P2Y Receptor Regulation of PAI-1 Expression in Vascular Smooth Muscle Cells

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Abstract—P2Y-type purine and pyrimidine nucleotide receptors play important roles in the regulation of vascular hemostasis. In this article, the regulation of plasminogen activator inhibitor-1 (PAI-1) expression in rat aortic smooth muscle cells (RASMCs) by adenine and uridine nucleotides was examined and compared. Northern analysis revealed that RASMCs express multiple P2Y receptor subtypes, including P2Y$_1$, P2Y$_2$, and P2Y$_6$. Treatment of RASMCs with UTP increased PAI-1 mRNA expression and extracellular PAI-1 protein levels by 21-fold ($P<0.001$) and 7-fold ($P<0.001$), respectively. The ED$_{50}$ for the effect of UTP on PAI-1 expression was $\approx 1 \mu$mol/L, and its maximal effect occurred at 3 hours. UDP stimulated a 5-fold increase ($P<0.005$) in PAI-1 expression. In contrast to these potent stimulatory effects of uridine nucleotides, ATP and 2-methylthioadenosine triphosphate (2-MeSATP) caused a small and transient increase in PAI-1 mRNA at 1 hour, followed by a rapid decrease to baseline levels. ADP produced only an inhibitory effect, reducing PAI-1 mRNA levels by 63% ($P<0.05$) at 3 hours. The relative nucleotide potency in stimulating PAI-1 expression is UTP$>$UDP$>$ATP=2-MeSATP, consistent with a predominant role of the P2Y$_6$ receptor. Further studies revealed that exposure of RASMCs to either ATP or ADP for 3 hours inhibited both UTP- and angiotensin II–stimulated PAI-1 expression by up to 90% ($P<0.001$). Thus, ATP induced a small and transient upregulation of PAI-1 that was followed by a strong inhibition of PAI-1 expression. These results show that extracellular adenine and uridine nucleotides exert potent and opposing effects on vascular PAI-1 expression. (Arterioscler Thromb Vasc Biol. 2000;20:866-873.)

Key Words: purinoceptors $\bullet$ nucleotides $\bullet$ plasminogen activator inhibitor $\bullet$ vascular smooth muscle cells $\bullet$ rats

Plasminogen activator inhibitor-1 (PAI-1) is the primary regulator of plasminogen activation. Elevated levels of PAI-1 shift the fibrinolytic balance toward thrombosis and may impair the turnover of extracellular matrix proteins.1,2 Reports from our laboratory and others have shown that vasoactive hormones may play an important role in regulating PAI-1 expression in vascular cells. Vasopressor hormones, including angiotensin II (Ang II) and arginine$^8$-vasopressin, are potent stimulators of PAI-1 expression in cultured vascular smooth muscle cells (VSMCs).3,4 Evidence for an important physiological role of the renin-angiotensin system in the regulation of PAI-1 has been provided by studies showing that inhibition of the angiotensin system reduces aortic PAI-1 expression and circulating PAI-1 levels.5–7 In addition, vasodilatory hormones, including nitric oxide and natriuretic factors, appear to reduce PAI-1 expression in VSMCs and endothelial cells.8,9 These findings suggest that the fibrinolytic balance may be coordinately regulated with vascular tone and blood pressure.

Extracellular purine and pyrimidine nucleotides regulate vascular tone and hemostasis by activating cell-surface P2-type receptors.10,11 Exogenous delivery of adenine nucleotides has been shown to increase forearm and cochlear blood flow12,13 and induce endothelium-dependent vasorelaxation in isolated coronary and cerebral arteries.14–16 In contrast, uridine nucleotides, acting primarily on smooth muscle cells, induce vasoconstriction in pulmonary and coronary arteries.17,18 The vascular actions of these nucleotides are mediated via 2 P2 receptor families, including the P2X ligand-gated cation channels and the P2Y G protein–coupled receptors.10,11 Multiple subtypes of these P2X and P2Y receptors are expressed by vascular cells, and specific receptor subtypes differ in their tissue distribution, regulation, and sensitivity to nucleotide agonists.11,19–22 These vascular P2 receptors are activated in an autocrine or paracrine manner by nucleotides that are released to the extracellular milieu from perivascular nerves, mechanically strained cells, and activated platelets.23–26 In addition to modulating vascular tone and blood flow, extracellular nucleotides have been shown to stimulate VSMC growth and endothelial permeability and chemotaxis and to inhibit glucose transport in cardiomyocytes.27–33

To examine the potential role of P2-type receptors in the regulation of the plasminogen system, we examined the
effects of extracellular adenine and uridine nucleotides in the regulation of PAI-1 expression in rat aortic smooth muscle cells (RASMCs). These studies have revealed that uridine nucleotides are highly potent stimulators of PAI-1 expression. In contrast, adenine nucleotides exert a combination of both stimulatory and inhibitory effects on PAI-1 expression. A role of the P2Y₄ receptor subtype in the regulation of PAI-1 expression in RASMCs is implicated.

Methods

Cell Culture
RASMCs were isolated from Sprague-Dawley rats, cultured in DMEM/100 mg/dL D-glucose (Gibco-BRL) with 10% FBS (Gibco BRL) as described previously, and used between passages 8 and 15. Confluent monolayers of cells were deprived of serum in DMEM containing 0.1% (wt/vol) BSA for 18 hours before stimulation. Cells were stimulated with uridine and adenine nucleotides (Sigma) in the absence or presence of additional stimulation with Ang II (Sigma), PD098059 (Biomol), and GF109203X (Calbiochem) as described.

Reverse Transcription–Polymerase Chain Reaction of P2Y Receptors From RASMCs

Total RNA was treated with RNase-free DNase, and cDNA was synthesized from total RNA by use of a random hexamer primer and a first-strand cDNA Synthesis Kit (Clontech). Then cDNA was used as template in the following polymerase chain reaction (PCR) reactions. The rat P2Y₁ receptor sequence was amplified with primers corresponding to 78 to 103 and 855 to 831 nucleotides, yielding a 777-bp product. The rat P2Y₂ receptor sequence was amplified with primers corresponding to 755 to 779 and 1506 to 1482 nucleotides, yielding a 751-bp product. The rat P2Y₆ receptor sequence was excised with EcoR1, agarose gel–purified, labeled with the Multiprime DNA labeling system (Amersham Corp), and used for Northern blot analysis. This study revealed P2Y₁ expression in total RNA from rat heart and poly A–enriched RNA from RASMCs (Figure 1). P2Y₂ expression was detected in total RNA from heart and aorta and poly A–enriched RNA from RASMCs. Northern blot analysis of P2Y₁, P2Y₂, and P2Y₆ expression in 20 µg total RNA from rat heart, aorta, and RASMCs or 12 µg poly A–enriched RNA from RASMCs.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated with TRI reagent (Molecular Research Center, Inc.) and used for Northern blot analysis. This study revealed P2Y₁ expression in total RNA from rat heart and poly A–enriched RNA from RASMCs. Northern blot analysis of P2Y₁, P2Y₂, and P2Y₆ expression in 20 µg total RNA from rat heart, aorta, and RASMCs or 12 µg poly A–enriched RNA from RASMCs.

Statistics

All statistical analyses were performed by 1-way ANOVA with SigmaStat (Jandel Scientific). Values of P<0.05 were considered significantly different.

Results

Northern blot analysis was performed to examine and compare P2Y₁, P2Y₂, and P2Y₆ receptor expression in cultured RASMCs, aorta, and heart left ventricle from Sprague-Dawley rats. This study revealed P2Y₁ expression in total RNA from rat heart and poly A–enriched RNA from RASMCs (Figure 1). P2Y₁ expression was detected in total RNA from heart and aorta and both total and poly A–enriched RNA from RASMCs. P2Y₆ mRNA expression was detected in total RNA from aorta and poly A–enriched RNA from RASMCs. These results demonstrate that RASMCs express low levels of P2Y₁ mRNA and comparably higher levels of P2Y₂ and P2Y₆ transcripts.

Because P2Y₂ and P2Y₆ are activated by both adenine and uridine nucleotides, the effects of these nucleotides on the regulation of PAI-1 expression in RASMCs were examined. Cells were grown to confluence, incubated for 18 hours in medium containing 0.1% BSA, and treated from 1 to 4 hours with 50 µmol/L UTP, ATP, or 2-methylthioadenosine triphosphate (2-MeSATP). After these treatments, RNA was harvested and PAI-1 mRNA levels were measured by Northern blot analysis. This study revealed that the effects of uridine and adenosine nucleotide triphosphates on PAI-1 mRNA differed in both kinetics and magnitude. UTP caused a sustained increase in PAI-1 mRNA, reaching 21-fold induction (P<0.001, ANOVA) after 3 hours (Figure 2A). In contrast, ATP and 2-MeSATP induced a small and transient increase in PAI-1 levels, reaching ~2-fold after 1 hour.
Figure 2. Effects of purine and pyrimidine nucleotides on PAI-1 mRNA expression in RASMCs. Cells were treated with 50 μmol/L of UTP (A), ATP or 2-MeSATP (B), and ADP or UDP (C) over the time courses indicated. After these treatments, total RNA was harvested, and PAI-1 and 36B4 mRNA levels were examined by Northern blot analysis. Results were visualized and quantified by PhosphorImager (Molecular Dynamics). Representative blots of PAI-1 and 36B4 mRNA expression and bar graph quantification of PAI-1 mRNA levels normalized to 36B4 mRNA from ≥3 separate experiments are shown. Significant differences from controls were determined by ANOVA and are indicated as *P < 0.05, **P < 0.005, or ***P < 0.001. Con indicates control.
followed by a return toward baseline after 3 to 4 hours (Figure 2B). The effects of adenine and uridine nucleotide diphosphates on PAI-1 expression were also examined. PAI-1 mRNA levels were measured in cells stimulated with 50 μmol/L ADP or UDP for 1 to 3 hours (Figure 2C). UDP increased PAI-1 levels by up to 5-fold at 2 hours. In contrast, ADP reduced PAI-1 mRNA levels after 2 and 3 hours by 58% and 63%, respectively (P<0.05). These results indicate that the relative potency of nucleotides on stimulating PAI-1 expression in RASMCs is UTP > UDP > ATP > 2-MeSATP.

The dose-response effect of UTP on PAI-1 expression was examined over a nucleotide concentration range from 100 nmol/L to 100 μmol/L. This study revealed that the ED₅₀ for the effect of UTP was ~1 μmol/L, and its maximal effect was observed at 10 μmol/L (not shown). Treatment of cells with 50 μmol/L GTP did not alter PAI-1 expression (not shown).

Because cells may be simultaneously exposed to adenine and uridine nucleotides, the combined effect of these nucleotides on PAI-1 expression in RASMCs was examined. Cells were treated with 50 μmol/L UTP in the absence or presence of a concentration range of 500 nmol/L to 50 μmol/L ATP or ADP for 3 hours. Treatment of cells with ATP alone induced a 10-fold increase in PAI-1 expression (Figure 3), as described in Figure 2A. Interestingly, addition of either ATP or ADP inhibited UTP-stimulated PAI-1 expression in a concentration-dependent manner (Figure 3). The IC₅₀s for the adenine nucleotides were ~5 μmol/L, and inhibitory effects at 50 μmol/L of ATP and ADP reduced PAI-1 levels by 93% and 90%, respectively (P<0.001).

To determine whether ATP can also inhibit PAI-1 expression induced by other agonists of PAI-1 expression, the effect of ATP on angiotensin II (Ang II)–stimulated PAI-1 was examined. Previous reports have shown that Ang II is a potent stimulator of PAI-1 mRNA expression in RASMCs and that the effect of Ang II is maximal at 3 hours. Cells were stimulated with 100 nmol/L Ang II for 3 hours either alone or with 50 μmol/L of either ATP or UTP. Stimulation of cells with Ang II alone increased PAI-1 mRNA levels by 13-fold (Figure 4). Treatment of cells with a combination of ATP and Ang II for 3 hours was 86% less effective than Ang II treatment alone in stimulating PAI-1. This inhibitory effect of ATP was also observed when ATP was added 1 hour before or 1 hour after the addition of Ang II (Figure 4). Thus, treatment of RASMCs with ATP for ≥2 hours inhibits both Ang II–stimulated and UTP-stimulated PAI-1 expression in RASMCs (Figures 3 and 4). In contrast to these inhibitory effects of ATP, treatment of cells with a combination of UTP and Ang II was 2-fold (P<0.001) more effective than Ang II alone in increasing PAI-1 expression (not shown).

Figure 3. Effects of adenine nucleotides on UTP-stimulated PAI-1 mRNA expression in RASMCs. Cells were stimulated for 3 hours with 50 μmol/L UTP in the absence or presence of the indicated concentrations of ATP or ADP. Representative blots showing PAI-1 and 36B4 mRNA expression and bar graph quantification of PAI-1 mRNA levels normalized to 36B4 mRNA are shown. Significant differences between UTP and UTP plus ATP or ADP are indicated as *P<0.05 and **P<0.001. Con indicates control.

Figure 4. Effects of ATP on Ang II–stimulated PAI-1 expression in RASMCs. Cells were stimulated with Ang II for 3 hours with or without concurrent stimulation with ATP for the times indicated. Representative blots and bar graph quantification from 4 experiments. Significant differences between ATP and ATP plus Ang II are indicated as **P<0.001, ANOVA) are indicated as ***. Con indicates control.
The effects of UTP and ATP on PAI-1 protein production were examined. Cells were stimulated with UTP or ATP (50 μmol/L) either alone or in combination with Ang II (100 nmol/L) for 18 hours, and PAI-1 protein released to the conditioned medium was measured by immunoblot analysis, as described previously. Conditioned media were separated by SDS-PAGE, and PAI-1 protein was quantified by immunoblot analysis with an anti–rat PAI-1 antibody. This study revealed that UTP increased PAI-1 protein production by 7-fold, which was similar to the 8-fold increase in PAI-1 induced by Ang II (Figure 5). The combined effect of Ang II and UTP on PAI-1 levels was 34% (P < 0.05) greater than that observed with UTP alone. Treatment of cells with ATP did not significantly alter PAI-1 protein levels either alone or in the presence of Ang II.

Previous studies have shown that ATP and UTP stimulation of P2Y receptors leads to the activation of the MAP kinase (ERK-1, -2) pathway and that activation of this pathway can increase PAI-1 transcription. To determine whether signaling differences of these nucleotides through the MAP kinase pathway may contribute their differential effects on PAI-1 expression, the abilities of ATP and UTP to activate the ERK-1, -2 were examined and compared. Activation of ERK-1 and -2 was assessed by measurement of phosphorylation at T202/Y204. Although both nucleotides activated this MAP kinase pathway, this study revealed that UTP was 2-fold (P < 0.001) more potent than ATP in increasing ERK-2 phosphorylation (Figure 6). To examine the role of the MAP kinase pathway in UTP-stimulated PAI-1 expression, cells were pretreated with MEK inhibitor PD098059 for 15 minutes, followed by stimulation with 50 μmol/L UTP for 3 hours. Northern blot analysis of PAI-1 mRNA expression showed that 30 and 100 μmol/L PD098059 inhibited UTP-induced PAI-1 expression by 34% and 51% (P < 0.05), respectively (not shown). These results suggest that the MAP kinase (ERK-1, -2) pathway partially contributes to UTP-stimulated PAI-1 expression in RASMCs. Thus, the greater stimulation of the MAP kinase pathway by UTP, compared with ATP, may contribute to its more potent effect in increasing PAI-1 expression.

To further characterize the mechanism of UTP-induced PAI-1 expression, the role of PKC was examined. Previous reports have shown that P2Y2 and P2Y6 receptors are G protein–coupled to phospholipase C, which leads to the generation of diacylglycerol and the elevation of cytosolic calcium, cofactors for protein kinase C (PKC). Because we have shown that PKC regulates PAI-1 expression in RASMCs, the effect of PKC inhibition on UTP-stimulated PAI-1 expression was examined. Pretreatment of cells with the PKC inhibitor GF109203X reduced UTP-stimulated PAI-1 expression by 58% (P < 0.001) (not shown). These results suggest that PKC contributes to the induction of PAI-1 by UTP.

**Discussion**

This report demonstrates that extracellular adenine and uridine nucleotides exert potent effects on PAI-1 expression in RASMCs. Exposure of these cells to UTP increased PAI-1 mRNA levels and extracellular PAI-1 protein by 20-fold and 7-fold, respectively. The magnitude of this induction of PAI-1 expression by UTP is comparable to that of other strong agonists of PAI-1, such as platelet-derived growth factor and Ang II. The time course of UTP-stimulated PAI-1 expression showed that elevation of PAI-1 mRNA
levels is rapid, being elevated by 10-fold after 1 hour and sustained over the 4-hour study. UDP also induced a rapid upregulation of PAI-1 expression, but its effect was less than that of UTP. The ED$_{50}$ of UTP-stimulated PAI-1 was between 1 and 10 $\mu$mol/L, which is comparable to the other signaling and biological effects of UTP. In addition, stimulatory effects of UTP on PAI-1 mRNA and protein expression appeared to be additive to that of Ang II. These findings suggest that these uridine nucleotides, which induce VSMC vasoconstriction, are also potent stimulators of PAI-1 expression in RASMCs.

In contrast to the pronounced induction of PAI-1 by uridine nucleotides, both ATP and the P2Y-agonist 2-MeSATP produced only small increases in PAI-1 mRNA after 1 hour, which were followed by a rapid decline to basal levels. Treatment of cells with ADP reduced PAI-1 levels without stimulating the transient increase in PAI-1 observed with ATP. Coincubation of cells with UTP and ATP or ADP revealed that both adenine nucleotides suppress PAI-1 expression in a concentration-dependent manner. Because ATP similarly inhibited Ang II-stimulated PAI-1 expression, the inhibitory effects of ATP are not due to antagonistic competition for pyrimidinoceptors. The ED$_{50}$ of ATP and the inhibitory effects of ADP on PAI-1 expression were $\approx 5$ $\mu$mol/L, which is similar to the ED$_{50}$ of other actions of these nucleotides. These results suggest that ATP exerts both stimulatory and inhibitory effects on PAI-1 expression, which are separated, at least in part, by kinetics.

Examination of P2Y receptor subtype expression in RASMCs derived from Sprague-Dawley rats revealed P2Y$_{1}$, P2Y$_{2}$, and P2Y$_{6}$ receptors. The expression of P2Y$_{1}$ receptors in RASMCs was detected in poly A–enriched RNA but not in total RNA, indicating that this receptor subtype is expressed at low levels in these cells. Although a previous report demonstrated that this receptor subtype is present in VSMCs, particularly in its synthetic phenotype, another report did not detect P2Y$_{1}$ in VSMCs derived from spontaneously hypertensive rats (SHR). Thus, expression of P2Y$_{1}$ may depend on both VSMC phenotype and species. Expression of P2Y$_{2}$ in RASMCs is consistent with a previous report showing expression of P2Y$_{2}$ in medial smooth muscle cells in rat aorta from Wistar rats and SHR-derived VSMCs. Detection of P2Y$_{6}$ in RASMCs is consistent with previous reports of this receptor in VSMCs. Comparison of the levels of P2Y subtype expression in RASMCs with that in rat aorta shows that P2Y$_{2}$ and P2Y$_{6}$ expression in vivo is similar to or higher than that observed in cultured cells.

The P2Y receptor subtypes described above differ in regard to their sensitivity to nucleotide agonists. P2Y$_{1}$ receptors are activated primarily by adenine nucleotides, with UTP having little or no effect. P2Y$_{2}$ and P2Y$_{6}$ receptors in rat are equally activated by ATP and UTP. The P2Y$_{6}$ receptor is the only P2Y receptor subtype in rat that is preferentially activated by uridine nucleotides compared with adenine nucleotides. Although the effects of uridine and adenine nucleotides on PAI-1 expression in RASMCs is most likely the result of the combined effect of P2Y receptors, the more potent effects of uridine nucleotides compared with adenine nucleotides is consistent with a predominant role of P2Y$_{6}$ in the regulation of PAI-1 in these cells. Because the P2Y$_{6}$ receptor is strongly expressed in aorta, this receptor may also contribute to the regulation of vascular PAI-1 expression in vivo. It is possible, however, that the potential effect of ATP-activated P2Y receptors on stimulating PAI-1 expression in RASMCs is underestimated because of the secondary inhibitory effects of ATP breakdown products, such as ADP and adenosine. Further characterization of the relative contributions of specific P2Y receptors in the regulation of vascular PAI-1 will require the development of subtype-specific receptor antagonists.

We have shown that UTP is a potent stimulator of ERK phosphorylation and that inhibition of PKC or MEK1,2 kinase partially inhibits UTP-induced PAI-1 expression. Because activation of PKC and the MAP kinase pathway has been shown to increase PAI-1 transcription, it is likely that UTP signaling through these elements similarly increases PAI-1 transcription. However, because inhibition of PKC and MEK1,2 was only partially effective in blocking UTP-stimulated PAI-1, it appears likely that additional signal processes contribute to P2Y-mediated upregulation of PAI-1 expression. Because ATP also activates the ERK pathway, it is likely that this pathway also contributes to the transient upregulation of PAI-1 mRNA by ATP. Comparison of the signaling effects of ATP and UTP showed that UTP was 2-fold more effective in increasing ERK phosphorylation. In contrast, UTP was 20-fold more effective than ATP in upregulating PAI-1 expression. These results suggest that the difference between the effects of ATP and UTP on PAI-1 is due to a combination of a smaller stimulation of RASMCs by ATP as well as a secondary inhibitory effect of ATP on PAI-1 expression. A possible mechanism for the inhibitory effects of ATP on PAI-1 expression could be related to its extracellular dephosphorylation to ADP and adenosine. VSMCs express ATP diphosphohydrolase and ecto-5'-nucleotidase, which convert adenine nucleotides to adenosine and thereby may lead to the activation of adenosine P1 receptors. We have found that treatment of cells with cyclopentyladenosine, a selective A$_1$ adenosine receptor agonist, reduced PAI-1 expression without affecting PAI-1 mRNA half-life (not shown). Because ATP and ADP exert greater inhibitory effects on UTP-stimulated PAI-1 than basal PAI-1 expression, it appears likely that adenosine nucleotides act, at least in part, by reducing PAI-1 transcription. Consistent with this, we observed that the rate of decrease in PAI-1 mRNA levels in ADP-treated cells (58% decrease in 2 hours) was similar to the decrease in PAI-1 mRNA we reported in actinomycin D–treated RASMCs (54% decrease in 2 hours).

The potent effects of uridine and adenine nucleotides on PAI-1 expression in RASMCs suggest that P2Y receptors may play an important role in the regulation of vascular PAI-1 expression. There are a number of physiological and pathophysiological conditions that regulate extracellular nucleotide levels in the vascular milieu. In cultured RASMCs, cyclic mechanical stretch has been shown to rapidly elevate extracellular ATP levels, which act in an autocrine manner via P2Y receptors to activate MAP kinases. This autocrine/paracrine activation of P2Y receptors in VSMCs may contribute to the rapid activation of MAP kinases and the elevation of PAI-1 expression reported in vessels after balloon-catheter stretch. In addition, endothelium disruption at sites of vascular injury may facilitate platelet adhesion and subsequent adenosine and uridine nucleotide release, which may locally stimulate VSMCs.
that interstitial ATP levels in rat heart increase by 10-fold after regional ischemia, and interstitial ATP and ADP levels have been shown to range from 2 to 6 μmol/L in exercising skeletal muscle. These reports indicate that extracellular levels of ATP and ADP are dynamically regulated and can reach micromolar concentrations that encompass the ED₉₀ of the inhibitory effects of ATP and ADP on PAI-1. Because interstitial ATP is rapidly dephosphorylated to ADP and adenosine, it is likely that this pool of nucleotides predominantly suppress PAI-1 expression.

In summary, we have shown that extracellular uridine and adenosine nucleotides regulate PAI-1 expression in RASMCs. Uridine nucleotides induce a prolonged induction of PAI-1 that is comparable to the effects of hormones and growth factors, such as Ang II and platelet-derived growth factor. In contrast, extracellular ATP had a bimodal effect that included a small transient upregulation of PAI-1 mRNA followed by a potent inhibitory effect. These results suggest that P2-type purinoceptors and pyrimidinoceptors may play an important role in the regulation of PAI-1 expression in the vasculature.

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