

Thrombin receptors in vascular smooth muscle cells – function and regulation by vasodilatory prostaglandins

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Summary

The vast majority of thrombin (>95%) is generated after clotting is completed, suggesting that thrombin formation serves purposes beyond coagulation, such as tissue repair after vessel injury. Two types of vascular thrombin binding sites exist: protease-activated receptors (PARs) and thrombomodulin (TM). Their expression is low in contractile vascular smooth muscle cells (SMC), the dominating subendothelial cell population, but becomes markedly up-regulated upon injury. In human SMC, PAR-1, PAR-3, and PAR-4 mediate thrombin-induced proliferation, migration and matrix biosynthesis as well as generation of inflammatory and growth-promoting mediators. Thrombin-responsive PARs are transcriptionally down-regulated in human vascular SMC by vasodilatory prostaglandins (PGI₂/PGE₂). For PAR-1 and PAR-3 this mechanism involves cAMP-dependent inactivation of the transcription factor NFAT. The human PAR-4 promoter does not possess NFAT recognition motifs suggesting involvement of other cAMP-regulated effec-

tors. Unlike PARs, TM is induced in SMC exposed to vasodilatory prostaglandins. Enhanced thrombin binding to TM might ameliorate PAR-mediated SMC stimulation. Also expressed in human SMC is the endothelial protein C receptor (EPCR), which serves as an anchor to facilitate generation of activated protein C (aPC) by TM-bound thrombin. Whether prostaglandins affect aPC-generation is not known. In SMC, thrombin and aPC act synergistically via PAR-1 to modify tissue remodelling, in contrast to their antagonistic interaction in the coagulation pathways. Overall, this will contribute to plaque stability and wound healing. The processes outlined here are likely to become clinically relevant after up-regulation of vascular cyclooxygenase2, the rate limiting step in vascular PGE₂/PGI₂ biosynthesis, such as in advanced atherosclerosis and acute coronary syndromes.

Keywords

Prostaglandins, thrombin, thrombomodulin, PAR, smooth muscle cell

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Introduction

Generation of thrombin from its zymogen prothrombin is the critical step of the clotting process, culminating in the generation of a platelet/fibrin thrombus and its fixation to the injured area of the vessel wall. Thrombus formation indicates that bleeding has been successfully stopped by the concerted actions of clotting factors, which incidentally are the same in both physiological and pathological haemostasis (thrombosis). However, in both situations, plug formation is not the true end of the haemostatic process. Vessel injury, specifically the local loss of endothelium and other structural elements required for sealing the vessel against blood loss, still persists. This damage must be repaired to allow for restoration of blood flow and recovery of antithrombotic/vasodilator properties of the vessel lining, in the optimal case, a *restitutio ad integrum*. This review discusses recent finding which shed

further light on how these events are regulated, namely the transcriptional control of thrombin receptor expression by the vasodilatory prostaglandins prostacyclin (PGI₂) and PGE₂.

Thrombin and the clotting process

Endothelial injury is associated with loss of barrier function and penetration of clotting factors into the subendothelium. This allows direct contact of tissue-factor bearing cells, such as subendothelial smooth muscle cells (SMC) and fibroblasts, with the thrombus and components of the streaming blood. The immediate consequence is the activation of the coagulation cascade. In this context, it should be noted that only a small proportion of thrombin, about 0.2% of total, is actually generated during the initiation

phase of the clotting process. The overwhelming component of thrombus-associated thrombin, more than 95%, is formed after clotting is complete and is continuously released by the mural thrombus (1). Human thrombi obtained at autopsy or surgery still contained an abundance of active thrombin (2). These findings suggest that the vast majority of thrombin generated during thrombus formation serves other purposes beyond clotting, and that high local levels of thrombin are the consequence, rather than the cause, of thrombus formation. The purpose is likely to be clot-related tissue repair in the vicinity of the thrombus. In other words, the platelet/fibrin clot, generated as a first step of haemostasis, stimulates in a second step the subsequent repair processes in the vessel wall.

The vascular smooth muscle cell (SMC) as a source and target of thrombin

The levels of circulating thrombin are controlled by soluble thrombin-binding factors in plasma, specifically antithrombin and heparin cofactor II (3, 4). After endothelial injury and thrombus formation, thrombin derived from the mural thrombus and/or circulating blood comes into contact with subendothelial SMC. These not only express prothrombin (5) but also tissue factor (TF),

the rate-limiting step of thrombin formation (6). SMC thus represent a potent source of thrombin formation and can generate large amounts of thrombin within a few minutes (7). Similar results have been reported in injured coronary arteries after transluminal angioplasty *in vivo* (8). Moreover, thrombin bound to the sub-endothelial extracellular matrix remains functionally active, localized and protected from inactivation by circulating inhibitors (9), thus surrounding cells are likely to be continuously exposed to high levels of thrombin.

Thrombin binding sites (receptors) in SMC

Protease-activated receptors (PARs)

The extent and intensity of cellular thrombin responses is determined by the number, affinity and activation mode of protease activated receptors (PARs) (10–12). All subtypes of thrombin-responsive PARs, specifically PAR-1, PAR-3 and PAR-4, are present and functionally active in human SMC (13, 14). These receptors differ in their affinities for thrombin, with EC_{50} values being lowest (0.05 nM) for PAR-1, followed by PAR-3 (0.2 nM) and PAR-4 (5 nM) (15). In normal arteries, thrombin receptor expression is

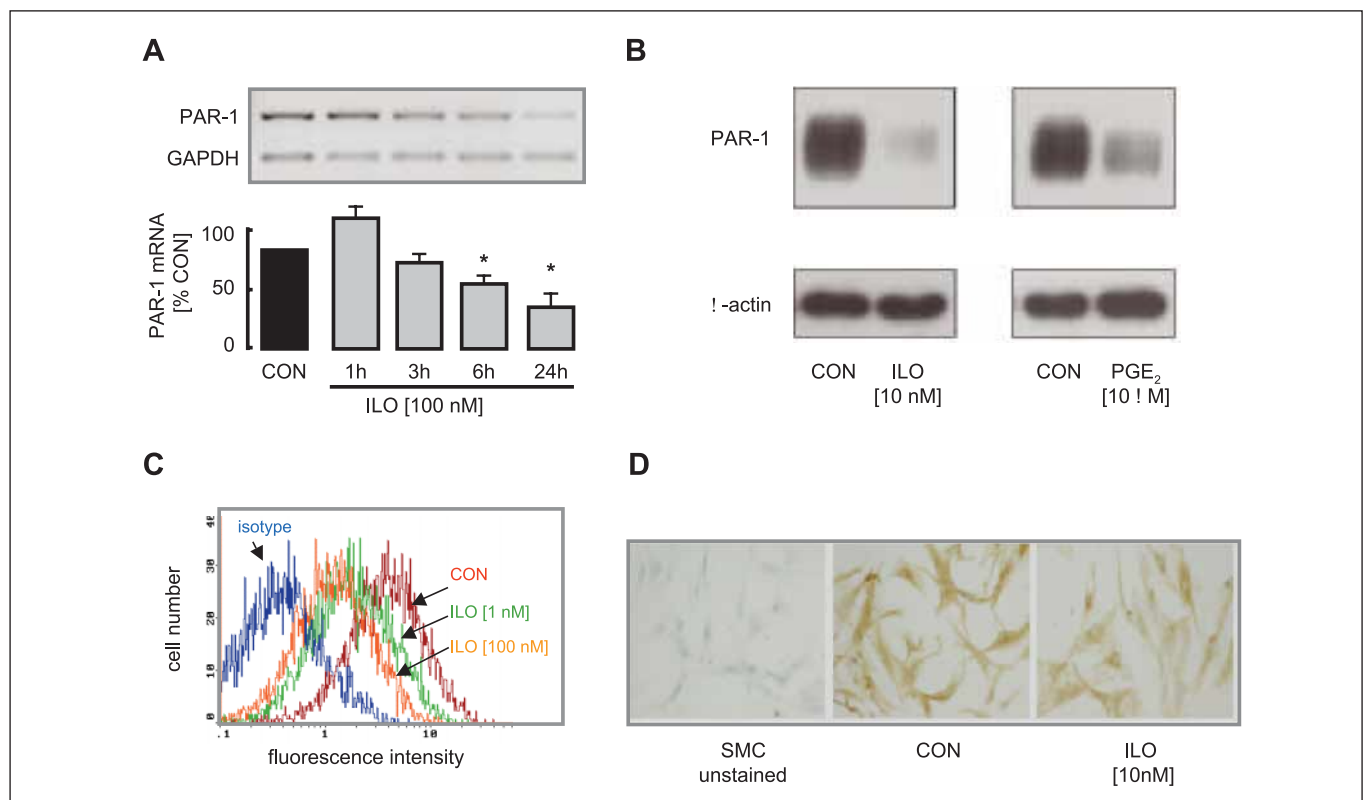


Figure 1: Transcriptional down-regulation of PAR-1 mRNA (A), protein (B) and PAR-1 receptor expression at the SMC surface (flow cytometry) (C) by iloprost (ILO). D) Inhibition of PAR-1 expression in SMC by ILO (immunohistochemistry). CON refers to constitutive PAR-1 expression in untreated samples. A) Time-dependency of ILO-induced PAR-1 down-regulation. B-D) Obtained after 24 h incubation with ILO or PGE₂. *; $p < 0.05$ (treatment vs. CON). Modified after (31).

mainly restricted to the endothelial layer. However, significant expression is found in atherosclerotic plaques, including areas which are rich in SMC (16). Moreover, thrombin receptor mRNA in SMC dramatically increases within hours of vessel injury (17, 18). Interestingly, thrombin does not appear to exert feedback control of its prototypic receptor subtype PAR1 in human SMC, while PAR-3 and PAR-4, which signal independently of PAR-1 in human SMC, are by contrast dynamically regulated upon exposure to thrombin ([14] and [Bretschneider et al, unpublished]). Such observations indicate a role for vascular PAR-3 and PAR-4 in the response to injury, which has to date been largely neglected.

Thrombomodulin (TM)

In addition to PARs, thrombin also binds to thrombomodulin (TM), another high-affinity binding site (K_D 0.5 nM) (3). Binding of thrombin to TM results in the loss of its procoagulant activities and conversion into anticoagulant properties by subsequent activation of the protein C/S pathway (3). TM, like the PARs, is mainly localised in the endothelium of healthy vessels, with negligible levels detected in the smooth muscle layer (19). After endothelial denudation or damage however, TM production by SMC is rapidly up-regulated (20). Moreover, TM generation is observed in cultured SMC, which resemble the secretory SMC phenotype *in vivo* (21). In contrast to endothelial TM, SMC-derived TM is increased rather than reduced by inflammatory cytokines (22) and thrombin (23). Induction of TM expression is possibly secondary to the up-regulation of cyclooxygenase (COX)-2 (24) and the subsequent increase in local prostaglandin formation (see below). Importantly, overexpression of TM on SMC reduces the proliferative response to thrombin and the thrombin receptor-activating peptide (TRAP). This effect is directly correlated with the TM-level at the cell surface (25). However, the precise role of TM expression on SMC and its function as a modulator of cellular effects of thrombin is still largely unknown.

Regulation of thrombin receptor expression by vasodilatory prostaglandins

Overall, these data suggest that SMC in the vascular subendothelium are both source and target of thrombin, which acts as a local injury-induced stimulus for SMC migration and proliferation, generation of cytokines and growth factors, and the production of extracellular matrix. These multiple and complex processes need to be tightly controlled and focussed to the area of vessel injury and/or atherosclerotic SMC transformations. Our recent studies define a new aspect of how this control mechanism works: the tight regulation of PAR- and TM-gene transcription by vasodilatory prostaglandins, specifically by PGI₂. This hypothesis is supported by the following arguments: (i) PGI₂, the main "antithrombotic" prostaglandin, is a functional antagonist of the prothrombotic ac-

tions of thrombin in platelets and of plasmatic coagulation via stimulation of the endothelial thrombomodulin/aPC pathway (26), in both instances acting via cAMP; (ii) PGI₂ formation is minimal in medial SMC due to their low COX-activity and hence low endogenous prostaglandin production (27), but this increases markedly in injured vessel walls and in cultured SMC (28) mimicking the secretory phenotype of SMC in vascular lesions (21, 29); (iii) vasodilatory prostaglandins, in particular PGI₂, are potent inhibitors of SMC proliferation, migration and matrix synthesis (30). Recent evidence supporting control of these processes at the level of thrombin receptor transcription is outlined below.

Transcriptional down-regulation of PAR-expression

We recently showed for the first time that vasodilatory prostaglandins such as PGI₂ and PGE₂, or the stable mimetics iloprost and cicaprost, transcriptionally down-regulate expression of the prototypic thrombin receptor PAR-1 in cultured human vascular SMC (31, 32). This regulatory effect is concentration- and time-dependent. It becomes significant at about 6 hours (h) and is translated into reduced expression of PAR-1 protein in both SMC homogenates and at the surface of intact SMC as shown by flow cytometry (► Fig. 1). Similar effects are seen with PAR-3 (33) and PAR-4 (unpublished data, not shown). Immunohistochemistry of these cells confirmed PAR-1-positive staining for SMC which was largely reduced after 24 h treatment with iloprost, a synthetic prostacyclin mimetic. Stimulation with iloprost for 1 h did not influence PAR-1 expression (not shown). This observation is at odds with a report in human lung fibroblasts, where PAR-1 appears to mediate a negative feedback control of its own expression via generation of PGE₂ (34). In human vascular SMC, we also found suppression of PAR mRNA and protein expression after stimulation of cAMP formation by forskolin, inhibition of its degradation by IBMX or with the stable cAMP-mimetic dbcAMP (31). Importantly, the protein kinase A inhibitor myristoyl-PKI completely prevented all of these actions of iloprost, confirming the critical involvement of cAMP-dependent signalling in the transcriptional control of PAR-1 (31). These data collectively suggest cAMP as the second messenger and PKA as the cellular target that mediates transcriptional down-regulation of PAR-1 by vasodilatory prostaglandins.

The role of NFAT

When we sought to identify possible downstream targets of PKA we looked for a protein which is related to cellular Ca⁺⁺-homeostasis and which is inactivated by phosphorylation. These requirements are exactly true for the nuclear factor of activated T-cells (NFAT). NFAT becomes activated by the phosphatase calcineurin while PKA-dependent phosphorylation promotes inactivation and nuclear export (35). Moreover, NFAT inhibition limits the proliferative response to vascular injury in experimental models (36). Interestingly,

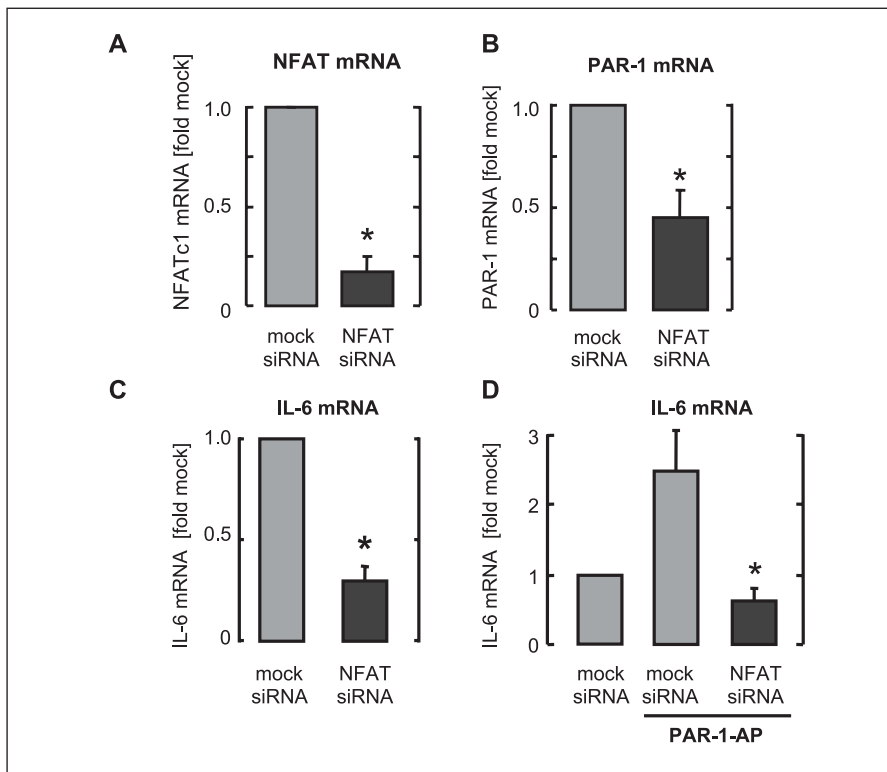


Figure 2: Nuclear factor of activated T-cells (NFAT)c1 siRNA (10 nM, 48 h) suppresses NFATc1 mRNA (A) and PAR-1 mRNA (B) expression. This is associated with significantly reduced protein expression (not shown) and down-regulation of interleukin-6 (IL-6) mRNA in both resting conditions (C) and after stimulation by PAR-1-AP (TFLLRN, 200 μ M, 3 h). *: $p < 0.05$ (NFAT siRNA vs. mock siRNA). Modified after (32).

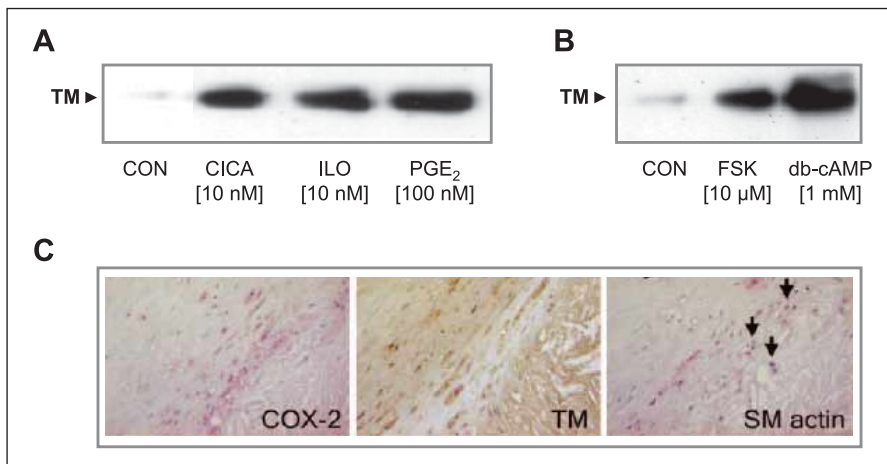


Figure 3: Up-regulation of thrombomodulin (TM) protein expression in human SMC by cicaprost (CICA), iloprost (ILO) and PGE₂ (A). Similar effects are obtained after stimulation of cAMP formation by forskolin (FSK) or the cAMP mimetic dibutyryl cAMP (db-cAMP) (B). Immunostaining of carotid atherectomy specimens shows positive staining for COX-2 and TM predominantly in areas of SMC but not macrophages (arrows) as seen from actin staining (C). Modified after (24).

the human PAR-1 promoter contains two putative NFAT-binding sites, one of these being adjacent to a consensus sequence for the transcription factor AP-1 with which NFAT often acts in tandem (37). We found that deletion of this NFAT binding sequence by site-directed mutagenesis suppresses PAR-1 promoter activity in a luciferase reporter assay, while siRNA against NFATc1 – the predominant NFAT isoform in vascular SMC – reduces PAR-1 mRNA and protein expression. Accordingly, the stimulatory effects of thrombin or PAR-1 activating peptide on SMC proliferation (not shown) and proinflammatory signalling, here induction of IL-6 (► Fig. 2), are abolished by pretreatment with NFAT1c siRNA. Similar loss of PAR-1 expression and functional responsiveness was seen with cyclosporine A, an inhibitor of calcineurin-dependent NFAT activation.

Thus NFAT, in particular the NFATc1 isoform, appears to be crucial for transcriptional control of PAR-1 (32).

Transcriptional upregulation of thrombomodulin-expression

The observation that vasodilatory prostaglandins negatively regulate expression of PARs raises the question whether they may also affect the transcription and expression of a further thrombin receptor, TM. This is indeed the case. However, in contrast to PARs, which are downregulated in human vascular SMC exposed to PGI₂ and PGE₂,

transcription of TM is up-regulated, as are TM protein expression and function (24) (► Fig. 3). This directional antagonism at the level of thrombin receptor regulation – suppression of PARs on the one hand, induction of TM on the other – is however likely to result in a net synergy of function. Because the affinities of thrombin for its targets TM and PARs are comparable, up-regulation of TM will effectively reduce the available amount of thrombin able to bind to PARs. Accordingly, TM-bound thrombin inhibits PAR-1-mediated proliferation of SMC. Potentially independent actions of TM in response to thrombin also contribute to the net antiproliferative effects of this non-PAR thrombin receptor (38). In agreement with this concept, thrombin-induced SMC proliferation in pig carotid artery is inhibited by recombinant TM (39). Similar results are obtained in rabbits after SMC-specific overexpression of TM (25). Although these findings could not be confirmed in other studies (40), the vast majority of available data suggests that up-regulation of TM in SMC will inhibit vascular proliferation.

The role of activated protein C (aPC)

There is yet a further aspect of TM/thrombin interactions: the binding of thrombin to TM will markedly (20,000-fold) enhance the activation of protein C (PC) (3), provided that the endothelial protein C receptor (EPCR) is present as an anchor. Activated PC (aPC) cleaves the endothelial PAR-1 receptor in the same way as thrombin, but as this effects requires much higher concentrations it is generally considered not to be physiologically relevant (41). In SMC we recently demonstrated for the first time the presence of a functionally active EPCR which is involved in aPC signalling (42). In SMC, aPC possesses independent mitogenic activities which are most likely mediated through PAR-1 and G_i activation (42). This is in keeping with reports that pertussis toxin-sensitive G_i and the downstream inhibition of adenylate cyclase promotes SMC proliferation (43). It is not known at present whether PGI_2 is able to up-regulate EPCR-expression and how EPCR exactly interacts with PAR-1 stimulation by thrombin.

Endogenous prostaglandins and control of cellular thrombin actions – the *in vivo* relevance

The beneficial effects of a synthetic PAR-1 antagonist in thrombus-related vascular stenosis (44) highlight the central importance of thrombin receptors in the proliferative and thrombotic responses to vascular injury. Endogenous factors which modify the expression and function of these receptors, such as the vasodilatory prostaglandins, therefore represent important components of a protective “braking” mechanism to limit the detrimental effects of thrombin.

Vessel injury and atherosclerosis are associated with not only increased TF levels, thrombin formation and PAR-expression (6),

but also with induction of COX-2 in SMC (45). Mechanistically, PGI_2 and phorbol ester up-regulate COX-2 and prostaglandin production via the cAMP-response element (CRE) and/or protein kinase C in the COX-2 promoter region (46). Immunohistochemistry of atherosclerotic plaque specimens of the human aorta also confirmed the expression of TM and COX-2 in SMC (► Fig. 3). Thus there is good evidence that our observations from cultured SMC can be extrapolated to the *in vivo* situation. A schematic overview of this hypothesis and the possible intracellular signalling pathways are shown in ► Figure 4.

Clearly vasodilatory prostaglandins represent a major control mechanism for vascular thrombin receptor expression, and this is likely to also be the case in clinical settings of atherosclerosis where generation of COX-2-derived prostaglandins by SMC is increased. Yet PAR-1 expression in particular is also high in atherosclerotic and damaged vessels. How can this apparent discrepancy be explained? Potentially the expression and atherothrombotic actions of PARs would be even higher if the endogenous inhibition by locally generated prostaglandins were absent. In support of this, selective COX-2 inhibitors which antagonise or prevent COX-2-dependent vascular

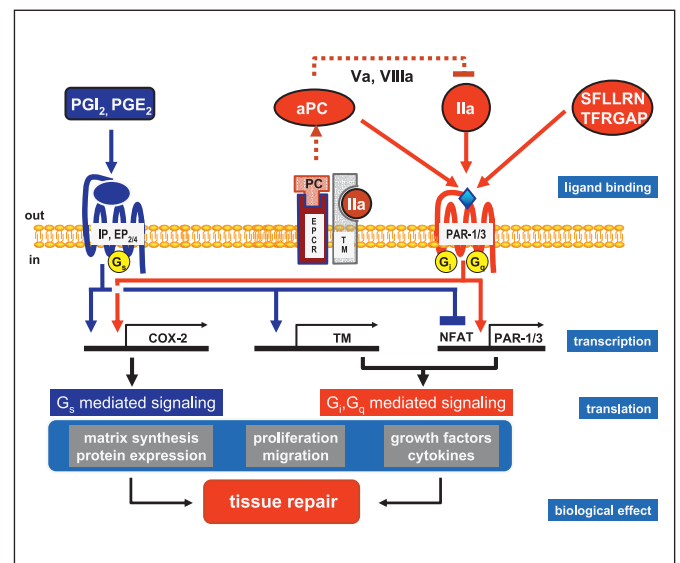


Figure 4: Transcriptional control of PAR and thrombomodulin (TM) expression in vascular smooth muscle cells by vasodilatory prostaglandins (PGI_2 , PGE_2). Thrombin (IIa) activates PAR-1 and PAR-3 by cleavage of the receptor and generation of a tethered ligand (♦). PAR1/3-activating peptides (S(T)FLLRN/TRFGAP) bind to these PARs and activated protein C (aPC) to PAR1, thereby stimulating G_q - and G_{12} -mediated signalling. aPC is generated after binding of thrombin to thrombomodulin (TM) and the endothelial protein C receptor (EPCR) and in addition to cellular effects also inhibits thrombin formation via inactivation of factor Va and VIIIa. PGI_2 transcriptionally down-regulates PAR-1 and PAR-3 expression via NFATc1 and up-regulates TM, promoting enhanced aPC activity. Both PGI_2 (G_s) and thrombin (G_q ?) up-regulate COX-2 and subsequent PGI_2 production. PGI_2 has opposing effects on PARs and TM: down-regulation of PARs and up-regulation of TM. In case of constitutive EPCR expression, these interactions allow for coordinated cell proliferation, cytokine production, matrix biosynthesis and tissue repair (further explanations see text).

PGE₂/PGI₂-production are strongly associated with an elevated incidence of thrombotic events, specifically in patients with existing atherothrombotic risk. Moreover, saphenous vein bypass grafts which are characterised by low endogenous capacity to generate PGI₂ (47) also exhibit reduced graft patency compared to arterial vessels (48). Removal of prostaglandins could conceivably reduce transcriptional control of PAR or TM expression in damaged vessels and thereby facilitate atherogenic and thrombotic processes. Part of this might be attributable to disinhibition of NFATc1-related mechanisms by cAMP downstream of prostanoid receptor activation. Accordingly, recent evidence suggests that NFAT might serve as a useful therapeutic target for the prevention of restenosis after vascular injury *in vivo* (49).

Overall, we are only just beginning to understand the finely tuned control of the local atherothrombotic, proliferative and inflammatory processes that occur after vascular injury. From a teleological point of view, transcriptional control of inducible genes such as PARs or TM, appears to be a more effective and specific approach than merely inhibiting activity. Future studies will tell whether these concepts also transfer into improved prevention and treatment of atherothrombosis.

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