Differential Effects of Vasodilatory Prostaglandins on Focal Adhesions, Cytoskeletal Architecture, and Migration in Human Aortic Smooth Muscle Cells


Objective—Cyclooxygenases 1 and 2 are expressed in atherosclerotic arteries, and local generation of prostacyclin and prostaglandin E\(_2\) (PGE\(_2\)) occurs. However, the role of cyclooxygenases and individual prostaglandins during plaque progression is currently uncertain. The present study characterizes the effect of vasodilatory prostaglandins on morphology, focal adhesion (FA) function, and migration in human aortic smooth muscle cells (SMCs).

Methods and Results—The stable prostacyclin analog iloprost transiently induced: (1) disassembly of FA and stress fibers, (2) partial retraction and rounding of SMCs, (3) hypophosphorylation of FA kinase (FAK) and paxillin, and (4) inhibition of platelet-derived growth factor-BB–induced migration. Inhibition of FAK phosphorylation and morphological changes were mimicked by forskolin, inhibited by H89, and prevented by the protein tyrosine phosphatase inhibitor vanadate and by calpeptin. PGE\(_2\) was by far less efficient with respect to all parameters investigated. This difference correlated with the respective cAMP induction in response to iloprost and PGE\(_2\).

Conclusion—Inhibition of FAK phosphorylation and FA function is a new target of vasodilatory prostaglandins, which might be causally involved in the antimigratory effects of prostaglandins. Importantly, prostacyclin analogs and PGE\(_2\) differ dramatically with respect to dephosphorylation of FAK and inhibition of migration, which might be of relevance for their respective functions in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:84-89.)

Key Words: focal adhesion kinase  ▪ extracellular matrix  ▪ prostaglandins ▪ atherosclerosis

Fundamental processes during neointimal hyperplasia and plaque progression are proliferation and migration of smooth muscle cells (SMCs) and the deposition of extracellular matrix (ECM) by SMCs. SMCs interact with ECM molecules via integrins, which cluster and induce the formation of focal adhesions (FAs) and the establishment of actin cytoskeleton. Furthermore, after ligation of integrins by ECM molecules, the multimolecular FA complex induces intracellular signaling, which is essential for survival, locomotion, and proliferation of SMCs.

See cover

Vascular endothelial cells and vascular SMCs synthesize various prostaglandins (PGs) by PG G\(_1\)/H\(_2\) synthase and by the subsequent activity of synthases specific for the individual prostaglandins (e.g., PG E synthase) and prostacyclin (PGI\(_2\)) synthase. PG G\(_1\)/H\(_2\) synthase is also known as cyclooxygenase (COX), which exists as a constitutive (COX-1) and an inducible (COX-2) form. Vasodilatory prostaglandins such as PGI\(_2\) and PG E\(_2\) (PGE\(_2\)) mediate various functions in vascular biology including regulation of vascular tone, thrombocyte function, and inflammatory responses. Furthermore, PGs are thought to participate in regulation of vascular remodeling during neointimal hyperplasia and atherosclerosis.

PGI\(_2\) activates the I-type prostanooid (IP) receptor, which is a heterotrimeric G-protein–coupled receptor (GPCR) that signals via activation of adenylate cyclase, cAMP, and protein kinase A (PKA). PGE\(_2\) activates four different GPCRs: E-type prostanooid (EP) receptors, EP\(_1\), EP\(_2\), and EP\(_3\) receptors couple to G\(_i\) and increase cAMP, EP\(_4\) receptors are coupled to G\(_i\) and EP receptors mostly to G\(_i\).

In atherosclerotic lesions, both COX isoforms are expressed and PGI\(_2\) and PGE\(_2\) are generated. However, the role of PGs during atherogenesis and the consequences of pharmacological inhibition of COX-1 or COX-2 with respect to plaque progression are still controversial. From in vitro studies and animal studies, it is evident that PGI\(_2\) inhibits SMC proliferation and migration and might therefore be antiatherogenic. PGE\(_2\) has also been shown to be inhibitory with respect to proliferation and migration of SMCs but has also been implicated in the inflammatory response and in upregulation of matrix metalloproteinases at the shoulder of human atherosclerotic plaques. Furthermore,
recent evidence from our laboratory suggested that in human atherosclerotic plaques, PGE\textsubscript{2} via EP\textsubscript{2} receptors promotes accumulation of hyaluronic acid, which is a proatherogenic ECM molecule.\textsuperscript{19} Thus, PGE\textsubscript{2} might be a mediator that supports the progression of atherosclerotic plaques.

With respect to the mechanisms controlling SMC migration, it is important to consider FA function because FAs regulate cytoskeletal organization and the dynamics of cell detachment and attachment during locomotion.\textsuperscript{2} Whether PGs interfere with FA function is not known.

In light of the uncertainty about the roles of COX-1 and COX-2 during the progression of atherosclerosis, further information about the effects of individual PGs on SMC phenotype is needed. Therefore, the aim of the present study was to use human aortic SMCs to compare the effects of PGI\textsubscript{2} and PGE\textsubscript{2} on FA formation and migration.

**Experimental Procedures**

**Reagents**

Iloprost and cicaprost were kindly provided by Schering AG (Berlin, Germany). PGE\textsubscript{2}, forskolin, dibutyl (db)-cAMP, 3-isobutyl-1-methylxanthine (IBMX), genistein, fluorescein isothiocyanate (FITC)-conjugated phalloidin, and Hoechst 33242 were purchased from Sigma. Platelet-derived growth factor-BB (PDGF-BB) was from PromoKine. H-89 was obtained from Calbiochem and calpeptin from Tocris. Phospho-FA kinase (FAK)-Tyr397 (rabbit polyclonal, 1:500) antibodies were purchased from Santa Cruz Biotechnology. FAK (mouse monoclonal, 1:1000) and paxillin (mouse monoclonal, 1:200) antibodies were from BD Biosciences and phospho-paxillin-FAK (mouse monoclonal, 1:1000) and paxillin (mouse monoclonal, 1:200) antibodies were from Biogenesis. Reagents and phospho-paxillin-Tyr-118 (rabbit polyclonal, 1:500) from Cell Signaling. The secondary antibodies for immunoblotting were horseradish peroxidase–linked horse anti-rabbit (1:5000) and goat anti-mouse (1:5000) from Vector Laboratories. The horseradish peroxidase–linked sheep anti-mouse (1:1000) from Sigma.

**Cell Culture and Stimulation**

Human aortic SMCs from Cascade Biologics were cultured according to manufacturer instructions in medium M231 supplemented with 5% SMC growth supplement at 37°C in a humidified atmosphere (5% CO\textsubscript{2}). To perform experiments, SMCs were seeded at 10 000 cells/cm\textsuperscript{2} in normal growth medium and used between passage 5 and 10. To achieve quiescence, growth medium was removed and substituted by medium M231 containing 0.1% FBS (BioWhittaker) for 48 hours. Subsequently, SMCs were stimulated with PDGF-BB (10 ng/mL) unless otherwise stated.

**RT-PCR of PG Receptors**

Total RNA from SMCs stimulated with PDGF-BB (10 ng/mL) was isolated using Trizol reagent. RT-PCR using 150 ng RNA was performed with Quagen OneStep RT-PCR kit (Quagen). Amplification was performed by 40 cycles: 30 sec at 95°C for denaturation, 30 sec at 55°C for annealing, and 90 sec at 72°C for extension. The primer sequences were as follows: IP receptor, sense 5’-gctgctctctggctgtgctggc-3’ and antisense 5’-gtgcctccctgtactggtgctgg-3’ (product, 384 bp); EP\textsubscript{1} receptor, sense 5’-gcgtgctcccttc-3’ and antisense 5’-gattgctcagctcagct-3’ (product, 441 bp); EP\textsubscript{2} receptor, sense 5’-gtgcgtgctcccttc-3’ and antisense 5’-aaaggacgctgaaggcttc-3’ (product, 450 bp); EP\textsubscript{3} receptor, sense 5’-tgctgccctctgttc-3’ and antisense 5’-agtggctggag gagcacagc-3’ (product, 591 bp); EP\textsubscript{4} receptor, sense 5’-actactgtgcaacgtagt-3’ and antisense 5’-tccagacgtttcagctgg-3’ (product, 575 bp). Products were resolved by electrophoresis on 2% agarose. PG receptor expression of PDGF-BB–stimulated human aortic SMCs was determined by semiquantitative RT-PCR (data polycarbonate filters (pore size 10 μm; Neuro Probe). The bottom chamber contained M231-medium without FBS. PDGF-BB (10 ng/mL) was used as a chemotactic agent. SMCs were trypsinized and seeded at 20 000 cells per well (250 000/cm\textsuperscript{2}) into the upper wells. SMCs were allowed to settle into the wells for 30 minutes before iloprost (100 nmol/L) or PGE\textsubscript{2} (100 nmol/L) were added into the wells. Experiments were stopped after 6 hours. The respective experimental conditions were repeated 6 to 12x in each 48-well migration assay, and nuclei in the center of the wells were counted at ×200 magnification using an Olympus BX 50 microscope. The mean of the replicates was used as n = 1.

**Morphological Analysis of the Actin Cytoskeleton and FAs**

SMCs were plated on 8-well chamber slides (LabTek; Nunc) at a density of 12 000/cm\textsuperscript{2}. At the end of the experiments (30 minutes and 18 hours), cells were fixed for 20 minutes (freshly made 3.7% paraformaldehyde), permeabilized (0.1% Triton X-100), and blocked for 1 hour (3% BSA) in PBS. Cells were then incubated with anti-paxillin IgG (1:200 in 1% BSA/PBS) for 1 hour and Cy3-conjugated secondary antibody (1:600 in 1% BSA/PBS) for 1 hour. Cells were washed with PBS between incubations and stained for filamentous actin (F-actin) by incubation with 1 μmol/L FITC-conjugated phallolidin in PBS for 20 minutes. Nuclear staining was achieved with Hoechst 33342. Finally, slides were mounted in Vectashield mounting medium (Vector Laboratories) and visualized with an Olympus BX 50 microscope using the appropriate filters. Images were taken and overlaid using a colorview II camera (Soft Imaging System) and SIS software (Soft Imaging System). cAMP Assay Quiescent SMCs in 24-well plates were washed with Hanks’ balanced salt solution (HBSS), supplemented with 1 mg/mL BSA, and preincubated in HBSS containing IBMX (1 mmol/L) at 37°C for 10 minutes. Thereafter, PDGF-BB (10 ng/mL), iloprost (100 nmol/L) or PGE\textsubscript{2} (100 nmol/L) were added. After 10 minutes, the supernatant was removed and the reaction was stopped by ice-cold ethanol (96%). After evaporation of ethanol, cells were overlaid with radioimmunoassay buffer (50 mmol/L Tris-HCl, 4 mmol/L EDTA, pH 7.5) and kept at −80°C for 24 hours. The supernatant was used for determination of cAMP by radioimmunoassay.\textsuperscript{25} Subsequently, cells were lysed with 0.1 N NaOH at 37°C for 30 minutes, and cell protein was measured according to Bradford. Levels of cAMP are expressed as pmol cAMP/mg protein×10 minutes. Measurements were performed in triplicates.

**Western Blotting**

SMCs were stimulated in 6-well plates as indicated and lysed in Laemmli buffer plus dithiothreitol (100 mmol/L) at the indicated times. Lysates were boiled, subjected to SDS-PAGE, and transferred to polyvinylidene fluoride-membrane (Millipore). Blots were blocked in 3% dry milk in PBS for 1 hour and incubated with the appropriate primary antibody in 0.5% dry milk/PBS at 4°C over-night. Thereafter, blots were washed 3x in 0.5% milk/PBS/0.05% Tween-20 before the secondary antibody (0.5% milk/PBS) was applied at room temperature for 1 hour. Protein bands were visualized by enhanced chemiluminescence using ECL-Plus (Amersham Biosciences) according to manufacturer protocol.

**Statistical Analysis**

Data are the mean±SEM of n independent experiments. Statistical analysis was performed by 1-way ANOVA followed by comparison of selected pairs (Bonferroni); \(P<0.05\) was considered significant.

**Results**

**Characterization of PG Receptor Expression Profile**

PG receptor expression of PDGF-BB–stimulated human aortic SMCs was determined by semiquantitative RT-PCR (data...
not shown). Strong expression of PG I_2_ receptor mRNA was detected. Furthermore, all PG E receptor subtypes (EP_1 to EP_4) were detected (data not shown).

**Morphology, Actin Stress Fibers, and FAs**
The morphology of serum-starved and PDGF-BB–stimulated cells is shown in Figure 1A and 1B, respectively. FAs were visualized by immunocytochemistry of paxillin (red), and the organization of actin stress fibers was detected by FITC-coupled phalloidin (green). Iloprost induced a dramatic change in morphology already within 30 minutes (Figure 1C). Actin stress fibers and focal contacts were disassembled and cells partially rounded up, leaving behind very thin branched extensions. The same effect on morphology was observed after 30 minutes of incubation with another specific IP receptor ligand cicaprost (Figure 1D). Within 18 hours, SMCs recovered despite the presence of iloprost, and spread again, they showed numerous paxillin-positive focal contacts and the reformation of actin stress fibers (details available online at http://atvb.ahajournals.org). In contrast, PGE_2 affected stress fibers, spreading, and FA, much less (Figure 1E). Furthermore, the same changes of cell morphology were observed 30 minutes after application of db-cAMP (1 mmol/L; Figure 1F) and forskolin (10 μmol/L; data not shown).

**Hypophosphorylation of FAK**
Ligation of integrins by ECM molecules induces FA formation and FAK phosphorylation. To investigate the mechanisms that underlie the morphological changes and the disassembly of FA demonstrated in Figure 1, the effect of iloprost on total FAK and FAK phosphorylation was analyzed by immunoblotting. The amount of total FAK was not affected by treatment with iloprost (Figure 2A), and no degradation products were observed (data not shown). However, FAK phosphorylation on tyrosine 397, which is the autophosphorylation site, was dramatically reduced. Reduction of FAK phosphorylation was detectable after 10 minutes and was maximal 30 minutes after addition of iloprost (Figure 2A). Subsequently, over a period of 18 hours, FAK phosphorylation recovered to the levels seen in the presence of PDGF-BB (Figure 2A). Thus, the hypophosphorylation of FAK on tyrosine 397 and the recovery were paralleled by the induction and recovery of the morphological changes of SMCs in response to iloprost.

According to these data, further characterization of the iloprost effect on FAK phosphorylation was performed always at 30 minutes. Hypophosphorylation of FAK in response to iloprost was concentration dependent occurring already at concentrations of 10 nmol/L (Figure 2B). cAMP dependency of FAK hypophosphorylation was indicated by the inhibitory effect of db-cAMP and forskolin on FAK phosphorylation (Figure 3A). In the presence of the PKA inhibitor H89 (100 nmol/L), the effect of iloprost on FAK phosphorylation was reduced (Figure 3B). The PGE_2 effect on FAK phosphorylation was significantly smaller compared with iloprost but could be enhanced in the presence of IBMX (Figure 3C and 3D). Furthermore, as shown in Figure 3E, paxillin phosphorylation at tyrosine 118 was reduced in response to iloprost, PGE_2, db-cAMP, and forskolin. This suggests that reduced autophosphorylation of FAK by cAMP-dependent pathways leads to reduced phosphorylation of downstream targets of FAK.

Incubation with vanadate (50 μmol/L) and pervanadate (200 μmol/L; data not shown), inhibitors of protein tyrosine phosphatases (PTPs), abolished the effect of iloprost on FAK phosphorylation.
morphology, FA assembly, and FAK phosphorylation on tyrosine 397 (Figure 4A and 4B). Genistein (100 μmol/L), an inhibitor of tyrosine kinases, had no effect on FAK hypophosphorylation (Figure 4B) but delayed the re-establishment of normal morphology of iloprost-treated cells (Figure 4C). Furthermore, FAK hypophosphorylation was prevented by calpeptin (20 μg/mL; Figure 4B), which has been shown to inhibit the PTP Shp-2.21

**Effects of Iloprost and PGE₂ on Migration**

To investigate whether the above-described effects on FA function correlate with the antimigratory effects of vasodilatory PGs, migration was analyzed in a 48-well microchemotaxis chamber using collagen type I–coated membranes and PDGF-BB (10 ng/mL) as a chemokine. Iloprost strongly inhibited migration in a concentration-dependent manner (Figure 5A). PGE₂ also inhibited PDGF-BB–induced migration but was significantly less effective compared with iloprost. The strength of the effect of iloprost and PGE₂ on migration correlated well to their respective ability to reduce FAK phosphorylation and FA assembly (Figures 1 and 3).

Furthermore, intracellular cAMP levels were determined in PDGF-BB–stimulated SMCs 10 minutes after addition of iloprost and PGE₂ (Figure 5B). Interestingly, 100 nmol/L iloprost induced a 10-fold higher increase of cAMP compared with 100 nmol/L PGE₂. Moreover, the effect of PGE₂ on PDGF-BB–induced migration was greatly enhanced in the presence of IBMX (data not shown).

**Involvement of IP Receptors**

Iloprost is also an agonist on EP₁ receptors at higher concentrations.22 To verify that the observed effects were mediated by the IP receptor, the specific IP receptor ligand cicaprost (Figure 1D) was also used in migration experiments and Western analysis. Cicaprost (100 nmol/L) strongly inhibits FAK pY397 and paxillin pY188 levels and migration (details available online at http://atvb.ahajournals.org).

**Discussion**

In atherosclerotic lesions, COX-1 and COX-2 are expressed, and generation of PGI₂ and PGE₂ has been demonstrated.10 Furthermore, during development of atherosclerotic lesions, the composition of ECM changes dramatically,23 which in turn causes changes in FA signaling. Because FA function, cytoskeletal organization, and SMC migration are intimately interrelated, analyzing the effects of individual PGs on these parameters in the same experimental system may help to understand the functional role of vasodilatory PGs in vascular remodeling and atherosclerosis. Therefore, in the present study, it was analyzed whether FA function is regulated by vasodilatory PGs in cultured human SMCs. For this purpose, iloprost and PGE₂ were used at equimolar concentrations (100 nmol/L) because the $K_i$ values of iloprost for the IP receptor (11 nmol/L) and of PGE₂ for the EP₁ receptor (12...
nmol/L) are similar. Moreover, the $K_i$ values of PGE$_2$ for the other EP receptors are in the same concentration range or lower (EP$_1$ 20 nmol/L; EP$_3$ 0.85 nmol/L; EP$_4$ 1.9 nmol/L).

**Disassembly of FAs and Inhibition of Migration**

Within 30 minutes after application of iloprost, the F-actin cytoskeleton became disassembled, and FA, visualized by paxillin staining, disappeared. FAK phosphorylation on tyrosine 397 was strongly reduced starting at 10 minutes and was maximal at 30 minutes, with complete recovery within 18 hours after addition of iloprost. Inhibition of FAK phosphorylation could explain the antimigratory effect of iloprost because it has been shown previously that the phosphorylation of tyrosine 397 and subsequent recruitment of Src family tyrosine kinases into the FA signaling complex are the first events required for PDGF-BB–induced migration. Further support for this hypothesis came from studies inhibiting FAK activity by overexpression of FAK-related nonkinase, which caused inhibition of locomotion in response to PDGF-BB.

The iloprost effect on FA assembly, FAK phosphorylation on tyrosine 397, and morphology were mediated by IP receptors as shown by cicaprost, and was cAMP/PKA dependent as shown by db-cAMP, forskolin, and H89. Interestingly, iloprost and PGE$_2$ markedly differed with respect to their effects on morphology and migration. This difference was likely because of less efficient elevation of intracellular cAMP in response to PGE$_2$. Concordantly, in combination with IBMX, a phosphodiesterase inhibitor causing accumulation of cAMP, PGE$_2$ caused strong dephosphorylation of FAK, severe morphological changes, and strong inhibition of migration. The relatively small increase in cAMP concentration in response to PGE$_2$ could be attributable to the inhibitory effect of the G$_i$-coupled EP$_3$ receptors, which were detected by RT-PCR. Together, the present data are consistent with the hypothesis that PGI$_2$, and to a lesser extent, PGE$_2$, inhibit SMC migration via cAMP-dependent inhibition of FAK phosphorylation at the autophosphorylation site leading to decreased phosphorylation of downstream targets of FAK such as paxillin and disassembly of FA and actin cytoskeleton.

**Involvement of PTPs**

The mechanism responsible for reduced FAK phosphorylation on tyrosine 397 in response to vasodilatory PGs is not known. The SH2 domain containing PTP 2 (Shp-2) has been shown to cause dephosphorylation of FAK (Y397), thereby regulating FA function, assembly of the cytoskeleton, and migration. Furthermore, Shp-2 has been shown to be activated by PKA via phosphorylation on serine in adrenocortical cells and in T-lymphocytes. Therefore, increased phosphatase activity in response to cAMP elevation might be considered the underlying mechanism responsible for the present observations. Experimental evidence for this hypothesis was provided by experiments using vanadate and calpeptin. Vanadate is an unspecific PTP inhibitor, and calpeptin has been shown recently to inhibit Shp-2 activity in fibroblasts, in human gastric epithelial cells, and in human platelets. The finding that both inhibitors prevented the effect of iloprost on morphology and FA assembly supports (1) the hypothesis that reduced FAK phosphorylation is indeed causally involved in the described PG effects; and (2) that activation of a phosphatase, possibly Shp-2, is upstream of the effect on FAK. Furthermore, consistent with decreased FAK phosphorylation as the primary reason for the morphological changes in response to vasodilatory PGs, the tyrosine kinase inhibitor genistein inhibited the re-establishment of FA and normal morphology.

In summary, the current study suggests that morphological changes and inhibition of cell migration in response to vasodilatory PGs are mediated by a new pathway of G$_s$-coupled PG receptor signaling involving the activation of
PTPs and subsequent hypophosphorylation of FAK. To our knowledge the present study is the first to identify FA as a target of PG receptor mediated signaling. Future studies are planned to identify the involved phosphatases and to analyze the molecular mechanism of PG-induced FAK hypophosphorylation in greater detail. Furthermore, significant differences between PGI₂ and PGE₂ with respect to their ability to inhibit FAK phosphorylation and SMC migration were observed. In atherosclerotic plaques, COX-1 and COX-2 are known to synthesize large quantities of PG G₂/H₂, the precursors of PGE₂ and PGI₂. Upon the basis of the present findings, it is possible that the balance between local PGE₂ and PGI₂ synthesis by the respective syntheses plays an important role in the regulation of SMC migration in atherosclerosis.

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References


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Fig. 1 online supplement. **Recovery of morphology, FA and actin stress fiber assembly.** FITC-phalloidin staining (green) and immunocytochemistry of paxillin (red); nuclear staining by HOECHST 33324 (blue). SMC were serum starved (48 hours) and stimulated with PDGF-BB (10 ng/ml) in the absence or presence of iloprost (100 nmol/L), PGE$_2$ (100 nmol/L) and db-cAMP (1 mmol/L). Cells were fixed and stained after 18 hours. **A**, control; **B**, PDGF-BB; **C**, PDGF-BB + iloprost; **D**, PDGF-BB + PGE$_2$; **E**, PDGF-BB + db-cAMP; original magnification 40x. Images are representative of 3 experiments.
Fig. 1