Review

Regulation of endothelial prostacyclin synthesis by Protease-activated receptors: mechanisms and significance

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Abstract:

The cellular actions of serine proteases are mediated through activation of a novel family of four G protein-coupled receptors known as protease-activated receptors (PARs). PARs are emerging as important modulators of diverse biological functions and there is evidence supporting roles for these receptors in both physiological and pathological settings in the cardiovascular system. Endothelial cells express all four known PARs but their specific roles as modulators of endothelial cell function are not well understood. One physiologically important response of the endothelium to PAR stimulation is the generation of prostacyclin (PGI_2) through cyclooxygenase (COX)-dependent pathways. Our studies have used selective PAR-activating peptides, endogenous PAR agonists, and pharmacological and molecular approaches to identify the mechanisms coupling PARs activation with endothelial PGI_2 synthesis and release. These mechanisms are differentially recruited by individual PARs but activation of the ERK1/2 and p38 families of mitogen-activated protein kinases (MAPK), as well as the nuclear factor kappa-B (NF-κB) pathway, play significant roles in controlling PAR-induced prostanoid formation through regulation of COX-2 induction and cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) activation. PAR agonists also modulate PAR expression by mechanisms that require p38^{mapk} as well as NF-κB. The defensive actions of PGI_2 in the vascular wall are well-established, and the ability of PARs to drive acute and chronic synthesis of this mediator suggests a potential role for these receptors in vascular protection. Our findings therefore have important implications for defining the vascular effects of current and future therapeutic agents that target COXs, PARs, and the signalling elements controlling their expression.

Key words:
endothelial cell, prostacyclin, protease-activated receptors, cyclooxygenases

Abbreviations: 6-keto-$PGF_{1\alpha}$ – 6-keto-prostaglandin $F_{1\alpha}$, AA – arachidonic acid; COX-1/-2 – cyclooxygenase-1/-2, cPLA$_2\alpha$ – cytosolic phospholipase $A_2\alpha$, ERK1/2 – extracellular signal-regulated kinase1/2, HUVEC – human umbilical vein endothelial cells, IκBα–inhibitory protein kappa Bα, IKK – IκBα kinase, IL-1α – Interleukin-1α, IP – receptor for prostacyclin, JNK – c-Jun NH$_2$-terminal kinase, LPS – lipopolysaccharide, MAPK – mitogen-activated protein kinase, MEK – mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, NF-κB – nuclear factor kappa B, PAR – protease-activated receptor, p38^{mapk} – p38 MAPK, PG – prostaglandin, PGI_2 – prostaglandin I_2 (prostacyclin), PGIS – prostacyclin synthase, TxA_2 – thromboxane A_2, TNF-α – tumor necrosis factor-α, VSMC – vascular smooth muscle cell
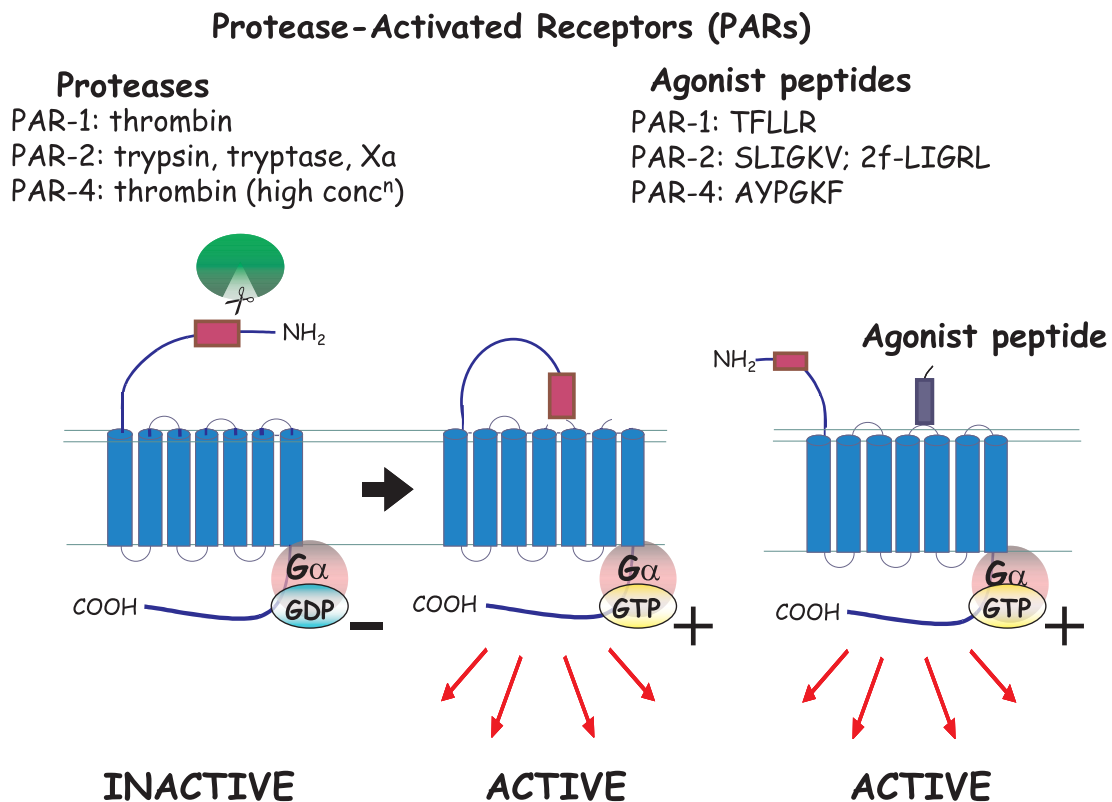
Introduction

Prostacyclin (PGI_2) generated through the sequential activities of cyclooxygenases (COX) and prostacyclin synthase (PGIS), is a strongly vasculoprotective prostanoid and is the predominant prostaglandin (PG) produced by endothelial cells. The central role of PGI_2 as a cardiovascular protectant is highlighted by its ability to inhibit platelet and lymphocyte adhesion to endothelium, promote vasodilatation, limit vascular

smooth muscle cell (VSMC) proliferation and migration, and counteract the production of pro-fibrotic growth factors. This mediator acts to oppose the effects of thromboxane A_2 (TxA_2), a potent vasoconstrictor released principally by platelets which is prothrombotic and promotes platelet activation, VSMC proliferation and endothelial cell apoptosis. This article examines the involvement of a unique family of G protein-coupled receptors (protease-activated receptors – PARs) in the control of acute and sustained endothelial PGI_2 synthesis, and explores the underlying cellular mechanisms.

Protease-activated receptors

Protease-activated receptors (PARs) are a novel family of seven transmembrane domain G protein-coupled receptors comprising PAR-1, PAR-2, PAR-3 and PAR-4. These receptors are activated by a broad range of serine proteases through a unique mechanism where cleavage of the PAR within its extracellular N-terminus generates a specific tethered ligand sequence that subsequently binds to the extracellular receptor domains and effects transmembrane signalling (see [7, 18, 32]) (Fig.1). Thrombin, the main effector protease of the coagulation pathway, has important actions on endothelial cells, stimulating von Willebrand factor secretion, prostanoid synthesis, adhesion molecule expression and increased proliferation [18–21, 50, 51]. Acute and/or prolonged exposure of the endothelium to elevated levels of thrombin and indeed other proteases is likely to be a feature of both normal and pathophysiology. Other proteases, particularly trypsin, are expressed by cultured endothelial cells and by endothelial cells of patients with coagulation disorders and cancer providing the potential for an additional signalling role for these enzymes [26]. Hence,

Protease-Activated Receptors (PARs)

Proteases
PAR-1: thrombin
PAR-2: trypsin, tryptase, Xa
PAR-4: thrombin (high conc^n)

Agonist peptides
PAR-1: TFLLR
PAR-2: SLIGKV; 2f-LIGRL
PAR-4: AYPGKF

Fig. 1. Mechanism of PAR activation by cleavage. Serine proteases cleave the extracellular domain of PARs, unmasking a new amino-terminal domain that acts as a tethered ligand and activates the receptor to effect transmembrane signalling and regulate functional outcomes. Agonist peptides that mimic the tethered ligand sequences activate the receptors in the absence of proteolysis. The activation state of PARs will depend upon the pathophysiological environment, the level of receptor expression and the range of activating protease ligands and their inhibitors present. PAR-3 (not shown) is thought to act as a co-factor for PAR-4 (see [32])
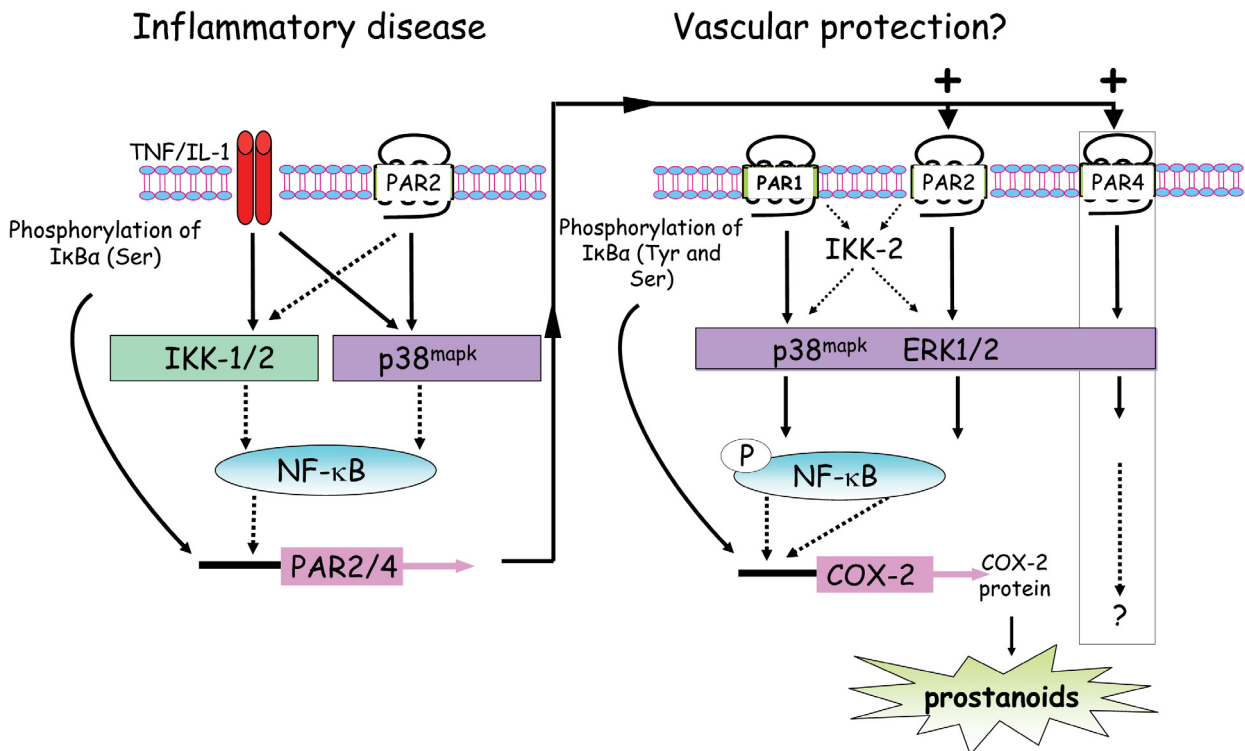
Fig. 2. Molecular regulation of PAR-mediated PGI_2 synthesis in endothelial cells. In unperturbed endothelial cells PAR-1 and PAR-2 utilise several signalling pathways, including MAPKs and NF-κB, to regulate COX-2 expression and prolonged PGI_2 synthesis. Activation of PAR-4, in contrast, does not promote PGI_2 formation but is capable of activating ERK1/2 and p38^{mapk}. During inflammation the local production of pro-inflammatory cytokines would trigger activation of these signalling elements to increase expression of PAR-2 (and PAR-4). This should then allow enhanced coupling of PAR-2 to COX-2 induction and efficient generation of vasculoprotective PGI_2

understanding the molecular mechanisms of protease-mediated endothelial cell activation will have consequences for the development of novel therapeutic strategies to influence the initiation and progression of cardiovascular and other diseases. Thrombin exerts its cellular effects in a number of systems principally through proteolytic activation of PAR-1, with a potential contribution from the low affinity thrombin receptor PAR-4 at high thrombin concentrations (see [32]). In endothelial cells thrombin is a recognised physiological activator of PAR-1 and indirect activation of PAR-2 by thrombin-cleaved PAR-1 may also be required for some responses [43].

In contrast, the endogenous proteases responsible for activating endothelial PAR-2 have not been definitively identified. It is known, however, that PAR-2 is not directly cleaved by thrombin, but instead is activated, at least *in vitro*, by a number of other serine proteases including trypsin, mast cell tryptase, the tissue factor-VIIa-Xa complex, and kallikreins [39, 43] supporting the likelihood that the relevant ligand pro-

tease(s) will vary depending upon the physiological or pathological setting. Although PAR-2 is generally associated with chronic inflammation, due in part to the recognised ability of pro-inflammatory cytokines to increase PAR-2 expression [38, 40], the high constitutive PAR-2 expression by normal unperturbed endothelial cells [40, 44] suggests that this receptor is likely to have physiological relevance.

While there are many potentially important endogenous PAR agonists, selective PAR activation both *in vitro* and *in vivo* can be achieved through the use of PAR-specific activating peptides which mimic the tethered ligand sequences generated by protease-mediated cleavage [17, 18, 41] (Fig. 2). We and others have used these tools to probe the selective involvement of PAR-1 *versus* PAR-2 in a range of endothelial cell functions. One major focus of our recent work has been to investigate the roles of endothelial PARs as regulators of PGI_2 synthesis and release, and to identify the underlying mechanisms.

Prostanoid synthesis by endothelial cells

Cleavage of arachidonic acid (AA) from the *sn*-2 position of phospholipids is the first step in the agonist-driven formation of prostanoids by endothelial cells and is catalysed by phospholipase A_2 (PLA$_2$) enzymes, principally cytosolic PLA$_2\alpha$ (cPLA$_2\alpha$). AA is converted into prostaglandin (PG) H_2 by the action of two isoforms of cyclooxygenase (COX), and a range of terminal synthases subsequently catalyse the conversion of PGH$_2$ into the various prostaglandins (see [1]).

At least two distinct forms of COX are expressed in mammalian tissues. These enzymes are the products of separate genes and evidence suggests that they subserve distinct physiological roles within the vasculature. Thus, COX-1 is constitutively expressed at low levels in most tissues and is thought to be necessary for PG production relating to normal homeostasis (see [14]). In contrast, COX-2, the product of a related gene, is not usually present in most normal tissues (although there are exceptions eg. [52]) but its expression is induced or enhanced by cytokines, tumor promoters and growth factors, its regulation occurs at both transcriptional and post-transcriptional levels, and its activity plays a central role in the production of PGs associated with the inflammatory response.

The differential expression of the terminal enzymes, some of which are also inducible (see [35]) ultimately determines the cell-specific profile of prostanoid production. Endothelial cells express high levels of PGI$_2$ synthase (PGIS) and hence generate large quantities of PGI$_2$. Our studies have shown that endothelial cells express both COX forms in the absence of inflammatory stimuli. Basal PGI$_2$ synthesis by human umbilical vein endothelial cells (HUVEC) is partially inhibited by the selective COX-2 inhibitor NS398, suggesting that both isoforms may contribute to PGI$_2$ synthesis in normal, unperturbed endothelium, at least *in vitro* [14, 19, 44].

PGI$_2$ participates in normal inflammatory responses by promoting vasodilatation and increasing vascular permeability. In addition to this role PGI$_2$ is critically important for protecting the vascular wall from pro-thrombotic and pro-atherogenic influences, and is also cardioprotective (eg. see [25]). In accordance with this, PGI$_2$ is a potent anti-platelet agent, and COX-2-derived PGI$_2$ is strongly thrombolytic in *in vivo* models of thrombolysis [15, 16]. It is also a well-established inhibitor of vascular smooth muscle cell differentiation, proliferation and migration (see [10]). PGI$_2$ limits fibrosis [12, 31] and suppression of COX-2-dependent PGI$_2$ synthesis, or deletion of the principal receptor for PGI$_2$ (IP), reduces vascular remodeling in experimental models of transplant arteriosclerosis or flow-induced remodeling [42]. Recent *in vitro* studies indicate that PGI$_2$ protects endothelial cells from oxidant-mediated apoptosis, a pro-atherosclerotic event associated with vascular injury [30], and PGIS overexpression *in vivo* limits neointimal formation after vascular injury [37]. Thus, understanding the mechanistic basis of endothelial PGI$_2$ production has important implications for both normal and pathophysiological control of the vasculature during inflammation and repair.

Mechanisms of PAR-mediated PGI$_2$ synthesis

Acute release

The molecular details of the events that couple PARs with functional outcomes in human endothelial cells are not well-defined. Our initial studies in this area utilised intact and electrically-permeabilised endothelial cells, together with a range of pharmacological tyrosine kinase inhibitors, to show that tyrosine kinases regulate rapid (10 min) thrombin- and PAR-1-peptide-stimulated PGI$_2$ synthesis by modulating a Ca^{2+}-dependent event upstream of COX activity [51]. Subsequent studies identified this step as activation of cPLA$_2\alpha$, a Ca^{2+}-sensitive member of the cPLA$_2$ enzyme family, and showed that indirect and direct kinase-mediated regulation of cPLA$_2\alpha$ phosphorylation status were key mechanisms for controlling acute PAR-mediated PGI$_2$ synthesis by human endothelial cells [20, 21, 49]. Mitogen activated protein kinases (MAPKs) are particularly important in this respect. These enzymes have been implicated in both acute and chronic responses to extracellular stimuli. Their functional relevance is highly dependent upon the cellular context, and on proximal signalling elements, but a role for MAPK signaling pathways in several aspects of endothelial cell biology is well established. A number of distinct groups of MAPKs have been identified in mammalian cells (see [47, 48]) but the

best characterised are the ERK1/2 family, and the related p38 and JNK (c-Jun NH$_2$-terminal kinases) families, also known as stress-activated protein kinases. The changes in gene expression (i.e. chronic responses) induced by activation of these pathways result from kinase-mediated phosphorylation/activation of a range of transcription factors [47]. Activation of the various MAPKs requires dual phosphorylation on tyrosine and threonine residues within the kinase activation loop. These phosphorylation events are regulated by the activities of upstream dual-specificity MAPK kinases which are in turn phosphorylated by MAPK kinase kinases. Endothelial cells express all elements of these pathways and we, and others, have shown that MAPK cascades are critical signalling components used in the physiological responses of endothelial cells to extracellular signals, including thrombin and activators of PAR-2 [13, 20, 21, 23, 24, 44]. In keeping with this, thrombin and the PAR-1-selective peptide (TFLLR) promote a robust activation of both ERK1/2 and p38^{mapk} in endothelial cells. Moreover, pharmacological inhibition of MEK, the direct upstream activator of ERK1/2, attenuates thrombin-induced PGI$_2$ synthesis, an effect mediated by blockade of ERK1/2-dependent cPLA$_2$α phosphorylation [20]. The exact roles of kinases upstream of MEK in this response are undefined [23, 24] but our data suggest that conventional protein kinase C isoforms are unlikely to be involved in rapid thrombin-stimulated PGI$_2$ formation [13]. Together, these findings emphasise the importance of ERK1/2-mediated cPLA$_2$α activation as a regulator of acute PAR-1-stimulated PGI$_2$ synthesis by endothelial cells.

Further investigation of the mechanisms mediating rapid PAR-mediated PGI$_2$ formation revealed that interplay between the MEK-ERK1/2 and p38^{mapk} pathways is also important [20], a mechanism that is now a recognized signaling paradigm operative in vascular and non-vascular cell types (e.g. [27]). In these studies blocking p38^{mapk} activation with a pharmacological inhibitor (SB203580) resulted in diminished phosphorylation and activation of MEK, ERK1/2, and cPLA$_2$α in thrombin-stimulated endothelial cells. This strongly suggests that SB203580-sensitive p38$^{mapk(s)}$ facilitate thrombin signaling through the MEK-ERK1/2 pathway to drive PGI$_2$ formation. In marked contrast, early activation of p38^{mapk} by interleukin-1α (IL-1α) serves to limit IL-1α-stimulated MEK-ERK-cPLA$_2$α activation and downstream acute PGI$_2$ production. While the failure of IL-1α to promote a rapid burst of PGI$_2$ undoubtedly relates to the inability of this cytokine to elevate intracellular Ca^{2+}, these studies nevertheless show that cross-talk between MAPKs is a critical determinant of acute PGI$_2$ formation by endothelial cells and emphasise mechanistic differences underlying regulation by PARs *versus* classic pro-inflammatory cytokines. Differential usage of signalling pathways by these agonists to regulate PGI$_2$ may have functional significance for vessel homeostasis since PAR stimulation is not associated with TxA$_2$ production whereas IL-1α induces Tx synthase and consequently produces large quantities of this mediator.

Prolonged release

It is generally accepted that thrombin promotes a rapid (min) and short-lived PGI$_2$ generation attributable to activation of cPLA$_2$α and subsequent AA metabolism through COX-1 activity (see above). In contrast, the prolonged and persistent prostanoid production caused by IL-1α in a number of vascular and non-vascular cell types results from induction of COX-2 and forms the basis for the effectiveness of COX-2 inhibitors as anti-inflammatory agents. We have shown, however, that in addition to acute release, activation of PAR-1 also promotes a sustained phase of PGI$_2$ synthesis accompanied by COX-2 induction equivalent to that of IL-1α [19, 44]. The PAR-1 tethered ligand peptide mimics the action of thrombin on COX-2 expression suggesting that thrombin acts through PAR-1 to induce COX-2. In accordance with this, non-selective and COX-2-selective inhibitors block prolonged thrombin-induced PGI$_2$ synthesis, demonstrating that sustained PGI$_2$ generation in thrombin-stimulated endothelial cells is through altered expression of COX-2 [44]. Importantly, relatively low concentrations of thrombin are capable of promoting significant COX-2 expression and PGI$_2$ release, and these concentrations are 500–1000 fold lower than those required for induction of other cytoprotective responses by thrombin in endothelial cells (eg. [28]). These results clearly show that PAR-1 activation in endothelium is coupled to increased COX-2 expression and sustained PGI$_2$ generation [5, 44].

The effects of activation of PARs, other than PAR-1, on endothelial prostanoid production have received little attention but we have shown that COX-2 expression is also induced by PAR-2-selective pep-

tides and that this occurs with kinetics distinct from induction by thrombin or PAR-1 peptide [19, 44]. In addition, examination of the kinetics of PGI_2 formation in PAR-1 *versus* PAR-2 agonist-stimulated cells revealed that in contrast to PAR-1, PAR-2 activation was not associated with acute PGI_2 release but that sustained synthesis co-incident with COX-2 induction was clearly apparent [44]. This does not seem to reflect an inability of PAR-2 agonists to increase intracellular Ca^{2+} since PAR-1 and PAR-2 activation results in equivalent Ca^{2+} elevations [6]. The mechanisms responsible for these differential PGI_2 release profiles are therefore unclear but most likely result from differences in the utilisation of signalling elements other than Ca^{2+} upstream of COX activation/expression. COX-2 induction and the associated changes in prostanoid synthesis are therefore functionally important responses of the endothelium to protease exposure acting through distinct PARs.

The signalling events that link thrombin receptors and PAR-2 with transcriptional events such as COX-2 expression are poorly understood, but the MAPKs are likely to play important roles and are associated with COX-2 induction by cytokines and growth factors in several systems. Indeed, our studies have implicated both ERK1/2 and p38^{mapk} in PAR-1- and PAR-2-mediated COX-2 expression [44]. Co-operative activation of these pathways leading to enhanced COX-2 expression could either result from activation of a common downstream kinase or from activation of distinct transcription factors leading to transcriptional effects on gene expression. One way in which MAPK signalling cascades could regulate COX-2 expression is through modification of NF-κB activation, since the human COX-2 promoter contains sites for NF-κB binding, as well as for several other transcription factors [2].

Transcriptional regulation of the COX-2 gene is tissue- and agonist-dependent, but recent studies have suggested that NF-κB activation is sufficient and essential for inflammatory gene expression in endothelial cells with other pathways exerting their effects through modulation of NF-κB activity [9]. In the absence of stimulation NF-κB is retained in the cytoplasm by association with IκBα, its inhibitory subunit. Following exposure to agonist, IκBα is serine phosphorylated which initiates its proteasomal degradation and allows translocation of NF-κB to the nucleus (see [45]). An alternative degradation-independent mechanism of NF-κB activation involves phosphorylation of IκBα on Tyrosine^{42} [4]. We examined the potential

role of NF-κB in PAR-mediated COX-2 expression. Our studies showed increased PAR-1 and PAR-mediated luciferase activity in endothelial cells infected with an NF-κB reporter adenovirus, and this, as well as PGI_2 synthesis, was inhibited by co-expression of wild-type IκBα or a mutated IκBα (IκBαY42F) suggesting involvement of degradation-dependent and -independent mechanisms mediating COX-2 expression and prostanoid generation. PAR-induced NF-κB activation was also associated with movement to the nuclear compartment as well as with its phosphorylation. In addition, COX-2 expression and PGI_2 release in PAR-1- and PAR-2-peptide-stimulated cells were attenuated in parallel by pharmacological blockade of NF-κB [44]. These findings suggest that NF-κB is a key signalling element regulating both PAR-1- and PAR-2-induced COX-2 induction. Precisely how the MAPK and NF-κB pathways interact is not known at present but the possibility exists that MAPK activation is upstream of PAR-1- but not PAR-2-mediated NF-κB activation [44] suggesting differences in the signalling pathways triggered by distinct PARs. Interestingly a PAR-4-selective peptide activates ERK1/2 and p38^{mapk} without modifying NF-κB activation or COX-2 expression providing preliminary evidence that this receptor, in contrast to PAR-2, does not participate in regulating prolonged PGI_2 generation.

The molecular mechanisms underpinning the effects of PAR-1 and PAR-2 activation on COX-2 expression are beginning to be unraveled, and these studies have demonstrated that human endothelial cells use MAPK cascades and NF-κB to functionally link PARs with COX-2 expression and sustained PGI_2 generation. The possibility that PAR-1 and PAR-2 activation promote distinct prostanoid release profiles, and the functional outcomes of this, remain to be determined.

Cellular responses to thrombin, and to other proteases, are determined not only by the absolute level of receptor expression, but also by the repertoire of PARs present on the cell surface. An additional mode of regulation of PAR signalling is therefore through control of PARs expression. Relatively little is known about how expression of PARs is regulated in the vasculature but inflammatory cytokines have been reported to increase endothelial PAR-2 expression [38] whereas prolonged exposure of HUVEC to laminar flow reduces PAR-1 expression and therefore blunts thrombin-mediated NF-κB nuclear translocation [29].

Recent studies have shown that PAR-2-specific peptides and putative PAR-2 agonists (eg. trypsin) upregulate PAR-2 and PAR-4 expression in HUVEC, and that enhanced expression is attenuated by inhibitors of p38^{mapk} but not of JNKs or the MEK-ERK1/2 pathway [40]. Stimulated PAR-2/-4 expression is also reduced by infection of endothelial cells with adenoviruses encoding IκBα, IKKα or IKKβ, suggesting that the canonical NF-κB pathway regulates PAR- and cytokine-stimulated PARs expression [40]. Thus, the extent of PARs expression appears to be regulated by some of the same mechanisms that control PAR-mediated COX-2 expression and PGI$_2$ production.

The knowledge that PGI$_2$ synthesis is dependent upon activation of MAPK and NF-κB pathways is important because components of these signalling cascades (like COX-2 itself) are targets for anti-inflammatory therapies (e.g. [45]), raising the possibility that such intervention could cause inappropriate inhibition of PGI$_2$ formation.

Physiology versus pathology

There is currently much debate over the vascular roles of COX-2 and particularly whether COX-2 expression is a feature of the 'normal' endothelium. There is now evidence for an increased risk of cardiovascular events in patients treated with COX-2-selective drugs which could, in part, be related to blockade of PGI$_2$ synthesis derived from the enhanced endothelial COX-2 expression likely associated with vascular inflammation in these individuals [14]. Whether this risk reflects an effect on endothelial COX-2- and/or COX-1-derived PGI$_2$ formation, or results from COX-independent actions of the drug(s) is not currently defined.

What is becoming clear, however, is that when COX-2 expression is enhanced in disease states, it is highly protective. For example, COX-2 deficiency in mice is associated with a dramatically decreased serum PGI$_2$ concentration and increased lipid deposition in the aorta [36]. Moreover, treatment with a COX-2-selective inhibitor (rofecoxib) resulted in accumulation of pro-inflammatory high density lipoprotein in wild type mice, suggesting an anti-atherogenic role for COX-2 [36]. COX-2 expression is also induced in the coronary arterioles of individuals with diabetes and this is coupled with increased

PG-mediated vasodilation which may ultimately assist in maintaining cardiac perfusion [46]. These and other studies therefore suggest that dysfunctional endothelial cells exhibit increased COX-2 expression as part of the inflammatory response but this in itself allows compensatory production of PGI$_2$, and probably other PGs, to maintain vessel patency and offset thrombosis, arteriosclerosis and vascular remodelling.

Sections of human atherosclerotic lesions show increased endothelial PAR-1/-2 expression [19, 32] as well as enhanced COX-2 expression [3] suggesting upregulation of PAR-COX-2 during atheroma progression. Indeed, in common with COX-2, expression of PAR-2 is induced on endothelial cells by a number of inflammatory mediators (eg. TNF-α, IL-1, LPS) and activation of PAR-2 has been implicated in pro-inflammatory immune responses including cytokine release by neutrophils and monocytes [34]. It has therefore been assumed that activation of this receptor is only functionally relevant during inflammation but there is now evidence that PAR-2 activation can trigger protective, anti-inflammatory responses [11], as well as contributing to normal inflammation. Our demonstration that human endothelial cells respond to PAR-2 activation with increased PGI$_2$ synthesis without the requirement for cytokine exposure [19, 44] is consistent with a physiological role for PAR-2 in the vasculature.

PARs activation on endothelial cells is also accompanied by increases in responses that are considered pro-thrombotic. For example, activation of either PAR-1 or PAR-2 causes exocytosis of von Willebrand factor-containing Weibel-Palade bodies together with surface expression of P-selectin, events which participate respectively in platelet adhesion to, and leukocyte rolling on endothelium. PAR-2 agonists have been reported to be less effective stimulants of P-selectin expression and release from the cell surface than thrombin [6], suggesting a diminished role for PAR-2 *versus* PAR-1 in pro-thrombotic/inflammatory responses. While in disease states such responses may exacerbate pro/anti-coagualant imbalance and favour inappropriate thrombosis, under normal conditions PAR-2-mediated von Willebrand Factor secretion may simply reflect a physiological role for this constitutive receptor in haemostasis.

In unperturbed endothelium, therefore, and under conditions of acute resolved inflammation the PAR-2-COX-2-PGI$_2$ axis is likely to be operative and generating large quantities of cardiovascular protectant.

In chronic inflammation it would be expected that the markedly increased expression of endothelial PAR-2, and perhaps other PARs [40], could result in enhanced coupling to this axis, ensuring maintained production of PGI_2 and thus optimal IP-mediated limitation of platelet activation, smooth muscle cell proliferation, and fibrosis (Fig. 2). Thus, while PAR-2 is likely to play an important role in the chronic inflammation associated with, for example, rheumatoid arthritis [22], its potential role as a vessel protector is exemplified by its ability to link key intracellular signals with COX-2 expression and PGI_2 synthesis.

Perspectives

It is clear that PARs are important players in endothelial PGI_2 production and that this reflects the ability of these receptors to couple to pivotal signalling events that control the activities and expression of key enzymes in the prostanoid synthetic pathway. Many questions remain unanswered. A role for PAR-3 as a modulator of protease-mediated signalling is beginning to emerge and recent work has shown that heterodimerisation of PAR-1 with PAR-3 regulates PAR-1-mediated signalling events [33]. Whether this interaction has significance for regulating prostanoid synthesis and endothelial function in general, and exactly how dimerisation influences intracellular signalling is currently unknown. We are beginning to identify the signalling events downstream of PARs activation that are relevant for their control of PGI_2 synthesis, but a complete picture awaits further study. For example, the details of MAPK-NF-κB interactions in PAR-dependent signal transduction await clarification. We have also shown that secreted PLA_2 enzymes ($sPLA_2$) cooperate with $cPLA_2\alpha$ in a MAPK-dependent manner to regulate PGI_2 generation [21]. The specific involvement of serine proteases in this cross-talk is not clear but it is conceivable that this may offer a further mechanism through which PAR-driven PGI_2 generation is controlled. Similarly, interactions between PARs and growth factor receptors (eg. [8]) are likely to be additional signalling modes with significance for controlling PGI_2 synthesis.

We know very little about the relevant endogenous PAR-activating proteases and how these may influence prostanoid synthesis and other responses of endothelial cells in physiology and pathology. PARs can also be 'disarmed' by proteases that cleave the receptor distal to the activating cleavage site and these also remain largely unidentified. The pattern of endothelial PARs expression in different vascular beds, and by endothelial cells of the macro *versus* microvasculature, is currently unknown and this knowledge will be a prerequisite for defining the precise *in vivo* roles of this receptor family.

PARs are an emerging therapeutic target for the management of inflammatory disorders [22]. However, there is growing evidence that these receptors exert protective actions in the cardiovascular system (eg. PGI_2 synthesis) suggesting that inhibition of PAR activation may not be an appropriate global anti-inflammatory strategy. Similarly, pharmacological inhibition of prostaglandin synthesis through blockade of COX activities poses a cardiovascular risk. Knowledge of the pathways that regulate COX-2 expression and the functions of its downstream products are therefore key to developing new approaches to prevent and treat cardiovascular disease. Continued investigations *in vitro* and *in vivo* should shed further light on these mechanisms and facilitate our understanding of the functional relevance of PARs and COX-2 in the cardiovascular system.

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