-modified oligonucleotides to rabbit P2Y2, were synthesized and purified by Integrated DNA Technologies (ADT). Sequences including translation initiation site were as follows: sense 5'-GGGAAGAGTGCCATTGCCC-3', antisense 5'-TGTCAGGTGATCCTCTTCCCCTACCTTGACA-3'. Sequences were checked for uniqueness with the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST).\(^{14}\) SMCs were incubated with 0.1 or 1 μmol/L P2Y2 sense or antisense S-oligonucleotides for 6 hours in serum-free medium containing 1.4% DOTAP liposomal reagent (Roche Diagnostics). Serum-free medium was added, and cells were cultured for an additional 20 hours. FITC-conjugated P2Y2 antisense and sense oligonucleotides were used to quantify cellular oligonucleotide uptake by SMCs. Cells were rendered quiescent by 24-hour incubation in serum-free medium before stimulation with nucleotides.

**Isolation of SMCs and Single-Cell Fura-2 Digital Imaging**

SMCs (identified by morphological differences from endothelial cells) expressing α-actin were isolated in enzymatic solution and used for single-cell digital imaging, as described previously.\(^8\) Myoelastic free Ca\(^{2+}\) (Ca\(^++\)) levels were measured with the InCa\(^++\) calcium imaging system (Intracellular Imaging Inc). Cells were incubated with 2.5 μmol/L fura-2-acetoxymethyl ester at 37°C for 25 minutes. Fura-2–loaded cells were placed on a coverslip inside a constant-flow superfusion chamber mounted on an inverted epifluorescence microscope (Nikon; model TMD). Fura-2 was excited by 340 and 380 nm of ultraviolet light, and emitted fluorescence (510 nm) was collected by a monochrome CCD camera (COHU; Inc). Data are expressed as a ratio of emitted light intensity at 340 and 380 nm. SMCs were continually superfused with physiological salt solution (PSS) containing (mmol/L) NaCl 140, KCl 5, CaCl2 2, MgCl2 1, HEPES 10, glucose 10, pH 7.4. Caffeine (1,3,7-trimethylxanthine) and 10, glucose 10, pH 7.4. Depolarizing solution (80K) was prepared with SigmaStat (Jandel Scientific Software). Significance, \(P<0.05\).

**Data Analysis**

Intimas were defined as regions between luminal endothelium and the center of internal elastic laminae. Cross-sectional areas of intima and media were measured at 400× magnification with computer-assisted color image analysis (PC-image Color; Foster Findlay Associates). Four sections were analyzed for each artery, and intimal and medial thicknesses at 20 random sites per section were averaged. Data are expressed as mean±SEM (n=number of arteries). Differences between collateral arteries that received UTP or PBS and controls (sham-operated arteries or segments proximal to collars) were evaluated by ANOVA and Student-Newman-Keuls test (significance, \(P<0.05\)).

**RNA Extraction and RT-PCR**

Isolation of RNA, cDNA synthesis, and PCR were performed as described previously.\(^{14}\) PCR was performed with rabbit P2Y2, mRNA oligonucleotide primers (Seye C, Weisman G; Oryctologus Cuniculus P2Y2 receptor partial sequence; GenBank No. AF510345): sense 5'-CATGTGGGCCATGAGGTCCACCAC-3' and antisense 5'-GCACGCTGTCTGTGAAAGGAAG-3'. Primers for human G3PDH were as follows: sense 5'-ACCTGGAAAGCCTCCACCA-3' and antisense 5'-GCACGCTGTCTGTGAAAGGAAG-3'. Primers for human G3PDH were as follows: sense 5'-ACCTGGAAAGCCTCCACCA-3' and antisense 5'-GCACGCTGTCTGTGAAAGGAAG-3'. Thirty-five amplification cycles were used, with annealing temperatures of 60°C for P2Y2, 55°C for P2Y1, and 55°C to 62°C for P2Y6. For G3PDH amplification, 25 cycles were used, with 55°C for annealing. PCR products were resolved by 2% agarose gel electrophoresis, and P2Y/G3PDH ratios were determined by densitometry.

**Collar Implantation**

Protocols conformed to Animal Care and Use guidelines of the University of Missouri-Columbia. New Zealand White rabbits (42 animals) were anesthetized subcutaneously with ketamine (33 mg/kg body weight) and xylazine (7 mg/kg body weight). Rabbids were maintained under respiration with oxygen and 0.2% isoflurane. A nonocclusive, biologically inert, flexible silicone collar (20 mm long; inlet/outlet diameter 1.8 mm; Silicone MED-4211, Nusil Technology) was placed around a carotid artery, and the contralateral artery was sham operated, ie, isolated from surrounding connective tissue and exposed to similar stretch. Arteries were collected at day 3 (n=12), 7 (n=14), or 14 (n=14). Two collars per animal were implanted in another series of experiments, and each was connected to an osmotic minipump (Alzet 2 ML2; Alza Corporation) implanted subdermally in the thoracic region. The pump continuously delivered (5 μL · h\(^{-1}\)) UTP (100 μmol/L) or PBS (mmol/L): NaCl 154, NaHPO\(_4\) 8, NaH\(_2\)PO\(_4\) 2, EDTA 0.2) for 3 (n=14), 7 (n=14), and 14 (n=14) days. After surgery, analgesic (Buprenex; 0.1 to 0.5 mg/kg body weight) was administered.

**Immunohistochemistry and In Situ Hybridization**

Rings cut from collar-wrapped or proximal and distal vessel segments outside controls (controls) were formalin fixed, paraffin embedded, or snap-frozen in liquid nitrogen for immunohistochemistry, RNA extraction, or Western analysis. Staining with hematoxylin/eosin was performed. Immunohistochemical detection of SMCs (α-actin; Sigma) and macrophages (CD68; Dako) was performed with monoclonal antibodies visualized by the indirect peroxidase-antibody conjugate technique. To generate P2Y2 riboprobes for in situ hybridization, a T7 promoter adapter was ligated to the 350-bp P2Y2 fragment amplified by reverse transcription–polymerase chain reaction (RT-PCR) with the no-cloning promoter addition kit (Ambion). Antisense and sense riboprobes were obtained by in vitro transcription with digoxigenin-UTP using DIG RNA labeling mix (Roche). Hybridization was performed overnight at 50°C in a humidified chamber, as described previously.\(^8\)

**Immunoblotting**

Cultured cells were solubilized in 2X Laemmli sample buffer (120 μmol/L Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 1 mmol/L EDTA, 50 mmol/L dithiothreitol, 0.003% bromophenol blue). Rabbit aortas were homogenized with a polytron homogenizer in buffer containing 0.9% NaCl, 20 mmol/L Tris-HCl, pH 7.6, 1 mmol/L PMSF in 0.2% Triton X-100. Equivalent amounts of protein (100 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Detection of OPN (Chem-
con International) was performed with rat anti-human OPN (1:1000 dilution) and horse radish-peroxidase–conjugated goat anti-rat IgG (1:1500 dilution) as primary and secondary antibodies, respectively. For signal normalization, membranes were reprobed with anti-mouse β/H9251-tubulin antibody (1:1000 dilution; Santa Cruz Biotechnologies).

Results

Upregulation of Functional P2Y2R in Collared Arteries

We examined changes in P2Y2, P2Y4, and P2Y6 mRNA expression in carotid media ex vivo before and after collar implantation. By RT-PCR, P2Y2 transcript was weakly amplified in control arteries and strongly amplified in collared arteries within 3, 7, and 14 days of surgery (Figure 1A). Cultured SMCs expressed high levels of P2Y2 mRNA (Figure 1A). In contrast, P2Y4 mRNA was equivalently expressed in sham-operated and collared arteries or cultured SMCs (Figure 1A), as reported for rat aortic SMC cultures.17 When several oligonucleotide primers were used, the P2Y6 transcript was not detected in carotid arteries or cultured SMCs (Figure 1A), as reported for rat aortic SMC cultures.17 In situ hybridization with sham-operated carotid arteries (a), and collared arteries at day 3 (b) and day 14 (c) were hybridized with antisense P2Y2 riboprobe (see Methods). Arrowheads indicate endothelial cells. Arrows indicate internal elastic lamina. Magnification ×400.

Single-cell calcium measurements confirmed that newly synthesized P2Y2Rs were functional. SMCs from control and collared (3-day) arteries were exposed to 80 mmol/L KCl to induce Ca2+ influx before treatment with UTP, ATP, or UDP (10 μmol/L) for 4 minutes followed by 3 minutes’ exposure to 5 mmol/L caffeine to release intracellular calcium stores.9,18 Figure 2 shows a typical fura-2 tracing for UTP with control and collared arteries. Percentages of cells responding to agonist in control arteries (Figure 3a) were as follows: UTP, 39% (23/59); ATP, 33% (17/52); and UDP, 2.5% (1/40). In these cells, Cam increased 0.35±0.05, 0.23±0.04, and 0.11±0.02 ratio units above baseline for UTP, ATP, and UDP, respectively (Figure 3b). In contrast, 71% (45/63) and 93% (50/54) of SMCs isolated from collared arteries responded to UTP and ATP, respectively (Figure 3b), with significant increases in Cam (0.62±0.07 and 0.72±0.09 ratio units, respectively) occurring with UTP and ATP, whereas UDP had no significant effect (Figure 3a). A responsiveness index was calculated to include percentages of responding cells and amplitude of responses (Figure 3c) for a more integrative analysis. These results indicate functional upregulation of P2Y2Rs in SMCs of collared arteries. The percentage of SMCs responding to 5 mmol/L caffeine (100%) was consistent in all samples, and amplitudes of caffeine-induced Ca2+ transients were similar between control (0.93±0.06) and collared (0.75±0.04) arteries, which indicates that intracellular Ca2+ stores were relatively equivalent and supports the functional coupling of P2Y2Rs to intracellular Ca2+ stores. Preexposure of SMCs to caffeine abolished the Ca2+ response to 10 μmol/L UTP or ATP (not shown), which confirms that these agonists act at caffeine-sensitive Ca2+ stores, as already reported.9
Effect of Local UTP Application

Intimal Thickening

Collar positioning for 14 days resulted in discrete intimal thickening that encompassed the entire inner surface of vessels (Figure 4), reaching 37.5±0.8 μm (n=14 arteries), whereas a continuous layer of anti-CD31–stained endothelial cells was maintained (not shown). No neointimas were apparent in sham-operated arteries (n=14). Control vessel segments proximal or distal to collars had normal appearance. Continuous delivery of UTP to vessel segments within collars strongly increased neointimal development by 7-fold (Figure 4), reaching 256±48 μm (n=14 arteries). In contrast, saline infusion (Figure 4) did not significantly increase intimal thickness above collaring alone (39.3±11 μm; n=14 arteries; ANOVA P<0.05).

Cell Composition

Collared arteries displayed discrete intimas composed of SMCs, as described previously.19–21 At day 14, macrophages were scattered throughout neointima, representing 2.1±0.1% of total cells (Figure 5A). Enlarged intimas evoked by UTP showed significant macrophage infiltration (12±0.9% of total cells) distributed in the upper neointima beneath the endothelium (Figure 5, A and B). Local saline delivery did not increase intimal macrophage numbers compared with collared arteries without infusion. A few macrophages were occasionally detected in adventitia of collared arteries. However, macrophage infiltration in adventitia was not increased by UTP or saline (Figure 5, A and B). Immunohistochemical staining for CD43 detected no lymphocytes in any collared segments (not shown).

OPN Expression

Immunoblots of sham-operated arteries indicated lack of OPN (Figure 6A, lane 5), whereas collared arteries at day 14 expressed OPN (Figure 6A, lane 2). Local UTP infusion greatly increased OPN expression (Figure 6A, lane 4) compared with PBS (Figure 6A, lane 3). OPN was not expressed at day 3 in sham-operated controls (Figure 6B, a) or collared arteries with (Figure 6B, b) or without (not shown) saline infusion. In contrast, OPN expression was increased in medial SMCs by local UTP application (Figure 6B, c) at day 3 after collaring before intimal thickening. At days 7 and 14, OPN was detected in intimal SMCs of collared arteries treated with saline (Figure 6B, c and d), whereas further increases in OPN expression were detected in intimal and medial SMCs of UTP-treated collared arteries (Figure 6B, f and g). SMCs and macrophages closest to the lumen showed the strongest OPN reactivity.

UTP-Induced OPN Expression Is Mediated by P2Y2Rs in SMCs

UTP exposure for 8 hours stimulated OPN expression in vascular SMCs (Figure 7A) in a dose-dependent manner (Figure 7B). ATP and 10% FBS (positive control) induced OPN expression, whereas ADP, 2-MeSATP, and UDP did not (Figure 7A), which suggests that P2Y2 Rs mediate OPN expression. To conclusively demonstrate the role of P2Y2 Rs, SMCs incubated with 0.1 or 1 μg of P2Y2 antisense or sense oligonucleotides were stimulated with 50 μmol/L UTP for 8 hours. Antisense (Figure 7C, lanes 5 and 6) but not sense (Figure 7C, lanes 2 and 3) P2Y2 oligonucleotides inhibited UTP-induced OPN expression in SMCs.

Discussion

Novel findings in this study demonstrate striking increases in P2Y2 mRNA in collared carotid arteries before intimal thickening. This temporal relationship provides strong evidence for the causal role of P2Y2R in development of intimal hyperplasia, compared with simple correlations obtained between severity of diabetes/atherosclerosis in diabetic, dyslipidemic pigs and functional expression of UTP-sensitive receptors.22 Collaring of rabbit carotid arteries induces discrete medial injury that leads to SMC activation.19–21 Previous studies indicated rapid upregulation of P2Y2 mRNA in
activated thymocytes, an immediate-early gene response. P2Y2Rs also are upregulated on ligation of salivary gland ducts. P2Y2Rs are activated equipotently by ATP and UTP, and the present results showed that activation by ATP or UTP strongly increased Ca\textsubscript{m} in SMCs of collared arteries. The pharmacological potency profile of upregulated P2Y receptors in rabbit carotid arteries does not resemble that of other uridine nucleotide receptors. The P2Y\textsubscript{4} receptor is activated preferentially by UTP, whereas P2Y\textsubscript{6} receptors have greater sensitivity to UDP than UTP. UDP is also a P2Y\textsubscript{4} receptor agonist, although its potency relevant to UTP varies in different studies. We found that very few SMCs responded to UDP, and SMCs from collared and control arteries were equally unresponsive to increases in Ca\textsubscript{m} caused by UDP, which rules out any significant contribution from functional P2Y\textsubscript{4} or P2Y\textsubscript{6} receptors. In addition, SMCs of control and collared arteries showed similar levels of P2Y\textsubscript{4} mRNA. These data strongly indicate that P2Y\textsubscript{2}Rs alone are functionally upregulated in collared arteries.

P2Y\textsubscript{2} R upregulation may relate to the phenotypic status of cells. SMCs of collared carotid arteries display an activated synthetic phenotype by electron microscopy and resemble SMCs of human atherosclerotic plaques. Partially dedifferentiated SMCs of angioplasty-induced rat aortic lesions express high levels of P2Y\textsubscript{2} mRNA, as do cultured SMCs that undergo dedifferentiation. Moreover, medial SMCs of rat embryo exhibit high levels of P2Y\textsubscript{2} expression similar to collared arteries. Finally, organ-cultured coronary SMCs display upregulation of UTP-preferring receptors associated with dedifferentiation and increased DNA synthesis. Thus, it is very likely that upregulation of P2Y\textsubscript{2}Rs in collared arteries is associated with SMC dedifferentiation. Increased P2Y\textsubscript{2} R expression may be sufficient to enhance local effects of extracellular nucleotides on SMC proliferation, thereby contributing to intimal thickening. Importantly, upregulation of P2Y\textsubscript{2}Rs is much greater in SMCs than in endothelium, which should promote SMC proliferation and migration to a greater extent than P2Y\textsubscript{2} R-mediated release of endothelium-derived relaxing factors that could counteract hyperplasia.

Reports of effects of extracellular nucleotides in vivo are limited. Because cellular ATP and UTP are presumably released during angioplasty, we examined whether activation of P2Y\textsubscript{2}Rs contributed to development of intimal hyperplasia. Intimal thickening of collared carotid arteries was greatly enhanced by in situ UTP application and was closely associated with OPN expression in medial SMCs by day 3, when neointimas were not apparent. OPN is chemotactic for SMCs and is associated with arterial SMC proliferation. The present data suggest a role for OPN in proliferation and migration of SMCs, processes involved in intimal thickening. Furthermore, UTP and ATP increased OPN expression in cultured SMCs, whereas ADP, UDP, and 2-MeSATP were ineffective, which suggests a role for P2Y\textsubscript{2} in which ATP and UTP are equipotent. Direct evidence for involvement of P2Y\textsubscript{2}R is provided by inhibition by P2Y\textsubscript{2} antisense oligonucleotides of UTP-induced OPN expression in cultured SMCs. Furthermore, UTP induced OPN expression in P2Y\textsubscript{2}-transfected human 1321N1 astrocytoma cells (not shown).

Figure 3. Percentage of responding cells and Ca\textsubscript{m} responses to nucleotides. SMCs from normal and collared arteries (day 3) were exposed to 80 mmol/L KCl for 3 minutes followed by washout (3 minutes) and treatment with 10 μmol/L UTP, ATP or UDP. a, Responding cells (%), defined as cells with responses ≥3 SD above baseline for 5% of agonist exposure time. Only responding cells were used. b, Increase in Ca\textsubscript{m} response above baseline. Only responding cells were used. c, Responsiveness index (% responding cells × amplitude of response × 100).
P2Y,Rs interact with α,β3-integrin and anti-α,β3-integrin antibodies inhibited UTP-induced migration of vascular SMCs associated with OPN expression. Recent reports indicate that UTP-induced SMC migration in vitro is mediated by P2Y,Rs. A hypothesis is that arterial injury caused by silicone collar placement induces P2Y,R expression, whereupon receptor activation increases OPN expression and activates α,β3-integrin to promote SMC migration in arteries.

Local UTP delivery induced intimal accumulation of macrophages similar to oxidized LDL. Macrophage distribution indicated that inflammatory responses induced by UTP were localized to neointima. Although mechanisms of UTP-induced monocyte/macrophage infiltration into intima remain unclear, leukocyte migration depends on activities of adhesion proteins (eg, selectins and integrins) on leukocytes and vascular endothelial cells. Preliminary data (C.I.S., unpublished results, 2001) indicate that UTP stimulates expression of vascular cell adhesion molecule-1 in human endothelial cells. Nucleotides also regulate leukocyte adhesion to endothelium, which suggests a role for UTP in early stages of leukocyte infiltration.

It is difficult to estimate locally effective concentrations of agonists in vivo. A tissue-free space corresponding to 100 μL is available inside the carotid artery collar, and with delivery of 100 μmol/L UTP at 5 μL/h, UTP concentrations of ~5 μmol/L should be maintained around carotid arteries. However, disappearance of UTP through hydrolysis and lymphatic...
Figure 7. Nucleotide-induced OPN expression in SMC cultures. Cells were stimulated with 10% FBS or (A) 50 μM nucleotide or (B) a nucleotide receptor agonist. After cell lysis, immunoblot analysis was performed with anti-OPN antibodies. Data represent 3 (A) or 5 (B) experiments. C, P2Y2R agonist-mediated OPN expression in SMCs. SMCs incubated with sense or antisense S-oligonucleotides for 6 hours in DMEM containing 1.4% DOTAP liposomal reagent were cultured for an additional 16 hours in serum-free medium before 50 μM UTP treatment. Cells were incubated with serum-free medium (lane 1); 0.1 (lane 2) or 1 (lane 3) μg of sense oligonucleotide; serum (lane 4); or 0.1 (lane 5) or 1 (lane 6) μg of antisense oligonucleotide.

drainage makes estimations suspect. Continuous periternal delivery of UTP within collars should enable UTP to reach SMCs in outer and inner medial layers. UTP (1 to 100 μM/L) can stimulate various cellular events, e.g., proliferation and migration of vascular cells.3–6 Although these concentrations appear high compared with reported circulating levels, such concentrations might be locally achieved in vivo on cell lysis that occurs during transmural angioplasty or atherosclerosis. Thus, UTP concentrations used in the present study may be relevant to in vivo arterial injury.

In conclusion, these studies demonstrate for the first time that extracellular UTP and P2Y2R contribute to development of arterial lesions associated with atherosclerosis and restenosis.

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