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Integration of endothelial protease-activated receptor-1 inflammatory signaling by ubiquitin

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Purpose of review
The maintenance and integrity of the endothelial barrier is essential for vascular homeostasis. Endothelial barrier dysfunction is mediated by various inflammatory factors, many of which act through G protein-coupled receptors including protease-activated receptors (PARs). PARs are expressed in multiple cell types in the vasculature and mediate cellular responses to thrombin, the key effector protease of the coagulation cascade. Thrombin activation of PAR1 induces endothelial barrier permeability through multiple pathways. Here, we discuss the mechanism by which thrombin activation of PAR1 promotes endothelial barrier breakdown and highlight recent advances that have provided new insight into molecular mechanisms that control endothelial barrier integrity.

Recent findings
Although the signal transduction pathways induced by thrombin activation of PAR1 in endothelial cells have been extensively studied, the key regulatory mechanisms remain poorly understood. Posttranslational modifications are integral to the regulation of PAR1 signaling and recent studies suggest a novel function for ubiquitination of PAR1 in regulation of endothelial barrier permeability.

Summary
An understanding of how endothelial barrier permeability is regulated by thrombin activation of PAR1 is important for the discovery of new drug targets that can be manipulated to control endothelial barrier permeability and prevent progression of vascular inflammation.

Keywords
arrestin, endosome, G protein-coupled receptor, p38 MAP kinase, TAB1, thrombin

INTRODUCTION
Diseases of the vasculature are the most common causes of morbidity and mortality in the United States. A hallmark of vascular inflammation is the breakdown of endothelial barrier integrity in the microvasculature that results in vascular leakage and tissue edema and is mediated by various factors, many of which act through G protein-coupled receptors (GPCRs) [1,2]. Thrombin, a coagulant protease, is generated during vascular injury and inflammation and elicits cellular responses through G protein-coupled protease-activated receptors (PARs). PARs are expressed in endothelial cells, platelets, smooth muscle cells and other cell types in the vasculature. There are four members of the PAR family including PAR1, PAR2, PAR3 and PAR4. The canonical mechanism of protease-activation for this subset of GPCRs has been established for PAR1. Thrombin binds to and cleaves the N-terminus of PAR1, which unmasks a new N-terminal domain that acts as a tethered ligand. Synthetic peptides that mimic the newly formed N-terminal tethered ligand sequence can activate PAR1 independent of thrombin cleavage. All PAR family members respond directly to thrombin with the exception of PAR2. Although PAR2 is not directly activated by thrombin, the N-terminus of PAR1 formed by thrombin cleavage can bind to an adjacent PAR2 in trans to activate the receptor in endothelial cells [3,4]. PAR1 is the major effector of thrombin signaling in most cell types including endothelial cells. However, PAR2, PAR3 and PAR4 are often coexpressed with PAR1 in endothelial cells and can...
influence thrombin-activated PAR1 signaling. Given that GPCRs are the largest class of drug targets for approved therapeutics, a thorough understanding of how endothelial barrier permeability is regulated by PAR1 may facilitate the discovery of new drug targets that can modulate endothelial barrier integrity and prevent the progression of vascular inflammation. Here, we discuss the mechanisms by which thrombin activation of PAR1 regulates endothelial barrier integrity and highlight new work implicating a role for ubiquitin in integrating p38 MAP kinase signaling and endothelial barrier dysfunction.

PAR1 signaling and endothelial barrier permeability

The generation of thrombin during vascular injury and inflammation induces a transient increase in endothelial barrier permeability [5,6]. Direct activation of PAR1 with synthetic agonist peptides has also been recently shown to increase vascular leakage in a murine model [7,8], implicating a specific role for PAR1. Disruption of the endothelial barrier induced by inflammatory mediators such as thrombin occurs through weakening of adherens junctions mediated by destabilization of adherens junction components and activation of actin–myosin contractility. Thrombin promotes endothelial barrier dysfunction through both of these mechanisms as discussed later. Once formed, thrombin is rapidly sequestered by thrombomodulin on the endothelial cell surface, which switches its substrate specificity from PAR1 to Protein C resulting in the generation of activated Protein C that diminishes proinflammatory signaling and promotes anti-inflammatory signaling and endothelial barrier stabilization [5].

PAR1 couples to multiple heterotrimeric G-protein subtypes including G q/11 and G 12/13 proteins that result in the rapid activation of signaling effectors that promote endothelial barrier permeability (Fig. 1). Activation of PAR1 by thrombin increases phospholipase C-β activity that leads to the generation of inositol phosphates and diacylglycerol and increases intracellular Ca 2+ concentrations and protein kinase C (PKC) activation. In addition, thrombin activation of PAR1 induces RhoA signaling through G 12/13 coupling to Rho guanine nucleotide exchange factors (GEEs) [9]. These signal transduction cascades converge to regulate phosphorylation of myosin light chain (MLC). MLC phosphorylation increases MLC phosphatase and thereby protects MLC from dephosphorylation and increases endothelial cell contraction. G q/11-dependent activation of PKC can also facilitate RhoA activation through phosphorylation of the Rho-GDP guanine nucleotide dissociation inhibitor [10]. RhoA can further promote Rho kinase-dependent phosphorylation of actin-depolymerizing proteins resulting in actin stress fiber formation [6]. Mice with endothelial-specific deficiency in G q/11 or G 12/13 exhibit diminished MLC phosphorylation induced by thrombin compared to wild-type littermate control mice [8]. Thrombin-induced MLC phosphorylation also correlates with reduced RhoA signaling in G 12/13 endothelial-deficient mice but not in G q/11 endothelial-specific knockout mice. In addition, vascular leakage induced by direct activation of PAR1 with agonist peptide is markedly reduced in G q/11 endothelial-deficient mice but not in G 12/13-deficient mice. Thus, although activation of PAR1 by thrombin promotes endothelial cell contractility through multiple G-protein subtypes resulting in interendothelial cell gaps and barrier disruption, the G q/11 pathway appears to be the predominant regulator of endothelial barrier permeability in vivo at least in mouse models. The MLC-dependent actin–myosin contractile forces are also important for the disruption of adherens junctions at endothelial cell–cell contact sites. Adherens junctions comprise of the major organizing transmembrane protein vascular endothelial-cadherin and cytoplasmic associated proteins including p120-catenin, β-catenin, α-catenin or plakoglobin [11]. The cytoplasmic tail of vascular endothelial-cadherin interacts with F-actin through binding to α-catenin, driving adherens junctions
disassembly during endothelial cell contraction. The retention of vascular endothelial-cadherin at the plasma membrane is mediated by the binding of p120-catenin to the juxta-membrane domain of vascular endothelial-cadherin. Thrombin stimulates PKC-\(\delta\)-dependent phosphorylation of p120 catenin and its disassociation from vascular endothelial-cadherin, which promotes vascular endothelial-cadherin internalization, adherens junction disassembly and enhances vascular permeability [12]. Intriguingly, endothelial barrier permeability is a reversible process and adherens junctions undergo rapid reassembly and barrier formation after thrombin stimulation. Recovery of endothelial barrier function following thrombin incubation is mediated by an increase Rac1 activity, mediated by the GEF protein Asef [13\textsuperscript{**}] and restoration of intracellular cAMP levels [14\textsuperscript{*}]. However, a recent study demonstrated that Src-dependent activation of Rap1 and afadin, a Rap1 downstream effector, also promotes adherens junction reassembly via recruitment of p120-catenin and reduces Rho signaling to facilitate endothelial barrier recovery. Rap1 further induces membrane localization of Tiam1, a Rac1-specific GEF and Rac1 activation resulting in resealing of intercellular endothelial gaps following exposure to thrombin [15]. Thus, thrombin-induced endothelial barrier permeability and recovery is intricately coordinated by the status of MLC phosphorylation and activities of RhoA, Rac1 and Rap1 in a finely tuned spatial and temporal manner.

**Role of p38 MAP kinase in thrombin-induced endothelial barrier permeability**

In addition to the well characterized pathways of thrombin-induced endothelial barrier permeability described earlier, p38 MAP kinase makes important contributions to endothelial barrier dysfunction. Thrombin stimulates p38 MAP kinase activation and NF-\(\kappa\)B activation, which increases intercellular...
adhesion molecule-1 (ICAM-1) expression in human umbilical vein endothelial cells [16]. A p38 MAP kinase-dependent increase in production of interleukin-6, interleukin-8 and monocyte chemotactic protein-1 cytokines as well as leukocyte recruitment is also observed in thrombin-stimulated endothelial cells [17]. Signaling by p38 MAP kinase has also been implicated in thrombin-induced endothelial barrier permeability via modulation of actin and microtubule cytoskeleton proteins. In bovine pulmonary artery endothelial cells, thrombin induced phosphorylation of the actin-binding protein caldesmon through a p38 MAP kinase-dependent pathway that appears to increase endothelial barrier permeability independent of MLC kinase activation [18]. In addition, several studies have shown that activation of p38 MAP kinase leads to MAP kinase-activated protein kinase-2 signaling, which phosphorylates heat-shock protein 27 and results in reorganization of the actin cytoskeleton and endothelial barrier dysfunction both in vitro and in vivo [19–21], but it is not known whether thrombin signaling is integrated in this pathway. In other recent work, p38 MAP kinase was shown to regulate the microtubule cytoskeleton and disrupt cell-cell junctions via modulation of microtubule-associated protein-4 (MAPK4) in human pulmonary microvascular endothelial cells [22]; however, the role of thrombin was not examined. Thus, thrombin activation of p38 MAP kinase appears to contribute to endothelial barrier dysfunction directly by modulating endothelial cytoskeleton proteins and indirectly by increasing the production of proinflammatory cytokines. However, the mechanism by which thrombin activation of PAR1 leads to induction of p38 MAP kinase activity in endothelial cells is not clear.

**Canonical and noncanonical p38 MAP kinase activation**

The activation of p38 MAP kinase occurs through a canonical three-tiered kinase cascade mediated by upstream MAP3Ks as well as a noncanonical pathway mediated by direct binding of transforming growth factor-β-activated protein kinase-1 binding protein 1 (TAB1). The canonical cascade converges on two MAP2Ks—MKK3 and MKK6—that phosphorylate and activate all four p38 MAP kinase isoforms (α, β, γ and δ) [23]. In contrast, the direct binding of TAB1 to p38α promotes a conformational change in p38α leading to autophosphorylation and activation [24,25]. The noncanonical pathway of p38 MAP kinase activation occurs in response to cytokines and stress inducers and bypasses the requirement for MAP2Ks. Our recent study demonstrates for the first time that PAR1 can activate p38 MAP kinase through noncanonical autophosphorylation mediated by TAB1 to regulate endothelial barrier permeability (Fig. 2) [7]. This study further defines an atypical ubiquitin-dependent pathway for PAR1-induced p38 MAP kinase activation, which is mediated by the TAB1-associated protein TAB2 on endosomes as described later.

**FIGURE 2.** Model of thrombin-activated PAR1 induction of noncanonical p38 MAP kinase signaling. Activation of PAR1 by α-thrombin promotes rapid coupling to heterotrimeric G proteins comprised of α and βγ subunits. Activated PAR1 is rapidly phosphorylated and ubiquitinated, the latter of which is mediated by the NEDD4–2 E3 ubiquitin ligase. Ubiquitination of activated PAR1 induces recruitment of TAB2 on endosomes. TAB2 associates with TAB1, which binds to p38 MAP kinase promoting autophosphorylation and activation following thrombin stimulation. Intriguingly, TAB1 protein is stabilized following recruitment to activated PAR1–TAB2–p38 MAP kinase signaling complex. Importantly, PAR1-stimulated noncanonical 38 MAP kinase activation causes a significant increase in endothelial barrier permeability through a poorly understood mechanism. PAR, protease-activated receptor; TAB, transforming growth factor-β-activated protein kinase-1 binding protein.
Diverse functions for ubiquitin in signaling and trafficking

Ubiquitin is covalently linked to lysine residues of substrate proteins by E3 ubiquitin ligases. In addition to ubiquitin’s role in protein trafficking, ubiquitin has been shown to function as a scaffold that facilitates assembly of signaling complexes important for inflammatory responses induced by cytokines [26,27]. This is best characterized for tumor necrosis factor-α and interleukin-1 mediated activation of NF-κB activation in which K63-linked ubiquitin conjugated to effector and adaptor proteins functions as docking sites for the ubiquitin-binding domain of TAB2 [28,29]. TAB2 forms a complex with TAB1 [30] and recruits kinases that phosphorylate IkB resulting in NF-κB activation [26,27]. For most GPCRs, ubiquitin is best known to serve as a signal for lysosomal sorting and degradation [31]. However, not all GPCRs including PAR1 require ubiquitination for lysosomal degradation, despite the fact that PAR1 is posttranslationally modified with ubiquitin [7,32]. These findings suggest that ubiquitination of certain GPCRs may serve a function distinct from lysosomal sorting.

Ubiquitin integrates PAR1 and p38 MAP kinase endosomal signaling

Ubiquitination of PAR1 facilitates recruitment of TAB2 to endosomes and promotes TAB1-dependent p38 MAP kinase activation independent of the canonical MKK3 and MKK6-mediated pathway. Activation of p38 MAP kinase by thrombin-activated PAR1 via the noncanonical pathway increases endothelial barrier permeability (Fig. 2). We found that activated PAR1 K63-linked ubiquitination is mediated by the E3 ubiquitin ligase neural precursor cell expressed developmentally downregulated protein 4–2 (NEDD4–2). This is consistent with NEDD4–2’s capacity to modify substrates with K63-linked ubiquitin [33]. NEDD4 family members are known to mediate ubiquitination of many GPCRs [34]. The Npl4 zinc finger (NZF) domain of TAB2 is required for binding to K63-linked ubiquitin [29] and is necessary for association with ubiquitinated PAR1 and p38 MAP kinase activation in response to thrombin exposure. In addition, wild-type TAB2 failed to bind to a PAR1 mutant that cannot be modified with ubiquitin and cannot signal to p38 MAP kinase activation [7*]. These findings support a role for ubiquitin in mediating PAR1-stimulated p38 MAP kinase activation. Confocal imaging in live cells also revealed rapid recruitment of TAB2 to activated PAR1 on early endosomes after thrombin stimulation. In contrast, neither a TAB2 NZF mutant defective in ubiquitin-binding nor a PAR1 mutant deficient in ubiquitination exhibited colocalization at endosomes. These findings suggest that PAR1 ubiquitination and the ubiquitin-binding capacity of TAB2 are required for formation of an endosomal p38 MAP kinase-signaling complex (Fig. 2). The G protein-coupled P2Y1 purinergic receptor utilizes a similar ubiquitin- and TAB1-TAB2-dependent pathway for p38 MAP kinase activation like PAR1 [7*], suggesting that a subset of GPCRs activate p38 MAP kinase through an ubiquitin-mediated and TAB1-mediated noncanonical pathway.

In addition to PAR1, the only other PAR shown to promote endosomal signaling is PAR2. Activated PAR2 forms a complex with β-arrestins that coregulates to endocytic vesicles and functions as a scaffold to promote early signal-regulated kinase 1 and 2 (ERK1/2) activation [35,36]. Interestingly, we found that in cytokine-treated endothelial cells PAR1 forms a dimer with PAR2, under these conditions PAR2 expression is markedly increased [4,37]. Thrombin-stimulated PAR1–PAR2 heterodimer cointernalizes, recruiting β-arrestins to endosomes and enhances ERK1/2 signaling [4]. Previous studies showed that the formation of the PAR1–PAR2 dimer in endothelial cells switches thrombin signaling from barrier-disruptive to barrier protective in a mouse model of sepsis [38], whereas the PAR1–PAR2 dimer mediates vascular smooth muscle hyperplasia in a vascular injury model [39]. Thus, signaling by the PAR1–PAR2 heterodimer is important for vascular disease progression. The contribution of ubiquitin to PAR1–PAR2 dimer-mediated β-arrestin ERK1/2 MAP kinase signaling is not known.

CONCLUSION

Our recent work demonstrates a role for TAB1-dependent autophosphorylation and activation of p38 MAP kinase-mediated induction of endothelial barrier permeability initiated by thrombin activation of PAR1 [39]. Several previous studies have also demonstrated an important role for TAB1-p38 MAP kinase signaling in various disease contexts including interleukin-12 production in macrophages [40], myeloid light-chain induced cardiotoxicity (amyloidosis) [41,42] and skin inflammation [43*]. Interestingly, the E3 ubiquitin ligase Itch was shown to regulate p38 MAP kinase activity through ubiquitin-mediated degradation of TAB1 during skin inflammation. TAB1 appears to be rapidly degraded by the proteasome in endothelial cells but is stabilized by p38 MAP kinase-mediated phosphorylation induced by thrombin [7*], but whether this involves ubiquitination of TAB1 remains to be determined. Finally, the precise mechanism by which thrombin-induced p38 MAP kinase signaling
from endosomes controls endothelial barrier permeability is not known and is an area of active investigation.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

15. This study demonstrates an important role for CAMP in regulating Rac1 activity and endothelial barrier recovery after thrombin stimulation.
24. This work shows a new role for the microtubule associated protein MAP4 phosphorylation mediated by p38 MAP kinase in endothelial barrier dysfunction.
46. This study shows that the E3 ubiquitin ligase Itch is a key regulator of TAB1-mediated p38 MAP kinase signaling in skin inflammation.