Editorial

Protease-Activated Receptor-1 Signaling
The Big Picture

Robert Flaumenhaft

Proteases serve an essential role in blood clotting, inflammation, angiogenesis, wound healing, and other vascular processes. The endothelium must be able to sense and respond to vascular proteases to coordinate these activities. Protease-activated receptors (PARs) are G-protein–coupled receptors responsible for sensing proteases, and PAR1 is a critical family member on endothelium. Like other PARs, endothelial PAR1 is able to sense the proteolytic environment by virtue of an unusual feature: an N terminus that serves as a substrate for many different proteases. Cleavage of the N terminus reveals a tethered ligand capable of activating the receptor via an intramolecular signaling mechanism (Figure).[1] More recent studies have shown that different proteases have unique cleavage sites, leading to distinct tethered ligands. Cleavage of endothelial PAR1 by thrombin results in apoptosis, release of granules, surface expression of inflammatory markers, and loss of barrier function.[3] In contrast, cleavage of PAR1 by activated protein C (APC) can result in protection from apoptotic stimuli, suppression of inflammatory markers, and fortification of barrier function.[3] These observations raise the complex question of how endothelial cells integrate PAR1 stimulation to mediate such varied responses.

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Strategies to define the intricate signaling pathways downstream of PAR1 have evolved over the years. Initial efforts defined the specific G proteins that couple to PAR1. Consistent with its ability to direct a broad range of cellular responses, PAR1 was found to couple with several G proteins, including Gαq, $G\alpha_{12/13}$, and Gαi, as well as Gβγ.[3] Later, PAR1 coupling to β-arrestins was demonstrated in the context of APC stimulation.[4] Many downstream phosphorylation events have been characterized primarily via the use of phospho-specific antibodies. Although this approach provides useful information regarding the kinetics and dose-dependency of endothelial PAR1 signaling, it focuses on only a small component of the signaling pathway without providing any sense of how the components interact. As a result, this approach cannot comprehensively compare signaling pathways induced by different PAR1 agonists nor can it comprehensively assess the effect of different PAR1 antagonists on downstream signaling.

van den Biggelaar et al[2,5] at Sanquin Research in Amsterdam have now applied a powerful global phosphoproteomic approach to evaluate signaling cascades activated after PAR1 stimulation in endothelial cells. To enable quantitative analysis of phosphorylated proteins, the authors used a 3-way reverse SILAC (stable isotope labeling with amino acids in cell culture) labeling strategy requiring metabolic labeling of blood outgrowth endothelial cells with light, medium, or heavy arginine and lysine, followed by phosphopeptide separation and analysis using high-resolution mass spectrometry. This rigorous approach enabled stringent criteria for quantifying phosphorylation events. The authors quantified 2553 phosphosites altogether and, importantly, 268 proteins (410 phosphosites) that demonstrated time-dependent changes (either increased or decreased) in phosphorylation after thrombin stimulation.[2]

The authors applied this phosphoproteomics strategy to address several issues in the PAR1 signaling field. First, they focused on an assertion arising from prior reports that thrombin modulates downstream signaling differently than SFLLRN (serine-phenylalanine-leucine-leucine-arginine-asparagine), a PAR1-activating peptide based on the tethered ligand produced by thrombin cleavage. A previous study showed that signaling through $G\alpha_{12/13}$ required lower concentrations of thrombin than signaling through Gαq, whereas SFLLRN-induced signaling showed the opposite pattern.[6] In contrast, the author's proteomic studies showed essentially identical phosphoprotein profiles after exposure to thrombin versus SFLLRN. One possible explanation for the apparent discrepancy is that the proteomic study was performed at near saturating concentrations of agonists. Nonetheless, the results provide investigators in the field assurance that this PAR1-activating peptide can be used as a reliable mimetic of thrombin with regard to PAR1 signaling function.

The authors also used the phosphoproteomic approach to compare and contrast PAR1 antagonists. The authors compared vorapaxar (an orthosteric inhibitor that associates with the shallow ligand-binding pocket of PAR1)[7] with parmodulin-2 (an antagonist that associates with the cytosolic face of PAR1 and blocks some, but not all, downstream pathways).[8–10] This analysis demonstrated differential inhibition of thrombin-mediated stimulation by parmodulin-2 compared with vorapaxar. Vorapaxar inhibited essentially all downstream signaling, buttressing the contention that PAR1 mediates the vast majority of thrombin signaling in endothelium. Parmodulin-2 blocked many PAR1-mediated phosphorylation events but was less effective at blocking dephosphorylation events that occurred in response to thrombin. This unexpected observation indicates that parmodulin-2 does not block many thrombin-activated phosphatase activities, providing additional complexity to the previously reported inhibitory bias of

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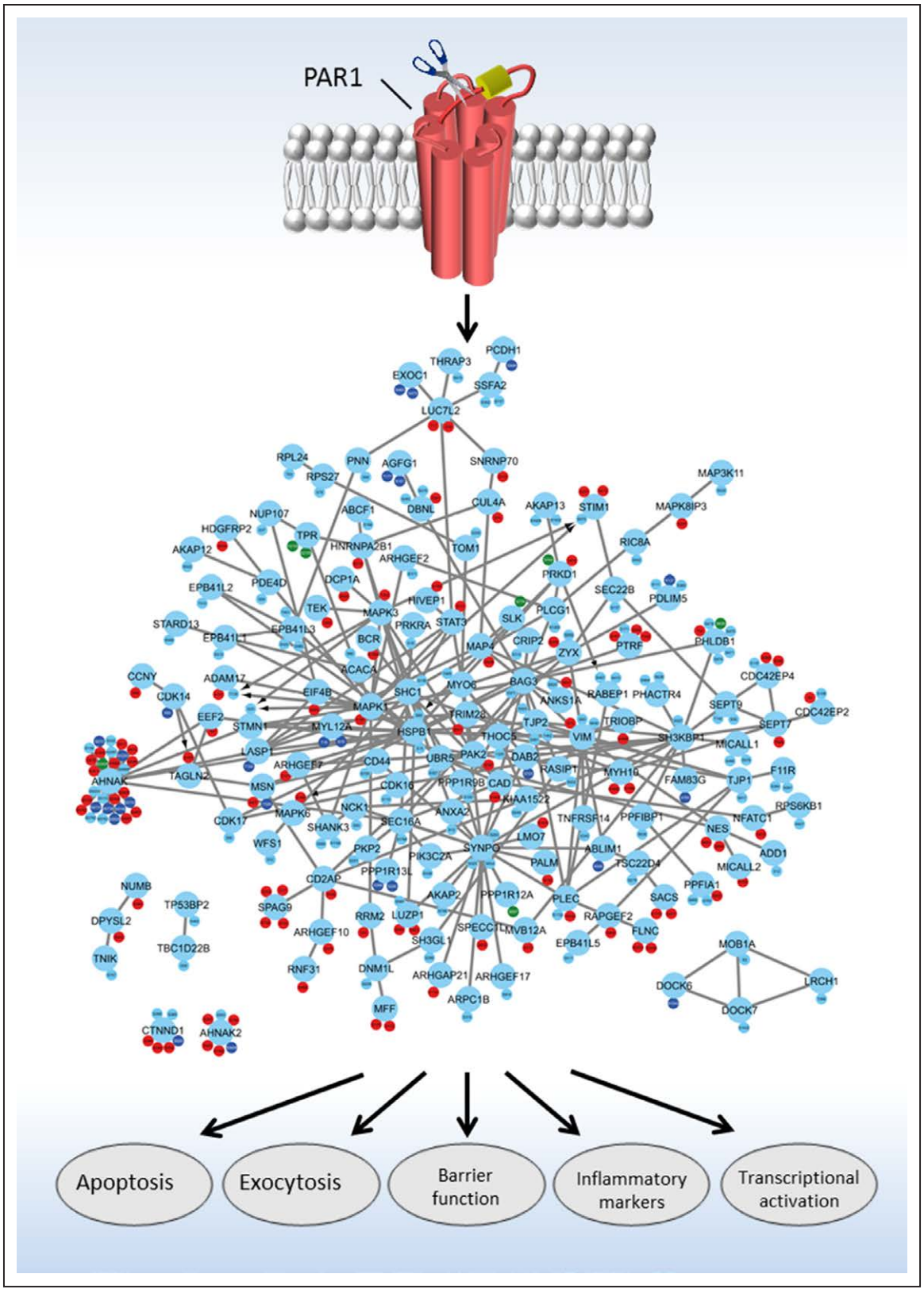

Figure. The N-terminus of protease-activated receptor (PAR1; red) is cleaved revealing a tethered ligand (yellow), which interacts with the ligand-binding site. Ligation of this binding site results in a conformational change in the receptor that signals to G-proteins and β-arrestin stimulating an intricate network of phosphorylation events (see van den Eshof et al[2] for details). PAR1 signaling coordinates several endothelial cell functions.

parmodulin-2.[10] These results underscore the value of global phosphoproteomics in assessing the mechanism of novel antagonists.

Another important area in PAR1 biology is biased signaling. For example, in explaining why cleavage of PAR1 by thrombin results in different endothelial responses than those observed after APC cleavage, some investigators have shown that thrombin-mediated signaling is biased toward G proteins, and APC-mediated signaling is biased toward β-arrestins.[3,11] APC, matrix metalloproteinase 1, proteinase 3, and neutrophil elastase all cleave PAR1 at distinct sites.[2] Peptides based on these noncanonical tethered ligands stimulate PAR1. When the authors compared the different PAR1-stimulating peptides to SFLLRN, they found that changes in the phosphoproteome induced by exposure to noncanonical peptides were minor compared with the robust changes induced by SFLLRN, and changes were generally in the same direction for both SFLLRN and noncanonical ligands. Surprisingly, only a few phosphosites were found that diverged from this pattern. This result is inconsistent with previous work using phosphorylation-specific antibodies that showed distinct phosphorylation events after exposure to noncanonical ligands compared with those after exposure to SFLLRN.

So how does one square previously reported results with these phosphoproteomic data? One possibility is timing. Because noncanonical peptide ligands can take longer to stimulate signaling, their distinct phosphorylation events may not have been captured. Another possibility is sensitivity (ie, phospho-specific antibodies may be more sensitive). Differences in cell type and origin could also contribute to the differences with previously published results. Finally, it is important to remember that not all signaling events are phosphorylation events. Differences in functional responses could be related to alternative second messenger systems. Nonetheless, these phosphoproteomic data warrant a critical re-evaluation of the nature of noncanonical PAR1 signal transduction in endothelial cells. Indeed, this rigorous phosphoproteomic approach provides investigators with a whole new way to look at PAR1 signaling—the big picture.

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