Protease-Activated Receptors in Cardiovascular Diseases
Andrew J. Leger, Lidija Covic and Athan Kuliopulos
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Abstract—Thrombosis associated with the pathophysiological activation of platelets and vascular cells has brought thrombin and its receptors to the forefront of cardiovascular medicine. Thrombin signaling through the protease-activated receptors (PARs) has been shown to influence a wide range of physiological responses including platelet activation, intimal hyperplasia, inflammation, and maintenance of vascular tone and barrier function. The thrombin receptors PAR1 and PAR4 can be effectively targeted in animals in which acute or prolonged exposure to thrombin leads to thrombosis and/or restenosis. In the present study, we describe the molecular and pharmacological basis of small-molecule inhibitors that target PAR1. In addition, we discuss a new class of cell-penetrating inhibitors, termed pepducins, that provide insight into previously unidentified roles of PAR1 and PAR4 in protease signaling. (Circulation. 2006;114:1070-1077.)

Key Words: arteries • endothelium • inhibitors • platelets • receptors • signal transduction • thrombosis

Protease-activated receptors (PARs) play critical roles in coagulation, inflammation, and vascular homeostasis.1–5 Proteases that are produced during vascular injury exert many of their cellular effects by cleaving and activating the PARs. Thrombin-dependent platelet activation and aggregation have been shown to be heightened in the setting of angioplasty and stenting, which may cause clinical complications including acute myocardial infarction and death.6–8 The high-affinity thrombin receptor PAR1 has long been recognized as an obvious candidate for therapeutic intervention in patients with acute coronary syndromes. It is not yet known, however, whether targeting only PAR1 will achieve sufficient therapeutic efficacy because of the presence of a more recently identified second thrombin receptor named PAR4.9–12 PAR1 and PAR2 (a trypsin but not a thrombin receptor) have also been shown to affect other cardiovascular functions such as vasoreactivity and cardiomyocyte hypertrophy.

The purpose of the present review is to help the clinical reader understand why PARs are essential for the maintenance of normal vascular integrity. This review will focus on the potential therapeutic utility of targeting the PARs in thrombosis, atherosclerosis, and restenosis. Historically, the PARs have been recalcitrant to the development of peptidomimetic-based antagonists; however, recent PAR1 drug candidates based on natural products are now entering large-scale clinical trials for treatment of patients with acute coronary syndromes. In an orthogonal approach, PARs have also been blocked on the inside of the cell with the use of cell-penetrating pepducins that prevent signaling to internally located G proteins.13–17 Proof-of-concept experiments in acute thrombosis models point to novel antiplatelet therapies that could potentially benefit patients at risk for acute thrombosis.

The Role of PARs in Normal Platelet Function

Platelets are essential for proper blood coagulation. Initiation of a platelet thrombus is triggered by a variety of stimuli including collagen, adenosine 5′-diphosphate (ADP), thromboxane, and epinephrine; the most potent activator of platelets, however, is thrombin. Thrombin triggers platelet aggregation through the coordinated actions of the PAR1 and PAR4 receptors (Figure 1).12 The activation of a PAR is a 2-step process. First, the cryptic ligand is unmasked by proteolytic cleavage of the receptor N-terminal domain; then, an intramolecular rearrangement allows the ligand and the receptor moieties to interact.18–21 PAR1 is a high-affinity receptor for thrombin by virtue of a hirudin (Hir)-like sequence that resides in its N-terminal extracellular domain.22–26 The Hir sequence allows PAR1 to compete with the much more plentiful fibrinogen, and, as a result, PAR1 is activated by thrombin at even subnanomolar concentrations. The von Willebrand factor (VWF) receptor, glycoprotein (GP) Ib/V/IX, also serves to deliver thrombin to PAR1 by focusing the activity of thrombin to the platelet surface.27 Recent studies indicate that after cleaving PAR1, thrombin may remain tethered to PAR1 through the Hir sequence, where it can cleave nearby thrombin receptors.17,21

Once cleaved, PAR1 rapidly transmits a signal across the plasma membrane to internally located G proteins, which culminate in the formation of platelet–platelet aggregates. PAR1 activation of G12/13 causes platelets to undergo a dramatic shape change characterized by spikelike projections...
that alter the hemodynamic properties of the platelet. $G_{12/13}$ also controls the release of platelet-dense granules. PAR1 stimulation of $G_q$ causes a rapid rise in intracellular calcium and activation of the GP IIb/IIIa fibrinogen receptor. PAR1-dependent formation of platelet–platelet aggregates through the GP IIb/IIIa receptor tends to be transient unless strengthened by additional inputs from the Gi-coupled P2Y12 ADP receptor or from the PAR4 receptor. PAR4 has evolved a different strategy for interacting with thrombin. Bereft of a high-affinity Hir-like sequence, PAR4 has instead optimized its interactions with the active site of thrombin and uses a negatively charged cluster of amino acid residues to slow dissociation from the positively charged thrombin molecule. Although also coupled to $G_{12/13}$ and $G_q$, thrombin signaling through PAR4 is quite distinct from PAR1. PAR4 is cleaved and signals more slowly but, despite its slower response, generates the majority of the intracellular calcium flux and does not require additional input from the P2Y12 ADP receptor to form stable platelet–platelet aggregates.

PAR3, the least understood of the protease receptors, acts as a high-affinity thrombin receptor in platelets from most animals other than primates. Rodent platelets lack PAR1 and instead use the PAR3 receptor to enhance thrombin cleavage of the lower-affinity PAR4. PAR3 in the vascular endothelium therefore complements the functions of platelet PAR1 during normal hemostasis by localizing the thrombus to the site of vascular injury. Endothelial PAR1 is also involved in acute inflammatory responses and in vessel repair. Akin to the $G_{12/13}$-dependent shape change in platelets, thrombin activation of PAR1 causes Rho-dependent cytoskeletal rearrangements in endothelial cells and induces cell contraction and rounding. Endothelial cell contraction destabilizes cell–cell contacts, causing a subsequent increase in vascular permeability that

**PARs in the Vascular Endothelium**

All 4 members of the PAR family, PAR1, PAR2, PAR3, and PAR4, are expressed in arterial and/or venous endothelial cells (Figure 1). Activation of PAR1 and PAR2 in mesenchymal cells of the vessel wall mediates responses involved in contractility, inflammation, proliferation, and repair. Like PARs expressed on platelets, endothelial PARs serve as sensors of extracellular proteases and transmit signals after cleavage by proteases such as thrombin and factors VIIa and Xa. Activation of endothelial thrombin receptors leads to calcium mobilization and secretion of Weibel-Palade bodies, which harbor vWF multimers and the P-selectin adhesion molecule. Exposure of endothelial cell–anchored vWF to circulating platelets provides an initial means of tethering platelets to the blood vessel wall. PAR1 in the vascular endothelium therefore complements the functions of platelet PAR1 during normal hemostasis by localizing the thrombus to the site of vascular injury.
facilitates the passage of molecules and cells from the blood into subendothelial compartments and exposure of tissue factor (TF) and collagen. Activation of PAR1 in the vascular endothelium also leads to increased surface expression of the adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1, P-selectin, and E-selectin and gene transcription of cytokines and chemokines such as interleukin-8. In the activated state, endothelial cells support the rolling, chemotaxis, and transmigration of leukocytes to the site of vascular damage.

Recent studies have indicated that coagulation factors upstream and downstream of thrombin can mediate activation of PAR1 and PAR2 in endothelial cells. Factor Xa activates both endothelial PAR1 and PAR2, whereas TF/VIIa activates PAR2 and to a lesser degree PAR1. The anticoagulant protease-activated protein C can activate PAR1 when in complex with the endothelial cell protein C receptor, which may account for much of the protective effects conferred by activated protein C in severe sepsis.50 The strength of PAR1 may account for much of the protective effects conferred by PAR1 and PAR2, whereas TF/VIIa activates both endothelial PAR1 and PAR2, whereas TF/VIIa activates PAR2 and to a lesser degree PAR1.40,41,48,49 The anticoagulant protease-activated protein C can activate PAR1 when in complex with the endothelial cell protein C receptor, which may account for much of the protective effects conferred by activated protein C in severe sepsis.50 The strength of PAR1 and PAR2 activation by thrombin, factor Xa, and activated protein C can either promote or protect against changes in vascular permeability depending on the status of the endothelium.51–53 Therefore, PARs may also serve as useful drug targets in disease states characterized by decreased barrier function, including sepsis and systemic inflammatory response syndrome.

PARs in Vascular Smooth Muscle Cells and Cardiomyocytes

Aberrant overexpression of PAR1 has been documented in the endothelium and vascular smooth muscle cells of human atherosclerotic arteries, including regions of intimal thickening.2 Activation of PAR1 triggers mitogenic responses in smooth muscle cells and fibroblasts.54 Neointimal thickening is an early process in lesion formation; targeting PAR1 with a blocking antibody reduced intimal hyperplasia by ≈50% in a catheter-induced injury model of restenosis.4 Atherosclerotic plaques also contain inflammatory cells such as macrophages that express and secrete cytokines. In addition, macrophages produce metalloproteases (MMPs) in vulnerable plaques,55,56 including the recently identified PAR1 agonist MMP-1.15 Plaques that had been assessed as unstable because of a thin fibrous cap demonstrated 8-fold higher MMP-1 levels than lesions that harbor a thicker, more stable cap structure.56 Therefore, targeting PAR1 downstream of MMP-1 may be efficacious in the control or reduction of plaque rupture in atherosclerotic disease.

Cardiomyocytes also contain functional PAR1 and PAR2. On stimulation with PAR1 and PAR2 agonists, cardiomyocytes undergo hypertrophy.57,58 Targeting cardiomyocyte PAR1 and PAR2 may therefore be of possible therapeutic interest in modulating the inflammatory and tissue repair responses to myocardial infarction when PAR protease agonists such as thrombin and TF/VIIa/Xa are elevated.

Role of PARs in Vasoreactivity

Treatment of blood vessel preparations with thrombin induces both vasodilatory and vasoconstrictive responses. Stimulation of intact coronary arteries with thrombin or PAR1-agonist peptides elicits relaxation.3,59 However, mechanical disruption of the intimal endothelium of canine coronary arteries completely abolishes PAR1-agonist peptide relaxation effects and instead induces contraction. Likewise, in human coronary arteries with minimal intimal proliferation, addition of the PAR1-agonist peptide SFLLRN results in endothelial-dependent relaxation that is blocked by endothelial disruption. Pretreatment of endothelium-intact coronary arteries with Nω-monomethyl-l-arginine or Nω-nitro-l-arginine methyl ester, inhibitors of endothelial nitric oxide synthase, attenuates the endothelium-dependent relaxation effects and unmasks the constrictor effects. This indicates that stimulation of PAR1 on endothelium in normal arteries causes production of diffusible nitric oxide, which mediates paracrine relaxation of the vascular smooth muscle cells. Conversely, in freshly harvested human coronary arteries with severe atherosclerotic lesions, stimulation of PAR1 failed to elicit relaxation and in some cases caused marked contraction. Therefore, PAR1 responses may vary greatly depending on whether the endothelium is normal or in the context of an atherosclerotic lesion.

Studies in mice and rats have shown that systemic intravenous administration of PAR2-agonist peptides results in marked hypotension due to arterial vasodilation without changes in heart rate.60,61 The systemic PAR1-agonist response is more complex, with an initial drop in arterial pressure and heart rate followed by hypertension. Selective activation of PAR2 results in more pronounced hypotensive responses than selective activation of PAR1. Gene knockout experiments revealed further distinct roles for PAR1 and PAR2 in the cardiovascular responses to various PAR agonists.61 In the absence of PAR2, systemic activation of PAR1 gave rise to accentuated hypotensive and bradycardic responses compared with wild-type mice, suggesting that PAR2 may dampen the vasoreactive response of PAR1. Surprisingly, adult mice that are deficient in PAR1 exhibit normal resting hemodynamic properties (mean arterial blood pressure, systolic and diastolic blood pressure, and heart rate), suggesting that PAR1 is not necessary for the maintenance of normal vascular tone.52 Selective stimulation of PAR4 with the AYPGKF peptide elicits a relatively minor vasorelaxation response compared with PAR1 in freshly isolated aortic rings from mice.57 It remains to be determined whether endothelial PAR4 plays a significant role in the regulation of vascular tone in animals.

High-Throughput Screening of Tethered Ligand-Based Antagonists

Thrombin is a primary mediator of platelet activation and aggregation in arterial thrombosis or subacute thrombosis associated with percutaneous coronary intervention.6–8 In African green monkeys, a PAR1 antibody inhibited the occlusion of damaged carotid arteries,63 demonstrating that directly targeting PAR1 was a feasible means of inhibiting platelet thrombus formation. The identification of PAR1 as a putative therapeutic target incited substantial efforts by the pharmaceutical industry to develop small-molecule and peptide-based inhibitors of the PAR1 tethered ligand for use in cardiovascular diseases.

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The PAR1 tethered ligand sequence SFLLRN was initially used as a starting template in structure–activity relationship screens to identify peptidomimetics with antagonist activity. A systematic substitution of amino acid residues with natural side chains or nonnatural chemical groups was tested in both radioisogand binding and platelet aggregation assays. This early screening effort led to potent compounds, such as BMS-200661, that blocked SFLLRN-dependent platelet aggregation with an IC_{50} of \( \approx 20 \text{ nmol/L} \) (Table). However, BMS-200661 was relatively ineffective in blocking PAR1 activity by the thrombin-generated tethered ligand.

Another structure–activity relationship screen used a topological approach with the use of essential pharmacophores from the PAR1 tethered ligand: the N-terminal amino group, phenyl group at position 2, and guanido group from the PAR1 tethered ligand. This early screening effort led to potent compounds, such as BMS-200661, that blocked SFLLRN-dependent platelet aggregation. Another recent study reported the development of an orally active PAR1 competitive antagonist, SCH205831 (Figure 2) inhibited ex vivo PAR1-dependent platelet aggregation in cynomolgus monkeys, suggesting its potential use as an antithrombotic agent. Evaluation of the safety and efficacy of this new orally active compound is now under way in phase II/III clinical trials.

**Pepducins**

**Blocking G Protein–Coupled Receptors From the Inside**

An entirely different strategy for targeting PARs and other G protein–coupled receptors (GPCRs) is to modulate receptor–G protein signaling on the inside surface of the receptor. Cell-penetrating pepducins (Figure 3) are lipidated peptides based on the intracellular loop sequences of the GPCR of interest. Pepducins are designed to bind to the receptor–G protein interface on the inner leaflet of the plasma membrane and have been studied extensively in the context of PAR1 and PAR4 signaling in platelets and in animal models of thrombosis, inflammation, and angiogenesis.

Pepducins can exhibit agonist or antagonist activity for their cognate receptor. For example, the full-length third intracellular (i3)–loop pepducin of PAR1, P1pal-19, is a full agonist of platelet signaling and aggregation. Interestingly, P1pal-19 is also a full agonist of the highly homologous PAR2 receptor and a partial agonist of the cholecystokinin B receptor, as seen in an activity screen of several GPCRs. An array of PAR1 i3-loop derivatives were further screened, and a shorter C-terminal PAR1 pepducin, P1pal-13, was found to be highly selective for PAR1 and did not cross-activate PAR2 or cholecystokinin B expressed in fibroblasts. Thus, although cross-activity can be observed with certain pepducins, it is possible to narrow the selectivity to the intended target by modification of the parent compound.

Other pepducins based on PAR1 function as full antagonists of PAR1-dependent platelet aggregation. Likewise, pepducins tailored for PAR4 fully inhibit PAR4 aggregation of human platelets. PAR1- and PAR4-directed pepducins also antagonize human platelet aggregation in response to thrombin (Table). In a systemic platelet activation model, the PAR4 pepducin P4pal-10 prevented activation of platelets and extended the bleeding times in mice, consistent with the phenotype observed with mice deficient in PAR4. Infusion of P4pal-10 also caused unsta-
ble hemostasis in mice, as demonstrated by a rebleeding phenotype,\textsuperscript{14} and inhibited occlusion of carotid arteries.\textsuperscript{76} Pepducin antagonists have shown efficacy in other disease models including cancer, inflammation, and sepsis.\textsuperscript{15,16,75} A PAR1-based pepducin, P1pal-7, significantly blocked tumor growth and angiogenesis of breast cancer xenografts in nude mice.\textsuperscript{15} Pepducins directed against the interleukin-8 receptors CXCR1 and CXCR2 conferred a marked survival benefit and reduced multiorgan failure and disseminated intravascular coagulation in septic mice, even when treatment was postponed 8 hours after the onset of peritonitis.\textsuperscript{16}

Pepducins have the potential to reveal previously unidentified receptor interactions, as evidenced by cross-inhibition of related receptor subtypes. For example, the i3-loop pepducin of PAR4, P4pal-10, can cross-inhibit PAR1-dependent aggregation of platelets (Figure 3B). This cross-inhibition predicts a direct interaction between PAR1 and PAR4. In the model shown in Figure 3, the i3 loop of PAR4 is positioned at the heterodimeric interface with PAR1. The i3-loop pepducin, P4pal-10, may bind at this heterodimeric interface to effect the cross-inhibition of PAR1. Because the PAR4 i1 loop resides on the opposite side of the complex relative to the i3 loop, it was hypothesized that an i1-loop pepducin based on PAR4 would not display cross-inhibition of PAR1. As predicted, the i1-loop–derived PAR4 pepducin, P4pal-i1, inhibited PAR4 but not PAR1 signaling. The PAR4-i1 pepducin completely blocked PAR4-dependent chemotaxis of HEK cells and prevented PAR4-dependent platelet aggregation but did not affect PAR1-dependent chemotaxis or aggregation (Figure 3B and 3C). In contrast, the PAR4 i3 loop-pepducin, P4pal-10, was able to partially inhibit PAR1-dependent chemotaxis in the absence of PAR4. Inhibition of PAR1 by P4pal-10 was enhanced on coexpression with PAR4. These data indicate that the PAR4 i3-loop pepducin can interact with PAR1, most likely at the PAR1–PAR4 receptor interface.

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Small-molecule inhibitors of PAR1. Small-molecule antagonists of PAR1 such as RWJ-58259 inhibit receptor activation by competing with the tethered ligand on the extracellular surface of the receptor. The orally active himbacine-based SCH205831 is postulated to be a competitive antagonist of the tethered ligand, PAR1 can also be antagonized on the inside surface with a cell-penetrating, membrane-tethered pepducin antagonist, P1pal-7, which prevents receptor signaling to internally located G proteins in the cell.}
\end{figure}
Validation that PAR1 and PAR4 exist as a heterodimeric or hetero-oligomeric complex was provided by direct biochemical and biophysical studies. PAR1 was found to copurify with PAR4 in immunoprecipitates from both human platelets and fibroblasts. Fluorescence resonance energy transfer studies revealed close molecular proximity (<100 Å) between PAR1 and PAR4 in fibroblasts. PAR1–PAR4 complexes were spatially resolved by confocal microscopy, which revealed a fluorescence resonance energy transfer signal between PAR1 and PAR4 that emanated from the plasma membrane and from intracellular regions.

The present study also compared the effects of monotherapy versus combination therapy with PAR1 and PAR4 inhibitors in a guinea pig carotid artery injury model. Inhibition of PAR1 with RWJ-56110 or P1pal-7 conferred partial protection against arterial thrombosis. Likewise, inhibition of PAR4 with P4pal-i1 gave partial blockade of arterial occlusion. However, combination inhibition of PAR1 and PAR4 gave significant protection against occlusive thrombus formation. These data indicate that inhibiting PAR1 and PAR4 or the PAR1–PAR4 complex might offer alternative routes to managing pathophysiological activation of platelets in acute settings such as percutaneous coronary intervention and stenting.

Conclusion
PARs expressed on platelets and the vascular endothelium play important roles in normal blood vessel biology. PARs also contribute to the pathogenesis of several cardiovascular diseases including atherosclerosis, restenosis, and thrombosis. PARs have recently garnered significant attention from the medical and pharmaceutical communities as potential therapeutic targets in the treatment of these cardiovascular diseases. Blocking PARs and PAR dimeric complexes presents a new paradigm in the development of inhibitors that could manage aberrant PAR signaling in acute and chronic diseases.

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Disclosures
None.

References


