Transcriptional Inhibition of Protease-Activated Receptor-1 Expression by Prostacyclin in Human Vascular Smooth Muscle Cells

Robert Pape, Bernhard H. Rauch, Anke C. Rosenkranz, Gernot Kaber, Karsten Schrö"or

Objective—Stimulation of protease-activated receptor-1 (PAR-1) by thrombin causes vascular smooth muscle cell (SMC) mitogenesis and has been implicated in the vascular response to injury. Vascular injury is also associated with enhanced formation of PGE2 and PGI1 (prostacyclin). This study investigates whether PGI1 and PGE2 modify the expression of PAR-1 and the cellular response to thrombin in human SMC.

Methods and Results—The PGI1-mimetic iloprost (1 to 100 nmol/L) attenuated mRNA, total protein, and cell surface expression of PAR-1. This was associated with inhibition of thrombin-induced mitogenesis and migration. Comparable inhibition of PAR-1 expression was observed with the selective IP-receptor agonist cicaprost, the adenyl cyclase activator forskolin, the phosphodiesterase inhibitor isobutylmethylxanthine and the PKA activator dibutyryl-cAMP. Similar effects of PGE2 required micromolar concentrations. The specific PKA-inhibitor Myr-PKI prevented PAR-1 downregulation by iloprost. The potential role of Rho family GTPases in PAR-1 regulation was also investigated. Iloprost decreased Rac1 mRNA and the Rac1 inhibitor NSC23766 mimicked the inhibitory effects of iloprost on PAR-1 protein—but not mRNA. The Rho kinase inhibitor Y27632 did not influence PAR-1 expression.

Conclusions—IP-receptor agonists may limit the mitogenic actions of thrombin in human SMC by downregulating PAR-1 via modulation of cAMP/PKA- and Rac1-dependent signaling pathways. (Arterioscler Thromb Vasc Biol. 2008;28:534-540)

Key Words: protease-activated receptor-1 (PAR-1) ■ smooth muscle ■ thrombin ■ prostaglandins ■ Rac1
tory actions of thrombin. However, this hypothesis is not established yet and mechanisms of this interaction are unknown, although transcriptional regulation of PAR-receptors is not unlikely because PG\(_I\) (iloprost) regulates many genes in vascular SMCs which are related to coagulation and tissue repair.\(^{27}\) Therefore, we examined whether transcriptional regulation of the thrombin receptor PAR-1 in human vascular SMCs contributes to the inhibitory effects of vasodilatory prostaglandins on thrombin-induced mitogenesis, and explored potential signaling pathways involved.

The present study reports that the PG\(_I\) analogue iloprost attenuates mRNA, total protein, and surface expression of PAR-1. This is associated with a reduced mitogenic response to thrombin. Iloprost-mediated suppression of PAR-1 depends critically on Gs/cAMP/PKA-dependent signaling, as well as on activation of the small GTPase Rac1, which was recently reported to be involved in PAR-1 trafficking.\(^{28}\) Taken together, this study provides first evidence that vasodilatory prostaglandins control the mitogenic response of thrombin at the level of PAR-1 expression in human SMCs.

Materials and Methods

Materials

Dulbecco’s modified Eagle medium (DMEM) and fetal calf serum (FCS) were from GibcoBRL. Purified α-thrombin was kindly provided by Dr J. Stürzebecher (Institut für Vaskuläre Medizin, Jena, Germany); iloprost and cicaprost by Schering AG. PGE\(_2\) was provided by Dr J. Stürzebecher (Institut für Vaskuläre Medizin, Jena, Germany); iloprost and cicaprost by Schering AG. PGE\(_2\) was recently reported to be involved in PAR-1 trafficking.\(^{28}\) Taken together, this study provides first evidence that vasodilatory prostaglandins control the mitogenic response of thrombin at the level of PAR-1 expression in human SMCs.

Cell Culture

Human SMCs were isolated from saphenous veins by the explant technique and cultured as described previously.\(^{29,30}\) For experiments, subconfluent cells at passages 4 to 10 were serum-deprived for 24 hours before treatment with the different substances for the indicated times.

Flow Cytometry

For analysis of surface expression of PAR-1, SMCs were seeded in 6-well plates and stimulated as indicated. After nonenzymatic detachment with citric saline buffer (0.135 mol/L potassium chloride, 0.015 mol/L sodium citrate) for 10 to 15 minutes at 37°C, cells were pelleted and resuspended in PBS. Cell suspensions (50 \(\mu\)L) were incubated with 10 \(\mu\)L PE-conjugated anti-human PAR-1 antibodies (Coulter-Immunotech) for 20 minutes at room temperature. After the dark, isotype-matched PE-conjugated antibodies were used to assess nonspecific binding. Samples were diluted with 500 \(\mu\)L IsoTune and immediately analyzed on an EPIC-XL cytometer (Beckman Coulter). SMC populations were identified according to forward and side scatter distributions. Detectors were set to logarithmic amplification and fluorescence was measured in 7500 cells using the System II (3.0) software. For quantification, the ratios of the mean fluorescence signals of PAR-1– and nonspecific IgG\(_1\)-stained cells were normalized to the unstimulated control.

Immunocytochemistry

SMCs, plated on 8-well chamber slides (LabTek; Nunc) at a density of 10,000 cells per \(\text{cm}^2\), were incubated as described above, fixed for 20 minutes (freshly made 3.7% paraformaldehyde) and blocked for 1 hour (3% BSA in PBS). Cells were then treated with anti–PAR-1 antibodies (ATAP2; Santa Cruz Biotechnology; 1:50 in 1% BSA/PBS) overnight followed by incubation with HRP-conjugated secondary antibodies (goat anti-mouse; Santa Cruz Biotechnology; 1:400 in 1% BSA/PBS) for 1 hour. Diaminobenzidine was used as chromogen. Nuclei were stained with hemalaun. Images were captured with a Colorview II camera and SIS software (Soft Imaging System) connected to an Olympus BX 50 microscope.

Semi-Quantitative RT-PCR and Real-Time PCR

Total RNA from SMCs was prepared with TriFast reagent (peqLab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer’s instructions. RT-PCR was performed with a One-Step RT-PCR kit (Qiagen, Hilden, Germany) using 250 ng RNA. Genespecific primers (0.6 \(\mu\)mol/L each) were: PAR-1 forward CAG CGG CAG ATG TGC TGT TTG, reverse TAG GCA GCC TCT GTG GTG GAA G; Rac1 forward CCC TAT CCT ATC GTG AAA CA, reverse CAG CAG GCA TTT TCT CTG CC. GAPDH primers were used as described.\(^{30}\) Thermal cycler conditions were: 1 minute 94°C, 1 minute annealing (58°C for PAR-1, 62°C for Rac1), 1 minute 72°C, and elongation at 72°C. After separation in agarose gels, PCR fragments were visualized and quantified on a Biorad GelDoc instrument. For real-time PCR, total RNA was reverse-transcribed into cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems). PAR-1 mRNA expression was determined using SYBR Green Master Mix (Applied Biosystems) and Quanti-Tect Primer Assay (Qiagen, Hilden, Germany) QT00230489 (PAR-1) and QT00199367 (ribosomal 18S as internal control) according to the manufacturer’s instructions. PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems). PAR-1 expression levels relative to 18S were determined using the \(\Delta\Delta C_t\) method\(^{31}\) and expressed relative to paired controls.

Western Blot Analysis

PAR-1 protein expression was detected in whole cell lysates by Western blotting using monoclonal anti–PAR-1-antibodies (ATAP2, Santa Cruz Biotechnology). After treatment with the indicated agents, cell lysates were resolved by SDS-PAGE as described previously.\(^{29}\) Bands were visualized by enhanced chemiluminescence (ECL; Amersham).

DNA Synthesis and Cell Migration

DNA synthesis was determined by [\(^3\)H] thymidine incorporation as described previously.\(^{29,30}\) For migration experiments, SMCs were serum-deprived for 24 hours with 0.5% serum and pretreated with iloprost for 24 hours. Cell migration was determined in Boyden chamber assays as described previously.\(^{30}\)

Statistical Analysis

Data are means±SEM from n experiments. Statistical analysis was performed using 1-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons procedure. \(P<0.05\) was considered significant.

Results

Effects of Iloprost and PGE\(_2\) on PAR-1 mRNA and Total Protein Expression

Exposure of cells to the stable PG\(_I\)-mimetic iloprost (100 \(\text{nmol/L}\)) time-dependently reduced expression of PAR-1 mRNA at 6 and 24 hours (Figure 1A). Nanomolar concentrations of iloprost (10 and 100 \(\text{nmol/L}\)) were sufficient to inhibit PAR-1 mRNA expression (Figure 1B, equivalent data obtained by semiquantitative RT-PCR are not shown). Iloprost also attenuated PAR-1 total protein at 24 hours as demonstrated by Western blotting (Figure 1C). Molecular identity of PAR-1 was confirmed by molecular size as well as by mobility shift of the protein band in SDS-PAGE after treatment of cells with thrombin, indicating proteolytic cleav-
Inhibition of PAR-1 Surface Expression by Iloprost and PGE$_2$

Changes in PAR-1 surface expression were assessed by flow cytometry. Incubation of cells with iloprost for 24 hours (1 and 100 nmol/L) reduced PAR-1 fluorescence intensity by 40 to 50% (Figure 2A). Fluorescence-labeled isotype-matched IgG were used as control. PGE$_2$ also suppressed cell surface expression of PAR-1 at higher concentrations (1 and 10 µmol/L, Figure 2B). Lower concentrations (1 and 100 nmol/L) were again ineffective (not shown). Immunocytochemistry of nonpermeabilized SMCs showed strong PAR-1 immunoreactivity in control cells, indicating the presence of PAR-1 at the cell surface (Figure 2C), which was markedly reduced after incubation with iloprost (10 nmol/L) for 24 hours.

Involvement of G$_s$/cAMP/PKA-Dependent Signaling Pathways in the Iloprost-Mediated Regulation of PAR-1 Expression

PGI$_2$ increases intracellular cAMP and activates protein kinase A (PKA) via stimulation of G protein (G$_s$)-coupled IP-receptors.$^{19}$ Treatment of human SMCs with either the selective IP-receptor agonist cicaprost (1 to 100 nmol/L) or the direct adenyl cyclase activator forskolin suppressed PAR-1 mRNA and cell surface protein expression (Figure 3A through 3C). The nonselective phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mmol/L) and the membrane-permeable PKA-activator dibutyryl-cAMP (db-cAMP, 1 mmol/L) mimicked the effects of iloprost on PAR-1 mRNA expression (Figure 3D). In contrast, preincubation with the highly specific PKA inhibitor Myr-PKI (5 µmol/L) prevented the downregulation of PAR-1 mRNA, total protein, and cell surface expression by iloprost (10 nmol/L, Figure 4). This confirms the significance of PKA signaling in iloprost-mediated regulation of PAR-1 expression in human SMCs.

Downregulation of PAR-1 Attenuates the Mitogenic Response to Thrombin

We next assessed whether the PG-induced suppression of PAR-1 surface expression is associated with an attenuated mitogenic response to thrombin or a selective PAR-1–activating peptide (PAR-1-AP). DNA synthesis, determined by [³H]-thymidine incorporation, demonstrated that in control cells, both thrombin (3 U/mL) and PAR-1-AP (TFLLRN, 200 µmol/L) induced approximately 3.5- and 2-fold increases in DNA synthesis, respectively (Figure 5A). These mitogenic responses were significantly reduced by pretreatment with iloprost for 24 hours, whereas preincubation for 1 hour was ineffective. The inhibitory effect of iloprost pretreatment was specific for PAR-1–dependent mitogenesis, as DNA synthesis induced by PDGF-BB (10 ng/mL) or FCS (10%) was not affected (Figure 5B). A comparable effect was observed for cell migration. Whereas preincubation with iloprost for 24 hours attenuated SMC migration toward thrombin PKA-dependently, PDGF-BB–induced migration was not affected (Figure 5C).

Role of Rho Family GTPases for the Regulation of PAR-1 Expression by Iloprost

The GTPase Rac1, a member of the Rho family of small G proteins, has recently been suggested to play a crucial role in

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Inhibition of PAR-1 mRNA and total protein expression in human SMCs. PAR-1 mRNA was determined by semi-quantitative RT-PCR (A) and real-time PCR (B) after incubation with iloprost. C, Immunoblot detection of PAR-1 after incubation with iloprost or PGE$_2$. D, PAR-1 mRNA after incubation with PGE$_2$; n=3 to 5, respectively, *P<0.05 vs con.
maintaining surface expression of PAR-1.28 Therefore, we investigated a possible role of Rac- and Rho-dependent signaling in iloprost-mediated regulation of PAR-1 expression. Iloprost significantly attenuated Rac1 mRNA within 24 hours (supplemental Figure IA). Selective inhibition of Rac1 activity with the cell-permeable pyrimidine compound NSC23766 (50 μmol/L)32 time-dependently reduced PAR-1 surface expression (supplemental Figure IB and ID) as well as total PAR-1 protein (supplemental Figure IE). In contrast, inhibition of Rho-associated protein kinase (ROCK) with Y27632 (10 μmol/L)15 did not affect PAR-1 surface expression (supplemental Figure IC and ID) or total PAR-1 protein (supplemental Figure IE). Neither inhibitor altered PAR-1 mRNA expression (supplemental Figure IE). Thus, modification of Rac1- but not Rho-dependent signaling appears to be involved in the regulation of PAR-1 surface expression by iloprost in human SMCs.

Discussion

PARs are the cellular targets of coagulation factor signaling on vascular cells.3 They play a crucial role in cardiovascular physiology and have been implicated in atherosclerosis and inflammation.11 Therefore, understanding the mechanisms that modulate PAR expression and function is of key importance for understanding the significance of coagulation factors for the vascular healing process. This study demonstrates for the first time that vasodilatory prostaglandins inhibit the mitogenic actions of thrombin in human vascular SMCs by downregulating PAR-1 expression.

Expression of PAR-1 was determined at the level of mRNA, total protein, and cell surface localization. We found that long-term exposure (24 hours) to iloprost inhibited surface expression of PAR-1 as demonstrated by flow cytometry. It is likely that decreased de novo synthesis of the receptor accounts for its decreased surface cell expression, because suppression of PAR-1 mRNA and total protein developed rather slowly. Although PAR-1 mRNA levels started to decline within 3 to 6 hours, the reduction was only significant at 6 and 24 hours (Figure 1). In agreement with this hypothesis, inhibitory effects on DNA synthesis in response to thrombin or PAR-1-AP were observed after 24 hours preincubation with iloprost, whereas short-term exposure (1 hour) was ineffective. A comparable effect of iloprost on thrombin-, but not PDGF-BB-, induced SMC migration, was observed (Figure 5). Of note is that thrombin-induced migration was inhibited by maximally 25% after incubation with iloprost. This suggests that PAR-1–independent chemotactic mechanisms such as activation of matrix metalloproteinases may be involved.29,34 Whether additional pathways such as increased internalization and degradation contribute to the reduction of total PAR-1 protein by prostaglandins needs to be elucidated in future studies.

Next, we were interested in elucidating signal transduction pathways involved in the prostacyclin-induced downregulation of PAR-1 expression. The data suggest that prostanoid-evoked inhibition of PAR-1 expression is mediated primarily via an increase in intracellular cAMP formation and subsequent PKA activation, as the effect was mimicked by cAMP-elevating agents like forskolin, isobutylmethylxanthine, or the membrane-permeable cAMP-analogue dibutyryl-cAMP, and was prevented with the cell permeable specific PKA inhibitor Myr-PKI.26 Similar data were obtained with the selective PKA inhibitor adenosine 3',5'-cyclic phosphorothioate-Rp isomer (Rp-cAMPs, not shown). These observations are in agreement with reports describing cAMP-dependent downregulation of PAR-1 in human lung fibroblast and mesangial cells.35,36 Moreover, we have used the selective IP receptor agonist cicaprost to distinguish between IP and possible EP receptor–mediated effects of the prostacyclin analog iloprost. The high efficacy of cicaprost suggests that stimulation of Gs-coupled IP receptors mediate inhibition of PAR-1 expression.

Whereas iloprost downregulated PAR-1 at nanomolar concentrations, equipotent inhibition by PGE2 required considerably higher concentrations (10 μmol/L). This is consistent with our recent observation that iloprost exerts a greater cAMP-stimulatory effect in human SMCs than PGE2,37 potentially because of simultaneous activation of cAMP-
lowering Gi-coupled EP3 receptors. Thus PGI₂ is likely to be the major prostanoid modulating cellular thrombin effects in vivo.

To further characterize the signaling pathways regulating PAR-1 in response to PGI₂, we examined the role of the Rho-GTPases Rac1 and Rho, which have been suggested to play an important role in regulation of receptor endocytosis. The use of the selective Rac1 inhibitor NSC23766 revealed that Rac-dependent pathways are involved in regulating PAR-1 expression at the cell surface but not at the mRNA level (supplemental Figure I). Thus Rac1 appears to control surface protein expression of PAR-1 via regulation of constitutive trafficking (e.g., internalization and degradation of receptor protein) and not via transcriptional changes. Our data further support a role for Rac1 in controlling constitutive surface expression of PAR-1 via regulation of constitutive trafficking (e.g., internalization and degradation of receptor protein) and not via transcriptional changes. Our data further support a role for Rac1 in controlling constitutive surface expression of PAR-1 via regulation of constitutive trafficking (e.g., internalization and degradation of receptor protein) and not via transcriptional changes.

Figure 3. Regulation of PAR-1 involves Gs-/cAMP-/PKA-dependent signaling pathways. PAR-1 mRNA was determined after incubation with cicaprost (A), forskolin (B), isobutylmethylxanthine (IBMX, 0.5 mmol/L) or dibutyryl-cAMP (db-cAMP, 1 mmol/L) (D). C, PAR-1 surface expression after treatment with forskolin (10 µmol/L) or cicaprost (1 nmol/L) for 24 hours; n=3 to 5, respectively, *P<0.05 vs con.

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To further characterize the signaling pathways regulating PAR-1 in response to PGI₂, we examined the role of the

Figure 4. Downregulation of PAR-1 by iloprost depends on PKA activation. Iloprost-induced downregulation of PAR-1 mRNA, total protein, and cell surface expression was inhibited by Myr-PKI (5 µmol/L) as determined by (A) real-time PCR (n=5), (B) Western blotting (n=7), and (C) flow cytometry (n=9), respectively, *P<0.05 vs con, #P<0.05 vs ilo.

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In addition to the effect of iloprost described here, determination of the link between cAMP and transcription factors involved in PAR-1 expression will be of future interest. Because no cAMP-response element (CRE) has been found in the PAR-1 promoter region, PKA-dependent control of other transcription factors may be involved in PAR-1 regulation. One transcription factor which has been reported to be involved in PAR-1 expression will be of future interest. PKA–dependent upregulation of AP-2 which then contributes to the transcriptional regulation of PAR-1.

In conclusion, we report for the first time that PAR-1 expression is inhibited by vasodilatory PG in human vascular SMCs. This suggests that in atherosclerotic or restenotic vessels, induction of PG synthesis via the COX-pathway in medial or neointimal SMCs, as well as long-term exposure of SMCs to PG from other sources such as macrophages, might result in reduction of cell responsiveness to PAR activation. This mechanism might serve to counteract the local mitogenic and other cellular actions of proteases such as thrombin generated at sites of vascular injury.

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Disclosures
None.

References


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Detailed Figure Legends

**Figure 1**

*Inhibition of PAR-1 mRNA and total protein expression in human SMC.* (A) PAR-1 mRNA levels determined by semiquantitative RT-PCR after incubation with iloprost for the indicated times. (B) Determination of PAR-1 mRNA by real-time PCR after incubation with increasing concentrations of iloprost for 6 or 24 hours. (C) Immunoblot detection of PAR-1 in total lysates of cells treated with or without iloprost or PGE$_2$ for 24 hours. Blots were reprobed for β-actin to confirm equal protein loading. (D) PAR-1 mRNA expression determined by real-time PCR after incubation with PGE$_2$ for 24 hours. Data are from n=3-5 independent experiments, *p<0.05 vs. con.

**Figure 2**

*Inhibition of PAR-1 surface expression.* Serum-deprived cells were treated with iloprost (A) or PGE$_2$ (B) for 24 hours at the indicated concentrations. Representative histograms are depicted on the left. Right panel shows quantitative analysis of the relative mean fluorescence intensities. Values are mean±SEM of 3 independent experiments, *p<0.05 vs. con. (C) PAR-1 expression was determined by immunolabeling with monoclonal anti-PAR-1 antibodies (ATAP2) in untreated cells and after incubation with iloprost (10 nmol/L) for 24 hours. No staining was observed in absence of primary antibody (negative control) as compared to PAR-1 staining (brown). Nuclei were stained with hemalaun (blue). Original magnification: x100. Images are representative of 3 independent experiments.
**Figure 3**

Regulation of PAR-1 expression by iloprost involves Gs-/cAMP-/PKA-dependent signaling pathways. (A) PAR-1 mRNA expression determined by real-time PCR after treatment of human SMC with cicaprost (1 - 100 nmol/L) for 6 or 24 hours (n=4-5). (B) Time-dependent downregulation of PAR-1 mRNA by forskolin (10 µmol/L) by semi-quantitative RT-PCR. (C) Flow cytometry analysis of PAR-1 surface expression in SMC treated with forskolin (forsk, 10 µmol/L) or cicaprost (cica, 1 nmol/L) for 24 hours. (D) Inhibition of PAR-1 mRNA expression by isobutylmethylxanthine (IBMX, 0.5 mmol/L) and dibutyryl-cAMP (db-cAMP, 1 mmol/L) after 24 hours. In B and D, representative RT-PCR experiments and the densitometric quantification (n=3) are shown. Bar graph in C shows quantitative analysis of the relative mean fluorescence intensity measured in at least 3 independent experiments, *p<0.05 vs. con.

**Figure 4**

Downregulation of PAR-1 by iloprost depends on PKA activation. SMC were incubated with the highly specific cell-permeable PKA inhibitor Myr-PKI (PKI, 5 µmol/L) for 30 minutes prior to incubation with iloprost (10 nmol/L) for 24 hours. Iloprost-induced downregulation of PAR-1 mRNA, total protein, and cell surface expression was inhibited by Myr-PKI as determined by (A) real-time PCR (n=5), (B) Western blotting (n=7) and (C) flow cytometry (n=9), respectively, *p<0.05 vs. con, #p<0.05 vs. ilo.
Figure 5

Downregulation of PAR-1 by iloprost attenuates the mitogenic and chemotactic response to thrombin. DNA synthesis was determined by $[^3H]$-thymidine incorporation. Where indicated, cells were pretreated with iloprost (ilo, 10 nmol/L) 1 hour or 24 hours prior to stimulation with (A) thrombin (3 U/ml) or PAR-1-activating peptide (PAR1-AP, 200 µmol/L) or with (B) PDGF-BB (10 ng/ml) or FCS (10%). SMC migration was determined in response to thrombin (3 U/ml) or PDGF-BB (10 ng/ml) (C). Cells were pretreated with iloprost for 24 hours in absence or presence of Myr-PKI. Values are expressed as fold of untreated controls (con), n=7 independent experiments, respectively, *p<0.05 as indicated, n.s. (not significant).

Figure 1

Rac- but not Rho-dependent signaling pathways are involved in regulation of PAR-1 surface expression. Expression of Rac-1 mRNA was determined after incubation with iloprost (10 nmol/L) for the indicated times (A). Serum-starved cells were treated with the Rac1 inhibitor NSC23766 (NSC, 50 µmol/L) or the Rho kinase inhibitor Y-27632 (Y, 10 µmol/L) for the indicated times. PAR-1 expression was analyzed by flow cytometry (B-D), Western blotting (E) and RT-PCR (F). Data are from at least 3 independent experiments, *p<0.05 vs. con.
Fig. I

A. Rac1 mRNA (% control)

B. Single parameter

C. PAR-1 surface expression

D. PAR-1 mRNA (% control)

E. PAR-1 and β-actin

F. PAR-1 mRNA (% control)