Regulation of Functionally Active P2Y12 ADP Receptors by Thrombin in Human Smooth Muscle Cells and the Presence of P2Y12 in Carotid Artery Lesions*

Bernhard H. Rauch, Anke C. Rosenkranz, Swen Ermler, Andreas Böhm, Julia Driessen, Jens W. Fischer, Atsuhiro Sugidachi, Joseph A. Jakubowski, Karsten Schrör

Objective—The platelet P2Y12 ADP receptor is a well-known target of thienopyridine-type antiplatelet drugs. This study is the first to describe increased transcriptional expression of a functionally active P2Y12 in response to thrombin in human vascular smooth muscle cells (SMC).

Methods and Results—On exposure to thrombin, P2Y12 mRNA was transiently increased, whereas total protein and cell surface expression of P2Y12 were markedly increased within 6 hours and remained elevated over 24 hours. This effect was mediated by activation of nuclear factor κB. Preincubation with thrombin significantly enhanced the efficacy of the P2Y receptor agonist 2-methylthio-ADP to induce interleukin 6 expression and SMC mitogenesis. Effects induced by 2-methylthio-ADP were prevented by RNA interference-mediated knockdown of P2Y12 and a selective P2Y12-antagonist R-138727, the active metabolite of prasugrel. In addition, positive P2Y12 immunostaining was shown in SMC of human carotid artery plaques and was found to colocalize with tissue factor, the rate-limiting factor of thrombin formation in vivo.

Conclusion—These data suggest that the P2Y12 receptor not only is central to ADP-induced platelet activation but also may mediate platelet-independent responses, specifically under conditions of enhanced thrombin formation, such as local vessel injury and atherosclerotic plaque rupture. (Arterioscler Thromb Vasc Biol. 2010;30:2434-2442.)

Key Words: atherosclerosis ■ thrombin ■ P2Y12 receptor ■ inflammation ■ vascular smooth muscle

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P2Y12 ADP receptor antagonists, such as the thienopyridines, are widely used as antiplatelet drugs.1,2 Besides their role as inhibitors of platelet aggregation, there is increasing evidence that these compounds also exert antiinflammatory actions. Reduced plasma levels of inflammatory markers, such as CD40 ligand, C-reactive protein, P-selectin, and platelet-leukocyte aggregates, have been shown with P2Y12 antagonists in atherothrombotic patients,3 and platelet P2Y12 has also been shown recently to influence vascular wall responses to injury and thrombosis.4 Thus far, these effects have been attributed solely to the antplatelet action and have led to the concept that antplatelet agents exert their antiinflammatory actions via platelet-related mechanisms.3

The ADP receptor P2Y12 was originally identified in platelets and in the brain.6 It is a member of the P2 receptor family, which consists of the ion-channel P2X and the G-protein-coupled P2Y receptors.7 P2 receptors are activated by the adenine nucleotides ATP and ADP, which can be released from platelets, endothelial cells, sympathetic nerve terminals, and immune cells.8 P2 receptors have been shown to be involved in a variety of inflammatory processes, such as allergen-driven lung inflammation.9 In addition, the P2Y12 receptor has been associated with enhanced cell growth in certain brain tumors.10 Recently, P2Y12 was found to be expressed in vascular smooth muscle cells (SMC),11 and a role in vessel contraction was suggested.12 However, no functional changes were seen after thienopyridine treatment.12 Whether P2Y12 is involved in inflammatory events in the atherosclerotic vessel wall and whether P2Y12 antagonists may exert direct antiinflammatory actions at injured sites independent of their antplatelet effects is unknown.

Here we report for the first time that the coagulation protease thrombin, which is generated in vessel injury, specifically at the surface of atherosclerotic plaques after exposure of tissue factor (TF),13 increases expression of P2Y12 in human vascular SMC and that this results in an enhanced proinflammatory and mitogenic response to ADP.

Methods

See the supplemental materials, available online at http://atvb.ahajournals.org for a detailed description of the methods.

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Cell Culture
Human saphenous vein and aortic SMC were isolated by the explant technique and cultured as described previously. Subconfluent cells at passages 4 to 9 were serum deprived for 48 hours before the experiments.

Semiquantitative and Quantitative Polymerase Chain Reaction
Isolation of mRNA, semiquantitative reverse transcription polymerase chain reaction (PCR), synthesis of cDNA, and quantitative PCR were performed as described. Real-time PCR assays for P2Y12 (TaqMan) and interleukin 6 (IL-6; SYBR Green) mRNA were performed on a Real-Time PCR System 7300 (Applied Biosystems). Data were normalized to GAPDH.

Immunoblotting
Immunoblotting was performed as described, with some modifications.

Flow Cytometry
Flow cytometry was performed as described previously, with minor modifications.

Immunofluorescence Microscopy
Immunocytochemistry was performed as described, with some modifications.

Immunohistochemistry
P2Y12, M-actin, and TF were detected in human carotid artery plaques. The study was approved by the local ethics committee. Primary antibodies were visualized by streptavidin/horseradish peroxidase–conjugated secondary antibodies. Nonspecific isotype-matched IgG was used to control for nonspecific staining.

cAMP Assay
Intracellular cAMP was determined by radioimmunoassay as described.

Chromatin Immunoprecipitation Assay
After fixation and cross-linking, nuclear factor κB (NF-κB) p65 was immunoprecipitated from cell lysates. From the precipitated DNA, the P2Y12 promoter region containing the NF-κB binding site was amplified by PCR. Products were resolved on ethidium bromide–containing agarose gels.

Figure 1. Thrombin induces expression of P2Y12 in human vascular SMC. A, P2Y12 mRNA was determined by TaqMan quantitative PCR after 1 hour of incubation with thrombin (30 nmol/L) or specific AP for PAR-1, -3, and -4 (AP1, AP3, and AP4, respectively; 100 μmol/L each). con indicates control. B, P2Y12 total protein was determined by immunoblotting using an antibody to the intracellular domain of P2Y12. SMC were incubated with thrombin for the indicated times. β-Actin was used as loading control. C, Cell surface expression of P2Y12 was determined by flow cytometry. Shown is a representative histogram illustrating isotype control, unstimulated expression level of P2Y12, and level after exposure to thrombin (30 nmol/L) for 6 hours. D, Time-course of P2Y12 cell surface expression after incubation with thrombin for the indicated times. Data are mean±SEM expressed as relative change to untreated control; n=6 (A) to 8 (B and D) experiments; *P<0.05 versus untreated controls.
Protein levels of IL-6 were determined by a commercially available ELISA kit (eBiosciences), which was used according to the manufacturer’s instructions.

**DNA Synthesis and Proliferation**
DNA synthesis was detected by incorporation of 3H-labeled thymidine as described.14,15 Cell proliferation was determined by cell counting.

**Statistical Analysis**
Statistical analysis was performed using 1-way ANOVA with the post hoc Bonferroni multiple comparisons test. \( P < 0.05 \) was considered significant.

**Results**

**IL-6 ELISA**
Protein levels of IL-6 were determined by a commercially available ELISA kit (eBiosciences), which was used according to the manufacturer’s instructions.

**DNA Synthesis and Proliferation**
DNA synthesis was detected by incorporation of 3H-labeled thymidine as described.14,15 Cell proliferation was determined by cell counting.

**Statistical Analysis**
Statistical analysis was performed using 1-way ANOVA with the post hoc Bonferroni multiple comparisons test. \( P < 0.05 \) was considered significant.

**Thrombin Induces Expression of P2Y12 in Human Vascular SMC**
Incubation of cultured human saphenous vein SMC with thrombin (30 nmol/L) transiently increased expression of P2Y12 mRNA, maximally at 1 hour (Supplemental Figure IA). SMC exposure to specific activating peptides (AP) (100 μmol/L each) for 1 hour showed that the thrombin response was mimicked by AP1 and AP3, AP1 showing the most significant effect (Figure 1A). Thrombin-induced expression of P2Y12 mRNA was associated with increased expression of total P2Y12 protein after 6 hours of stimulation, and this effect was maintained over 24 hours (Figure 1B). Immunoblotting was performed with 2 different antibodies to P2Y12, which detected either an extracellular epitope of the P2Y12 receptor (Supplemental Figure IB) or its intracellular domain (Figure 1B). Induction of P2Y12 expression by thrombin was also observed in human aortic SMC (Supplemental Figure IC). Consistent with the immunoblotting data, thrombin also caused an increased expression of P2Y12 at the cell surface (Figure 1C and 1D). Induction of P2Y12 expression by thrombin was further validated by immunofluorescence microscopy. A significant and time-dependent increase in P2Y12 immunostaining was observed in thrombin-stimulated SMC after 6 and 24 hours (Figure 2). In comparison, no expression of P2Y12 mRNA was detected in endothelial cells isolated from human saphenous veins (not shown), suggesting that this action was restricted to vascular SMC.

**P2Y12 Is Expressed in Human Carotid Artery Plaques**
To determine whether P2Y12 is also expressed in human SMC in vivo and to assess its potential role in vascular function, carotid artery plaques from patients undergoing carotid artery thrombectomy were analyzed for P2Y12 expression. In comparison with an isotype control (Figure 3A and 3B), P2Y12-specific immunostaining was consistently detected in atherosclerotic tissue from 10 different patients (Figure 3C and 3D). In Western blots, the same antibody detected multiple bands in human SMC and platelet lysates, which may correspond to differently glycosylated forms of the receptor (Supplemental Figure IIA).17 The specificity of the antibody used was confirmed by gene-specific knockdown of expression of P2Y12, using small interfering RNA (Supplemental Figure III). Interestingly, P2Y12 expression was particularly pronounced at sites of plaque rupture (Supplemental Figure IIB to IIG) and colocalized with M-actin, indicating association with SMC (Figure 3E and 3F). Because initiation of thrombin generation is strictly dependent on availability of TF and increased TF expression has been observed in atherosclerotic plaques,18 human carotid artery plaque tissue was also stained for TF (Figure 3G and 3H). TF expression was evident at SMC-rich areas and colocalized with M-actin (Supplemental Figure IV). In contrast, no staining was observed for [Image 129x259 to 489x484]
the platelet marker CD42 in P2Y12-positive plaque areas, indicating that the P2Y12 protein detected was not from platelets (Supplemental Figure V) and suggesting TF bearing SMC as the major source of thrombin-induced P2Y12 upregulation.

Thrombin Regulates Expression of P2Y12 via Activation of NF-κB

An analysis of the P2Y12 gene promoter region using BLAST (Basic Local Alignment Search Tool) and TRANSFAC...
(Transcription Factor Database) identified one binding site for the transcription factor NF-κB, a well-known inflammatory mediator. We have previously shown that thrombin activates NF-κB in SMC, suggesting that thrombin-induced transcriptional upregulation of P2Y12 might involve NF-κB activation. This hypothesis was evaluated by chromatin immunoprecipitation assay using antibodies to the NF-κB p65 subunit. Incubation with thrombin for 20 minutes induced specific binding of NF-κB to the P2Y12 promoter region containing the NF-κB binding motif (Figure 4A). In addition, a specific inhibitor of NF-κB activation (NF-κB-I) suppressed thrombin- and AP1-induced upregulation (Figure 4B), as well as cell surface expression (Figure 4C) of the P2Y12 protein. These data suggest that thrombin-induced transcription of P2Y12 in human SMC requires activation of NF-κB.

Thrombin-Induced P2Y12 Modulates cAMP Levels in Human SMC

Next, we determined whether thrombin-induced P2Y12 expression also reduces cAMP levels in human SMC via activation of Gi. To mimic the action of endogenous prostaglandin, which is assumed to contribute to the antiplatelet effects of P2Y12 inhibition in vivo, intracellular cAMP was increased by stimulation with the prostacyclin analog iloprost (1 nmol/L). Only in cells that had been pretreated with thrombin for at least 6 hours did the P2Y selective agonist 2-methylthio-ADP (2-MeSADP) significantly reduce cAMP levels. This effect was prevented by the specific P2Y12 antagonist R-138727 (Figure 5A), suggesting Gi-mediated inhibition of cAMP levels after upregulation of P2Y12 by thrombin.

Thrombin-Induced P2Y12 Enhances IL-6 Expression in Human SMC

To determine a possible proinflammatory function of the P2Y12 receptor in human SMC, we investigated whether increased expression of P2Y12 by thrombin influences transcription of IL-6. Expression of IL-6 mRNA was markedly increased when cells were pretreated with thrombin for 6 hours before stimulation with the P2Y receptor agonist 2-MeSADP (Figure 5B). This effect was observed at 30 minutes and 3 hours of 2-MeSADP stimulation but not at 1 hour, suggesting a possible biphasic response on IL-6 mRNA expression. The P2Y12 antagonist R-138727 prevented the additional increase in IL-6 mRNA expression induced by 2-MeSADP in thrombin-pretreated SMC (Figure 5C). In cells that had not been pretreated with thrombin before stimulation with 2-MeSADP, R-138727 was ineffective (Figure 5C). To determine whether the observed effects at the level of IL-6 mRNA were also translated to an enhanced IL-6 protein expression, IL-6 protein was determined in the medium. Thrombin increased the levels of IL-6 in the incubation medium. This effect was markedly enhanced by 2-MeSADP after pretreatment with thrombin for 6 hours and inhibited by R-138727 (Figure 5D). When 2-MeSADP was added together with thrombin, no additive effect was seen (not shown). These data collectively suggest that thrombin-induced inflammatory responses in human SMC, such as induction of IL-6 expression and secretion, involve ADP-dependent signaling through upregulation of P2Y12.

Thrombin-Regulated P2Y12 Enhances Proliferation of Human SMC

Another important function of thrombin in tissue repair is stimulation of cell proliferation. To study a functional involvement of thrombin-induced P2Y12 expression in SMC mitogenesis, DNA synthesis was determined by incubation of SMC with [3H]thymidine. In cells not preexposed to throm-
bin, 2-MeSADP added simultaneously with thrombin did not modify mitogenic responses (Figure 6A). However, DNA synthesis was significantly enhanced when 2-MeSADP was added after preincubation of cells with thrombin for 6 hours (Figure 6B). This 2-MeSADP-induced increase in mitogenesis was prevented by the P2Y12 antagonist R-138727, which did not affect SMC mitogenesis by itself (Figure 6A and 6B). A similar inhibitory effect was seen with another selective P2Y receptor antagonist, 2-Methylthio-AMP23 (Supplemental Figure VIA). The increase in DNA synthesis after incubation of thrombin-pretreated cells with 2-MeSADP also resulted in an increased cell number after 4 days of incubation (Figure 6C). In addition to the selective inhibitors R-138727 and 2-Methylthio-AMP, gene-specific knockdown of P2Y12 expression confirmed that the effect of 2-MeSADP was P2Y12 mediated (Supplemental Figure VIB). These data suggest that a thrombin-induced increase in P2Y12 receptor expression enhances the proliferation of human vascular SMC.

**Discussion**

The P2Y12 receptor is recognized as a platelet ADP receptor that mediates platelet secretion and aggregation. Its contribution to thrombus formation has been appreciated by the efficacy of P2Y12 antagonists in preventing platelet clot formation on vascular injury. However, novel studies have suggested an involvement of platelet P2Y12 in the development of atherosclerosis.4 In addition, systemic antiinflammatory effects of antiplatelet therapy have been described.5 The data presented here suggest for the first time that P2Y12 not only is important for platelet activation but also directly mediates proinflammatory and atherogenic actions in the vessel wall that are platelet independent.
Exposure of cultured human vascular SMC to thrombin resulted in an increased expression of P2Y12 mRNA, total P2Y12 cellular protein, and cell surface expression. Thrombin-induced proinflammatory and mitogenic responses in vascular cells are mediated by activation of the G protein–coupled protease-activated receptor (PAR)-1, PAR-3, and PAR-4.14,15,24–27 Our data indicate that predominantly the prototypic thrombin receptor PAR-1 is involved. Immunoblot analysis confirmed a marked 2- to 3-fold upregulation of P2Y12 after 6 hours of stimulation with thrombin, which was maintained over 24 hours. Identical results were obtained with 2 different antibodies to P2Y12, detecting either an extracellular or an intracellular epitope of P2Y12. These findings in human saphenous vein SMC were confirmed in human aortic SMC, suggesting that both arterial and venous SMC are capable of increased P2Y12 expression after thrombin stimulation.

Human carotid artery plaques from patients undergoing carotid artery thrombectomy were analyzed to determine the expression of P2Y12 in vivo. This suggests TF bearing SMC as the source and target of thrombin-induced P2Y12 expression. Immunostaining for CD42 and CD68 (not shown), excluding the possibility that the observed P2Y12 expression was from other sources, such as platelets or macrophages. Interestingly, P2Y12 expression appeared to be focused locally to areas of tissue injury. For example, as shown in Figure 3E and 3F, intense P2Y12 signals were seen in the vicinity of a necrotic plaque core, whereas Supplemental Figure II shows locally restricted P2Y12 expression in a lesion area possibly prone to rupture. Such increased local P2Y12 expression could be due to increased local thrombin formation at unstable plaque sites. In agreement with this hypothesis, we observed TF enrichment at the vulnerable surface of the plaques, ie, the site where thrombin generation occurs.18,28 Whether P2Y12 expression in SMC is actually associated with lesion rupture or may even facilitate acute events (eg, by affecting expression of destabilizing factors, such as matrix metalloproteinases29) needs to be addressed in future studies.

Figure 6. Thrombin-induced P2Y12 enhances mitogenesis in human SMC. A, DNA synthesis was determined by incorporation of [3H]thymidine. Cells were incubated with 2-MeSADP (1 μmol/L) and R-138727 (10 μmol/L) alone and together, and in the presence of thrombin (thr, 30 nmol/L) for 24 hours. B, In an additional setting, cells were preincubated with thrombin for 6 hours before stimulation with 2-MeSADP (1 μmol/L) and R-138727 (10 μmol/L). Shown are mean±SEM of n=6 experiments (A and B); *P<0.05 as indicated. C, Cell count after incubation for 4 days with thrombin (30 nmol/L), 2-MeSADP (1 μmol/L), and R-138727 (10 μmol/L) as indicated. Shown are mean±SEM of n=4 experiments; ‡P<0.05. D, Scheme of PAR-1-regulated P2Y12 expression and their functional interaction in human SMC.

Exposure of cultured human vascular SMC to thrombin resulted in an increased expression of P2Y12 mRNA, total P2Y12 cellular protein, and cell surface expression. Thrombin-induced proinflammatory and mitogenic responses in vascular cells are mediated by activation of the G protein–coupled protease-activated receptor (PAR)-1, PAR-3, and PAR-4.14,15,24–27 Our data indicate that predominantly the prototypic thrombin receptor PAR-1 is involved. Immunoblot analysis confirmed a marked 2- to 3-fold upregulation of P2Y12 after 6 hours of stimulation with thrombin, which was maintained over 24 hours. Identical results were obtained with 2 different antibodies to P2Y12, detecting either an extracellular or an intracellular epitope of P2Y12. These findings in human saphenous vein SMC were confirmed in human aortic SMC, suggesting that both arterial and venous SMC are capable of increased P2Y12 expression after thrombin stimulation.

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After establishing the existence of thrombin-regulated P2Y12 in human SMC, the next step was to identify major signaling pathways. Thrombin activates the proinflammatory transcription factor NF-κB in vascular SMC, and the P2Y12 gene promoter region contains a binding site for NF-κB. Chromatin immunoprecipitation analysis demonstrated that thrombin induced binding of NF-κB to the P2Y12 promoter region, whereas inhibition of NF-κB activation also suppressed thrombin-induced expression of P2Y12. Thus, thrombin-induced transcriptional regulation of P2Y12 does require NF-κB activation in human vascular SMC.

Signaling of the G<sub>i</sub>-coupled P2Y12 receptor occurs via inhibition of adenylate cyclase and subsequent reduction of platelet cAMP levels. Thus, we determined a possible effect of thrombin-induced P2Y12 expression on cAMP levels in human SMC. cAMP levels were enhanced by iloprost to mimic the action of endogenous prostacyclin, which is assumed to contribute to the antiplatelet effects of P2Y12 inhibition. In cells that had been pretreated with thrombin for at least 6 hours, the P2Y agonist 2-MeSADP reduced cAMP levels by ∼25%. This was prevented by the specific antagonist R-138727, indicating a P2Y12-mediated reduction of cAMP levels via G<sub>i</sub>. The functional impact of this inhibition of cAMP levels by thrombin-induced P2Y12 expression, such as secretory functions of SMC in atherosclerotic plaques, remains to be determined.

In addition to their role in platelets, ADP receptors mediate a range of inflammatory effects in nonplatelet tissues and have been shown to enhance the proliferative responses to certain growth factors, such as platelet-derived growth factor. Genetic variability of P2Y12, such as the H2 haplotype, has also been associated with advanced atherosclerosis and may be a determinant of atherogenic SMC responses. Therefore, we sought to determine a possible functional consequence of thrombin-induced P2Y12 expression for mitogenic and proinflammatory responses in human SMC. IL-6 is a key inflammatory mediator involved in the pathogenesis of atherosclerosis. Elevated IL-6 levels have been proposed to predict an increased cardiovascular risk rate. Because both thrombin and activation of the P2Y receptors have recently been shown to induce IL-6 production, we investigated whether increased expression of P2Y12 by thrombin may influence IL-6 transcription. Expression of IL-6 mRNA by 2-MeSADP was markedly enhanced in thrombin-preexposed cells and antagonized by R-138727. This effect also translated to an increased IL-6 secretion into the extracellular space (Figure 5D). These observations indicate that thrombin-induced inflammatory actions in human SMC may involve ADP-dependent signaling through upregulation of P2Y12. Increased P2Y12 expression may therefore be considered a thrombin-triggered proinflammatory signal in human atherosclerotic plaque tissue, which potentially could trigger chemotaxis of SMC or monocytes to sites of injury.

Another function of increased P2Y12 expression was stimulation of thrombin-induced mitogenesis. Comparable to IL-6 expression, preexposure to thrombin significantly enhanced the mitogenic response of human SMC to 2-MeSADP, eventually resulting in increased cell numbers. This promitogenic response was blocked by the P2Y12 antagonist R-138727 or by another selective inhibitor of P2Y receptors, 2-Methylthio-AMP. The specificity of the inhibitor data was confirmed by gene-specific knockdown of P2Y12 with small interfering RNA (Supplemental Figure V1). These observations collectively suggest that a thrombin-induced increase in P2Y12 receptor expression amplifies proinflammatory and mitogenic responses in human SMC.

Taken together, our data indicate that thrombin increases expression of functionally active P2Y12 ADP receptors in human SMC. This is due to enhanced, mainly PAR-1-mediated P2Y12 transcription via activation of NF-κB and eventually results in increased proinflammatory and mitogenic responses (Figure 6D). These findings reflect clinical observations that P2Y12 antagonists exert antiinflammatory actions, which to date have been attributed solely to inhibition of platelet-mediated effects. Our data suggest that these newly described actions of P2Y12 antagonists may also be relevant to patients, especially in the presence of enhanced thrombin formation in situations, such as acute coronary syndromes, and might contribute to their overall clinical benefit.

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**Disclosures**

Dr Sugidachi is an employee of Daiichi Sankyo Co Ltd, and Dr Jakubowski is an employee of Eli Lilly and Company.

**References**


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Regulation of functionally active P2Y₁₂ ADP receptors by thrombin in human smooth muscle cells and its presence in carotid artery lesions

Bernhard H. Rauch, Anke C. Rosenkranz, Swen Ermler, Andreas Böhm, Julia Driessen, Jens W. Fischer, Atsuhiro Sugidachi, Joseph A. Jakubowski and Karsten Schrör

Extended Materials and Methods

Materials

Dulbecco’s modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Invitrogen. Purified α-thrombin was from Dr. J. Stürzebecher (Institut für Vaskuläre Medizin, Jena, Germany). 2-(Methylthio)adenosine 5’-diphosphate (2- MeSADP), 2-(Methylthio)adenosine 5’-monophosphate (2- MeSAMP) and rabbit anti-human antibodies detecting the intracellular domain (P6997) and for the extracellular loop (P4871) of P2Y₁₂ were from Sigma-Aldrich. Activating peptides for PAR-1 (AP1, TFFLRN), PAR-3 (AP3, TFRGAP), and PAR-4 (AP4, AYPGQV) were from Biosynthan (Berlin, Germany). R-138727 was from Eli Lilly and Company (Indianapolis, Indiana, USA). Goat anti-human tissue factor antibody (4501) was from American Diagnostica, monoclonal anti-human M-actin (clone HHF35) from DAKO, and monoclonal anti-human CD42 (8226) antibodies were from Abcam. Secondary antibodies were from Santa Cruz Biotechnology, alkaline phosphatase-conjugated secondary antibody was from Abcam. NFκB activation inhibitor (6-amino-4-(4-phenoxy-phenylethylamino)quinazoline) was from Calbiochem.
Cell Culture

Human saphenous vein and aortic SMC were isolated by the explant technique and cultured as described previously. Subconfluent cells at passages 4 to 9 were serum-deprived for 48 hours prior to the experiments.

Semiquantitative and quantitative PCR

Isolation of mRNA, semiquantitative reverse transcriptase PCR, synthesis of cDNA and qPCR were performed as described. Gene specific primers for P2Y were from Invitrogen: forward: GAAGACCACCAGGCCATTTA; reverse: GTGTAAGGAATTCCGG GCAAA. TaqMan® assays were used on a Real-Time PCR System 7300: Hs_P2RY12_3SG for P2Y and Hs99999905_m1 for GAPDH as endogenous control (Applied Biosystems). IL-6 mRNA was determined using SYBR Green Master Mix (Applied Biosystems) and Quanti-Tect Primer Assays for IL-6 and GAPDH (Qiagen). Data were analyzed with Sequence Detection Software v1.2.3 and Ct values were normalized to GAPDH as described.

Immunoblotting

Cell extracts were prepared in sodium dodecyl sulfate (SDS) lysis buffer (4% w/v SDS, 20% glycerol, 0.0625 M sodium dihydrogen phosphate/disodium hydrogen phosphate, pH 7.0, 100 mM dithiothreitol, 0.1% bromophenol blue). Separation of proteins by SDS polyacrylamide gel electrophoresis and immunoblotting was performed as described previously. Density of the band was quantified by the Gel-doc System GS-800 from BioRad using Quantity One analysis software. Data are shown as fold increase relative to unstimulated controls.
Flow cytometry

Flow cytometry of human SMC was performed as described previously with minor modifications. SMC were seeded in 6-well plates and stimulated as indicated. After nonenzymatic detachment with citrate saline buffer (0.135 M potassium chloride, 0.015 M sodium citrate) for 15 minutes at 37°C, cells were pelleted and resuspended in PBS. Cell suspensions (50 µL) were incubated with anti-human P2Y12 antibodies targeting an extracellular loop (P4871) for 15 minutes at room temperature, followed by incubation with FITC-labeled secondary antibody in the dark for 15 minutes. Isotype-matched primary antibody with FITC-labeled secondary antibody was used to assess nonspecific binding. Samples were analyzed on an EPIC-XL cytometer (Beckman Coulter). Data are expressed as relative mean fluorescence of unstimulated controls.

Immunofluorescence microscopy

Immunocytochemistry was performed on human SMC as described with some modifications. Anti-human P2Y12 antibody (P4871) (1:100 in 1% BSA/PBS) was incubated with the fixed and blocked cells for 1h at room temperature. Binding to P2Y12 was detected by using FITC-labeled secondary anti-rabbit antibodies. Nuclei were stained with Hoechst-33342 (Invitrogen). Images were taken with a Nikon Eclipse TE 2000 PFS microscope equipped with NIS Elements Software V. 3.0.

Immunohistochemistry

Immunohistochemistry was performed in section of human carotid artery plaques obtains during endarterectomy. The study was approved by the local ethics committee and the material obtained after informed written consent. Tissue sections
(5 μm) were deparaffinized in xylene, rehydrated in ethanol, and washed with PBS. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed in 0.1 mM Na-citrate/0.1 M citric acid (pH 6) in distilled water at 96°C for 20 minutes. Sections were blocked by 10% FCS/1% BSA for 60 min at room temperature. Antibodies to P2Y12 (rabbit anti-human P2Y12 antibody P6997, 1:100), TF (goat anti-human, 1:40), M-actin (mouse monoclonal, 1:40) and CD42 (mouse monoclonal anti-human, 1:40) were applied in 1% BSA/PBS. Primary antibodies were visualized by streptavidin/horseradish peroxidase (HRP)-conjugated secondary antibodies (1:400 in PBS) and the DAB Substrate Kit (Zytomed Systems). In double stained sections, primary antibodies were first detected by HRP/DAB and subsequently with an alkaline phosphatase-conjugated secondary antibody using Fast Red (DAKO, Germany) as chromogen. Nuclei were stained with hemalaun. Nonspecific isotype-matched IgG with secondary antibodies were used to control for unspecific staining. Images were taken with a Colorview II camera and SIS software (Soft Imaging System) connected to an Olympus BX 50 microscope.

**Cyclic-AMP assay**

Intracellular cAMP was determined by radioimmunoassay as described.3

**Chromatin immunoprecipitation (ChIP) assay**

Human SMC (2 × 10^6 each) were fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped with 0.125 M glycine. Cells were pelleted and hypotonically lysed. Nuclei were collected and sonicated to desired chromatin length (~ 500 bp). The chromatin was precleared by addition of Protein G PLUS-Agarose
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(Santa Cruz Biotechnology, INC) prior to immunoprecipitation with antibodies specific to NF\textsubscript{κ}B p65 subunit (Santa Cruz Biotechnology, INC) at 4°C overnight. Protein-antibody complexes were collected by addition of Protein G PLUS-Agarose for 16 h and the beads extensively washed. The protein-DNA cross-links were eluted and reversed. DNA was purified by phenol/chloroform/isoamylalcohol extraction, precipitated with ethanol and amplified by PCR. NF\textsubscript{κ}B primers were: 5'-GATCGCTTGTCCTCTAGCTCTT-3' (forward) and 5'-TTGTTGTGTAAACAACAGTGCT-3' (reverse) amplifying a 258 bp region of the human P2Y\textsubscript{12} promoter containing the NF\textsubscript{κ}B binding site. Negative control primers were: 5'-ATGGTTGCCACTGGGTCT-3' (forward) and 5'-TGCAACACTGGAAGTAAGTTGA-3' (reverse), amplifying a 174 bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 1.8% agarose gel in the presence of ethidiumbromide.

**IL-6 enzyme-linked immunosorbent assay (ELISA)**

Human SMC were seeded in 24-well plates and serum-deprived for 24 hours. Cells were incubated in 300 µL serum-free medium with thrombin and/or 2-MeSADP simultaneously for 24 hours or 2-MeS-ADP was added 6 hours after thrombin. Media were collected and cell lysates (in 300 µl of 25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton-X100, 1% saturated PMSF solution in isopropanol) were stored at -80°C. Protein levels of IL-6 were determined by a commercially available ELISA kit (eBiosciences, cat #88-7066-88) which was used according to the manufacturer’s instructions.
DNA synthesis and proliferation

DNA synthesis was determined by incorporation of [³H]-labeled thymidine as described.¹² Proliferation of human SMC was determined in 96-well plates in quadruplicates. Cells were seeded at about 5,000 cells /cm². The following day, cells were serum-deprived and stimulated with thrombin about 6 hours later. 2-MeS-ADP was added after 6 hours preincubation with thrombin. Media and stimuli were renewed after 48 hours. After 96 hours, media were removed and cells were fixed with paraformaldehyde for 2 hours prior to staining with Hoechst 33342 for 15 minutes. Images were taken at 40x magnification and nuclei were counted using Image J analysis software.

Transfection with siRNA

P2Y12-specific and non-silencing control siRNA was from Qiagen (Hilden, Germany). Cells were transfected with 30 nmol/L siRNA, respectively, using RiboJuice™ siRNA transfection reagent (Merck) in DMEM with 10% FCS according to the manufacturer’s instruction for 24 hours. Prior to experiments, cells serum-deprived for 48 hours. Efficacy of knockdown was controlled by Western blotting.

Statistical analysis

Data are presented as means±SEM from n independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons test. P<0.05 was considered significant.
References


Supplemental Data

Supplemental Figure I

Enhanced expression of P2Y₁₂ by thrombin in human vascular SMC. (A) P2Y₁₂ mRNA was determined in human saphenous vein SMC by semiquantitative reverse-transcriptase PCR after incubation with thrombin for the indicated times, n = 6, *p<0.05 vs. unstimulated control (con). (B) P2Y₁₂ total protein was determined in human saphenous vein SMC by immunoblotting after incubation with thrombin for the indicated times. An antibody (P4871) detecting the extracellular loop of human P2Y₁₂ was used. Human platelet lysates (PL) are shown for comparison and β-actin is used as loading indicator, n = 6, *p<0.05 vs. unstimulated control (con). (C) P2Y₁₂ total protein was determined in human aortic SMC by immunoblotting after incubation with thrombin (thr, 30 nmol/L) for 16 hours (shown in duplicate). An antibody (P6997) detecting the intracellular domain P2Y₁₂ was used. β-Actin was used as loading indicator. (D) Quantification of P2Y₁₂ protein expression in human aortic SMC, n = 8, *p<0.05 vs. unstimulated control (con).
**Supplemental Figure II**

**Detection of P2Y_{12} in human SMC.** (A) Band pattern of P2Y_{12} in human cultured SMC observed in immunoblots with the rabbit anti-human antibody (P6997), which detects the intracellular domain of P2Y_{12}. Multiple bands were observed in human SMC and platelet lysates (PL), in accordance with the literature, presumably corresponding to differently glycosylated forms of the receptor. (B-G) Representative staining (brown) with anti-P2Y_{12} antibody P6997 human carotid artery plaques. Positive staining was mostly observed in association with ruptured plaques sides. Nonspecific rabbit IgG with HRP-conjugated secondary antibody was compared to anti-P2Y_{12} staining at original magnifications of (B, C) 40x, (D, E) 100x, and (F, G) 400x.
Supplemental Figure III

Gene-specific knockdown of P2Y\textsubscript{12} protein expression by small-interfering RNA (siRNA). Human vascular smooth muscle cells were transfected with P2Y\textsubscript{12}-specific siRNA (30 nmol/L) or non-silencing control RNA (siCon). Effect on P2Y\textsubscript{12} protein was determined by Western blotting using the same antibody as for the immunohistochemistry staining. Quantification shows mean from n = 5 independent experiments, *p<0.05 vs. untreated control cells (Con) and cell treated with non-silencing control siRNA (siCon).
Supplemental Figure IV

Representative staining of tissue factor (TF) in human carotid artery plaques obtained after carotid endarterectomy. (A, B) Nonspecific IgG and HRP-conjugated secondary antibodies were used to control for nonspecific staining. Magnifications shown are 40x (A) and 200x (B). (C, D) TF was immunostained (brown) with goat anti-human antibody. Shown is a plaque at 40x (C) and 200x (D) magnification. (E, F) TF protein was co-stained with M-actin (red) as marker for smooth muscle cells. Double staining resulted in a red-brownish staining pattern indication the presence of both proteins. Shown are original magnifications of 100x (E) and 400x (F).
Supplemental Figure V

Representative staining of platelet CD42 in human carotid artery plaques obtained after carotid endarterectomy. (A, B) Isotopic-control IgG and HRP-conjugated secondary antibodies were used to control for nonspecific staining. Magnifications shown are 40x (A) and 400x (B). (C, D) CD42 was immunostained with monoclonal anti-human antibody. No positive staining pattern was seen. Shown is a plaque at 40x (D) and 400x (C) magnification. (E) Staining of CD42 (brown) in recalcified human platelet-rich plasma adherent to fibrin fibers after coagulation. CD42 positive staining is indicated by arrows. Shown is an original magnification of 1000x.
Supplemental Figure VI

Knockdown of the P2Y\textsubscript{12} receptor attenuates the mitogenic response to 2-MeSADP in thrombin-pretreated human SMC. Incorporation of \[^{3}\text{H}]\text{thymidine}\ was determined in human SMC treated with (A) the selective P2Y inhibitor 2-MeSAMP (10 \(\mu\text{mol/L}\)) or (B) with siRNA specific for P2Y\textsubscript{12} (siP1Y12, grey bars) or cells treated with non-silencing control siRNA (siCon, black bars) after incubation with thrombin (30 nmol/L, 6 hour preincubation) or/and 2-MeSADP (1 \(\mu\text{mol/L}\)); \(n = 7\) (A) or 10 (B) experiments, \(^{*}p<0.05\) vs. thrombin alone, \(^{#}p<0.05\) vs. thr + 2-MeSADP.