Regulation of protease-activated receptor-1 by vasodilatory prostaglandins via NFAT

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Aims We recently reported that prostacyclin suppresses protease-activated receptor-1 (PAR-1) in human vascular smooth muscle cells (VSMC) via cyclic AMP and protein kinase A. This study examines the downstream mechanisms, particularly the role of nuclear factor of activated T-cells (NFAT).

Methods and results Human saphenous vein VSMC were exposed to phorbol 12-myristate 13-acetate (PMA) to induce endogenous cyclooxygenase-2-dependent prostaglandin generation. This was found to attenuate PAR-1 expression; similar suppression was seen with the EP2-prostaglandin receptor agonist butaprost. Stimulation of the 'exchange protein directly activated by cyclic AMP' (EPAC) was without effect. The NFAT inhibitor cyclosporin A (CsA) or NFAT2 siRNA both reduced PAR-1 mRNA and protein expression and prevented the stimulatory effects of thrombin or PAR-1 activating peptide (TFLLRN) on ERK1/2 phosphorylation and interleukin-6 expression. CsA or mutation of the NFAT binding motif in the PAR-1 promoter also blunted PAR-1 promoter activity (luciferase reporter assay). These inhibitory actions of CsA were comparable to those of the prostacyclin-mimetic iloprost, and both CsA and iloprost similarly attenuated nuclear NFAT2 localization and binding to the PAR-1 promoter (chromatin immunoprecipitation assay).

Conclusions This study provides the first evidence that NFAT2 contributes to the transcriptional control of PAR-1 in human VSMC and that PKA-dependent NFAT2 inhibition represents a mechanism by which vasodilatory prostaglandins regulate the vascular actions of thrombin.

KEYWORDS
Prostaglandins; Vascular smooth muscle; Thrombin receptors; Nuclear factor of activated T-cells; Cyclosporine

1. Introduction

The coagulation factor thrombin exerts direct cellular effects on the vessel wall via protease-activated receptors (PAR)-1, PAR-3, and PAR-4. Proteolytic cleavage by thrombin generates an N-terminal tethered ligand that auto-activates the receptor.1 Although we have shown that PAR-1, PAR-3, and PAR-4 are expressed in human vascular smooth muscle cells (VSMC),2,3 PAR-1 is the prototypic thrombin receptor and the major isoform involved in VSMC mitogenesis, differentiation and matrix synthesis leading to neointimal formation and restenosis in vivo.4,5 Upon stimulation, PARs are rapidly internalized and degraded through phosphorylation-dependent mechanisms. Reappearance at the cell surface requires de novo synthesis or delivery from intracellular stores,6 and transcriptional control of PAR expression therefore represents an important mechanism to regulate the thrombogenic and proliferative actions of thrombin in vivo. We recently reported that prostacyclin (PGI₂) reduces PAR-1 expression and mitogenic signalling in human saphenous vein SMC.7 This could contribute to the reduced patency of saphenous vein bypass grafts, which compared with arterial vessels exhibit an impaired endogenous capacity to generate PGI₂.6

Our previous study identified cyclic AMP/protein kinase A (PKA)-dependent signalling as a key mechanism of PAR-1 downregulation, but the downstream mediators of this effect have not been elucidated. The human PAR-1 promoter does not possess a functional cyclic AMP response element consensus site; however, transcription factor binding analysis (www.genomatix.de) identified two potential nuclear factor of activated T-cells (NFAT) binding sites (TGGAAAA) in the human PAR-1 promoter. One of these is adjacent to a consensus sequence for the transcription factor AP-1, with which NFAT often acts in tandem.8 NFAT represents an attractive candidate regulator of PAR-1, since its activity is negatively regulated by cyclic AMP/PKA.9,10 The NFAT family consist of the classical members NFAT1 (also known as NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), and NFAT4 (NFATcX or NFATc3) as well as the non-classical NFAT5 (TonEBP).9 NFAT2 (NFATc1) is the predominant member in human VSMC and modulates migration, differentiation, and proliferation.12,13 NFAT inhibitors such as cyclosporin A (CsA) or VIVIT have recently been shown

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to be beneficial in restenosis, but the regulatory influence of NFAT on PAR-1 thrombin receptors is not known. We therefore investigated the potential role of NFAT, particularly NFAT2, as a regulatory target for the PKA-mediated regulation of PAR-1 in human VSMC in response to prostaglandin receptor activation.

2. Methods

2.1 Cell culture and incubations

Human saphenous veins were obtained from the Department of Cardiac Surgery at the University Hospital Düsseldorf. VSMC were isolated by the explant technique after Fallier-Becker and cultured in Dulbecco's Modified Eagle Medium supplemented with 15% foetal calf serum (both from GibcoBRL, Rockville, MD, USA) as described. The investigation conforms with the principles outlined in the Declaration of Helsinki and granted by our local University ethics review board (approval reference #3199). VSMC at passages 5–8 were serum-deprived for 48 h prior to stimulation with study drugs: butaprost (Cayman Chemical Company, An Arbor, MI, USA), phorbol myristoyl acetate (PMA, Alexis, Grünberg, Germany) and the cyclooxygenase 2 inhibitor etoricoxib (Laboratorien Berlin Aldersdorf, Germany), 8CPT-2Me-cAMP (BioLog Life Science Institute, Bremen, Germany), CsA, nitrendipine (both from Tocris, Ellisville, MO, USA), iloprost (kindly provided by Schering AG, Berlin, Germany) and a 11920 bp HI fragment containing the PAR-1 promoter was ligated into a pGL3bas luciferase reporter vector (Promega, Mannheim, Germany). The resultant constructs were transfected into VSMC seeded in 24-well plates using Lipofectamine2000 (Invitrogen, Karlsruhe, Germany). Iloprost or CsA were added 24 h following transfection and cell lysates were collected at 48 h for the measurement of luciferase reporter activity using the Luciferase Assay System (Promega).

2.2 Quantitative real-time PCR

Target gene mRNA expression was determined relative to the housekeeping genes ribosomal 185 or GAPDH as described. Effects of treatment were expressed as fold change vs. controls.

2.3 Western blot analysis

Total protein expression was detected in cell lysates by western blotting. Monoclonal primary mouse anti-human PAR-1 (sc-13503) and NFAT2 (sc-7294) were obtained from Santa Cruz Biotechnology (CA, USA); rabbit anti-human phosphorylated and total ERK1/2 antibodies (sc-9101, sc-9102) were from Cell Signalling Technology (MA, USA); rabbit anti-human phosphorylated and total ERK1/2 antibodies (sc-9101, sc-9102) were from Cell Signalling Technology (MA, USA). Horseradish peroxidase-attached anti-mouse secondary antibodies were also from Santa Cruz Biotechnology. Bands were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and quantified by densitometry (BioRad GelDoc8, visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK)). The specificity of NFAT binding to the PAR-1 promoter was validated a 171 bp region of genomic DNA between the GAPDH gene and the PAR-1 promoter was isolated and cloned into a pSK Bluescript vector. DNA from transformed E. coli was isolated using Midiprep Kits (Qiagen, Hilden, Germany) and correct clones identified by restriction enzyme digestion. A 5181 bp KpnI/Smal fragment retaining the NFAT/AP-1 site of the human PAR-1 promoter was ligated into a pGL3bas luciferase reporter vector (Promega, Mannheim, Germany). The resultant constructs were transfected into VSMC seeded in 24-well plates using Lipofectamine2000 (Invitrogen, Karlsruhe, Germany). Iloprost or CsA were added 24 h following transfection and cell lysates were collected at 48 h for the measurement of luciferase reporter activity using the Luciferase Assay System (Promega).

2.4 NFAT siRNA

VSMC were transfected with 10 nmol/L NFAT2 FlexiTube HP-Validated siRNA or the green fluorescent (Alexa 488)-labelled AllStars negative RNAi control (Qiagen, Hilden, Germany) by electroporation (GenePulser II, BioRad, München, Germany). Successful transfection was monitored by fluorescence of cells transfected with negative control RNA and validated by western blotting. Cells were used 48 h after transfection.

2.5 PAR-1 luciferase reporter assay

PAR-1 promoter activity was determined in VSMC transfected with a luciferase reporter vector in which the luciferase reporter gene is under the control of the human PAR-1 promoter. For this purpose, the human PAR-1 genomic clone RZPD8737G01022D (pBeloBAC11 vector from RZPD, Heidelberg, Germany) was used to transform E. coli (DH10B strain). DNA was isolated using the BigBAC DNA Isolation Kit (Princeton Separations, Philadelphia, NJ, USA) and a 11920 bp BamHI fragment containing the PAR-1 promoter was isolated and cloned into a pSK Bluescript vector. DNA from transformed E. coli was isolated using Midiprep Kits (Qiagen, Hilden, Germany) and correct clones identified by restriction enzyme digestion. A 5181 bp KpnI/Smal fragment retaining the NFAT/AP-1 site of the human PAR-1 promoter was ligated into a pGL3bas luciferase reporter vector (Promega, Mannheim, Germany). The resultant constructs were transfected into VSMC seeded in 24-well plates using Lipofectamine2000 (Invitrogen, Karlsruhe, Germany). Iloprost or CsA were added 24 h following transfection and cell lysates were collected at 48 h for the measurement of luciferase reporter activity using the Luciferase Assay System (Promega).

2.6 NFAT/AP-1 consensus site mutation

To validate the importance of the NFAT/AP-1 consensus site at positions for PAR-1 transcription, a 2 bp mutation (TC→GG) was introduced into the NFAT motif of the complementary strand (5′-TTTCCACTGTGTTTCA-3′) using the Quikchange XL site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according to manufacturer's instructions. Sequences for mutagenic primers (synthesis and HPLC validation by Invitrogen) were: TGTC ATATGTGTTTATTTG6GACTGTGTTTCACTATTGATCAA (forward) and TTGAGAATCTGAAGACACAGCTGGCATAATAAACACATGAGCA (reverse). Underlined bases indicate Mutation sites. Successful mutation was confirmed by sequencing (MWG-Biotech, Ebersberg Germany) and alignment analysis (http://www.ebi.ac.uk/cgi-bin/clustalw/).

2.7 NFAT2 nucleo-cytosolic translocation

Nucleo-cytosolic shuttling of NFAT2 was determined as a measure of inactivation. VSMC were treated with iloprost (10 nmol/L) or CsA (10 μmol/L) prior to extraction of cellular fractions as described. Preliminary studies indicated significant cytosolic accumulation at 2 h, this interval was utilized for subsequent experiments. The PKA inhibitor myr-PKI (5 μmol/L) was added for 30 min prior to treatment with iloprost. The purity of cytosolic and nuclear fractions was confirmed by reprobing of western blot membranes with antibodies (Santa Cruz Biotechnology) against the nuclear and cytosolic markers p300 (sc-584) and HSP70 (sc-1060), respectively.

2.8 ChIP assay

The specificity of NFAT binding to the PAR-1 promoter was validated by a modified ChIP assay. VSMC (2 × 10⁶ each) were stimulated for 2 h with CsA (10 μmol/L) or iloprost (10 nmol/L) + myr-PKI (5 μmol/L), then fixed with 1.5% formaldehyde. Cross-linking was stopped with 0.125 M glycine. Cells were pelleted and hypotonically lysed; nuclei were collected and sonicated. Chromatin was preclared with Protein G PLUS-Agarose (Santa Cruz Biotechnology) and immunoprecipitated with NFAT2 antibody (4°C, overnight). Protein–antibody complexes were collected by the addition of Protein G PLUS-Agarose for 16 h, and the beads were extensively washed. Protein–DNA cross-links were eluted and reversed. DNA was purified by phenol/chloroform/isoamylalcohol extraction and precipitated with ethanol.

PCR with NFAT-specific primers [5′-GTCAGATTCCACATGGGGAGG-3′ (forward), 5′-CCATGTTGTTAAACACACTTCAAA (reversed)] amplified a 171 bp region of human PAR-1 promoter containing the NFAT binding site. Negative-control primers, binding to the 3′ UTR region of GAPDH were: 5′-ATGGTGGGACCTGGGATCT-3′ (forward) and 5′-TGCCCAATGCTTTCTATGAGGAA-3′ (reverse), which amplified a 174 bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. Cycler conditions were five cycles of 94°C/30 s, 72°C/60 s; five cycles of 94°C/30 s, 70°C/30 s, 72°C/60 s, then 35 cycles 94°C/30 s, 58°C/30 s, 72°C/60 s. PCR products were resolved on a 1.8% agarose/ethidiumbromide gel.
2.9 Statistical analysis
Data are expressed as mean ± SEM, normalized to paired controls. Each ‘n’ represents an independently replicated experiment performed in a separate cell preparation, with 1–3 replicates for each treatment group in each individual study. Statistical analysis utilized one-way analysis of variance (ANOVA) or ANOVA on ranks with Dunnet’s or Tukey’s post hoc multiple comparisons procedure applied as appropriate. A P < 0.05 was accepted as significant.

3. Results
3.1 Gs-coupled prostaglandin receptor activation but not EPAC regulates PAR-1 in human VSMC
We previously found that the PGI₂ analogues iloprost and cicaprost downregulate PAR-1 expression in human venous SMC via activation of Gs-coupled IP receptors and subsequent stimulation of cyclic AMP/PKA signalling. We now examined if this effect is shared by vasodilatory prostaglandins generated endogenously. PMA (100 nmol/L), which we previously reported to induce COX-2 expression and endogenous PGI₂ formation in human VSMC, markedly reduced PAR-1 mRNA (n = 5, P < 0.05, Figure 1A). This effect was completely prevented the selective COX-2 inhibitor etoricoxib (10 μmol/L, n = 4); similar regulation of PAR-1 was seen at the protein level (Figure 1B, n = 4, P < 0.05). PMA-induced suppression of PAR-1 at earlier time points was not significant (data not shown). The EP2 receptor agonist butaprost (1 μmol/L) also significantly attenuated PAR-1 mRNA (Figure 1A) and protein (Figure 1B) expression at 24 h (both n = 4–5, P < 0.05).

3.2 NFAT inhibition downregulates PAR-1 in human venous SMC
The transcription factor NFAT represents a downstream target for PKA. The NFAT inhibitor CsA time and concentration dependently suppressed PAR-1 mRNA expression in human VSMC, significantly at 3–10 μmol/L and at 16–24 h (all n = 5, P < 0.05 vs. control, Figure 2A and B). PAR-1 protein expression was concentration dependently attenuated at 24 h (n = 6, P < 0.05, Figure 2C). Calcium channel blockers have also been reported to suppress NFAT activity in VSMC, and the dihydropyridine-type calcium antagonist nitrindipine (1 μmol/L) also markedly reduced PAR-1 protein expression by 44 ± 14% at 24 h (n = 5, P < 0.05, Figure 2D).

As a measure of the cellular responsiveness to thrombin, induction of the inflammatory cytokine interleukin-6 (IL-6), which mediates thrombin-stimulated VSMC migration and mitogenesis, was examined. In untreated cells, thrombin (3 IU/mL, 30 min) and the PAR-1 activating peptide TFLLRN (200 μmol/L, 3 h) significantly increased IL-6 mRNA expression to 2.6 ± 0.2- and 2.2 ± 0.2-fold of control,
respectively ($n = 5$, Figure 2E). Preliminary studies showed maximal IL-6 induction by thrombin or TFLLRN at these time points. When PAR-1 had been downregulated by previous (24 h) exposure to CsA (10 μmol/L) or iloprost (10 nmol/L), the stimulatory effects of thrombin or TFLLRN were lost (all $n = 4–5$, $P < 0.05$ vs. untreated, Figure 2E). Washout of iloprost prior to stimulation with thrombin or TFLLRN did not affect the inhibitory action (n = 3, data not shown) confirming that the suppression of the IL-6 response was not due to a direct inhibitory interaction.

3.3 NFAT2 knock-down reduces functional responsiveness to PAR-1 activation

Specific siRNA knocked down NFAT2 mRNA by ~80% (Figure 3A) and total protein by ~50% (Figure 3B). This was accompanied by significant suppression of PAR-1 mRNA (Figure 3C) and total protein (Figure 3D, all $n = 5$, $P < 0.05$). In mock-transfected VSMC, acute stimulation with thrombin or TFLLRN significantly enhanced phosphorylation of the mitogenic protein kinase ERK1/2 by thrombin or TFLLRN, and these stimulatory actions were blunted when PAR-1 was previously downregulated by NFAT2 siRNA (all $n = 5$, $P < 0.05$ vs. mock, Figure 3E). Induction of IL-6 mRNA in response to thrombin or TFLLRN (to $4.4 \pm 1.0$, and $2.5 \pm 0.6$-fold of control, respectively in mock-transfected VSMC) was also suppressed by NFAT siRNA (all $n = 5$, Figure 3F, all $n = 5$, $P < 0.05$).

3.4 NFAT inhibition or iloprost attenuate human PAR-1 promoter activity

PAR-1 promoter activity was examined by luciferase reporter assay in human VSMC transfected with a pGL3 vector containing the luciferase reporter gene under control of the human PAR-1 promoter. Transfection significantly increased basal luciferase activity about eight-fold (all $n = 6$, $P < 0.05$ vs. Figure 4A). This was suppressed by CsA (10 μmol/L, 24 h) and by site-directed mutagenesis of the NFAT/AP-1 consensus site (all $n = 6$, $P < 0.05$ vs. wild-type PAR-1 promoter activity, Figure 4A). Iloprost (10 nmol/L, 24 h) also blunted PAR-1 promoter activity, and the PKA inhibitor myr-PKI reversed this effect (all $n = 5$, $P < 0.05$, Figure 4B). PGE2 and butaprost showed similar results as iloprost (n = 2, data not shown).

3.5 Iloprost suppresses NFAT and DNA binding via PKA

PGI2 has not previously been reported to influence NFAT activity. Shuttling of nuclear NFAT2 to the cytosol was therefore determined by western blotting as a measure of inactivation. CsA (10 μmol/L) reduced the ratio of nuclear:cytosolic NFAT2 to 0.35 ± 0.1-fold of control; similarly, iloprost (10 nmol/L) suppressed nuclear localization of NFAT2 to 0.51 ± 0.1-fold of control in a myr-PKI-sensitive manner (restored to 0.83 ± 0.1-fold, all $n = 4$, $P < 0.05$, Figure 5A). PGE2 and butaprost showed similar results as iloprost (n = 2, data not shown).

Total cellular NFAT2 was not affected by any of the treatments (data not shown). To confirm the purity of cytosolic and nuclear fractions, membranes were reprobed with antibodies to nuclear and cytosolic markers p300 and HSP70, respectively.

The influence of iloprost on NFAT2 binding to the PAR-1 promoter was validated by ChIP assay (Figure 5B). A basal level of NFAT2 binding was detected in nuclei of untreated control SMC, this was near completely blocked by iloprost and restored by addition of myr-PKI. Specific NFAT/DNA binding was totally suppressed by CsA. Negative-control PCR for GAPDH confirmed absence of contaminating non-specific DNA in all samples. PGE2 and butaprost showed similar results as iloprost (n = 2, data not shown).

4. Discussion

This study provides the first evidence that NFAT contributes to transcriptional control of PAR-1 in human VSMC and that PKA-dependent inhibition of NFAT2 is a key mechanism leading to PAR-1 downregulation by which vasodilatory prostaglandins oppose the vascular effects of thrombin.

The mechanisms controlling the prototypic thrombin receptor PAR-1 are highly relevant for the pathological consequences of vascular injury, where thrombin generation is enhanced. At the same time, vascular injury is associated with induction of COX-2 and increased local generation of PGI2 and PGE2. We recently found that PGI2 suppresses PAR-1 expression and mitogenic signalling in human VSMC via cyclic AMP and PKA and now show that PAR-1 regulatory effects are shared by vasodilatory prostaglandins generated endogenously in human VSMC. The phospholipid PMA, which we have shown to elicit an upregulation of COX-2 and thereby increase prostaglandin production in human
VSMC,\textsuperscript{21,22} reduces PAR-1 mRNA and protein expression to a similar extent as we previously observed with the PGI\textsubscript{2}-mimetics iloprost and cicaprost.\textsuperscript{7} The effects of PMA are prevented by the COX-2 inhibitor etoricoxib, confirming the critical role of COX-2 in PAR-1 expression, which suggests that basal COX-2 dependent prostaglandin generation is minimal and that regulatory influences on PAR-1 become particularly apparent when COX-2 is induced.

Exogenous PGE\textsubscript{2} exerts similar inhibitory effects on PAR-1 expression as the PGI\textsubscript{2}-mimetics iloprost and cicaprost, but only at micromolar concentrations,\textsuperscript{7} possibly due to concurrent activation of multiple EP receptor subtypes, of which only EP2 and EP4 couple to Gs. Specific EP2 receptor activation with butaprost is now shown to also suppress PAR-1 mRNA and protein expression, in agreement with a recent report in human lung fibroblasts.\textsuperscript{28} Overall, this suggests a general action of vasodilatory prostaglandins acting via Gs-coupled receptors as transcriptional regulators of the human PAR-1. Autocrine formation of COX-2-derived prostaglandins by SMC in atherosclerotic vessels\textsuperscript{27} may represent an endogenous negative-feedback mechanism to control the proliferative effects of thrombin and to focus these to the site of injury. Our observations could explain in part the low patency of venous bypass grafts, which compared with arterial bypass vessels exhibit an impaired endogenous capacity to generate PGI\textsubscript{2}.\textsuperscript{8}

The effectors downstream of PKA have not been defined. In this study, we observed no influence of EPAC activation on PAR-1 expression, mirroring observations in lung fibroblasts.\textsuperscript{28} One attractive candidate is the transcription factor NFAT, which is controlled by both cyclic AMP/PKA\textsuperscript{11} and the small GTPase Rac\textsubscript{1},\textsuperscript{29} which we showed is suppressed in human VSMC upon exposure to iloprost.\textsuperscript{7} In this study, CsA, an inhibitor of calcineurin-dependent NFAT activation, was found to downregulate PAR-1 mRNA and protein in a time- and concentration-dependent manner, and to suppress PAR-1 promoter activity. Similar loss of PAR-1 promoter activity could be achieved by site-directed mutagenesis of the NFAT/AP-1 consensus site. Accordingly, NFAT inhibition with CsA also blunts the functional responsiveness to thrombin and the PAR-1 activating peptide TFLLRN, determined by induction of IL-6 mRNA. IL-6 is an inflammatory cytokine that mediates VSMC migration and proliferation and is highly expressed in atherosclerotic lesions.\textsuperscript{30,31} Downregulation of PAR-1 through NFAT inhibition may therefore prove beneficial in limiting inflammatory and proliferative responses to the site of vascular injury.

The CsA-induced loss of PAR-1 responsiveness is comparable to that induced by the PGI\textsubscript{2}-analogue iloprost, which also suppresses PAR-1 expression levels over this time course. Washout of iloprost immediately prior to stimulation does not affect inhibitory effects on PAR-1 expression, confirming that suppression of the IL-6 response results from transcriptional effects rather than a direct inhibitory interaction. This is in agreement with our previous observation that iloprost blunts PAR-1 mediated VSMC mitogenesis after 24 h preincubation but not after acute (1 h) exposure.\textsuperscript{7} Thus NFAT as well as PGI\textsubscript{2} control vascular PAR-1 expression, and this novel observation may explain at least part of the recently reported antirestenotic and vascular
antiproliferative effect of NFAT inhibition.\textsuperscript{13-15} PAR-1 down-regulation is also observed with the L-type calcium channel blocker nitrendipine. Dihydropyridines also suppress activation of NFAT in VSMC,\textsuperscript{23} and subsequent modulation of thrombin receptor function might contribute to the reduced severity of atherosclerosis reported in dihydropyridine-treated primates.\textsuperscript{32}

NFAT2 is the predominant NFAT isoform implicated in human VSMC migration, differentiation, and proliferation.\textsuperscript{12,13} We now demonstrate that knockdown of NFAT2 by siRNA attenuates basal PAR-1 expression to a comparable degree as both CsA and iloprost and abolishes functional responsiveness of VSMC to thrombin or the PAR-1 synthetic ligand TFLLRN. Both induction of the inflammatory cytokine IL-6 and activation of the mitogenic kinase ERK1/2 are reduced, suggesting that new generation NFAT inhibitors such as VIVIT peptide might prove beneficial in preventing the PAR-1-mediated atherosclerotic and thrombotic effects of thrombin in the vessel wall. Interestingly, although PAR-1 expression was suppressed ~50%, the functional responsiveness to thrombin and PAR-1 activating peptide was near completely abolished. The reason for this apparently disproportionate effect is not clear, but might reflect our earlier observation that a similar downregulation of PAR-1 by iloprost almost totally abolishes the mitogenic response to receptor activation.\textsuperscript{7}

Clearly, both PGI\textsubscript{2} and NFAT inhibition exert similar regulatory effects on PAR-1 expression and function in human VSMC. What is not known is if NFAT indeed represents an effector target for PGI\textsubscript{2}. We here show for the first time that iloprost, like the NFAT inhibitor CsA, promotes shuttling of NFAT2 from the nucleus to the cytosol, which indicates a direct influence on NFAT activation status by PGI\textsubscript{2}. This effect is sensitive to the PKA inhibitor PKI, reflecting our previous findings that iloprost regulates PAR-1 via this signalling pathway. Further, these data are consistent with reports that PKA promotes nuclear export of NFAT\textsuperscript{10,11} Phosphorylation of the NFAT regulatory domain masks the nuclear localization sequences to promote cytoplasmic sequestration.\textsuperscript{33,34}

Iloprost-induced suppression of PAR-1 luciferase reporter activity is also blunted by PKA inhibition. The hypothesis that NFAT2 actually binds to the PAR-1 promoter, and that such an interaction is suppressed by iloprost in a PKA-dependent manner, is validated by ChIP assay. The translocation and ChIP assays both indicate that a certain amount of NFAT is localized to the nucleus and bound to the PAR-1 promoter under basal conditions. This, together with the high degree of basal PAR-1 promoter activity seen in the luciferase assay, is in keeping with the robust constitutive expression of PAR-1 thrombin receptors in human VSMC.

Collectively, these observations demonstrate for the first time that PAR-1 is transcriptionally regulated in human VSMC by vasodilatory prostaglandins in a PKA- and NFAT2-dependent manner. This counter-regulatory mechanism is likely to be of physiological relevance despite the rapid phosphorylation-dependent desensitization of IP prostacyclin receptors.\textsuperscript{35,36} Given the recent report by Kasza \textit{et al.}\textsuperscript{34} that IP activation can induce COX-2 induction and hence the further release of prostacyclin by adjacent SMC layers in which IP receptor had not been activated. This positive-feedback loop, dependent on PKA, could then propagate the protective effects of prostacyclin through the vessel media, despite receptor densitization.

Other additional mechanisms are no doubt likely to contribute. The proposed signalling cascade is summarized in Figure 6. This finding suggests an endogenous protective mechanism by which induction of COX-2-dependent generation of vasodilatory prostaglandins may limit local atherothrombotic and inflammatory processes after vascular injury.

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\section*{Conflict of interest:} none declared.

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