

# Protease-Activated Receptor PAR-4: An Inducible Switch between Thrombosis and Vascular Inflammation?

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## Abstract

Thrombin triggers activation of platelets through protease-activated receptor 1 (PAR-1) and PAR-4. Both receptors are widely expressed and exert multiple platelet-independent functions. PAR signalling contributes to healing responses after injury, by promoting cytokine activity and cellular growth and mobility. Uncontrolled PAR activation, however, can prevent timely resolution of inflammation, enhance thrombogenic endothelial function and drive adverse remodelling. The specific role of PAR-4 in thromboinflammatory vascular disease has been largely underestimated, given the relatively limited expression of PAR-4 in non-platelet cells under healthy conditions. However, unlike PAR-1, PAR-4 expression adapts dynamically to numerous stimuli associated with thromboinflammation, including thrombin, angiotensin II, sphingosine-1-phosphate (S1P), high glucose and redox stress, suggesting expression is switched on ‘at need’. Prostacyclin negatively regulates PAR-4 expression at the post-transcriptional level, which may serve to fine-tune thrombin responses and limit these to the injury site. PAR-4 elicits inflammatory, mitogenic and proliferative actions not only in response to thrombin but also to numerous other inflammatory proteases, and can cross-talk with other receptor systems such as S1P and adenosine receptors. Accordingly, PAR-4 has emerged as a candidate player in vessel disease and cardiac post-infarction remodelling. Currently, PAR-4 is a particularly promising target for safer anti-thrombotic therapies. Recent studies with the PAR-4 antagonist BMS-986120 lend support to the concept that selective antagonism of PAR-4 may offer both an effective and safe anti-thrombotic therapy in the acute thrombotic setting as well as an anti-inflammatory strategy to prevent long-term progressive atherosclerotic disease in high-risk cardiovascular patients.

## Keywords

- ▶ thrombin
- ▶ thrombo-inflammation
- ▶ protease-activated receptor
- ▶ vascular
- ▶ antagonist

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## Introduction

The serine protease thrombin is the key enzyme of the blood coagulation cascade activated upon vessel or tissue injury. A rapidly initiated chain of inflammatory and wound-healing processes serves to prevent further blood loss and restore function. At rest, a non-coagulant barrier surface is provided by the endothelium, which also releases anti-thrombotic factors such as the tissue factor pathway inhibitor and binds (and inactivates) the majority of circulating thrombin by binding to thrombomodulin. As a result of injury or inflammation, exposure of sub-endothelial collagen to blood components enables the first adhesion of circulating platelets to the site of injury via von Willebrand factor, resulting in a platelet aggregate or 'plug' that stops bleeding. During this initiation phase of coagulation, activation of endothelial cells, blood leukocytes and sub-endothelial vascular smooth muscle cells (SMC) and fibroblasts increases surface expression of the potent procoagulant trigger tissue factor (TF). Engagement of circulating coagulation factor (F) VII by TF initiates the sequential proteolysis and activation of a cascade of coagulation factors, culminating in the generation of active factor X (FXa) and thrombin. In the amplification phase, adherent platelets are activated by thrombin and form a platelet aggregate, providing a surface for the activation of other procoagulant factors and procoagulant microparticles. Concomitantly, thrombin sustains and further enhances coagulation factor activation. During the subsequent propagation phase, generation of active thrombin on the surface of activated platelets is amplified, allowing for sufficient production of insoluble fibrin that is cross-linked together with the aggregated platelets into a stable clot. The details of the finely tuned haemostatic pathway have been excellently reviewed recently.<sup>1</sup>

## Thrombin Receptors in Platelet Activation

Platelet activation is a key initiating event in blood coagulation, and thrombin is the strongest known trigger of this process. Human platelet activation by thrombin is mediated through two receptors, protease-activated receptor 1 (PAR-1) and

PAR-4.<sup>2,3</sup> PARs belong to a family of four known G protein-coupled receptors (GPCRs) with a unique mode of receptor activation. Rather than the ligand engagement typical of other GPCR, PAR activation by thrombin depends on proteolytic cleavage of the N-terminal domain of the receptor, generating a new amino terminus that acts as a tethered ligand to activate the receptor. Diverse serine proteases from immune cells, the coagulation cascade and digestive tract, amongst others, have been reported to activate PAR with different specificities (► **Table 1**).<sup>4</sup> PAR-1 was first described, and is the prototypical thrombin receptor on platelets and other cells. PAR-2 is conventionally not considered as a thrombin receptor, responding instead to tryptic enzymes, although high levels of thrombin in excess of 10 U/mL can reportedly activate PAR-2.<sup>5</sup> PAR-3 is cleaved by thrombin, but the ability of this receptor to signal autonomously is controversially discussed. It appears to act primarily as a cofactor for the other thrombin-activated PAR, specifically PAR-4 in rodent platelets.<sup>6</sup>

PAR-4 itself is a low-affinity thrombin receptor. The EC<sub>50</sub> of PAR-4 for thrombin is 100-fold higher than that of PAR-1<sup>7</sup> due to the lack of a specific hirudin-like binding domain. The signalling and desensitization/re-sensitization profiles of PAR-4 are distinct from PAR-1 and hence the PAR-4 component of the platelet response to thrombin is slower but more prolonged. Intriguingly, depending on the activating protease, stimulation of PAR-1 and PAR-4 may elicit different responses, providing functional plasticity that may allow for the development of specific PAR-targeting therapies.<sup>8</sup> A further remarkable feature of PAR-4 is the ability to interact functionally and physically with other receptors, such as PAR-1 or the ADP receptors P<sub>2</sub> × 1 or P<sub>2</sub>Y<sub>12</sub>.<sup>8-13</sup> Such heterodimer formation generally supports mutual receptor activation beyond the individual signalling capacity of each receptor alone, which likely underlies the sustained platelet signalling required for thrombus stabilization. P<sub>2</sub>Y<sub>12</sub> is the pharmacological target of thienopyridine-type anti-platelet agents such as clopidogrel and prasugrel,<sup>14</sup> and appears to contribute critically to the steady and sustained rise in calcium and α<sub>IIb</sub>β<sub>3</sub> activation evoked by PAR-4. The response to PAR-1 activation by contrast involves an acute and short-lived rise in cytosolic calcium and

**Table 1** Main characteristics of the known PAR family members

	Signalling	Activating proteases	Cellular expression
PAR-1	G <sub>q</sub> , G <sub>12/13</sub> , G <sub>i</sub>	Thrombin, FVIIa/Xa trypsin, elastase, plasmin, chymase, protease3, activated PC, granzyme A, gingipains	Platelets (h), endothelial and vascular smooth muscle cells, leukocytes, epithelial cells, fibroblasts, neurons, mast cells, myocytes, astrocytes
PAR-2	G <sub>q</sub> , G <sub>12/13</sub> , G <sub>i</sub>	FVIIa/Xa, trypsin, trypsin, elastase, thrombin? <sup>5</sup>	Endothelial and vascular smooth muscle cells, leukocytes, epithelial cells, fibroblasts, neurons, mast cells, myocytes, astrocytes, adipocytes
PAR-3	?	Thrombin	Platelets (m), endothelial and vascular smooth muscle cells, astrocytes
PAR-4	G <sub>q</sub> , G <sub>12/13</sub> (G <sub>i</sub> ?)	Thrombin, cathepsin G, factors Xa and VIIa, trypsin, gingipains-R	Platelets (h, m) leukocytes, endothelial and vascular smooth muscle cells, myocytes, astrocytes, adipocytes

Abbreviations: H, human; m, mouse.

Note: For detailed reviews, see Han et al,<sup>18</sup> Shah,<sup>22</sup> Coughlin,<sup>29</sup> Napoli et al,<sup>33</sup> Mahajan et al,<sup>39</sup> and Traynelis and Trejo.<sup>138</sup>

$\alpha_{IIb}\beta_3$  activation, independently of this ADP receptor.<sup>9,15–17</sup> Accordingly,  $P_2Y_{12}$  receptor inhibition with, e.g. clopidogrel may additionally add to the net response and suppress PAR-4-mediated platelet aggregation, while PAR-1-mediated effects are largely unaltered.<sup>15</sup> This intra-platelet phenomenon, attributable presumably to purinergic feedback, will likely occur primarily when thrombin levels are high, and the functional contribution of PAR-4 becomes more important. While the ability for receptor cross-talk of PAR-4 (and PAR-1) adds a further level of fine-tuning platelet responses, it at the same time represents another factor to be considered in therapeutic drug development. Current antagonists target receptor monomer function, and it remains to be seen how the formation and signalling of dimers or oligomers are influenced.

### Thrombin Receptor Function in Vascular Inflammation and Remodelling

As detailed above, coagulation culminates in the formation of a fibrin clot, which seals the injury site to prevent further blood loss. However, damage to the surrounding endothelium and structural matrix must also be repaired for restoration of function.<sup>18</sup> Of the total amount of thrombin generated in the context of blood coagulation, the vast majority is actually formed *after* clotting is completed,<sup>19</sup> indicating additional roles. Clotting human plasma generates and releases active thrombin and FXa in sufficient levels to elicit mitogenic and inflammatory responses in cultured vascular SMC.<sup>11</sup> Both processes are essential for healing and protection from infection. The thrombin-PAR signalling system encompasses multiple functions extending far beyond platelet activation. Direct platelet-independent actions of thrombin on cells within injured tissue, particularly in the vicinity of thrombi, are increasingly seen to contribute to local wound-healing and remodelling responses. As evident from ►Table 1, PAR-1 and PAR-4 are widely expressed and respond to a broad range of ligands. Accordingly, involvement in many diverse processes has been reported, including embryonic development, neuropathology, angiogenesis, tumour biology, tissue regeneration, immunity, acute and chronic inflammation, and cardiovascular diseases.<sup>1,8,21–33</sup>

Several endogenous braking systems tightly control thrombin actions, including several serine protease inhibitors that rapidly inactivate circulating thrombin. At the cellular level, thrombomodulin and the endothelial activated protein C receptor (EPCR) cooperate as a tandem ‘response converter’ to switch thrombin/PAR-1 interactions from a procoagulant to an anti-coagulant interaction<sup>in18</sup>, at least in the settings of an intact, functional endothelium. The endothelial PAR-1/activated protein C (aPC) interaction is a classical example of biased signalling, whereby activation of the same receptor can context dependently result in different signalling responses. PAR-1 activation by thrombin in the absence of aPC elicits a constellation of proinflammatory responses<sup>8,34</sup> The mechanisms of the signalling bias may be related to the 10,000-fold greater efficiency for PAR-1 cleavage over aPC cleavage, the requirement for receptor partitioning into caveolar microdomains for effective aPC

signaling and distinct intracellular signalling and desensitization events downstream of PAR-1.<sup>8,34</sup>

Despite the numerous inactivating mechanisms, active thrombin can continue to be released from mural thrombi for up to a week, and high levels of active protease have even been detected within clots at surgery or autopsy.<sup>8,34</sup> Moreover, thrombin bound to the sub-endothelial extracellular matrix remains functionally active, localized and protected from inactivation by circulating inhibitors;<sup>36</sup> thus, PAR expressed by nearby cells are likely to be continuously exposed to high levels of thrombin. While increased cytokine activity and cellular growth and mobility are essential to achieve healing and restoration of function, an overshooting or sustained PAR activation conceivably prevents resolution of inflammation, enhances thrombogenic endothelial function and promotes excessive vascular remodelling. For PAR-1, this is supported by the findings of the TRA 2°P-TIMI 50 trial.<sup>37</sup> In a subset of patients with peripheral artery disease (PAD), PAR-1 inhibition with the selective antagonist vorapaxar significantly reduced acute limb ischaemia and peripheral revascularization, an effect that appears to be largely attributable to direct effects on vascular or inflammatory cells and independent of anti-platelet actions.

The contribution of PAR-4 to thromboinflammatory vascular pathophysiology has by contrast been underestimated, given the relatively limited expression of PAR-4 in non-platelet cells under healthy conditions.<sup>18,31,33,38–43</sup> In earlier studies, we provided the first evidence for the functional expression of all thrombin receptors—PAR-1, PAR-3, PAR-4 and thrombomodulin/EPCR—in human vascular SMC and could demonstrate independent contribution of each receptor to the net migratory, proliferative and inflammatory profile of SMC.<sup>23,44–53</sup> Intriguingly, PAR-4 appears to be subject to a differential regulation by its primary agonist thrombin, when added at concentrations approximating those released by clotting plasma.<sup>20</sup> While constitutive expression of PAR-1 remains steady in human vascular SMC of either arterial or venous origin, PAR-4 mRNA levels respond dynamically to thrombin.<sup>44,48</sup> One speculative possibility is that different thrombin receptors serve distinct functions that are switched on ‘at need’.

### PAR-4 Regulation as a Novel Stress Response Signal

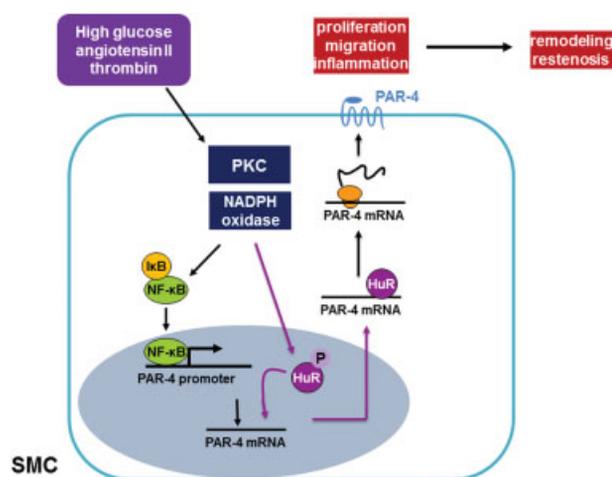
Besides thrombin, other stimuli characteristic of vascular injury and inflammation also promote a differential thrombin receptor regulation.<sup>23,48,54–62</sup> PAR-4 seems particularly sensitive to such signals, suggesting the receptor may represent a type of sensor for pathological stress that responds with marked upregulation from a normally low level of expression under resting conditions. Human arteries, for example, do not respond to PAR-4 activation *in situ* unless previously stimulated with a cytokine cocktail containing interleukin (IL)-1 $\alpha$  or tumor necrosis factor alpha.<sup>63</sup> Similarly, we have observed striking induction of PAR-4—but not PAR-1 or PAR-3—in human vascular SMC exposed to elevated extracellular glucose, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or angiotensin II (Ang II).<sup>23,48</sup>

Additional findings point to a highly dynamic regulation of PAR-4 occurring also in human monocytes exposed to proinflammatory lipid mediators such as sphingosine-1-phosphate (S1P). S1P is released from activated platelets and may enhance responsiveness to thrombin in monocytes via elevated PAR-4 expression resulting in increased levels of cyclooxygenase (COX)-2 at sites of injury.<sup>61</sup> This unique adaptability in response to stress or inflammatory triggers, i.e. high glucose or S1P, clearly sets PAR-4 apart from other thrombin receptors, which show no such upregulation either at the expression level or in terms of function. Accordingly, high glucose-pre-treated SMC show greater sensitivity to the PAR-4 component of the thrombin response in terms of intracellular calcium mobilization, migration and inflammatory gene expression, while PAR-1-mediated responsiveness remains unaltered.<sup>48</sup>

The precise explanation for the unique regulatory control of PAR-4 is slowly being unravelled. One reason might be the distinct chromosomal location of PAR-4 on chromosome 19p12, while the other thrombin-responsive PAR map to chromosome 5q13.<sup>64</sup> We have explored the cellular mechanisms of PAR-4 regulation in response to diverse stressors, and have identified a dual control system involving both a transcriptional and post-transcriptional component (summarized in ►Fig. 1). Stress-activated PAR-4 transcription occurs in a rapid and sustained manner,<sup>23,48</sup> converging upon protein kinase C (PKC)-dependent activation of the nuclear factor kappa B (NF-κB). Transcription factor binding analysis identified recognition motifs for NF-κB in the promoter region of PAR-4, but not of other thrombin-sensitive PAR.<sup>48</sup> This essentially implies a potentially unique role for PAR-4 in settings of inflammatory, redox and/or hyperglycemic stress and in response to certain growth factors. Stabilization of the PAR-4 transcript serves a second checkpoint for selectively regulating PAR-4 abundance. We could identify an important role of the mRNA-binding protein human antigen R (HuR) in this regard. HuR is a ubiquitously

expressed mRNA stabilizing factor that is strongly implicated in diverse disorders associated with redox imbalance, inflammation and fibroproliferation.<sup>65-67</sup> HuR is a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, which bind AU-rich elements (AREs) in the 3'-untranslated region (UTR) of target mRNAs. These usually mark the mRNA for rapid degradation. Competitive binding by RNA-binding proteins either stabilizes or destabilizes the mRNA, depending on the upstream input signal. HuR delays target mRNA decay by binding directly to several classes of ARE, including AUUUA repeats, AU regions with interspersed AUUUA and U-rich sequences). A strict requirement for mRNA stabilization is nuclear-cytoplasmic shuttling of the HuR-bound mRNA to the translational machinery, directed by a nuclear shuttling sequence (HNS) and the interaction with nuclear export/import adaptor proteins. We could identify putative ARE in the 3'UTR of human PAR-4, while no such motifs exist in the PAR-1 mRNA. Accordingly, HuR activators such as Ang II and H<sub>2</sub>O<sub>2</sub><sup>68,69</sup> upregulate PAR-4 in human vascular SMC, and HuR-mediated mRNA stabilization contributes also to the stimulatory effects of high glucose on PAR-4 expression in these cells.<sup>23</sup>

Suppression of HuR activity by contrast is seen upon stimulation of cyclic AMP signaling.<sup>52,70</sup> Accordingly, functional PAR-4 expression in both unstimulated and high glucose-challenged SMC is attenuated by the prostacyclin analogue cicaprost, which impairs PAR-4 transcript stabilization, HuR binding and subsequent cytosolic shuttling in a protein kinase A (PKA)-dependent manner.<sup>23</sup> At this point, it is worth mentioning that prostacyclin has been for a long time recognized as a functional antagonist of thrombin.<sup>18</sup> By opposing thrombin's prothrombotic actions as well as its stimulatory effects on cell proliferation, migration, cytokine release and matrix production, prostacyclin helps to control the extent and the site of thrombin action. As we now know, prostacyclin can also dynamically fine-tune thrombin responses by transcriptional and post-transcriptional receptor regulation.<sup>23,52,53</sup> Induction of COX-2 with subsequent increase of prostaglandin production might serve as an early response after injury,<sup>71,72</sup> to control the local actions of thrombin and prevent excessive vessel remodelling beyond the injury site. Interestingly, PAR signalling per se can upregulate COX-2 in several cell types including vascular SMC,<sup>73</sup> endothelial cells<sup>74</sup> and monocytes,<sup>61</sup> adding a further, self-limiting, level of regulation.



**Fig. 1** Schematic summary of PAR-4 regulation in human vascular SMC. PAR-4 expression is controlled by a dual mechanism involving transcriptional upregulation through NF-κB and mRNA stabilization by the mRNA-binding protein HuR. As a consequence, migratory, proliferative and inflammatory responses to PAR-4 activation are augmented, with implications for remodelling and restenosis.

### PAR-4 at the Nexus of Inflammatory Signalling Systems

The dynamic, context-dependent regulation of PAR-4 in cell types associated with stress responses—including endothelial cells, neutrophils, monocytes and macrophages as well as CD4 T cells and B lymphocytes<sup>56,75-79</sup>—could conceivably direct responses to coagulant protease generated upon injury or to proteases released from inflammatory cells. PAR-4 activation is a candidate checkpoint for the recruitment of neutrophils<sup>78,80</sup> and T cells<sup>76</sup> and hence plays an important role in the inflammatory response to tissue damage and the defence against possible infection. PAR-4 is thus ideally positioned as

potential functional switch in settings of thromboinflammation,<sup>81</sup> a concept further supported by its ability to respond to a spectrum of inflammatory activating proteases and to interact with other receptor systems associated with proinflammatory and prothrombotic processes. For example, besides thrombin and FXa, PAR-4 responds to the mannan-binding lectin-associated serine protease-1 (MASP-1). The MASP-1/PAR-4 interaction has been particularly well-defined in the context of endothelial activation during microbial defence, triggering inflammatory reactions as part of the immune response against extracellular pathogens.<sup>82,83</sup> Cathepsin G, a serine protease released from neutrophils and mast cells, is a further inflammatory activator of PAR-4,<sup>81,84</sup> During enhanced neutrophil mobilization and activation, cathepsin G, via PAR-4, promotes thrombin-independent platelet activation,<sup>84,85</sup> cellular proliferation<sup>86</sup> and monocyte-endothelial interaction.<sup>87</sup>

PAR-4 is increasingly recognized to interact at the functional level with other thromboinflammatory systems such as the S1P pathway or the P<sub>2</sub>Y<sub>12</sub>-ADP receptor. We have found that the signalling lipid S1P selectively increases PAR-4 transcription and cell surface expression in human monocytes, while PAR-1 is regulated only at the transcriptional level. Thus, solely PAR-4 contributes to the increased chemotactic and inflammatory function of monocytes in the context of elevated S1P.<sup>61,88</sup> S1P is incidentally released by platelets and cells of the vessel wall in response to thrombin and FXa, and is thus closely linked to thromboinflammatory states.<sup>89</sup> Besides PAR-4-mediated monocyte migration, S1P also amplifies thrombin-induced expression of COX-2 in human monocytes.<sup>61</sup> Perhaps PAR-4, rather than PAR-1, is in fact the predominant thrombin receptor directing monocyte recruitment and enhancing local inflammatory responses to thrombin. This hypothesis is supported by a finding made some 30 years ago, i.e. long before PAR-4 was identified. In this study, the chemotactic response to  $\alpha$ -thrombin was reported to be suppressed by anti-thrombin III, while blocking the active centre or generating gamma-thrombin via tryptic proteolysis of the procoagulant exosite did not abrogate chemotactic activity.<sup>90</sup> Since then, gamma-thrombin, an auto-proteolytic product of  $\alpha$ -thrombin, has become known as a selective activator of PAR-4.<sup>91</sup>

A cooperative PAR-4/P<sub>2</sub>Y<sub>12</sub> interaction occurring also in other cells besides platelets is plausible, but has not been demonstrated to date. The contribution of purinergic receptors to chronic or acute vascular inflammation, atherosclerosis, restenosis, endotoxaemia and sepsis has been excellently reviewed elsewhere.<sup>89,92,93</sup> Thienopyridine P<sub>2</sub>Y<sub>12</sub> antagonists reduce systemic levels of several inflammatory markers in atherothrombotic patients, presumably to a large extent via their anti-platelet action.<sup>94-98</sup> However, P<sub>2</sub>Y<sub>12</sub> receptors have been detected in vascular SMC,<sup>99-101</sup> and we have shown transcriptional upregulation of P<sub>2</sub>Y<sub>12</sub> in SMC response to thrombin. As a consequence, cyclic AMP elevation, IL-6 expression and secretion, and proliferation induced by the P<sub>2</sub>Y agonist 2-MeSADP are augmented in human SMC pre-exposed to thrombin. This regulatory effect on P<sub>2</sub>Y<sub>12</sub> expression is mediated predominantly through PAR-1 and does not appear to involve PAR-4.<sup>102</sup> However, PAR-4, but not PAR-1, can specifically modify P<sub>2</sub>Y<sub>12</sub> function through direct inter-

action.<sup>9,103</sup> This is likely to become relevant under conditions of thrombosis and inflammation where both extracellular nucleotides and PAR-4 are increased and expressed by the overwhelming majority of circulating and vascular cells.

### Potential Involvement of PAR-4 in Immune Cell Responses in Thromboinflammation

PAR-4 is expressed and dynamically regulated on monocytes, neutrophils and T-cells,<sup>61,104,105</sup> and acts as a strong chemottractant and trigger for proinflammatory signaling.<sup>105-108</sup> Direct PAR-4-mediated actions are therefore likely to contribute to the inflammatory component of repair and remodelling responses after injury, as well as immune defence. Additionally, PAR-4 will conceivably modulate immunity and inflammation indirectly, through platelet-dependent processes.

The idea of platelets as cells of the innate immune system has received increasing attention in recent years.<sup>109-112</sup> Platelets can interact directly with diverse pathogens including bacteria, fungi and protozoa, or envelop them in a platelet-fibrin clot. This can, for example, limit microbial propagation and increase exposure to neutrophil attack<sup>113</sup> and reduce the viability and virulence of various fungal species.<sup>114,115</sup> Yet envelopment of microorganisms by platelets and thrombi might also provide protection from innate immune processes and enable their transport to distant sites. Whether procoagulant and inflammatory interactions between platelets and microorganisms thus outweigh the beneficial immune defence function is therefore context-dependent, particularly in settings of anti-platelet therapy which will modulate platelet function and granule content release and accordingly impact on immune responses.<sup>116</sup> The PLATO study indicated that acute coronary syndrome patients treated with ticagrelor developed fewer lung infections and exhibited a lower mortality risk following pulmonary adverse events and sepsis than patients receiving the less potent anti-platelet agent clopidogrel.<sup>117</sup> It is, however, currently unknown to what extent adenosine-related actions of ticagrelor, unrelated to its P<sub>2</sub>Y<sub>12</sub> inhibitory effects, contribute to these actions. By contrast, preoperative use of dual anti-platelet therapy (aspirin plus clopidogrel) has been associated with an increased risk of infection after coronary artery bypass surgery.<sup>118</sup>

Clearly, the contribution of platelet-dependent effects to the net immune response is strongly context-dependent. How PAR-4 and the novel PAR-4 blockers fit into this picture remains to be seen. Although the differential pattern of PAR-1 versus PAR-4 release profiles is not clear,<sup>119</sup> there is evidence that specifically PAR-4 activation favours the release of certain factors that directly and indirectly influence inflammatory and immune responses.<sup>120-122</sup> PAR-4-activated platelets, for example, release gremlin-1, which subsequently enhances monocyte migration, adhesion and macrophage differentiation.<sup>123</sup> PAR-4-triggered release of platelet factor 4 (PF4, chemokine CXCL4) has been implicated in the host response to pneumococcal pneumonia; PAR-4<sup>-/-</sup> mice accordingly show higher bacterial loads and more pulmonary inflammation, attributable to enhanced cytokine release by the pathogen.<sup>124</sup> By stimulating release of PF4, PAR-4 may, moreover, direct

macrophage polarization towards the inflammatory M4 phenotype. These exhibit distinct phenotypic and functional characteristics, such as complete loss of the haemoglobin-haptoglobin (Hb-Hp) scavenger receptor CD163. Lack of CD163 renders M4 macrophages unable to upregulate the atheroprotective enzyme heme oxygenase-1 (HO-1) in response to heme stress and fail to effectively clear haemoglobin after plaque hemorrhage.<sup>125,126</sup> In this way, PAR-4 may be indirectly involved in the platelet/macrophage-dependent inflammatory component of diseases such as atherosclerosis by disturbing the heme stress response. Conceivably, PAR-4 blockade will limit the derailment of macrophage differentiation in this direction.

### PAR-4 in Diabetic Vascular Disease

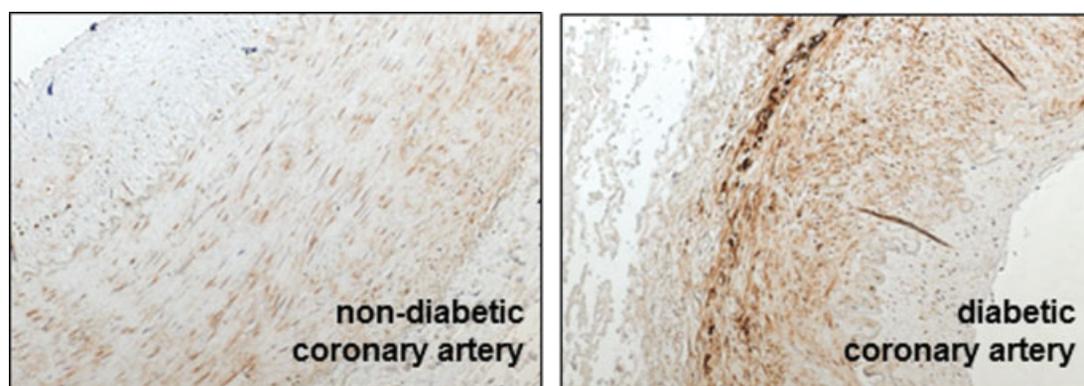
Elevated extracellular glucose levels are causally involved in the macro- and microvascular complications, in part by promoting SMC proliferation, matrix synthesis and inflammation in diabetics.<sup>127–130</sup> Hypercoagulability associated with increased thrombin generation and risk of thrombotic vessel occlusion is also a characteristic of diabetes.<sup>131–135</sup> Our observation that PAR-4 appears to be selectively upregulated by thrombin, high glucose, Ang II and redox-stress, and that high glucose selectively augments PAR-4-mediated migration and inflammatory gene expression in vascular SMC,<sup>23,48</sup> suggested its unique role in the context of diabetes. We confirmed the selective increase in PAR-4 abundance, with no change in PAR-1, in vessels obtained from diabetic patients at bypass and atherectomy<sup>23,48</sup> (►Fig. 2). Interestingly, we detected greater PAR-4 receptor expression in venous bypass vessels compared with arterial samples which—in addition to other factors—might contribute to the early thrombus formation in venous bypass grafts.<sup>136</sup>

In a proof-of-concept study, we examined the role of PAR-4 during neointimal hyperplasia in mice with streptozotocin-induced type 1 diabetes. Much as we saw with our human samples, diabetic C57Bl/6 wild-type mice exhibited marked increases in vascular PAR-4 abundance (►Fig. 3a,b) and, in the carotid artery ligation model, developed more profound neointimal remodelling than non-diabetic control animals,

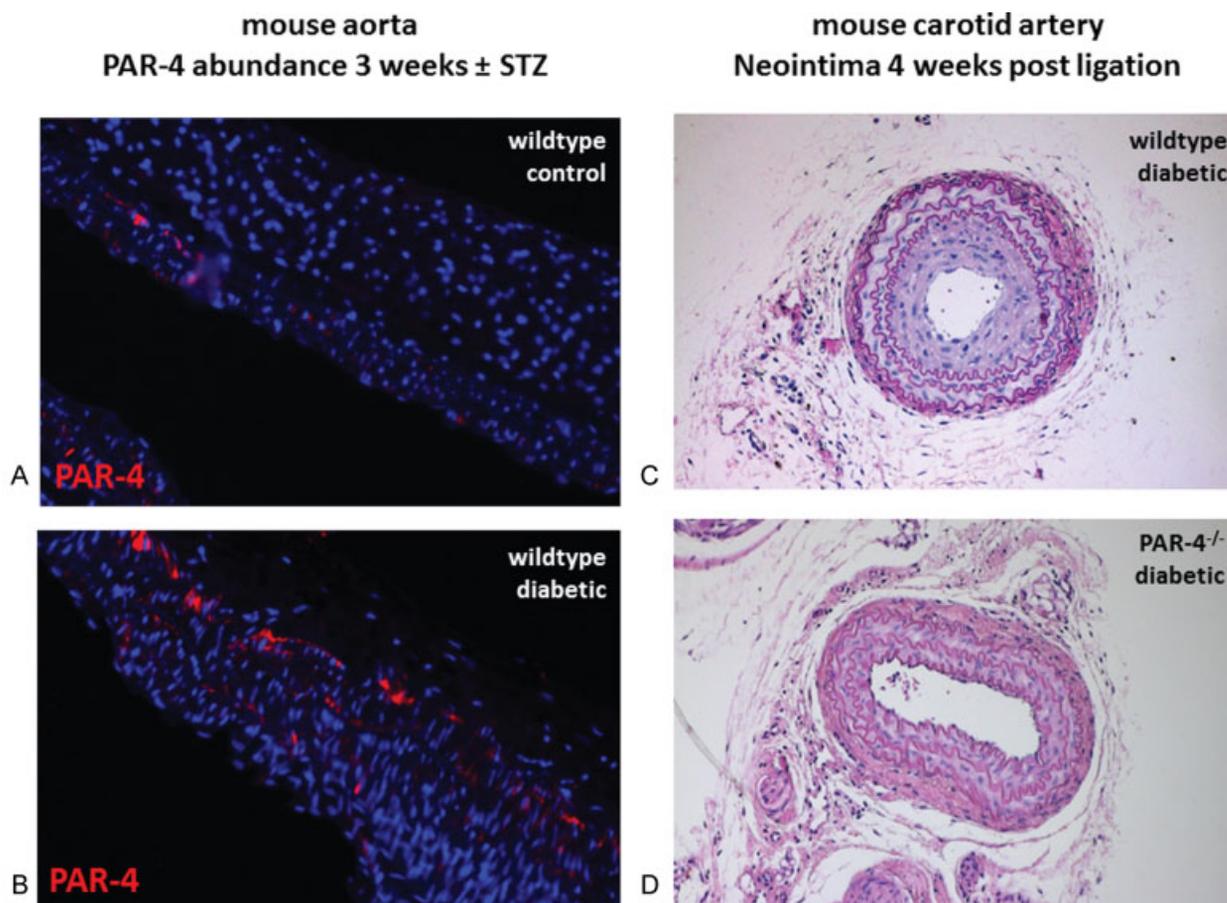
while PAR-4-deficient diabetic mice did not exhibit such a strikingly augmented neointimal formation (►Fig. 3c,d).<sup>23</sup> In comparison with their wild-type diabetic littermates, PAR-4<sup>-/-</sup> diabetic mice showed reduced numbers of proliferating cells and less macrophage infiltration.<sup>23</sup> Non-diabetic PAR-4-deficient mice were essentially comparable to non-diabetic wild-types. These observations support the concept that PAR-4 is minimally involved in vascular inflammatory and proliferative processes under resting, normoglycemic conditions, but becomes functionally relevant when expression increases in pathologically challenged cells, presumably vascular SMC. A contribution of platelets can of course not be excluded since PAR-4 is the predominant thrombin receptor in rodent platelets<sup>6</sup> and streptozotocin treatment reportedly increases platelet sensitivity to PAR-4, explaining in part the increased propensity for thrombosis in this setting.<sup>137</sup> Presumably, inhibition of augmented PAR-4 platelet function will contribute to the vascular protection seen in the PAR-4-deficient diabetic mice.

### PAR-4 in Post-infarction Remodelling

The interplay between PAR-4 and several vascular and immune cells centrally involved in thromboinflammation is depicted in ►Fig. 4. One setting in which this intercellular PAR-4 network has received recent attention is myocardial ischaemia/reperfusion (I/R) injury. Reperfusion is essential to prevent irreversible injury of the ischemic myocardium and permanent organ dysfunction. However, reperfusion itself induces an acute inflammatory response that is a requirement to initiate healing but can also promote an overshooting fibrotic remodelling that ultimately worsens cardiac function. Mice deficient in PAR-4 display reduced infarct size and better functional recovery in the acute phase following I/R, which could be attributed in part to prosurvival signalling and reduced apoptosis in cardiac myocytes.<sup>21</sup> Similar protection has also been reported in models of cerebral I/R injury.<sup>30,138</sup> In this setting, parameters of vascular inflammation such as rolling and adhesion of platelets and leukocytes were suppressed and oedema and blood–brain barrier function were improved. Strikingly, focal transient ischaemia strongly



**Fig. 2** Increased PAR-4 abundance in human diabetic coronary artery. Representative immunohistochemistry (unpublished,  $n = 3$ ) for PAR-4 (brown) in tunica media of human non-diabetic and diabetic coronary artery sections (patients described in Shpacovitch et al<sup>23</sup>). PAR-4 was visualized with primary antibody ab66103 (1:50, Abcam, Cambridge, UK) and secondary goat anti-rabbit antibody sc2004 (Santa Cruz Biotechnology, Santa Cruz, CA) plus DAB Substrate Kit (Zytomed Systems).



**Fig. 3** Diabetes augments vascular PAR-4 expression and neointimal hyperplasia after carotid artery ligation in mice. Representative PAR-4 immunofluorescence (red) in aorta of non-diabetic (a) and diabetic mice (b), 3 weeks after treatment  $\pm$  streptozotocin (200 $\times$  magnification). Neointimal hyperplasia of carotid arteries from diabetic (streptozotocin) wildtype (c) and PAR-4<sup>-/-</sup> mice (d), 4 weeks after ligation, visualized by haematoxylin and eosin (H&E) staining. Representative H&E sections taken 500  $\mu$ m proximal to the ligation site (100 $\times$  magnification). All  $n = 6$ , unpublished images (AF); detailed procedures described in Shpacovitch et al.<sup>23</sup>

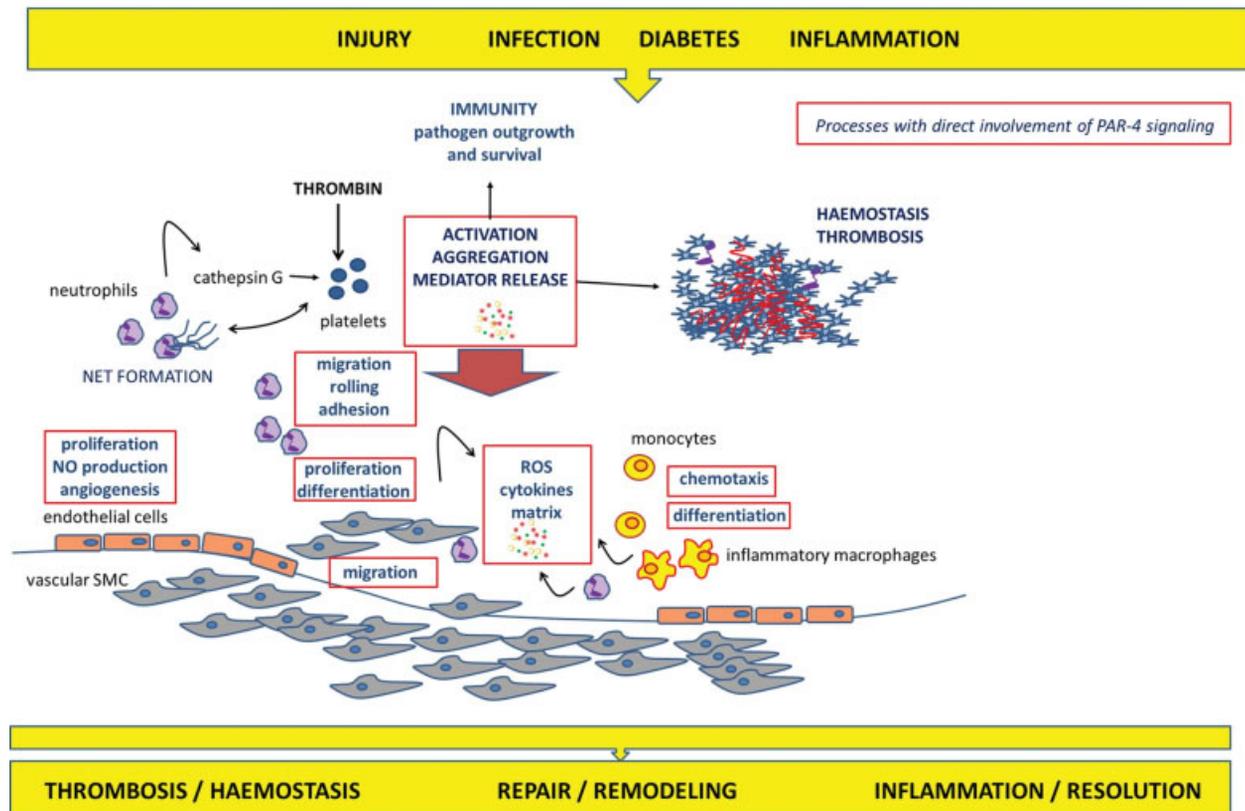
increased cerebral PAR-4 expression, while other PAR were only moderately and transiently influenced or, in the case of PAR-1, significantly downregulated.<sup>138</sup> This observation is in keeping with a specialized role of PAR-4 in settings of inflammation-associated injury and remodelling, at least in mouse models. Interestingly, certain single nucleotide polymorphisms in human PAR-4 have been associated with perioperative myocardial injury, with patients homozygous for the rs773857 risk allele showing increased platelet count and hyperactivity.<sup>139,140</sup> Thus, PAR-4 may also emerge as a target for strategies to limit clinical I/R injury.

### Therapeutic Targeting of PAR-4

The prototypical thrombin receptor PAR-1 has been the focus of intense research as a therapeutic target for novel anti-thrombotic drugs. PAR-4, by contrast, was for a long time seen as a mere co-factor for PAR-1 or 'back-up' receptor, with functional relevance only at high thrombin levels. Accordingly, PAR-4-deficient mice show no overt phenotype beyond prolonged bleeding time and protection from experimental thromboembolism.<sup>141</sup> Although PAR-4 responds to thrombin with an about 100-fold lower potency than PAR-1 ( $EC_{50} = 5$  nM vs.

0.05 nM),<sup>16,142</sup> both receptors have the capacity to fully activate platelets<sup>2,143</sup> and appear to play equally critical but distinct roles in platelet activation. By virtue of its slower on/off kinetics, PAR-4 ensures a sustained calcium signalling response and, according to a recent study, is more important than PAR-1 for the procoagulant effect of thrombin on platelets during prolonged thrombus formation.<sup>144,145</sup> PAR-4 has thus emerged as a promising candidate target for new anti-thrombotic therapies. This approach could be particularly suited in patients in whom complete thrombin inhibition or selective PAR-1 blockade leads to excessive bleeding or other undesired effects, as well as in patients with myocardial damage or diabetes who display heightened platelet PAR-4 reactivity. Currently, the development of selective PAR-4 antagonists is still in its infancy. Available to date are the trans-cinnamoyl-YPGKF-amide (tc-Y-NH<sub>2</sub>), based on the PAR-4 tethered ligand sequence, the pepducin P4pal10 (palmitoyl-SGRRYGHALR-amide), a cell-penetrating lipopeptide and YD-3, a non-peptide indole compound.<sup>146,147</sup> Only one PAR4 inhibitor, BMS-986120, has been applied in humans (clinicaltrials.gov registry number NCT02208882)<sup>148</sup> and will be discussed further on.

Another consideration with the use of selective PAR-1 blockers is the central role of the receptor in endothelial barrier



**Fig. 4** Simplified schematic of PAR-4 contribution to thromboinflammation. In settings of injury and stress, such as diabetes, inflammation and infection, generation of the primary PAR-4 activators thrombin and cathepsin G is enhanced, leading to augmented (PAR-4-mediated) platelet activation. This triggers platelet activation and aggregation, culminating in the haemostatic response and, depending on context, thrombosis. Cross-talk with neutrophils and induction of NETosis further amplifies this. Release of platelet-derived mediators such as thrombin, PF4, gremlin-1, reactive oxygen species, various growth factors, adhesion molecules, sphingolipids and others, moreover, promotes cellular signalling responses that support immune defence as well as vascular repair and remodelling, inflammation and resolution. Many of these effects, specifically neutrophil and monocyte recruitment and inflammatory/oxidative mediator release, as well as vascular smooth muscle cells (SMC) proliferation, migration, differentiation and release of cytokines and inflammatory/oxidative mediator release, are directly mediated through PAR-4 expression, which is dynamically upregulated in numerous cell types by injury, diabetic or inflammatory stress, or infection.

integrity and protection, in response to aPC. Protein C is activated to aPC via cleavage by the thrombin-thrombomodulin complex, which then elicits a combination of anti-coagulant and cytoprotective, anti-inflammatory cell signalling responses. The latter require engagement of the EPCR with secondary activation of PAR-1, while the anti-coagulant action of aPC depends on proteolytic inactivation of coagulation factor Va.<sup>149,150</sup>

One clinical condition in which aPC is of particular importance is sepsis, a systemic host response to microbial infection associated with a high mortality, due to the mutual potentiation of inflammation and coagulation. Sepsis leads to a rapid decline in PC, and low levels of PC correlate with poor prognosis. Recombinant aPC was successfully evaluated some 16 years ago (PROWESS Study) and approved for adult severe sepsis. However, the drug was subsequently withdrawn from the market after failing to show benefit in paediatric sepsis, non-severe adult sepsis and acute lung injury in follow-up trials (APACHE II, RESOLVE, PROWESS-SHOCK). This might in part be due to dosing. Clinically used aPC doses result in plasma concentrations that are still much lower than endogenous PC levels, and which may be inadequate to compete with endo-

genous PC for EPCR binding. An upper dosing limit is, however, set by adverse bleeding due to the strong anti-coagulant response to aPC. This dilemma, and which patients will yet benefit from aPC therapy, has recently been discussed in detail elsewhere.<sup>151,152</sup> How PAR-4 fits in this picture remains to be defined. In a canine model of bacterial infection and inflammation, platelet activation in response to PAR-4 agonists was disturbed, although not at the level of receptor expression.<sup>153</sup> In a mouse endotoxemia model, prasugrel inhibited PAR-4-dependent platelet activation and platelet-leukocyte interactions.<sup>154</sup> However, other mouse studies failed to support an important role for any PARs in endotoxemia; in fact, PAR-4<sup>-/-</sup> mice showed decreased survival.<sup>155,156</sup>

Besides its designated role in the endothelium, EPCR is also expressed in human vascular SMC, where it positively modulates the mitogenic actions of thrombin in the presence of aPC.<sup>46</sup> This could potentially contribute to local repair processes after injury. Suppression of protective EPCR-dependent functions could conceivably limit the net usefulness of selective PAR-1 blockers. PAR-4 by contrast is functionally independent of the aPC/thrombomodulin/EPCR signalling system. The increased functional relevance of PAR-4 in

thromboinflammatory settings suggests that this receptor in particular may be a candidate to switch away from timely and controlled resolution of inflammation. This feature of PAR-4, together with its relevance at pathological rather than physiological thrombin levels, makes the receptor an attractive target for novel therapies.

## Clinical Implications

Selective PAR-4 blockers will limit thrombin-inducible, PAR-1-independent actions. They, therefore, will not interfere in constitutive thrombin functions mediated through PAR-1, such as the protective effects demonstrated by vorapaxar in PAD patients. Activation of PAR-4 appears to drive thrombin production to a greater extent than PAR-1;<sup>121</sup> therefore, functional upregulation of PAR-4 in conditions of inflammation or diabetes could explain in part the enhanced prothrombotic risk that occurs in these settings. By extension, selective PAR-4 blockade might be expected to show a greater efficacy and safety in certain patients than inhibition of constitutively expressed PAR-1. PAR-1 antagonism has been evaluated in randomized controlled trials (RCTs) of vorapaxar for the secondary prevention of major adverse cardiovascular events (MACE) in high-risk cardiovascular patients.<sup>157,158</sup> Although vorapaxar showed enhanced efficacy to reduce MACE compared with dual anti-platelet therapy including acetylic salicylic acid plus clopidogrel, bleeding risk in certain subgroups, in particular in patients with a history of cerebrovascular events, remains a major concern.

Results of a randomized, blinded, placebo-controlled study including *in vivo* experiments with clopidogrel and the selective PAR-4 antagonist BMS-986120 in monkeys were published recently.<sup>159</sup> The primary end points were thrombus weight reduction, integrated blood flow and increased bleeding time. This study also comprised *in vitro* experiments using human blood. Human platelet-rich plasma aggregation in response to gamma-thrombin was fully inhibited *in vitro* by BMS-986120. In the animal study, BMS-986120 produced dose-dependent, selective and reversible (within 24 hours after last dose) inhibition of platelet aggregation and reduction of thrombus weight without relevant signals on bleeding times. Compared with the long half-life of the PAR-1 antagonist vorapaxar, which inhibits platelets for several weeks after a single loading dose in humans, explaining some of the adverse safety effects in RCTs, the pharmacodynamics of currently evaluated PAR-4 antagonists make the compound attractive in high-risk scenarios for thrombosis and bleeding.

In conclusion, it is tempting to speculate that compared with current, mainly platelet-directed, strategies, selective antagonism of PAR-4 offers both an effective and safe anti-thrombotic strategy in the acute thrombotic setting as well as anti-inflammatory strategies to prevent long-term progressive atherosclerotic disease in high-risk cardiovascular patients. Future studies are warranted to better interpret reported variabilities and mechanisms of PAR-4 responses in humans and the pharmacodynamic effects of PAR-4 antagonists.

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