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Regulation of Human Vascular Protease-Activated Receptor-3 through mRNA Stabilization and the Transcription Factor Nuclear Factor of Activated T Cells (NFAT)

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ABSTRACT

Thrombin promotes vascular smooth muscle cell (SMC) proliferation and inflammation via protease-activated receptor (PAR)-1. A further thrombin receptor, PAR-3, acts as a PAR-1 cofactor in some cell-types. Unlike PAR-1, PAR-3 is dynamically regulated at the mRNA level in thrombin-stimulated SMC. This study investigated the mechanisms controlling PAR-3 expression. In human vascular SMC, PAR-3 siRNA attenuated thrombin-stimulated interleukin-6 expression and extracellular signal-regulated kinases 1/2 phosphorylation, indicating PAR-3 contributes to net thrombin responses in these cells. Thrombin slowed the decay of PAR-3 but not PAR-1 mRNA in the presence of actinomycin D and induced cytosolic shuttling and PAR-3 mRNA binding of the mRNA-stabilizing protein human antigen R (HuR). HuR siRNA prevented thrombin-induced PAR-3 expression. By contrast, forskolin inhibited HuR shuttling and destabilized PAR-3 mRNA, thus reducing PAR-3 mRNA and protein expression. Other cAMP-elevating agents, including the prostacyclin-mimetic iloprost, also down-regulated PAR-3, accompanied by decreased HuR/PAR-3 mRNA binding. Iloprost-induced suppression of PAR-3 was reversed with a myristoylated inhibitor of protein kinase A and mimicked by phorbol ester, an inducer of cyclooxygenase-2. In separate studies, iloprost attenuated PAR-3 promoter activity and prevented binding of nuclear factor of activated T cells (NFAT2) to the human PAR-3 promoter in a chromatin immunoprecipitation assay. Accordingly, PAR-3 expression was suppressed by the NFAT inhibitor cyclosporine A or NFAT2 siRNA. Thus human PAR-3, unlike PAR-1, is regulated post-transcriptionally via the mRNA-stabilizing factor HuR, whereas transcriptional control involves NFAT2. Through modulation of PAR-3 expression, prostacyclin and NFAT inhibitors may limit proliferative and inflammatory responses to thrombin after vessel injury.

Introduction

The clinical success of venous bypass grafting is often limited by constrictive vascular remodelling. This involves vascular smooth muscle cell (SMC) proliferation, migration, and inflammation (Forrester et al., 1991), in which the clotting factor thrombin plays a central role (Schrör et al., 2010). Thrombin acts via protease-activated receptors (PARs), which are proteolytically cleaved to create a new NH_2-terminal domain that autoactivates the receptor (Coughlin, 2000). Of four known PARs, PAR-1, PAR-3, and PAR-4 are activated by thrombin, responding with EC_{50} values of 50 pM, 0.2 nM, and 5 nM, respectively (Steinberg, 2005; Schrör et al., 2010). PAR-1 is the prototypical receptor mediating most thrombin actions (Wilcox et al., 1994; Coughlin, 2000), whereas PAR-4 mediates mouse and human platelet activation (Coughlin,

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ABBREVIATIONS: SMC, smooth muscle cell; CsA, cyclosporin A; EPAC, exchange protein directly activated by cAMP; ERK1/2, extracellular-regulated kinase 1/2; HuR, human antigen R; IBMX, isobutyl-1-methylxanthine; IL-6, interleukin-6; NFAT, nuclear factor of activated T cells; PAR, protease-activated receptor; PGI_2, prostacyclin; PKA, protein kinase A; PKI, myristoylated protein kinase A inhibitor; PMA, phorbol 12-myristate 13-acetate; VASP, vasodilator-stimulated phosphoprotein; VSMC, vascular smooth muscle cell; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; UTR, untranslated region; IP, immunoprecipitation; siRNA, short interfering RNA; db-cAMP, dibutyryl cAMP; 8CPT-2Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP, sodium salt; COX-2, cyclooxygenase-2.

2000; Ofosu, 2003). PAR-3 acts as a cofactor for PAR-4 in mouse platelets (Ishihara et al., 1997) and as an allosteric modulator of PAR-1 in endothelial cells (McLaughlin et al., 2007). In other cells by contrast, PAR-3 counteracts PAR-1 signaling (Wysoczynski et al., 2010) or may even signal autonomously (Wang et al., 2002; Ostrowska and Reiser, 2008).

We and others have reported that PAR-3 is also expressed in human VSMC (Bretschneider et al., 2003; Vidwan et al., 2010). It is noteworthy that PAR-3, unlike PAR-1, is dynamically regulated by thrombin in these cells (Bretschneider et al., 2003). In human lung fibroblasts by contrast, PAR-3 is down-regulated upon prolonged exposure to thrombin (Sokolova et al., 2005). Such adaptive regulation of PAR-3, a modulator of thrombin/PAR-1 signaling, could serve to fine-tune cellular responses to thrombin, which would be of particular relevance in settings of high thrombin generation or modified PAR-1 activity (i.e., patients receiving selective PAR-1 inhibitors).

The factors controlling PAR-3 expression remain to be defined. Potential candidates include the vasodilatory prostaglandin prostacyclin (PGI_2). This inhibits SMC proliferation and dedifferentiation (Fetalvero et al., 2006); hence, increased generation of PGI_2 in atherosclerosis (Belton et al., 2000) may serve to limit the actions of thrombus-derived thrombin near the site of vessel injury. We have demonstrated that PGI_2 transcriptionally regulates vascular PAR-1 (Pape et al., 2008; Rosenkranz et al., 2009). Activation of G_s-coupled prostaglandin I_2 receptors stimulates cAMP formation and the downstream effectors protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC), and cAMP response elements in target genes. Moreover cAMP regulates gene expression through inhibition of the mRNA-stabilizing protein human antigen R (HuR) (Klöss et al., 2004) or of transcription factors such as nuclear factor of activated T cells (NFAT) (Sheridan et al., 2002). The NFAT2 isoform plays a central role in the control of vascular PAR-1 expression (Rosenkranz et al., 2009). The human PAR-3 promoter also contains two potential NFAT consensus sites (TGGAAAA; http://www.genomatix.de), but it is not known whether NFAT2 can regulate this receptor.

Thus, PAR-3, unlike PAR-1, is subject to a dynamic regulation by thrombin (Bretschneider et al., 2003), which might be particularly important during the vascular response to injury. This study investigated the regulation of PAR-3 in human SMC and examined the role of major cAMP targets, including HuR, PKA, and NFAT2 in this effect.

Materials and Methods

Compounds. Isobutyl-1-methylxanthine (IBMX), forskolin, and dibutyryl cAMP (db-cAMP) were all from Sigma-Aldrich (Taufkirchen, Germany); phorbol 12-myristate 13-acetate (PMA) was from Alexis (Grünberg, Germany); etoricoxib was from Laboratorien Berlin Aldersdorf (Berlin, Germany); EPAC activator 8CPT-2Me-cAMP was from BioLog Life Science Institute (Bremen, Germany); cyclosporin A (CsA) was from Tocris Bioscience (Ellisville, MO); iloprost was kindly provided by Bayer-Schering (Berlin, Germany); and myristoylated protein kinase A inhibitor (PKI) and actinomycin D were from Calbiochem (San Diego, CA). α-Thrombin was a gift from the late Dr. J. Stürzebecher (Zentrum für Vaskuläre Biologie und Medizin, Jena, Germany).

VSMC Culture and Incubations. VSMCs were isolated from human saphenous vein specimen obtained through the Department of Cardiac Surgery at the University Hospital Düsseldorf by the explant technique (Fallier-Becker et al., 1990) and cultured in Dulbecco's modified Eagle's medium (15% fetal calf serum; Invitrogen, Carlsbad, CA). The study was performed with institutional Human Ethics Commission approval, and informed consent of donors and was performed in accordance with the Declaration of Helsinki. VSMCs (passages 5–8) were serum-deprived 48 h before stimulation.

Real-Time Reverse Transcription-PCR. Target gene expression was determined by real-time PCR as described previously (Pape et al., 2008; Rosenkranz et al., 2009).

Western Blot Analysis. Protein expression was determined in total cell lysates by Western blotting (Rauch et al., 2002) using primary rabbit anti-human PAR-3, mouse anti-human NFAT2, and goat anti-human vasodilator-stimulated phosphoprotein (VASP) antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human total and phospho-ERK1/2 antibodies, rabbit anti-phospho VASP antibodies (all from Cell Signaling Technology, Danvers, MA), and horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology). Expression was normalized to β-actin (mouse monoclonal antibody from Sigma-Aldrich) after stripping (0.2 N NaOH) and reprobing of membranes.

HuR Cytosolic Shuttling. Cytosolic HuR accumulation as a measure of its activation was determined by western blotting of cytosolic and total cellular fractions prepared from VSMCs (Rosenkranz et al., 2009) using monoclonal primary mouse anti-human HuR antibody (Santa Cruz Biotechnology).

Pull-Down PCR. HuR binding to PAR-3 mRNA was determined by immunoprecipitation (IP)-PCR assay as described previously (Doller et al., 2008). In brief, VSMCs were stimulated for 2 h before extraction of cytosolic fractions (Rosenkranz et al., 2009). After all samples were adjusted for equal protein concentration, aliquots were taken as input control and the remainder divided for immunoprecipitation (4°C overnight) with anti-HuR antibody or mouse IgG. Protein G-Sepharose CL-4B beads (Santa Cruz Biotechnology) were added for further 2 h at 4°C. Beads were successively washed with low- and high-salt buffers, and then total RNA was extracted from input controls and HuR-IP and IgG-IP samples for real-time PCR analysis (Rosenkranz et al., 2009). IP samples were analyzed for PAR-3 expression levels and normalized to 18S rRNA expression in input controls.

Knockdown via siRNA. For knockdown of PAR-3 or HuR, VSMCs were transfected with 30 nmol control or specific siRNA (Santa Cruz Biotechnology) using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For knockdown of NFAT2, VSMCs were transfected with 10 nmol green fluorescent (Alexa 488)-labeled control siRNA or NFAT2 siRNA (both from QIAGEN, Valencia, CA) by electroporation (Genepulser II; Bio-Rad, München, Germany). Successful transfection was monitored by fluorescence and validated by Western blotting. Cells were used 48 h after transfection.

Chromatin Immunoprecipitation Assay. VSMCs were stimulated for 2 h with study drugs, and the specific binding of NFAT2 to the PAR-3 promoter was examined by a modified chromatin immunoprecipitation (ChIP) assay essentially as described previously (Rosenkranz et al., 2009). Forward (5′-CCACTCAAAAGCCAGGTTCT-3′) and reverse (5′-CCTTTCCTTTATCTTCCCAGC-3′) NFAT primers amplified a 190-base pair region of the human PAR-3 promoter containing the two NFAT binding sites. Negative control primers binding to the 3′-untranslated region (UTR) region of glyceraldehyde-3-phosphate dehydrogenase were 5′-ATGGTTGCCACTGGGGATCT-3′ (forward) and 5′-TGCCAAAGCCTAGGGGAAGA-3′ (reverse). Cycler conditions were 5 cycles of 94°C for 30 s and 72°C for 60 s; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 60 s; and then 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. PCR products were resolved on a 1.8% agarose/ethidium bromide gel.

PAR-3 Luciferase Reporter Assay. A DNA fragment containing the PAR-3 promoter was isolated from human PAR-3 genomic clone RZPDB737C121013D (Heidelberg, Germany) and cloned into a

pSK⁻ Bluescript vector. DNA from transformed K12 *Escherichia coli* (DH10B strain) was isolated using Midi-Prep Kits (QIAGEN), and correct clones were identified by restriction enzyme digestion. A 3467-base pair KpnI/Esp3I fragment retaining the two putative NFAT binding sites was ligated into the KpnI/SmaI cut pGL3basic luciferase reporter vector (Promega, Mannheim, Germany) after filling in the 5′-protruding end by PCR. The resultant constructs were transfected into VSMC using Lipofectamine2000 (Invitrogen). Study drugs were added 24 h after transfection, and cell lysates were collected at 48 h for measurement of luciferase reporter activity using the Luciferase Assay System (Promega).

DNA Synthesis. DNA synthesis was determined as a measure of mitogenesis by [^{3}H]thymidine incorporation as described previously (Rauch et al., 2002).

Statistical Analysis. Data are expressed as mean ± S.E.M, normalized to controls. Statistical analysis used one-way analysis of variance with Dunnett's multiple comparisons procedure. $P < 0.05$ was accepted as significant.

Results

PAR-3 Contributes to Thrombin Effects in Human VSMC. Transfection of human VSMCs with siRNA against PAR-3 resulted in a knockdown of receptor protein by almost 80% compared with transfection with control siRNA ($n = 4$, $P < 0.05$; Fig. 1A). In these cells, the stimulatory effect of thrombin (3 U/ml) on IL-6 mRNA expression was attenuated (3-h stimulation, $n = 4$; Fig. 1B). Thrombin-induced ERK1/2 phosphorylation in VSMC was modestly reduced in VSMCs transfected with PAR-3 siRNA, but this was not significant (10-min stimulation, $n = 4$, Fig. 1C).

Post-Transcriptional Regulation of PAR-3. PAR-1 regulation is reported to be independent of mRNA stabilization (Sokolova et al., 2008); thus, selectivity in the regulatory action of thrombin upon its receptors could be due to differences in mRNA-stabilizing effects. The decay of PAR-1 and PAR-3 mRNA was determined in VSMCs pretreated with actinomycin D (5 μg/ml, 30 min) to prevent de novo transcription and then stimulated with thrombin (3 U/ml). PAR-1 mRNA half-life was approximately 5 h under both control and thrombin-stimulated conditions ($n = 4$; Fig. 2A). PAR-3 mRNA degradation was more rapid, with a half-life of approximately 2 h, which was significantly slowed by thrombin at 2 to 5 h ($n = 4$, Fig. 2B). Over a similar time course, exposure to thrombin resulted in an accumulation of the mRNA-stabilizing protein HuR in the cytosol, with no change in total cellular HuR, which was consistent ($n = 4$, $P < 0.05$, Fig. 2C). Maximal HuR shuttling was seen at 2 h; therefore, this incubation interval was chosen for subsequent analysis of HuR binding to PAR-3 mRNA by pull-down PCR. The amount of cytosolic PAR-3 mRNA immunoprecipitated by the anti-HuR antibody was markedly increased by thrombin to approximately 3-fold ($n = 4$, $P < 0.05$; Fig. 2D). Only minimal PAR-3 transcripts were detected after pull-down with an IgG control antibody. The critical role of HuR in thrombin-regulated PAR-3 expression was investigated with HuR siRNA. This suppressed endogenous HuR protein by approximately 65% compared with control siRNA ($n = 4$, $P < 0.05$, Fig. 2E) and abolished the stimulatory effects of thrombin (3 U/ml, 3h) on PAR-3 mRNA expression ($n = 4$, Fig. 2F).

Regulation of PAR-3 via cAMP. Forskolin (10 μM) reduced cytosolic HuR shuttling in a time-dependent manner ($n = 4$, $P < 0.05$ at 1 h; Fig. 3A), with a return to baseline within 6 h. Forskolin accelerated PAR-3 mRNA decay in the presence of actinomycin D (5 μg/ml, 30 min, $n = 4$, $P < 0.05$; Fig. 3B) and in the absence of actinomycin D significantly suppressed basal PAR-3 mRNA expression within 3 h. This inhibition was sustained to 24 h ($n = 4$, $P < 0.05$; Fig. 3C). PAR-3 protein expression in human VSMC was suppressed significantly at 24 h ($n = 4$, $P < 0.05$; Fig. 3D). The inhibitory actions of forskolin on PAR-3 mRNA expression at 6 h were mimicked by the phosphodiesterase inhibitor IBMX (0.5 mM) or the cAMP analog db-cAMP (1 mM, all $n = 5$, $P < 0.05$; Fig. 3E). Activation of the EPAC with 8CPT-2Me-cAMP (200 μM) did not influence PAR-3 mRNA ($n = 3$, Fig. 3F) or total protein expression ($n = 3$, data not shown).

Prostacyclin Analog Iloprost Suppresses PAR-3 via PKA. The potential regulatory impact of the cAMP stimulus iloprost, a PGI_2-mimetic, on PAR-3 was examined. Iloprost (10 nM) suppressed PAR-3 mRNA and total protein expression (both 24-h incubation, $n = 5$, $P < 0.05$; Fig. 4, A and B). PAR-3 suppression was also seen with the IP-selective agonist cicaprost (10 nM; Schering AG, Berlin, Germany), exogenous PGE_2 (1 μM; Cayman Chemical, Ann Arbor, MI), and the EP_2-selective receptor agonist butaprost (1 μM, Cayman Chemicals; all $n = 4$, $P < 0.05$, data not shown). Inhibitory actions of iloprost on PAR-3 expression were prevented by the cell-permeant PKA inhibitor myristoyl-PKI. Complete inhibition of PKA by myristoyl-PKI was validated by Western blotting for total and VASP. Both forskolin (10 μM) and iloprost (10 nM) induced VASP phosphorylation, as seen by the appearance of phospho-VASP bands and a shift in the bands for total VASP, and this was prevented by myristoyl-PKI ($n = 4$; Fig. 4C). Iloprost also attenuated HuR binding to PAR-3 mRNA in a pull-down PCR assay (2h incubation, $n = 3$, $P < 0.05$; Fig. 4D). To extrapolate the inhibitory effects of iloprost to PGI_2 generated endogenously, the influence of the cyclooxygenase-2 inducer PMA (Debey et al., 2003; Rabausch

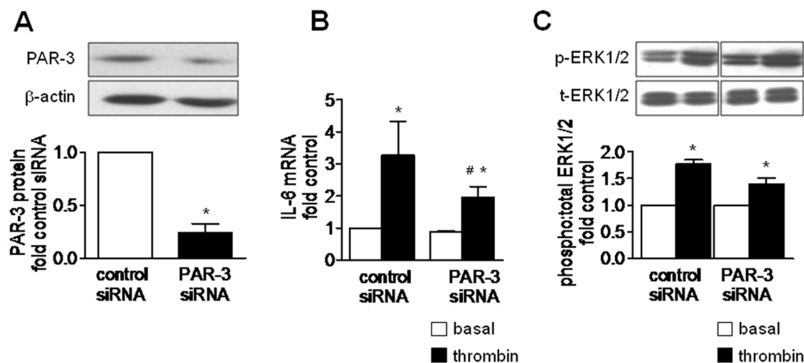

Fig. 1. PAR-3 contributes to thrombin responses in human VSMC. A, suppression of PAR-3 protein expression with siRNA against PAR-3 versus transfection with control siRNA and the impact on the stimulatory actions of thrombin (3 U/ml) on IL-6 mRNA expression (3-h stimulation) (B) and ERK1/2 phosphorylation (10-min stimulation) (C). All, $n = 4$; *, $P < 0.05$ versus control.

et al., 2005) was examined. PMA (100 nM, 24 h) significantly suppressed basal PAR-3 mRNA (Fig. 4E) and total protein expression (Fig. 4F; both $n = 5$, $P < 0.05$), which were both restored by the COX-2 selective inhibitor etoricoxib (10 μM, 30 min prior).

Transcriptional Regulation of PAR-3 via NFAT. As described above, the effects of forskolin on HuR shuttling and PAR-3 mRNA stability were normalized by 6 h, yet PAR-3 mRNA remained suppressed at 24 h. This indicates involvement of additional cAMP-dependent mechanisms, potentially at the transcriptional level. NFAT2 was investigated as a candidate regulator of PAR-3 downstream of cAMP given its central role in VSMC proliferation and dedifferentiation (Yellaturu et al., 2002). Specific siRNA against NFAT2 suppressed NFAT2 protein by >50% compared with control siRNA ($n = 5$, $P < 0.05$; Fig. 5A). In these cells, basal PAR-3 mRNA and total protein expression was reduced ($n = 5$, $P < 0.05$; Fig. 5, B and C). The NFAT inhibitor CsA also attenuated PAR-3 mRNA and protein expression in a concentration-dependent manner over 24 h ($n = 5$, $P < 0.05$ at 3–10 μM; Fig. 5, D and E).

Iloprost inhibits the transcriptional activity of NFAT2 in human VSMCs via cAMP/PKA (Rosenkranz et al., 2009), and here, iloprost (10 nM, 2 h) suppressed the binding of NFAT2 to the PAR-3 promoter in a modified ChIP assay ($n = 3$; Fig. 6A). This action was prevented by the PKA inhibitor PKI and was comparable with the effect of CsA. Negative control PCR for glyceraldehyde-3-phosphate dehydrogenase confirmed minimal contamination by nonspecific DNA in all samples. Transfection of VSMC with a pGL3 vector containing the luciferase reporter gene under control of a portion of the human PAR-3 promoter increased basal luciferase activity approximately 7-fold; this was attenuated by iloprost and CsA, and the inhibitory effect of iloprost was prevented by PKI ($n = 5$; Fig. 6B).

Discussion

The distinct functions of PAR-3, a dynamically regulated thrombin receptor, in the human vasculature has not been fully defined. We now show that expression of PAR-3 is controlled at the transcriptional level via NFAT2 and post-transcriptionally through HuR-mediated mRNA stabilization. Via these mechanisms, strategies targeting cAMP and

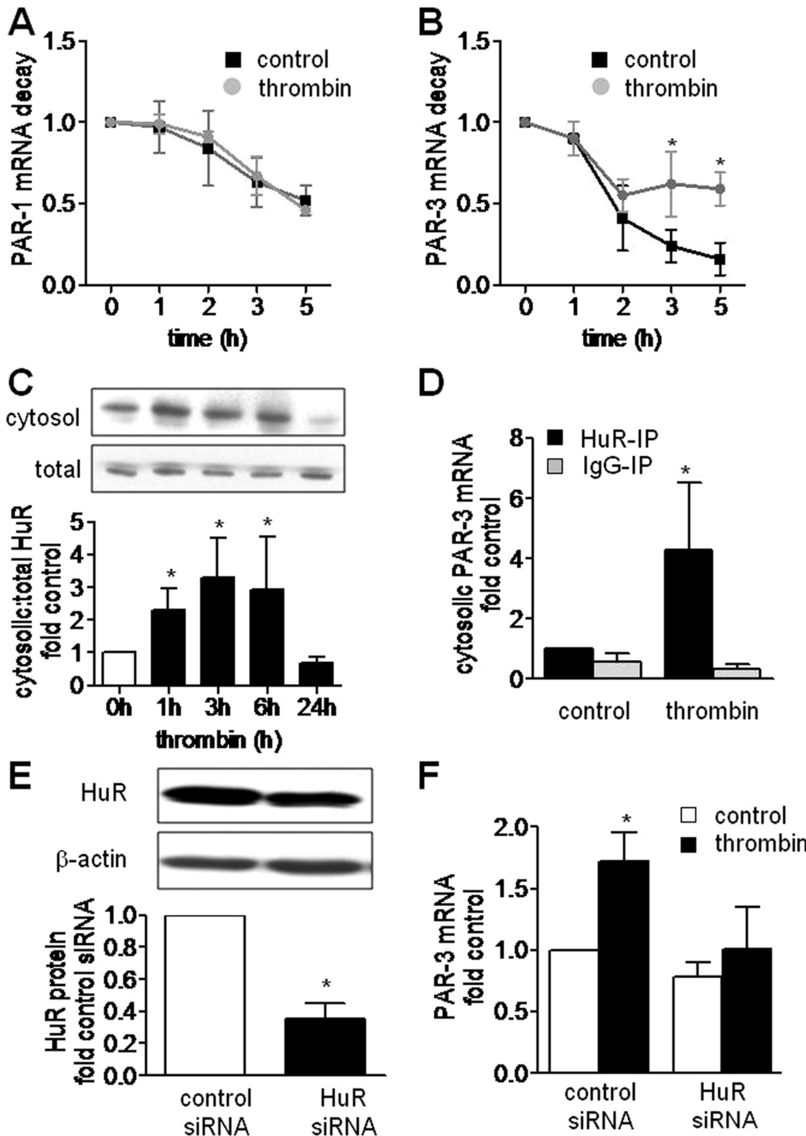

Fig. 2. PAR-3 mRNA stabilizing effects of HuR in thrombin-stimulated human VSMC. PAR-1 (A) and PAR-3 (B) mRNA decay in human VSMCs treated with actinomycin D (5 μg/ml, 30 min) before stimulation with thrombin (3 U/ml). C, cytosolic HuR accumulation in response to thrombin (3 U/ml), normalized to total cellular HuR. D, binding of HuR to PAR-3 mRNA in unstimulated or thrombin-stimulated (3U/ml, 2 h) VSMC. Shown is PAR-3 mRNA expression in cytosolic fractions after immunoprecipitation with HuR (■) or IgG control antibody (□). E, HuR protein expression in VSMC transfected with control (□) or HuR siRNA (■) by siRNA, normalized to β-actin. F, influence of HuR siRNA on thrombin (3 U/ml, 3 h)-stimulated PAR-3 mRNA expression (thrombin shown in ■). All, $n = 4$; $*$, $P < 0.05$ versus control.

NFAT2 signaling, including iloprost and CsA, negatively regulate PAR-3 expression and may therefore modulate cellular responsiveness to thrombin.

PAR-3 distribution and functionality exhibit marked species- and cell type-specific differences. Intrinsic PAR-3 signaling is reported in some (Wang et al., 2002; Ostrowska and Reiser, 2008) but not in all cell systems (Ishihara et al., 1997; Nakanishi-Matsui et al., 2000), whereas in other cells, PAR-3 may either counteract (Wysoczynski et al., 2010) or potentiate PAR-1 responses (McLaughlin et al., 2007). In the vasculature, expression of PAR-3 is generally believed to be limited to the endothelium (McLaughlin et al., 2007; Martorell et al., 2008; Borissoff et al., 2009). However, we have reported that PAR-3 is expressed in human VSMC (Bretschneider et al., 2003), and this has since been reported by others (Vidwan et al., 2010). Abundant expression of PAR-3 is also found in rat lung VSMCs (Jesmin et al., 2007).

In the present study, the cellular responsiveness to thrombin was attenuated by siRNA against PAR-3, indicating that this receptor contributes to the net thrombin response in human vascular SMC. This raises the possibility that inhibitory strategies against PAR-1 alone may be insufficient to prevent vascular actions of thrombin after injury. VSMCs also express low levels of PAR-4; however, in preliminary studies, PAR-4 activating peptide (200 μM) failed to increase either DNA synthesis (n = 4) or IL-6 mRNA expression in human VSMCs (n = 3). Thus, the contribution of PAR-4 to the effects observed here are negligible.

In healthy arteries, expression of PAR-1 is minimal but increases during vascular lesion formation, predominantly in

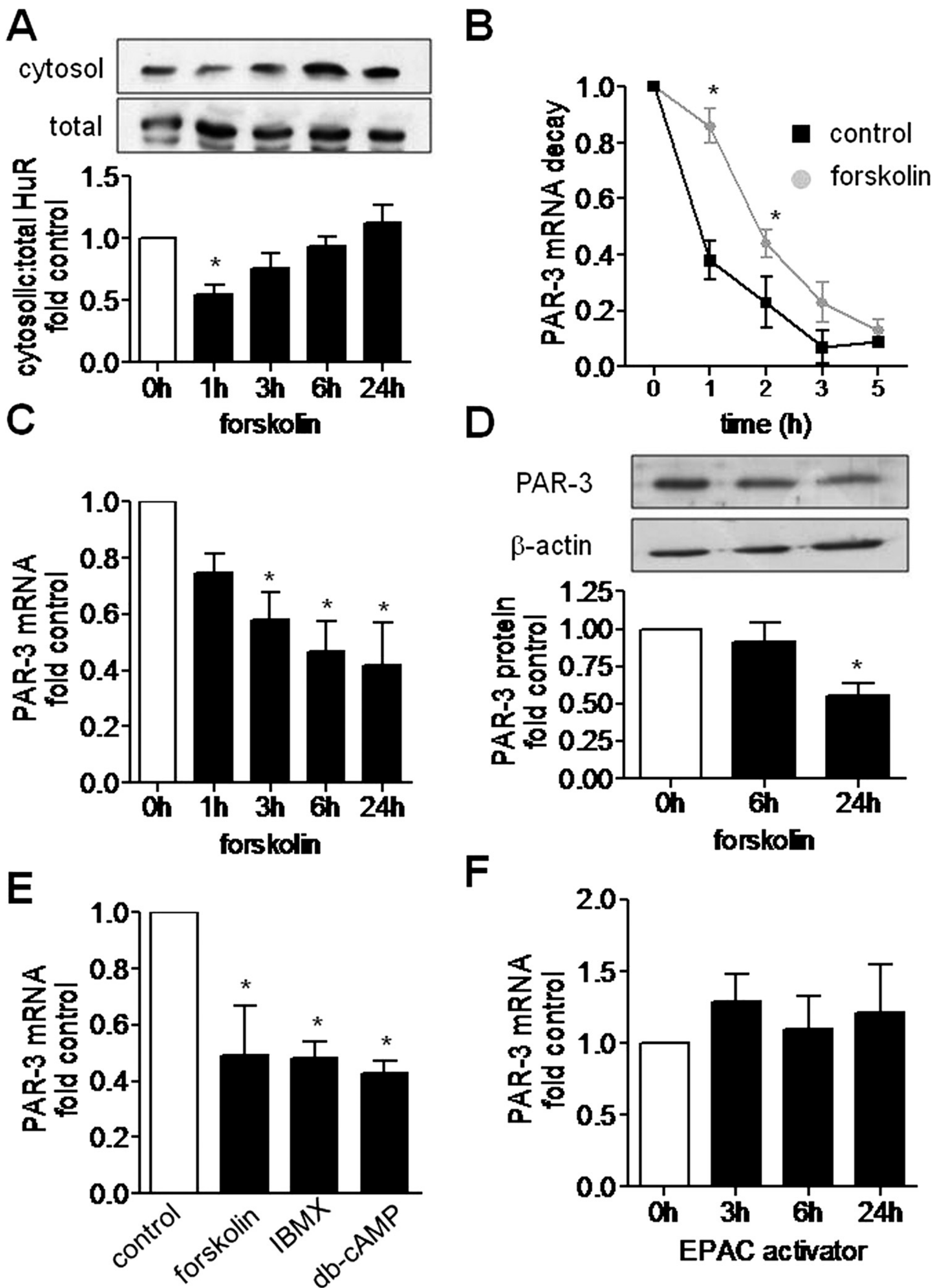

Fig. 3. PAR-3 mRNA destabilizing effect of forskolin through inhibition of HuR. A, forskolin-induced cytosolic HuR accumulation, normalized to total cellular HuR. B, decay of PAR-3 mRNA in VSMCs pretreated with actinomycin D (5 μg/ml, 30 min) in the presence (▲) and absence (○) of forskolin (10 μM). Time-dependent suppression of PAR-3 mRNA (C) and PAR-3 protein (D) by forskolin (10 μM, 24 h). E, PAR-3 mRNA regulation by 6-h exposure to forskolin, IBMX (0.5 mM), or db-cAMP (1 mM). F, EPAC activator 8CPT-2Me-cAMP (200 μM) does not influence PAR-3 expression in human VSMCs. All, n = 4–5 (EPAC, n = 3); *, $P < 0.05$ versus control.

areas of cell proliferation (Wilcox et al., 1994). This reflects a relatively low turnover of PAR-1 in quiescent cells. In the present study, the mRNA half-life of PAR-1 was approximately 5 h, whereas that of PAR-3 was 2 h, indicating a more rapid turnover of this receptor mRNA. Thrombin slowed the decay of PAR-3 mRNA with no influence on PAR-1. This is in accordance with a report in human lung fibroblasts that PAR-1 is not regulated via mRNA stabilization (Sokolova et al., 2008) and confirms our previous observation that PAR-3 but not PAR-1 mRNA is dynamically regulated by thrombin in human VSMCs (Bretschneider et al., 2003). Post-transcriptional regulation via mRNA stabilization is a feature of inducible genes with rapid turnover and involves the actions of mRNA-stabilizing proteins such as HuR, which recognize AU-rich elements in the 3′-UTR of target mRNAs (Misquitta et al., 2001; Doller et al., 2008). Numerous such motifs exist in the 3′-UTR of human PAR-3. Here, the slowed degradation of PAR-3 mRNA in thrombin-stimulated VSMCs coincided with the accumulation of cytosolic HuR, and HuR siRNA abolished thrombin-regulated PAR-3 expression. Moreover, thrombin enhanced binding of HuR to PAR-3 mRNA in a pull-down PCR assay. This is the first evidence that expression of a thrombin receptor is controlled in part via HuR. The high abundance of HuR in atherosclerosis, neointima forma-

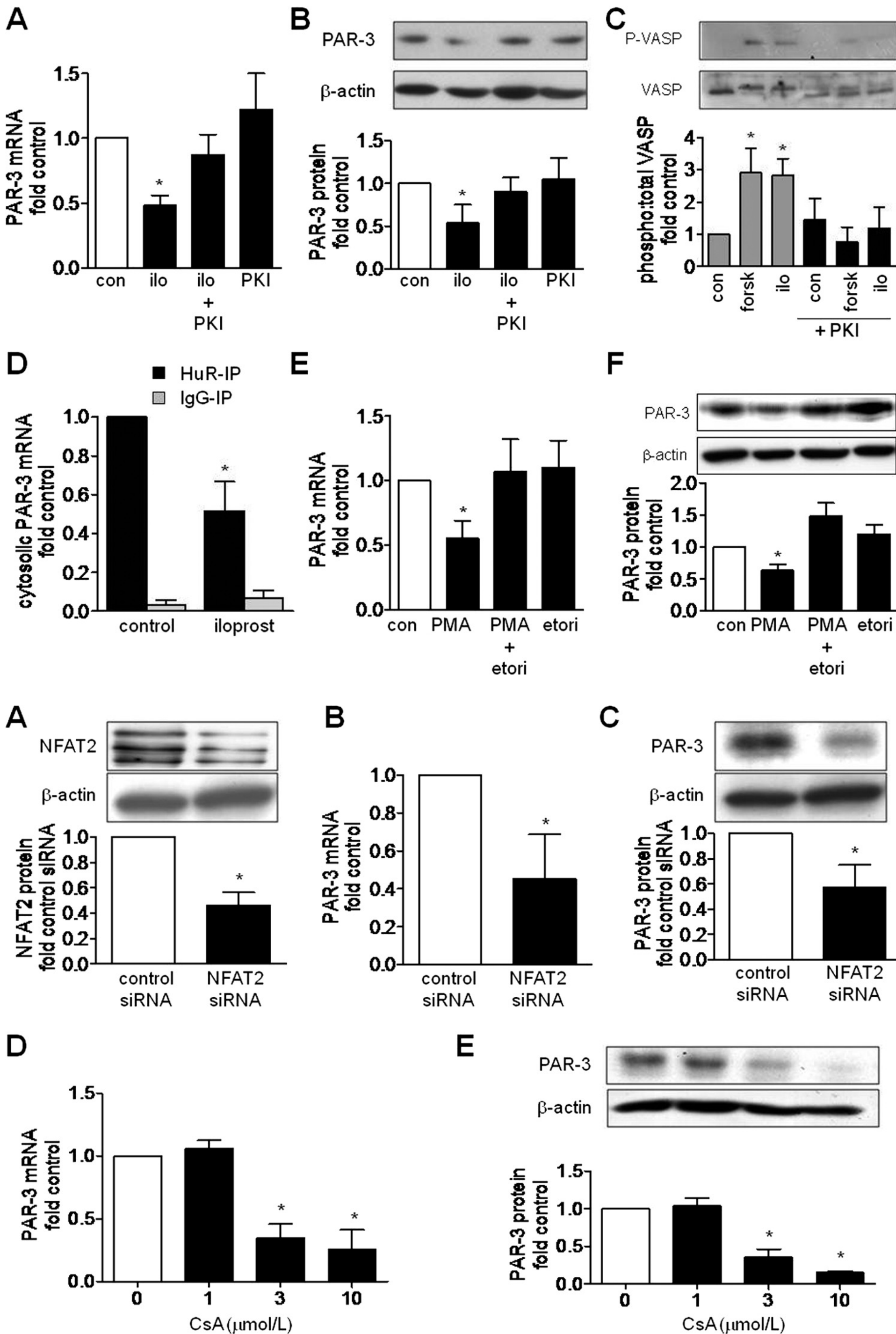

Fig. 4. Prostacyclin suppresses PAR-3 expression and function in human VSMCs. A, PAR-3 mRNA and B, PAR-3 protein expression in VSMC stimulated 24 h with iloprost (ilo, 10 nM) and/or the PKA inhibitor PKI (5 μM). C, validation of PKA inhibition by PKI: shown is phosphorylated and total VASP protein in VSMC stimulated with forskolin (10 μM) or iloprost (10 nM) for 30 min ± PKI. D, iloprost (10 nM, 2 h) suppresses binding of HuR to PAR-3 mRNA in human VSMCs. Shown is PAR-3 mRNA expression in cytosolic fractions after immunoprecipitation with HuR (■) or IgG control antibody (▨). Influence of the COX-2 inducer PMA (100 nM, 24 h) and/or the COX-2 inhibitor etoricoxib (etori, 10 μM) on PAR-3 mRNA (E) and total protein expression in human VSMCs (F). All, n = 4 to 6; $*$, $P < 0.05$ versus control.

Fig. 5. Suppression of PAR-3 expression and function via NFAT2. NFAT2 siRNA (10 nM, 48 h)-induced suppression of NFAT2 protein (A), PAR-3 mRNA (B), and PAR-3 protein expression (C) in human VSMC. Time-dependent suppression of PAR-3 mRNA (D) and PAR-3 protein (E) by cyclosporine A (CsA, 10 μM) in human VSMCs. All, n = 5 to 6; $*$, $P < 0.05$ versus control.

tion, and venous grafts (Misquitta et al., 2001) may augment the local actions of thrombin through stabilization of PAR-3, highlighting the potential importance of this regulated receptor in vascular pathology.

HuR is inhibited by cAMP (Klöss et al., 2004). In this study, forskolin attenuated cytosolic HuR shuttling, destabilized PAR-3 mRNA, and induced a time-dependent suppression of PAR-3 mRNA and total protein expression. Other agents elevating intracellular cAMP, including IBMX, db-cAMP, and the prostacyclin analog iloprost also suppressed PAR-3 mRNA expression. Iloprost moreover reduced the binding of HuR to PAR-3 mRNA. Thus, cAMP could represent an endogenous switch regulating the expression of the PAR-3 thrombin receptor.

cAMP activates a number of effectors, including EPAC and PKA. A direct activator of EPAC did not influence PAR-3 expression. By contrast, inhibition of PKA with the selective inhibitor myristoyl-PKI completely abolished the ability of iloprost to suppress PAR-3 mRNA and protein expression. PKA may thus control PAR-3 first by regulating its expression and second through its recently reported ability to induce desensitization of calcium signaling (Porter et al., 2006).

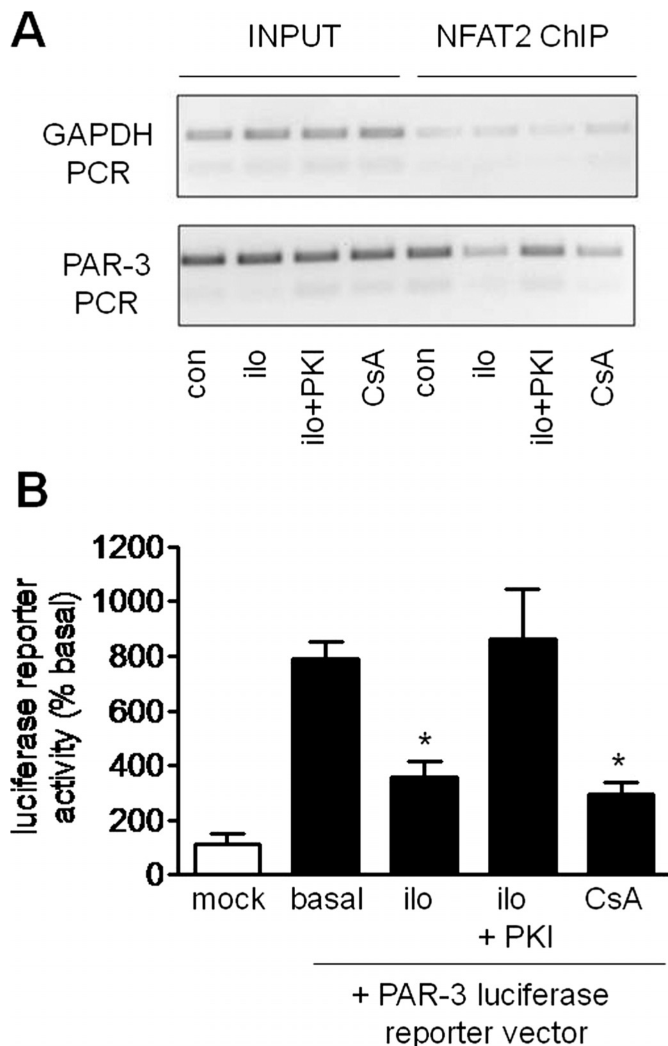

Fig. 6. Transcriptional control of PAR-3 by iloprost via inhibition of NFAT2/PAR-3 promoter binding. A, ChIP assay showing NFAT2/PAR-3 promoter binding in VSMCs stimulated for 2 h with iloprost (ilo, 10 nM) ± the PKA inhibitor PKI (5 μM) or CsA (10 μM). Representative of n = 3. B, PAR-3 luciferase reporter assay in human VSMCs stimulated with iloprost (24 h) ± PKI or CsA, n = 5, $P < 0.05$ versus basal.

In atherosclerosis, proliferating VSMCs themselves generate substantial amounts of PGI_2 (Belton et al., 2000). Expression of COX-2 and generation of endogenous prostacyclin can be induced in VSMCs in a protein kinase C-dependent manner with the phorbol ester PMA (Debey et al., 2003; Rabausch et al., 2005). Exposure of human VSMCs to PMA suppressed PAR-3 mRNA and total protein expression, an effect completely prevented by the COX-2-selective inhibitor etoricoxib. These observations suggest that endogenously generated PGI_2, after COX-2 induction, is sufficient to regulate PAR-3 in an autocrine manner. Other agonists acting at G_s-coupled prostanoid receptors, such as the IP agonist cicaprost and the EP_2 agonist butaprost, as well as exogenous PGE_2, mimicked PAR-3-regulatory effects of iloprost and PMA. This suggests a general action of vasodilatory prostaglandins acting at G_s-coupled receptors in controlling PAR-3 expression and hence the vascular response to thrombin. Perhaps this could explain in part the low patency of venous bypass grafts, which compared with arterial vessels possess impaired capacity to generate PGI_2 (Subramanian et al., 1986).

A significant suppression of PAR-3 mRNA expression by forskolin was still evident at 24 h, although the destabilizing effects of forskolin on PAR-3 mRNA and its inhibitory action on HuR shuttling were normalized within 6 h. Additional cAMP-elicited events are therefore likely to contribute to the regulation of PAR-3. Sequence analysis of the human PAR-3 promoter identified two binding motifs for NFAT, a transcription factor known to be regulated by cAMP/PKA (Sheridan et al., 2002). The NFAT2 isoform is of particular interest, being the predominant isoform modulating VSMC migration, differentiation, and proliferation (Yellaturu et al., 2002). NFAT2 siRNA attenuated PAR-3 mRNA and protein expression in human VSMC, as did the NFAT inhibitor CsA. We demonstrated previously that NFAT2 is inhibited by iloprost, acting via PKA (Rosenkranz et al., 2009). In the present study iloprost attenuated NFAT2 binding to the PAR-3 promoter and suppressed PAR-3 luciferase reporter assay to a similar degree as the positive control CsA. These observations identify NFAT2 as a key factor controlling thrombin receptor expression in human VSMC, which may contribute to the beneficial antiproliferative actions of NFAT inhibitors in vascular injury models in vivo (Yu et al., 2006).

Together, our findings highlight an important contribution of cAMP and its effectors PKA, HuR, and NFAT2 to the control of PAR-3 expression in human VSMC. But what is the purpose of this regulation? PAR activation is irreversible, and the magnitude and kinetics of the thrombin response are controlled primarily through classic desensitization pathways. The current concept, at least for PAR-1, is that each activated receptor signals, generates a defined amount of second messenger, and then shuts off. This involves phosphorylation- and arrestin-mediated pathways leading to internalization and lysosomal sorting. A renewed responsiveness to thrombin therefore requires de novo synthesis of receptors, regulated at the level of transcription and mRNA stability (Vouret-Craviari et al., 1995; Coughlin, 2000; Trejo, 2003). Perhaps PAR-3, which unlike PAR-1 is dynamically regulated by thrombin but can modulate responses mediated through PAR-1, serves to fine-tune the net thrombin re-

sponse. Upon activation, both PAR-1 and PAR-3 are likely to be shut off from signaling. Within hours, enhanced NFAT2-mediated transcription and HuR-mediated mRNA stabilization of PAR-3 will replace internalized receptors to enable renewed responsiveness to thrombin. Secondary events such as induction of COX-2 and subsequent generation of endogenous prostacyclin will probably counteract the positive regulation of PAR-3 by inhibiting NFAT and HuR. The balance of these processes will ultimately control the net effects of thrombin in the vessel.

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Authorship Contributions

Participated in research design: Rosenkranz, Rauch, Doller, and Schrör.

Conducted experiments: Rosenkranz, Rauch, Böhm, and Bretschneider.

Contributed new reagents or analytic tools: Doller, Eberhardt, and Bretschneider.

Performed data analysis: Rosenkranz and Böhm.

Wrote or contributed to the writing of the manuscript: Rosenkranz, Rauch, Doller, Eberhardt, Böhm, Bretschneider, and Schrör.

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