

## P2 receptors in atherosclerosis and postangioplasty restenosis

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**Abstract** Atherosclerosis is an immunoinflammatory process that involves complex interactions between the vessel wall and blood components and is thought to be initiated by endothelial dysfunction [Ross (Nature 362:801–809, 1993); Fuster et al. (N Engl J Med 326:242–250, 1992); Davies and Woolf (Br Heart J 69:S3–S11, 1993)]. Extracellular nucleotides that are released from a variety of arterial and blood cells [Di Virgilio and Solini (Br J Pharmacol 135:831–842, 2002)] can bind to P2 receptors and modulate proliferation and migration of smooth muscle cells (SMC), which are known to be involved in intimal hyperplasia that accompanies atherosclerosis and postangioplasty restenosis [Lafont et al. (Circ Res 76:996–1002, 1995)]. In addition, P2 receptors mediate many other functions including platelet aggregation, leukocyte adherence, and arterial vasomotricity. A direct pathological role of P2 receptors is reinforced by recent evidence showing that upregulation and activation of P2Y<sub>2</sub> receptors in rabbit arteries mediates intimal hyperplasia [Seye et al. (Circulation 106:2720–2726, 2002)]. In addition, upregulation of functional P2Y receptors also has been demonstrated in the basilar artery of the rat double-hemorrhage model [Carpenter et al. (Stroke 32:516–522, 2001)] and in coronary artery of diabetic dyslipidemic pigs [Hill et al. (J Vasc Res 38:432–443, 2001)]. It has been proposed that upregulation of P2Y

receptors may be a potential diagnostic indicator for the early stages of atherosclerosis [Elmaleh et al. (Proc Natl Acad Sci U S A 95:691–695, 1998)]. Therefore, particular effort must be made to understand the consequences of nucleotide release from cells in the cardiovascular system and the subsequent effects of P2 nucleotide receptor activation in blood vessels, which may reveal novel therapeutic strategies for atherosclerosis and restenosis after angioplasty.

**Keywords** Atherosclerosis · Inflammation · Migration · Nucleotide receptors · Proliferation · Restenosis · Smooth muscle cell

### Introduction

Atherosclerosis is a pathological phenomenon primarily affecting the large conduit arteries, for example the aorta, coronary, carotid iliac, and femoral arteries. The development of atherosclerotic lesions in arteries involves the intimal recruitment of smooth muscle cells (SMC) within the blood vessel wall, and also the infiltration of blood-derived cells [1]. This process necessitates the proliferation and migration of SMC from the underlying media and the endothelial adhesion of leukocytes and their infiltration into the subendothelium. A similar intimal accumulation of SMC also takes place during the postangioplasty restenotic process. Although the factors involved in intimal cell recruitment are not clearly identified, it is becoming evident that endothelial dysfunction is a key factor in the development of vascular disease. Experimental evidence suggests that an intact endothelium plays a central role in maintaining a low proliferative state of SMC under normal conditions [10]. In arterial injury, endothelial cells, SMC,

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and various blood cells can release chemotactic factors and mitogens, including ATP and other nucleotides (see [4] for review). Activation of P2 nucleotide receptors has been shown to induce not only the proliferation and migration of vascular SMC but also apoptosis, a process involved in the evolution of the atherosclerotic plaque [11]. In addition, P2 receptors mediate both vasorelaxation and vasoconstriction of arteries that may be involved in the vascular remodeling accompanying atherosclerosis and postangioplasty restenosis [5]. A better understanding of the causative agents and mechanisms of proliferation and migration of vascular SMC as well as recruitment of blood-derived cells by the endothelium could lead to prevention, attenuation, or even reversal of intimal thickening, which may dramatically reduce morbidity and mortality from vascular diseases such as atherosclerosis and restenosis after angioplasty. In this respect, a better understanding of the physiological role of P2 receptors in both normal and pathological blood vessels could potentially lead to a breakthrough in the fight against vascular disease.

### P2 receptors in the cardiovascular system

Extracellular nucleotides bind to cell surface receptors known as P2 receptors, which are present in many tissues. To date, these receptors have been classified into two main families: the P2X receptors that are ligand-gated ion channels comprised of homo- or hetero-oligomers [12] and P2Y receptors that are seven membrane spanning receptors coupled via G proteins ( $G_{q/11}$  or  $G_{i/o}$ ) to phospholipase C (PLC) and/or adenylate cyclase [12–14]. In turn, PLC activation generates inositol 1,4,5-triphosphate ( $IP_3$ ), a mediator of  $Ca^{2+}$  release from intracellular stores, and diacylglycerol, an activator of protein kinase C (PKC), whereas adenylate cyclase generates cyclic adenosine monophosphate (cAMP), an activator of protein kinase A (PKA). The cloning of seven P2X (P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, P2X<sub>5</sub>, P2X<sub>6</sub>, P2X<sub>7</sub>) and eight P2Y (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, P2Y<sub>14</sub>) receptor subtypes has made it possible to use molecular and pharmacological approaches to study the distribution and functional properties of specific P2 receptor subtypes at the tissue and cellular level.

### P2 receptors in vascular cells

The normal arterial wall consists of three layers: the intima, media, and adventitia. The single layer of endothelial cells facing the vessel lumen is a very important component of the vascular wall in terms of releasing both vasodilators such as nitric oxide (NO) and prostacyclin ( $PGI_2$ ) and

vasoconstrictors like thromboxane  $A_2$  and endothelin. The principal P2Y receptor subtypes that have been functionally characterized in endothelial cells are P2Y<sub>1</sub> and P2Y<sub>2</sub>, but mRNAs for P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors have also been detected [4]. Endothelium-dependent vasorelaxation has been attributed to the release of NO and  $PGI_2$  after binding of nucleotides to P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors in endothelial cells [15], whereas vasoconstrictor effects in SMC result from the action of nucleotides on P2Y<sub>2</sub> and P2X receptors [16, 17]. In most blood vessels, P2Y<sub>1</sub> receptors for ADP are present on the endothelium and regulate vasodilatation by  $Ca^{2+}$ -dependent (PLC-mediated) activation of nitric oxide synthase (NOS) and generation of endothelial-dependent relaxing factor (EDRF) (see [4] for review). Endothelial prostacyclin production is also stimulated by P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors, but this seems to play a minimal role in vasodilatation, at least under physiological conditions (see [18] for review). Recent studies have indicated that in the aorta of P2Y<sub>2</sub>-null mice the endothelium-dependent relaxation by ATP and ATP $\gamma$ S was inhibited, demonstrating the role of the P2Y<sub>2</sub> receptors, but that a relaxation by UTP and UDP was maintained, suggesting the additional involvement of P2Y<sub>6</sub> receptors [19]. The majority of the cells in intact blood vessels are SMC, which occupy most of the media, and are involved in vasoconstriction and vasorelaxation of the vessel. P2Y<sub>2</sub>Rs in SMC mediate the induction of immediate-early and delayed-early cell cycle-dependent genes, consistent with a role for P2Y<sub>2</sub>Rs in vascular proliferation of SMC [20, 21]. A recent study demonstrated that P2Y<sub>2</sub> is the predominant functional receptor that responds to ATP and UTP in rat aortic SMC [22]. In human cerebral arteries, P2Y<sub>6</sub> seems to be the predominant subtype and induces vasoconstriction when activated by UDP/UTP [23, 24]. This is consistent with findings from rat pulmonary and mesenteric arteries [25, 26]. In addition, a recent study on P2X<sub>1</sub> knockout mice further supports the prominent contractile effect of the P2Y<sub>6</sub> subtype in mesenteric arterial trees [27]. Taken together, it seems that the principal receptor mediating UTP/UDP-induced contractile responses in blood vessels might be the P2Y<sub>6</sub> subtype. Other studies have reported the presence of both P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors in rat aortic SMC [21, 28, 29]. P2Y<sub>1</sub> receptors are expressed in SMC of a number of blood vessel types and like their EC counterparts mediate vasodilatation most likely through the activation of  $K^+$  channels (see [18] for review). The presence of several P2X receptor subtypes also has been reported in human saphenous vein SMC, including P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> receptors [30]. The outermost layer of the blood vessel consists of connective tissue and fibroblasts, which have not been appreciated in the regulation of vascular tone. A recent study showed that fibroblasts can migrate into the neointima, suggesting their

possible involvement in the development of vascular diseases such as atherosclerosis and restenosis after angioplasty [31]. Since human and rat fibroblasts are known to express P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> receptors [32], further investigation is needed to establish the role of fibroblast P2 receptors in either physiological or pathophysiological conditions.

### P2 receptors in blood cells

G protein-coupled P2Y receptors whose activation leads to intracellular calcium mobilization have been observed in neutrophils [4, 33, 34], and turkey erythrocytes express both P2Y<sub>1</sub> and P2X<sub>7</sub> receptors [35, 36]. ATP and UTP act as secretagogues by binding to P2Y<sub>2</sub>Rs to enhance exocytosis of primary granules in neutrophils [37]. In macrophages, ATP activates a P2X<sub>7</sub> receptor that differs from other ligand-gated ion channel P2X receptors by its ability to generate plasma membrane pores when activated [38]. Human T lymphocytes also have been shown to express P2X<sub>7</sub> receptors [39]. Human monocytes and macrophages coexpress P2X<sub>1</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> receptors, whereas granulocytes only express P2X<sub>7</sub> receptors [40]. P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>6</sub> receptors also are expressed in monocytes, B lymphocytes, and polymorphonuclear granulocytes [41]. Human platelets express P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2X<sub>1</sub> receptors [42–44]. Thus, the diversity of P2 receptor expression in blood cells, as well as endothelial cells, SMC, and fibroblasts, suggests that this receptor superfamily plays a significant role in the regulation of cardiovascular functions.

### P2 receptors regulate nucleotide-induced vascular smooth muscle cell proliferation

Proliferation of SMC is a hallmark of vascular diseases such as atherosclerosis and restenosis following angioplasty. ATP and other nucleotides are released by aggregating platelets and damaged vascular cells, such as endothelial cells and SMC in pathological or stress conditions [45]. Extracellular nucleotides can act via P2X and P2Y receptors to induce acute responses such as the regulation of vascular tone [46]. However, it is generally believed that the ionotropic P2X receptors do not mediate the chronic responses of nucleotides such as cell proliferation. The mitogenic effect of extracellular nucleotides on vascular SMC (VSMC) has been known for years [47]. However, a potent antiproliferative effect of UTP on VSMC also has been reported in human arterial and venous SMC [48]. In either case, the P2 receptor subtype(s) responsible for these effects on the proliferation of VSMC has not been determined. Earlier studies have shown that ATP or UTP

increased DNA and protein synthesis in subcultured rat aortic VSMC [49, 50]. In the same cell culture model, however, Malam-Souley et al. [51] were unable to detect increases in DNA synthesis after ATP/UTP stimulation, although ATP or UTP upregulated the expression of mRNA to several cell cycle progression-related genes. Because P2X receptor agonists were essentially inactive, it was concluded that a P<sub>2U</sub>-like receptor (now termed P2Y<sub>2</sub>) was responsible for the mitogenic effects of ATP/UTP. However, the role of a P2Y<sub>4</sub> receptor cannot be excluded, because the nucleotide agonist profile between rat P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors is essentially indistinguishable [52]. Indeed, Harper et al. [53] suggested that the P2Y<sub>4</sub> receptor mediated ATP/UTP-induced proliferation of rat aortic VSMC. Recent studies indicated that ATP, UTP, or ITP, three agonists of the cloned porcine P2Y<sub>2</sub> receptor, increased DNA and protein synthesis and cell number in coronary artery SMC [54]. Indeed, treatment of pig coronary artery SMC with UTP, ATP, or ITP caused a concentration-dependent increase in DNA and protein synthesis and cell number, whereas UDP only caused a small increase in protein synthesis. Intriguingly, ATP was much more potent and efficacious than UTP, ITP, and UDP in increasing DNA synthesis and expression of PCNA, a protein marker of cell proliferation, suggesting that another receptor may contribute to the proliferative response [54]. In vivo experiments have shown that intimal thickening of collared rabbit carotid arteries was greatly enhanced by in situ UTP application and was closely associated with osteopontin expression in medial SMC [6]. Osteopontin is chemotactic for SMC and is associated with arterial smooth muscle cell proliferation [55]. Moreover, both UTP and ATP increased osteopontin expression in cultured SMC, whereas ADP, UDP, and 2-MeSATP were ineffective, which suggests a role for the P2Y<sub>2</sub>R in which ATP and UTP are equipotent. Direct evidence for involvement of the P2Y<sub>2</sub>R was provided by inhibition of UTP-induced osteopontin expression in cultured SMC by P2Y<sub>2</sub> antisense oligonucleotides [6]. P2Y<sub>6</sub> receptors also have been shown to mediate proliferation of SMC in rat aorta [56].

### Role of P2 receptors in the migration of vascular SMC

Recent studies indicate that the extracellular nucleotides ATP, ADP, UTP, and UDP serve as directional cues for the migration of rat aortic SMC [57]. At identical concentrations, the most powerful migratory response induced by these nucleotides was elicited by UTP. Nucleotide-induced migration of SMC is the consequence of both chemotaxis and chemokinesis and may result either from the activation of one particular P2 nucleotide receptor subtype or from activation of several P2 receptor subtypes. The ability of

UTP at submicromolar levels to stimulate migration of SMC supports the hypothesis that this response could have physiological consequences and is essentially mediated by P2Y<sub>2</sub> receptor activation without excluding participation of other P2Y receptor subtypes. The difference in the capacity of UTP and ATP to elicit migration of SMC could be due to the inhibition of nucleotide-induced cell migration by adenosine generated from ATP catabolism by cell surface ectonucleotidases. Indeed, ATP and UTP were equally effective in causing migration of SMC when ATP was prevented from degradation by addition of the ectonucleotidase inhibitor  $\alpha,\beta$ -methylene-ATP [57]. Several P2Y receptor subtypes could be involved in nucleotide-induced migration of SMC. It has been shown in rat aortic SMC that the P2Y<sub>2</sub> receptor is the predominant P2Y receptor subtype [28, 58], whereas lower levels of P2Y<sub>4</sub> and P2Y<sub>1</sub> receptor mRNA were detected [58]. The very low level of P2Y<sub>1</sub> receptor mRNA expression was consistent with the absence of ADP-induced migration of cultured rat aortic SMC, demonstrating that P2Y<sub>1</sub> is not involved in this process [58]. In addition, the same study showed that a commercially available solution of hexokinase-treated UDP (UTP-free) induced cell migration equally well as untreated UDP, thereby demonstrating that UDP is chemotactic for aortic SMC by activation of the P2Y<sub>6</sub> receptor. Conversely, migration of rat aortic SMC induced by UTP occurred even when UTP degradation by nucleoside diphosphate kinase was inhibited, demonstrating the involvement of P2Y<sub>2</sub> and/or P2Y<sub>4</sub> receptor(s) [58].

The increased migration of SMC in response to extracellular nucleotides could be related to increases in extracellular matrix (ECM) protein expression. Indeed, previous studies have shown that UTP induces osteopontin expression in rat and rabbit aortic SMC [51, 6]. Increased expression of osteopontin, an RGD-containing ECM protein, is associated with the activation of rat arterial SMC in vitro and in vivo [57]. The increase in osteopontin expression plays a key role in UTP-induced migration of rat aortic SMC, since a monoclonal antibody against osteopontin fully abolished UTP-induced migration [57], whereas an antibody against vitronectin, another ECM protein also involved in migration of human SMC [59], had no effect on the migration of rat aortic SMC [57]. UTP induces increases in osteopontin mRNA expression by increasing both osteopontin mRNA stabilization and *osteopontin* promoter activity [60]. Recent studies have shown that activation of an AP-1 binding site located 76 bp upstream of the transcription start in the rat *osteopontin* promoter is involved in UTP-induced osteopontin expression. Using a luciferase promoter deletion assay, Renault et al. [61] identified a new region of the rat *osteopontin* promoter (-1837 to -1757) that is responsive to UTP. This region contains an NF $\kappa$ B site located at -1800 and an Ebox located at -1768. Supershift electrophoretic mobility

shift and chromatin immunoprecipitation assays identified NF $\kappa$ B and USF-1/USF-2 as DNA binding proteins induced by UTP. Using dominant negative mutants of I $\kappa$ B kinase and USF transcription factors, it was confirmed that NF $\kappa$ B and USF-1/USF-2 are involved in the UTP-induced expression of osteopontin.

This ability of nucleotides to act as chemoattractants for rat arterial SMC in a concentration range potentially found in pathological vessels [62] and the findings of previous studies demonstrating the mitogenic activity of extracellular nucleotides for these cells suggest that nucleotides released from mechanically stretched vascular or damaged cells during the angioplasty process may participate in arterial wall remodeling.

### Role of P2 receptors in nucleotide-induced vascular inflammation

In addition to their mitogenic effects, extracellular nucleotides may also cause cell recruitment by inducing lymphocyte and macrophage adhesion to human pulmonary artery endothelial cells as demonstrated in vitro [63]. Nucleotides can also modulate rat aortic smooth muscle cell adhesion and migration by increasing the expression of osteopontin [51, 57], a protein involved in both processes. Moreover, extracellular nucleotides may play a role in intra-arterial attraction of monocytes by inducing an increased expression of monocyte-chemoattractant protein-1 by arterial SMC [21]. Stimulation of P2 receptors is coupled to the release of the proinflammatory cytokines interleukin (IL)-1 $\beta$ , IL-1 $\alpha$ , IL-8, and tumor necrosis factor (TNF)- $\alpha$  (see [4] for review) that are of obvious relevance to inflammation in atherosclerosis. Activation of P2X<sub>7</sub> receptors on monocytes/macrophages enhances release of proinflammatory cytokines that modulate NO production and expression of inducible nitric oxide synthase (iNOS) (see [64] for review), mediators of immune cell activation that is an early step in atherosclerotic lesion development.

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 [65, 66]. VCAM-1 expression is induced or upregulated by proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  in cellular components of the arterial wall including endothelial cells, smooth muscle cells, and fibroblasts [67–69]. ATP and UTP have been shown to induce cell-cell adhesion in a human monocyte/macrophage lineage and neutrophil adherence to human endothelial cell monolayers [63, 70].

Recent studies have shown that local UTP delivery via an osmotic pump to collared rabbit carotid arteries induced

intimal accumulation of macrophages, similar to oxidized low-density lipoprotein (LDL), a response that was mediated by activation of P2Y<sub>2</sub> receptors [6]. Leukocyte migration depends on the activities of adhesion proteins (e.g., selectins and integrins) on leukocytes and vascular endothelial cells. We have demonstrated that activation of P2Y<sub>2</sub> receptors in endothelial cells causes the expression of VCAM-1 that mediates the adherence of monocytes to vascular endothelium [71], leading to their penetration into the vessel wall to promote arterial inflammation associated with atherosclerosis.

Recent studies have revealed that a Src homology-3 (SH3) binding domain in the C-terminal tail of the P2Y<sub>2</sub> receptor promotes the nucleotide-induced association of Src with the P2Y<sub>2</sub> receptor, leading to the transactivation of growth factor receptors, such as the epithelial growth factor (EGF) and vascular endothelial growth factor (VEGF) receptors, and nucleotide-induced upregulation of VCAM-1 [72, 73]. Since leukocyte infiltration and migration are key processes involved in atherosclerosis, these findings suggest that P2Y<sub>2</sub> receptors represent a novel target for reducing arterial inflammation associated with cardiovascular disease.

## P2 receptors in vascular apoptosis

Apoptosis (programmed cell death) has been reported to occur in various vascular diseases such as atherosclerosis, restenosis, and hypertension [74, 75]. The major cell types undergoing apoptosis in human atherosclerotic lesions are arterial SMC [11, 76–78] and macrophages [79]. In restenosis following balloon angioplasty, there is a peak in the proliferation and apoptosis of rat vascular SMC 14 days postangioplasty [76]. Furthermore, apoptosis of arterial SMC has been described in animal models of intimal thickenings [80] and probably takes part in the normal process involved in the control of hyperplasia. In contrast, apoptosis of SMC in advanced human atherosclerotic plaques may destabilize the fibrous lesion to promote plaque rupture and its clinical consequences.

As a mediator of cell-to-cell communication, ATP can trigger a variety of biological responses after being rapidly released in large amounts from various sources, including activated platelets, endothelial cells, nerve terminals, antigen-stimulated T cells, and other cell types following hypoxia, stress, and tissue damage. For example, in human umbilical cord vein endothelial cells (HUVEC), a substantial release of ATP (and UTP) is induced by shear stress [81], which may lead to alterations in the balance between proliferation and apoptosis regulated by P1 adenosine and P2 (particularly P2X<sub>7</sub>) nucleotide receptors [82]. P2X<sub>7</sub> and P1 receptors have been previously linked to apoptosis in other cell types, including immune cells, astrocytes, and thymocytes [83–85].

The P2X<sub>7</sub>R also has been shown to mediate ATP-induced cell death in human embryonic kidney cells [86], human cervical epithelial cells [87], and primary rat cortical neurons [88]. In human arterial SMC, adenosine-induced apoptosis is essentially mediated via the A<sub>2b</sub>-adenosine receptor subtype and involves a cAMP-dependent pathway [89].

As an important constituent of atherosclerotic plaques, fibroblasts share several features with smooth muscle cells. In human fibroblasts, P2X<sub>7</sub> was identified as the main nucleotide receptor involved in the high glucose concentration-dependent responses modulated by ATP, including morphological changes, enhanced apoptosis, caspase-3 activation, and IL-6 release [90]. In the immune system, ATP also plays important roles through nucleotide receptors in leukocyte functions. P2X<sub>7</sub> receptor-mediated apoptosis has been demonstrated in various types of leukocytes, including a lymphocytic cell line, murine thymocytes, murine peritoneal macrophages, human macrophages, mesangial cells, dendritic cells, and microglial cells [83, 91–96]. Extracellular ATP acting via the P2X<sub>7</sub> receptor activates the transcription factor NFκB by selectively targeting NFκB p65 (Rel A) in the N9 mouse microglial cell line [97]. It also has been reported that the P2X<sub>7</sub> receptor modulates macrophage production of TNF-α, IL-1β, and nitric oxide (NO) following lipopolysaccharide (LPS) exposure [98], consistent with a role for the P2X<sub>7</sub> receptor in inflammation. In HUVEC, TNF-α markedly increases apoptotic cell death via the activation of caspase-3 [74]. Recent reports indicate that ATP/ADP activate NFκB and induce apoptosis probably through P2X<sub>7</sub> receptors in porcine aortic endothelial cells [99]. These studies have provided compelling evidence suggesting a role for P2 and P1 receptor-mediated apoptosis in vascular diseases; however, further studies are needed to determine the precise pathways involved and to accumulate direct evidence that these pathways contribute significantly to the development of atherosclerosis, hypertension, and restenosis.

## Modulation of P2 receptors in vascular injury

Experimental arterial intimal hyperplasia can be induced by balloon angioplasty or by the perivascular placement of a silicone collar around an artery. An influx of leukocytes precedes the migration and proliferation of vascular SMC into the intima in both these models [100]. In normal adult rat aorta, P2Y<sub>2</sub> mRNA was found in the endothelial cell lining, while a sustained expression of P2Y<sub>2</sub> mRNA was detected in few medial SMC [20]. In contrast, P2Y<sub>2</sub> mRNA was detected in all medial SMC of rat fetal aortas and in most of the aortic SMC of intimal lesions after balloon angioplasty, with an overexpression in cells lining the lumen both 1 and 3 weeks after injury.

In the collar model, neointimal formation appears to be triphasic [100]. The first phase is characterized by vascular infiltration of leukocytes beginning 2 h after collar placement around a rabbit carotid artery. The second phase begins within 12 h of collar placement and is characterized by medial replication of SMC. The third phase is characterized by the appearance, beginning at day 3 after collar placement, of subendothelial SMC. *In situ* hybridization with sham-operated rabbit carotid arteries indicated that P2Y<sub>2</sub> mRNA expression was localized to CD31-positive aortic endothelial cells and not medial SMC [6]. High levels of P2Y<sub>2</sub> mRNA were detected in medial SMC 3 days after collar placement, before appearance of neointima. At day 14, all intimal and medial SMC were P2Y<sub>2</sub> positive. Fura-2 digital imaging of single SMC, used to measure changes in myoplasmic calcium concentration in response to P2Y receptor agonists, confirmed an increase in P2Y<sub>2</sub> receptor activity. However, the same study showed that P2Y<sub>4</sub> mRNA was equivalently expressed in sham-operated and collared arteries or cultured rabbit carotid SMC, whereas P2Y<sub>6</sub> mRNA was not detected in carotid arteries or cultured SMC. In a more recent study, it was shown that P2Y<sub>2</sub> receptor upregulation occurs in stented porcine coronary artery, a clinically relevant model of arterial injury [54]. P2Y<sub>2</sub> receptor mRNA levels were significantly increased in coronary SMC dispersed from stented segments of coronary arteries 3 weeks after stent angioplasty compared with SMC from unstented segments. There was no significant difference observed in levels of P2Y<sub>6</sub> mRNA in the stented and unstented artery segments, whereas P2Y<sub>4</sub> receptor mRNA was undetectable.

Upregulation of functional P2Y receptors also occurs in the basilar artery of the rat double-hemorrhage model [7], in the coronary artery of diabetic dyslipidemic pigs [8], and in human atherosclerotic lesions (Seye and Desgranges, unpublished data). It has been proposed that upregulation of P2Y receptors could be a potential diagnostic indicator for the early stages of atherosclerosis [9]. Interestingly, a more recent study showed that high shear stress, associated with vascular diseases, can selectively upregulate P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors in perfused arterial SMC [101]. P2X<sub>1</sub> and P2X<sub>4</sub> receptors have been shown to be upregulated in rabbit intimal thickenings [102]. Taken together, these findings strongly suggest that at least some P2 receptor subtypes (most notably P2Y<sub>2</sub>) are implicated in the development of vascular disease.

### Pathophysiological significance of P2 receptor modulation in vascular injury

Migrating and proliferating SMC in the arterial media, together with infiltrating macrophages and T lymphocytes,

are the main cell types that comprise atherosclerotic and restenotic lesions [1]. In the early stages of intimal hyperplasia, SMC are modified from a differentiated, contractile phenotype to an immature, synthetic phenotype, and this enables them to migrate into the intima, to proliferate, and to secrete extracellular matrix components. In many respects, this shift in phenotype is a reversal of the normal differentiation pattern of vascular SMC during fetal and early postnatal life [103, 104]. However, there is still a lack of knowledge concerning the phenotypic regulation of SMC during vasculogenesis and vascular disease. A high level of P2 receptor expression could be related to the altered phenotype of SMC in intimal thickenings. Partially dedifferentiated SMC are found in rat arterial intimal lesions after balloon angioplasty [105, 106], and their phenotype has been compared with that of newborn rat aortic SMC [107]. Interestingly, it has been reported that medial SMC of rat embryonic aorta also exhibit high P2Y<sub>2</sub> expression, similar to intimal thickenings [20]. Other studies have shown that P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor transcripts are strongly upregulated with phenotypic changes in rat SMC, whereas P2X<sub>1</sub> mRNA is completely downregulated, and P2Y<sub>4</sub> and P2Y<sub>6</sub> mRNA levels are unchanged [58, 28]. Taken together, these and other results suggest that P2Y<sub>2</sub> receptor expression is upregulated in the entire cell population of intimal thickenings and is closely associated with a poorly differentiated phenotype of SMC. The dramatic increase in P2Y<sub>2</sub> mRNA expression observed in balloon angioplasty-induced intimal lesions would suggest increased activity of extracellular nucleotides with consequent enhancement of cell proliferation and vasoreactivity. Indeed, extracellular nucleotides, particularly ATP and UTP, have been shown to induce cell cycle progression and proliferation of cultured arterial SMC [21, 49, 50, 51] and vasoconstriction in the absence of endothelial cells [17, 108, 109]. Since both neointimal hyperplasia and vasoconstrictive remodeling have been found to be involved in postangioplasty restenosis [1, 5, 107, 110], these findings suggest that extracellular nucleotides may play a significant role in this process, at least as long as functional endothelial cells, which regulate intimal thickening [111, 112] and nucleotide-induced vasorelaxation [113, 114], are not regenerated.

Increased P2Y<sub>2</sub> receptor expression in the neointima may by itself be sufficient to enhance the local effects of extracellular nucleotides on the proliferation of SMC. Although the expression of other P2 receptors has been described in arterial SMC [21, 29, 115], the P2Y<sub>2</sub> receptor seems to be more specifically involved in the response of SMC to ATP and UTP [116], particularly in the potentiation of proliferation [21, 50]. The effects of extracellular nucleotides are not only dependent on the nature and number of P2 receptors present on target cells, but also on the local concentrations of nucleotide agonists. Although

in vivo concentrations of extracellular nucleotides are difficult to measure, various in vitro experiments suggest that extracellular nucleotides are released from blood and vascular cells exposed to various physicochemical conditions, e.g., stress, hypoxia, and other factors [117, 118, 119] associated with the angioplasty process.

P2Y<sub>2</sub> receptors in SMC are involved in nucleotide-induced constriction of normal arteries [17, 108, 109]. Long lasting alterations in vasomotricity after endothelial cell denudation, resulting in increased sensitivity to vasoconstrictive substances, have previously been demonstrated [113, 114]. It appears that like other receptors for vasoconstrictive factors such as angiotensin II [120], endothelin [121], and platelet-derived growth factor (PDGF) [122], which are overexpressed in neointima, P2Y<sub>2</sub> receptors may play an important role in controlling the vasoactive properties of pathological arteries, particularly in chronic constriction at the lesion site that is postulated to be one of the processes leading to postangioplasty restenosis [5, 110].

## Conclusion

P2 receptor subtypes, including P2Y<sub>2</sub>, P2X<sub>1</sub>, and P2X<sub>4</sub>, appear to play a role in responses to endothelial injury that are thought to be key events in the initiation of atherosclerosis and restenosis after angioplasty. P2Y<sub>2</sub>R upregulation and activation in endothelial cells and SMC promote leukocyte transmigration and intimal thickening in arteries of animal models of vascular injury, suggesting a possible regulatory role for this receptor in the mechanisms leading to neointimal hyperplasia after angioplasty. Although there are no selective P2Y<sub>2</sub> receptor antagonists yet available, recent progress in siRNA technology has made it possible to design small RNA interference molecules that can selectively inhibit P2 receptor subtype expression. Such molecules can be efficiently delivered into the vessel wall using adenoviral vectors. In addition, P2 receptor transgenic mice (i.e., mice in which the relevant receptor subtype has been deleted or overexpressed) will be valuable tools to substantiate the role that nucleotides and P2 receptors play in the etiology of cardiovascular disease. Further delineation of the signaling pathways involved in these P2 receptor-mediated processes may help limit or prevent vascular diseases such as atherosclerosis and restenosis after angioplasty.

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