Paradigm of Biased PAR1 (Protease-Activated Receptor-1) Activation and Inhibition in Endothelial Cells Dissected by Phosphoproteomics

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Objective—Thrombin is the key serine protease of the coagulation cascade and mediates cellular responses by activation of PARs (protease-activated receptors). The predominant thrombin receptor is PAR1, and in endothelial cells (ECs), thrombin dynamically regulates a plethora of phosphorylation events. However, it has remained unclear whether thrombin signaling is exclusively mediated through PAR1. Furthermore, mechanistic insight into activation and inhibition of PAR1-mediated EC signaling is lacking. In addition, signaling networks of biased PAR1 activation after differential cleavage of the PAR1 N terminus have remained an unresolved issue.

Approach and Results—Here, we used a quantitative phosphoproteomics approach to show that classical and peptide activation of PAR1 induce highly similar signaling, that low thrombin concentrations initiate only limited phosphoregulation, and that the PAR1 inhibitors vorapaxar and parmodulin-2 demonstrate distinct antagonistic properties. Subsequent analysis of the thrombin-regulated phosphosites in the presence of PAR1 inhibitors revealed that biased activation of PAR1 is not solely linked to a specific G-protein downstream of PAR1. In addition, we showed that only the canonical thrombin PAR1 tethered ligand induces extensive early phosphoregulation in ECs.

Conclusions—Our study provides detailed insight in the signaling mechanisms downstream of PAR1. Our data demonstrate that thrombin-induced EC phosphoregulation is mediated exclusively through PAR1, that thrombin and thrombin–tethered ligand peptide induce similar phosphoregulation, and that only canonical PAR1 cleavage by thrombin generates a tethered ligand that potently induces early signaling. Furthermore, platelet PAR1 inhibitors directly affect EC signaling, indicating that it will be a challenge to design a PAR1 antagonist that will target only those pathways responsible for tissue pathology.

Visual Overview—An online visual overview is available for this article. (***Arterioscler Thromb Vasc Biol*. 2017;37: 1891-1902. DOI: 10.1161/ATVBAHA.117.309926.)**

Key Words: endothelial cells ■ mass spectrometry ■ phosphorylation ■ platelet aggregation inhibitors ■ proteomics ■ receptors, protease-activated ■ thrombin

Thrombin is a plasma protein that functions as a key serine protease in the coagulation cascade by activating a variety of coagulation proteins and converting soluble fibrinogen into insoluble fibrin strands. In addition, thrombin mediates cellular responses by activation of PARs (protease-activated receptors), a family of 4 G-protein–coupled receptors: PAR1, PAR2, PAR3, and PAR4. These receptors are activated by proteolytic cleavage of their extracellular N terminus, resulting in the formation of a novel N terminus that serves as a tethered ligand (TL) folding back into the ligand-binding pocket of the receptor.[1,2] PARs are expressed on a large number of cells, including endothelial cells (ECs), platelets, monocytes, T lymphocytes, and smooth muscle cells.[3] The physiological role of PAR activation in hemostasis is linked to platelet and EC activation. Thrombin activation of platelets induces shape change and release of α-granules, whereas thrombin activation of ECs leads to exocytosis of the von Willebrand factor containing Weibel–Palade bodies and a decreased endothelial barrier function that promotes leukocyte extravasation.[3–5]

See accompanying editorial on page 1809

Using quantitative phosphoproteomics, we unraveled the complex signaling pathway of thrombin in ECs, revealing thousands of dynamically regulated phosphorylation sites (phosphosites).[6] Although PAR1 is the predominant thrombin receptor in ECs, it has remained unclear whether the extensive thrombin-induced phosphoregulation is mediated exclusively via PAR1 or whether other receptors, including PAR2, also contribute.[7]

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Nonstandard Abbreviations and Acronyms

APC	activated protein C
EC	endothelial cell
MMP1	matrix metalloproteinase-1
NE	neutrophil elastase
PR3	neutrophil protease 3
PAR	protease-activated receptor
PLCB3	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase β-3
PRKD1	serine/threonine-protein kinase D1
SILAC	stable isotope labeling with amino acids in cell culture
STAT3	signal transducer and activator of transcription 3
TL	tethered ligand

Like many G-protein–coupled receptors, PAR1 is subject to ligand-dependent preferential activation of downstream targets, a process known as biased signaling.[8] Several mechanisms have been described,[9] including (1) enzymatic and peptide activation,[2,10–13] (2) PAR1 inhibitor-induced differential G-protein activation,[14,15] (3) concentration-dependent effects of thrombin,[16,17] (4) differential proteolytic cleavage,[9,18–20] and (5) G-protein versus β-arrestin–mediated signaling.[21,22] The molecular mechanism of these distinct modes of biased PAR1 activation has remained an unresolved issue.

The most well-known mechanisms of PAR1 activation are enzymatic activation by thrombin and activation by soluble peptides that mimic the TL that is formed after N-terminal proteolytic cleavage. It has been suggested that although thrombin–TL peptide (SFLLRN-NH2) mimics thrombin activation of PAR1,[2,10] in comparison to thrombin it favors downstream activation of $G\alpha_q$ over $G\alpha_{12/13}$.[11–13] Therefore, it is still under debate whether classical activation of PAR1 by thrombin and peptide activation by thrombin–TL peptide induce the same effects in ECs.

Another form of biased PAR1 activation has been attributed to PAR1 inhibitors. Platelet PAR1 has become a target for antithrombotic therapies, and several inhibitors have been developed that target PAR1. The orthosteric PAR1 inhibitor vorapaxar prevents activation of PAR1 by occupying the ligand-binding pocket[23] and has recently been approved by the Food and Drug Administration.[24] However, treatment with vorapaxar was associated with an increased bleeding risk,[24–26] which has been suggested to be linked to global inhibition of PAR1 on cells other than platelets, most notably ECs.[27] New approaches to prevent side effects in targeting of PAR1 have led to the development of several allosteric PAR1 inhibitors that target its cytoplasmic face (pepducins[28,29] and parmodulins[14,15]). It has been suggested that parmodulin-2 promotes biased PAR1 signaling by preferentially blocking $G\alpha_q$- over $G\alpha_{12/13}$-mediated PAR1 signaling.[15] However, detailed understanding of the global effects of PAR1 inhibition on ECs as well as the effect of PAR1 inhibitors on thrombin-mediated endothelial signaling is lacking.

Recently, yet another mechanism of biased PAR1 has been described. In addition to PAR1 cleavage by thrombin (canonical PAR1 cleavage at Arg-41), several other proteases have been described to cleave PAR1 (noncanonical PAR1 cleavage), resulting in biased signaling in ECs.[9] The cytoprotective effects of APC (activated protein C) are mediated via PAR1 (cleavage at Arg-46)[18] and also MMP1 (matrix metalloproteinase-1), NE (neutrophil elastase), and PR3 (neutrophil protease 3) are able to cleave the N terminus of PAR1 in proximity to the canonical thrombin cleavage site.[19,20] However, little is known about the signaling networks that are activated after noncanonical cleavage of PAR1. Finally, thrombin itself has also been suggested to induce biased signaling in ECs depending on its concentration; at high concentrations (>100 pmol/L), thrombin causes a barrier-disruptive response in ECs, whereas at low concentrations (50 pmol/L), the effect of thrombin is thought to be barrier protective.[16,17] Although the effects of thrombin at high concentrations have been well characterized, the molecular details of thrombin at low concentrations have remained an unresolved issue.

To dissect the various mechanisms of biased PAR1-mediated signaling, we have used a quantitative phosphoproteomics approach. Our results demonstrate that PAR1 mediates all thrombin-induced phosphoregulation in ECs and that classical and peptide activation of PAR1 result in highly similar signaling. Furthermore, thrombin at low concentrations initiates only limited phosphoregulation. Thrombin-mediated signaling in ECs is completely blocked by vorapaxar, whereas thrombin seems to induce biased activation of PAR1 in the presence of parmodulin-2. Finally, we show that, in contrast to the noncanonical PAR1 TL peptides, only canonical PAR1 cleavage by thrombin generates a TL that potently induces early signaling in ECs.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Vorapaxar and Parmodulin-2 Completely Block Thrombin-Mediated Endothelial Barrier Disruption

PAR1 inhibitors vorapaxar and parmodulin-2 were used to determine the role of PAR1 in the thrombin-induced effects in ECs. First, we determined their effect on thrombin–TL peptide–mediated platelet aggregation. Both PAR1 inhibitors completely inhibited thrombin–TL peptide–mediated platelet aggregation, with IC_{50} values (vorapaxar: ($2\pm1\times10^{-8}$ M (mean±SD), parmodulin-2: $7\pm1 \times 10^{-6}$ M) close to their reported IC_{50} values[15,30] (Figure 1A). Next, specificity of both inhibitors for blocking PAR1-mediated platelet aggregation was confirmed (Figure 1B). Thrombin–TL peptide–mediated platelet aggregation was specifically blocked, whereas PAR4 peptide- and U46619-mediated platelet aggregation were unaffected. Next, PAR1 inhibitors were used to determine the contribution of PAR1 in thrombin-induced endothelial barrier disruption by Electric Cell-substrate Impedance Sensing system. Vorapaxar and parmodulin-2 completely inhibited thrombin-induced endothelial barrier disruption, with IC_{50} values close to those for blocking thrombin–TL peptide–mediated platelet aggregation (vorapaxar: $2\pm1\times10^{-8}$ M, parmodulin-2: $2\pm1\times10^{-6}$ M (Figure 2A through 2C). In addition, the role of other receptors than PAR1 in the thrombin-mediated

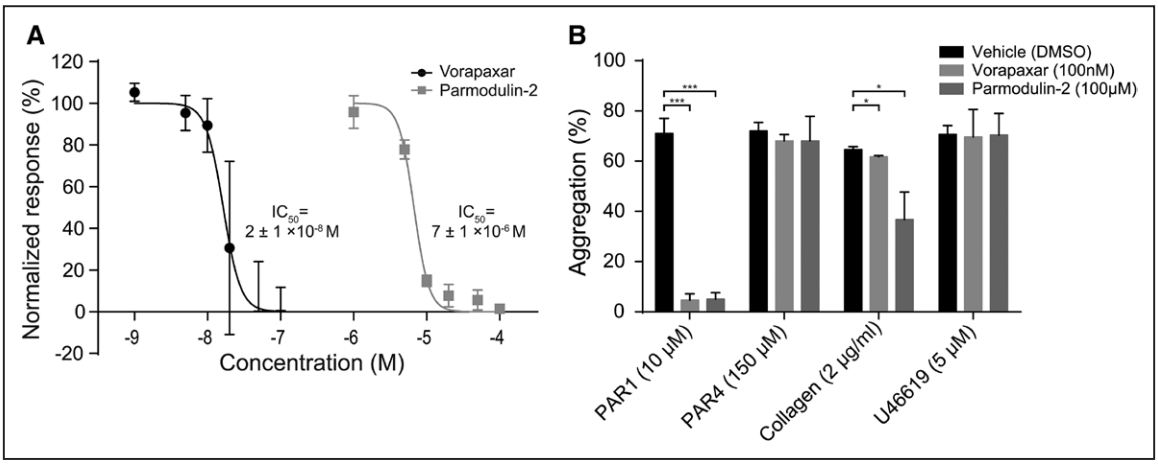

Figure 1. Vorapaxar and parmodulin-2 specifically inhibit thrombin-tethered ligand (TL) peptide–mediated platelet aggregation. **A**, Platelets were preincubated for 10 min with indicated concentrations of vorapaxar, parmodulin-2, or vehicle (DMSO; dimethyl sulfoxide), followed by triggering platelet aggregation with 10 μmol/L thrombin-TL peptide (SFLLRN-NH2). Normalized response was calculated per sample by normalizing the maximal aggregation to the maximal aggregation in the vehicle control. Data and IC_{50} values represent mean±SD (n=3). IC_{50} values were calculated using log(inhibitor) vs normalized response–variable slope (Graphpad Prism version 6.04). **B**, Specificity of vorapaxar and parmodulin-2 for blocking thrombin–TL peptide–mediated platelet aggregation was determined. Platelets were preincubated for 10 min with indicated concentrations of vorapaxar, parmodulin-2, or vehicle (DMSO), followed by triggering platelet aggregation with the following agonists: thrombin–TL peptide (10 μmol/L), PAR4 peptide (AYPGKF-NH2; 150 μmol/L), collagen (2 μg/mL), or thromboxane A2 receptor agonist U46619 (5 μmol/L). Data represent mean±SD (n=3). *P<0.05, ***P<0.001 by 2-way ANOVA with post hoc Tukey multiple comparison test (Graphpad Prism version 6.04).

endothelial barrier disruption was determined. Thrombin-mediated endothelial barrier disruption was completely blocked by vorapaxar, even at a thrombin concentration of 100 nmol/L (Figure 2D and 2E). Taken together, these data show that PAR1 inhibitors completely block thrombin-mediated endothelial barrier disruption.

Quantitative Phosphoproteomics of Thrombin- and PAR1-Stimulated ECs

To determine the contribution of PAR1 and other receptors in the thrombin-induced signaling in ECs, we used a quantitative phosphoproteomics approach. To this end, phosphoregulation by thrombin at high (10 nmol/L) and low (50 pmol/L) concentrations was compared with phosphoregulation by thrombin–TL peptide. In addition, the effects of PAR1 inhibitors vorapaxar and parmodulin-2 on thrombin-induced phosphoregulation in ECs were studied. For all these conditions, we performed a time-resolved phosphoproteomic analysis of stimulated blood outgrowth endothelial cells by using a 3-way reverse SILAC (Stable Isotope Labeling with Amino acids in Cell culture) labeling strategy (Figure I and Materials and Methods in the online-only Data Supplement). Time points of stimulation were chosen based on our previous study of thrombin signaling.[6] Mass spectrometry data were analyzed using the MaxQuant computational platform,[31] which identified 8306 accurately localized (class I[32]) phosphosites, of which 332 (4.0%) have not been described before (PhosphoSitePlus 20 March 2017[33]) (Table I in the online-only Data Supplement). In total, 2553 phosphosites were accurately quantified, and 410 phosphosites, localized on 268 proteins, were found to be regulated over the time course of our experiment (Figure I, Materials and Methods, and Table II in the online-only Data Supplement). The relatively low number of quantified class I phosphosites compared with those that were identified can be explained by our diversity of stimulations (n=5) and time-resolved analysis (2 time points per stimulation). To check the consistency of the quantified phosphosites, the thrombin/PAR1 inhibitor data set was compared with our previously published thrombin data set[6] (Figure IIA in the online-only Data Supplement). Although, for reasons mentioned above, the current data set contains a lower number of quantified phosphosites compared with the published thrombin data set (2553 versus 7793 phosphosites), 816 new phosphosites were quantified. Notably, 87% of the thrombin-regulated phosphosites were found to be regulated in both data sets. Because of the short time course of our phosphoproteomic experiment, we expected that the proteomic content of the blood outgrowth endothelial cells did not change over the time course of the experiment.[6]

Thrombin and Thrombin–TL Peptide Induce Highly Similar Phosphoregulation

To compare the phosphoproteomic changes induced by the 5 stimulation conditions, we performed a principal component analysis of the quantified phosphosites. The phosphoproteomes of ECs stimulated with 10 nmol/L thrombin and thrombin–TL peptide clustered together and were clearly distinct from the those of ECs stimulated with 10 nmol/L thrombin in the presence of vorapaxar or parmodulin-2 and ECs stimulated with 50 pmol/L thrombin (Figure 3A). Next, temporal phosphoregulation of the 5 stimulation conditions was analyzed by hierarchical clustering of the regulated phosphosites (Figure 3B). This revealed that most phosphoregulation was induced by 10 nmol/L thrombin and thrombin–TL peptide, followed by 10 nmol/L thrombin in the presence of parmodulin-2, and only limited phosphoregulation was induced by 10 nmol/L thrombin in the presence of vorapaxar

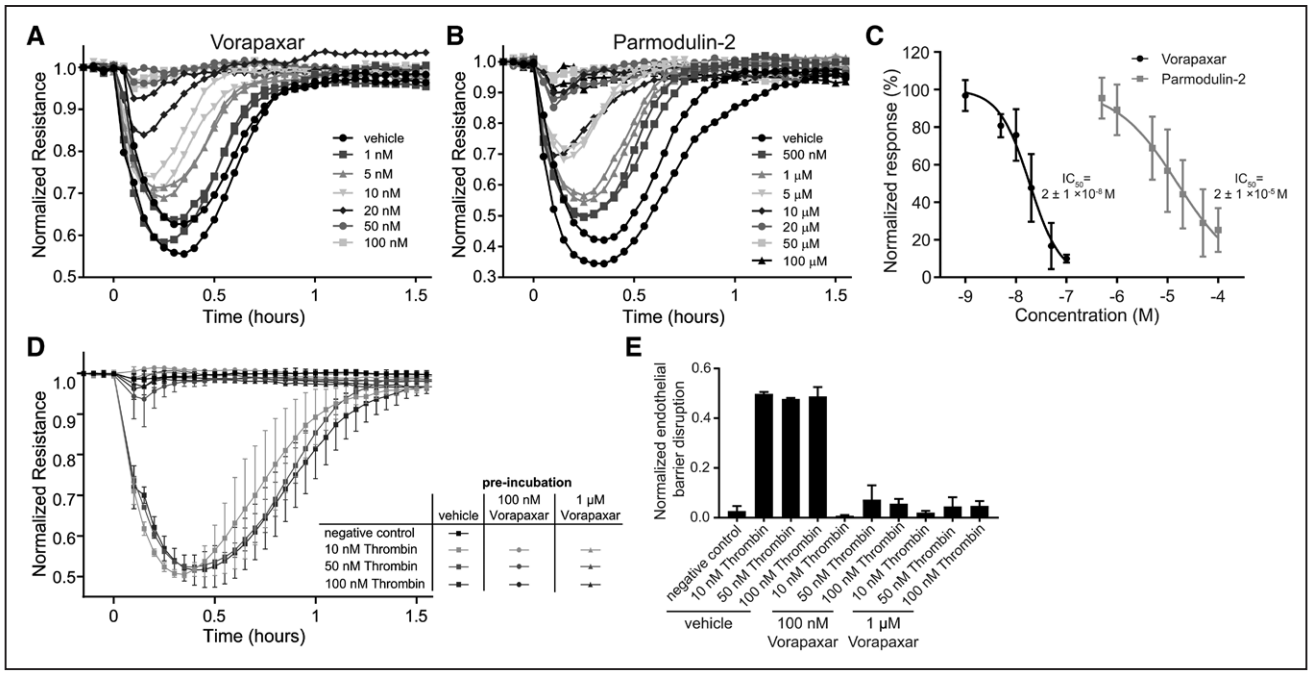

Figure 2. Vorapaxar and parmodulin-2 completely inhibit thrombin-induced endothelial barrier disruption. **A** and **B**, Blood outgrowth endothelial cells (BOECs) were preincubated for 1 h with indicated concentrations of vorapaxar (**A**), parmodulin-2 (**B**), or vehicle control (DMSO; dimethyl sulfoxide), followed by stimulation with 10 nmol/L thrombin (1 U/mL thrombin=10 nmol/L). Representative tracings of transendothelial electrical resistance after addition of thrombin (*t*=0 h) are shown. Values were normalized per sample to the resistance 9 min before the addition of thrombin. Experiments were performed in duplicates. **C**, Effect of vorapaxar and parmodulin-2 on thrombin-induced endothelial barrier disruption. Normalized response was calculated per sample by normalizing the minimal resistance after thrombin addition to the minimal resistance after thrombin addition in the vehicle control. Data and IC_{50} values represent mean±SD (n=3). IC_{50} values were calculated using log(inhibitor) vs normalized response–variable slope (Graphpad Prism version 6.04). **D**, BOECs were preincubated for 1 h with indicated concentrations of vorapaxar or vehicle control (DMSO), followed by stimulation with the indicated concentrations of thrombin (1 U/mL thrombin=10 nmol/L). Values were normalized per sample to the resistance 9 min before the addition of thrombin (*t*=0 h). Data represent mean±SD (n=3). **E**, Effect of vorapaxar preincubation on thrombin-mediated endothelial barrier disruption. Data as in **D**, endothelial barrier disruption was calculated per sample by calculating the minimal resistance after thrombin addition. Data represent mean±SD (n=3).

and in ECs stimulated with 50 pmol/L thrombin (Figure 3B). Regulated phosphosites included phosphosites with increased (241 sites; clusters 1–3) and decreased abundance (169 sites; clusters 4–6). Remarkably, phosphoregulation by thrombin and thrombin–TL peptide demonstrated a high degree of similarity, in terms of phosphorylation site, phosphorylation or dephosphorylation status, as well as temporal profile. This similarity was further supported by the Pearson correlation of the SILAC ratios (log_2) (Figure 3C). The appearance of minor differences between the impact of thrombin and thrombin–TL peptide was analyzed; however, no apparent differences in phosphoregulation were found. All together, these data show that thrombin and thrombin–TL peptide induce highly similar phosphoregulation. To study the thrombin concentration-dependent activation of PAR1, we compared phosphoregulation by thrombin at high (10 nmol/L) and low (50 pmol/L) concentrations. In sharp contrast to the extensive phosphoregulation by thrombin at high concentrations (279 regulated phosphosites, localized on 201 proteins), at low concentrations, thrombin only induced limited phosphoregulation (Figure III in the online-only Data Supplement). Although we cannot rule out that that the effects of low thrombin occur on a different time scale (hours), little evidence was found for biased PAR1 signaling by thrombin at low concentrations.

Vorapaxar and Parmodulin-2 Demonstrate Distinct Antagonist Properties for PAR1

To exclude potential direct side effects of PAR1 inhibition on ECs, we determined the effect of vorapaxar and parmodulin-2 on the EC steady state (phospho)proteome. We found no apparent changes in ECs at the (phospho)proteome level after 1 hour of preincubation with vorapaxar or parmodulin-2 (Tables III and IV in the online-only Data Supplement), indicating that PAR1 inhibition alone does not induce (short-term) protein expression differences or initiate signaling in ECs. Next, we determined the effect of both PAR1 inhibitors on thrombin-induced endothelial signaling. Vorapaxar almost completely blocked the extensive phosphoregulation by thrombin in ECs, whereas parmodulin-2 showed partial inhibition (Figure 3B; Table II in the online-only Data Supplement). To further dissect this observed differential antagonistic property, the phosphosites that were still regulated by thrombin in the presence of one or both PAR1 inhibitors (remaining regulated phosphosites) were analyzed (Figure 4). This showed that vorapaxar and parmodulin-2 differentially inhibit thrombin-mediated signaling (35 versus 100 remaining regulated phosphosites, respectively). Remaining regulated phosphosites in the presence of vorapaxar mainly showed opposing phosphoregulation compared with thrombin alone (Figure 4A), whereas those that remained

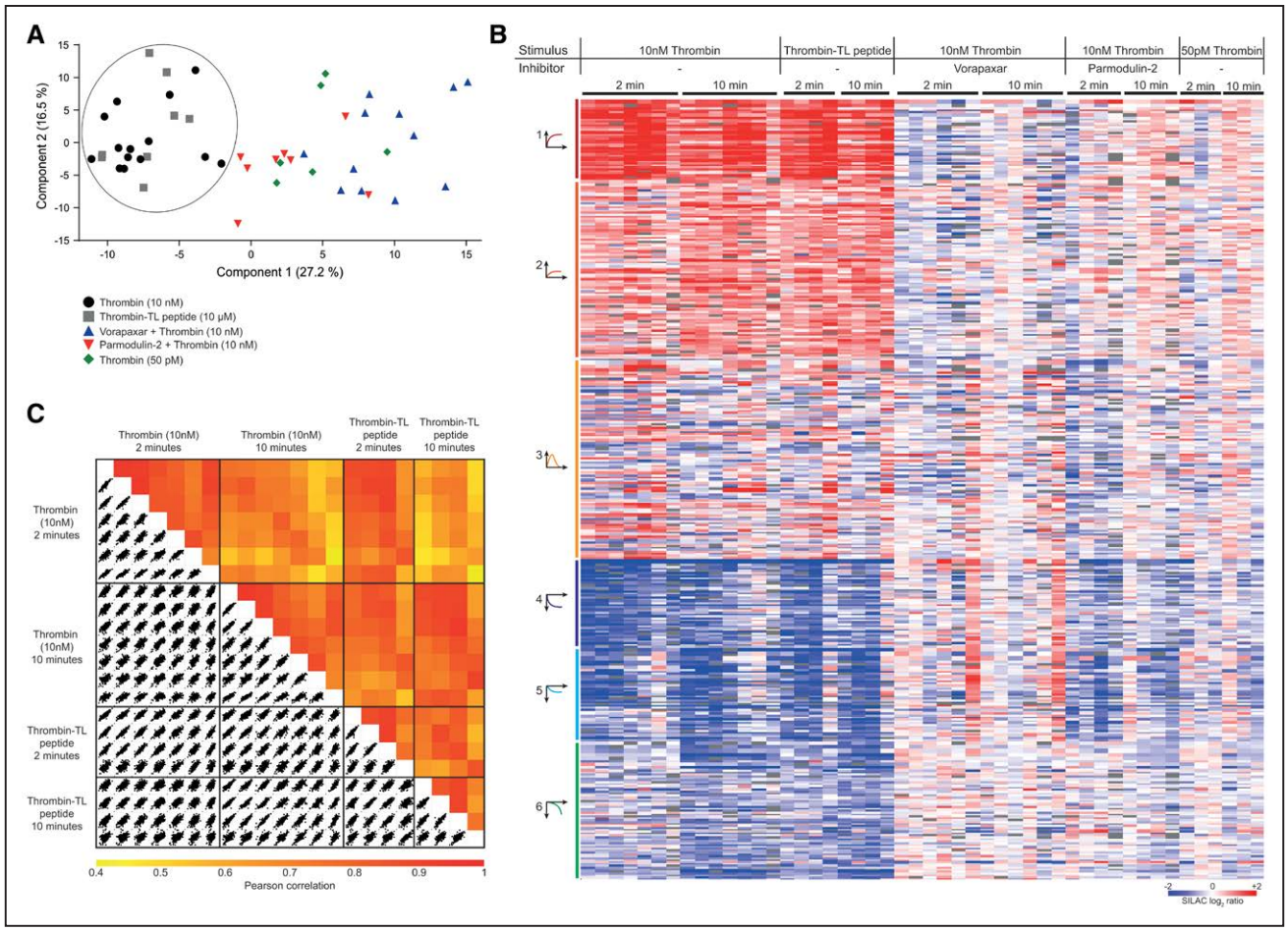

Figure 3. Thrombin and thrombin–tethered ligand (TL) peptide induce highly similar temporal phosphoregulation. **A**, Two components that capture 43.7% of the total variance of the blood outgrowth endothelial cell (BOEC) phosphoproteome changes during the 5 different stimulation conditions (thrombin alone [10 nmol/L or 50 pmol/L]; thrombin-TL peptide; and 10 nmol/L thrombin in the presence of either vorapaxar or parmodulin-2 [see legend]) are shown. Principal component analysis was performed on the quantified SILAC (Stable Isotope Labeling with Amino acids in Cell culture) ratios (n=2553). Replicates of both time points are shown per stimulation condition. Replicates of thrombin and thrombin–TL peptide cluster together, indicated by sphere. **B**, Heatmap and hierarchical clustering (based on average Euclidean distance and preprocessed with k-means) based on the SILAC $\log_2$ ratio of the regulated phosphosites (n=410). The 6 clusters discriminate between phosphosites with increased (clusters 1–3) and decreased abundance (clusters 4–6). Heatmap colors (see legend) are based on the SILAC $\log_2$ ratios reported in Table II in the online-only Data Supplement; gray indicates not quantified. **C**, Correlation plots and heatmap of Pearson correlation coefficients of thrombin and thrombin–TL peptide stimulated BOECs. Pearson correlation coefficients were calculated for each replicate and time point and were based on the SILAC $\log_2$ ratios of the regulated phosphorylation sites (n=410). Correlation plots (**lower left** half) and a heatmap of the Pearson correlation coefficients (**top right** half) are shown. Heatmap colors (see legend) are based on the Pearson correlation coefficient.

regulated in the presence of parmodulin-2 mainly resembled thrombin-mediated phosphoregulation (Figure 4B). Notably, the latter mainly concerned dephosphorylation events. To provide insight into pathways that were blocked by the PAR1 inhibitors, we constructed a PhosphoPath interaction map (Figure IV in the online-only Data Supplement). No unique pathway was revealed that was differentially affected by vorapaxar and parmodulin-2 (Figure IV in the online-only Data Supplement). However, there were clearly phosphosites that were completely blocked (Figure VA in the online-only Data Supplement) or differentially blocked (Figure VB in the online-only Data Supplement) by the PAR1 inhibitors. Because it has been described that parmodulin-2 selectively blocks $G\alpha_q$- over $G\alpha_{12/13}$-mediated activation,[15] we reconstructed the $G\alpha_q$ pathway based on the interactors of PRKD1 (serine/threonine-protein kinase D1; Figure 5). This revealed that early downstream effectors of $G\alpha_q$ are completely inhibited by vorapaxar, but only partially by parmodulin-2. Remarkably, phosphorylation of the downstream effectors of PRKD1, including Heat shock protein β-1 and Rab GTPase-binding effector protein 1 was completely inhibited by both PAR1 inhibitors, whereas phosphorylation of catenin δ-1, another potential downstream target of PRKD1, showed differential inhibition. Thrombin-specific phosphorylation of the transcription factor STAT3 (signal transducer and activator of transcription 3) on Ser-727[34] was completely inhibited by both PAR1 inhibitors (Figure VA in the online-only Data Supplement). Taken together, these data indicate that most, if not all, thrombin-mediated signaling in ECs is dependent on PAR1 activation and that PAR1 inhibitors vorapaxar and parmodulin-2 show differential antagonistic properties that result in biased activation of PAR1 by thrombin.

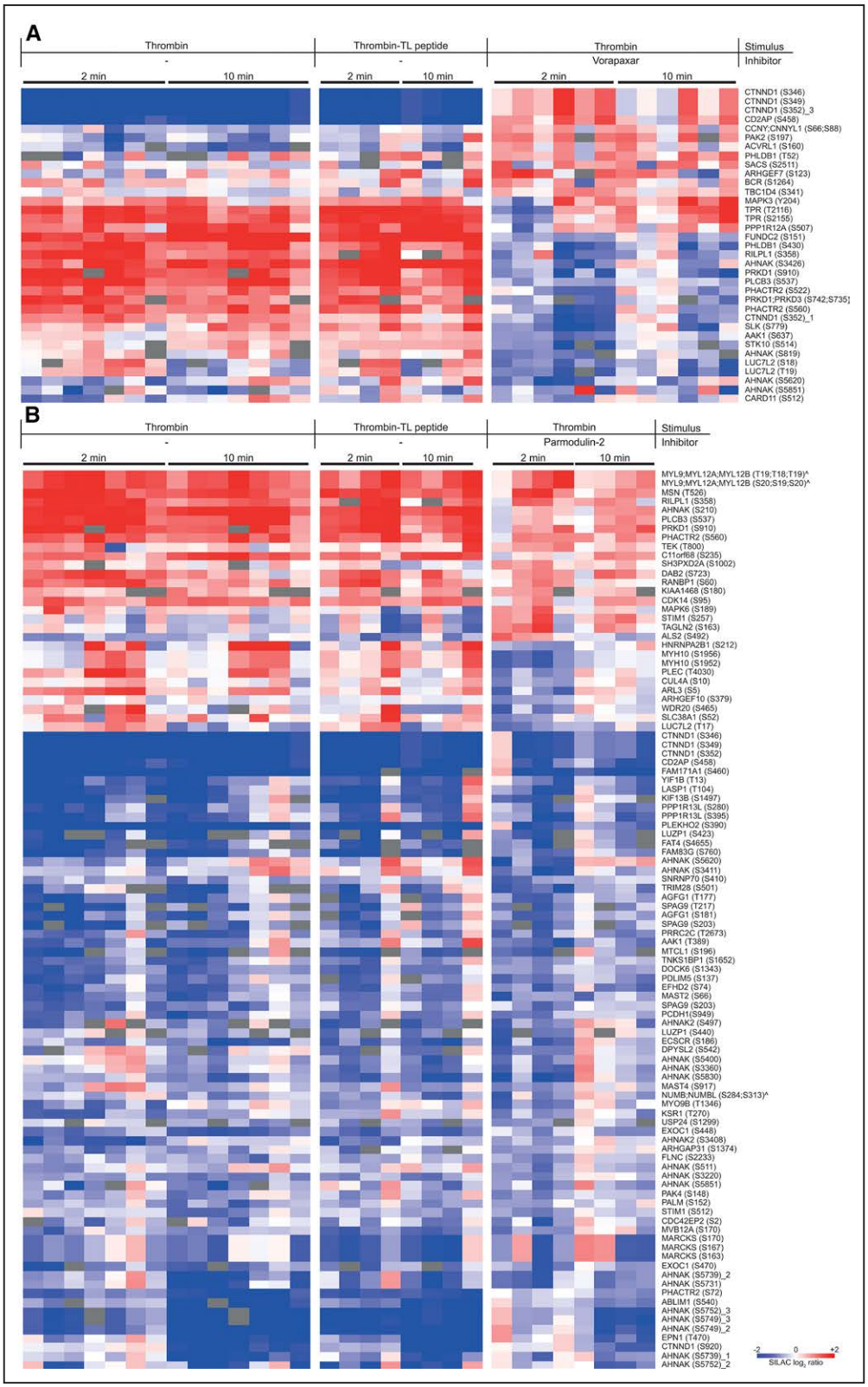

Figure 4. Remaining regulated phosphosites reveal distinct antagonistic properties for vorapaxar and parmodulin-2. Heatmaps based on the SILAC (Stable Isotope Labeling with Amino acids in Cell culture) $\log_2$ ratio of regulated phosphosites by 10 nmol/L thrombin (1 U/mL thrombin=10 nmol/L) in the presence of (**A**) vorapaxar (35 phosphosites) or (**B**) parmodulin-2 (100 phosphosites). SILAC $\log_2$ ratios of 10 nmol/L thrombin alone and 10 μmol/L thrombin–TL peptide are also shown. Gene names with modified amino acid are shown on the **right**. The reported amino acid position refers to the protein within the protein group for which a reference number (referred to as UniProtKB) was found in the PhosphoSitePlus database. Heatmap colors (see legend) are based on the SILAC $\log_2$ ratios reported in Table II in the online-only Data Supplement; gray indicates not quantified; ^, redundant site, _1, _2, or _3 indicate if the quantification of the phosphorylation site was based on singly (_1), doubly (_2), or multiply (_3) phosphorylated peptides.

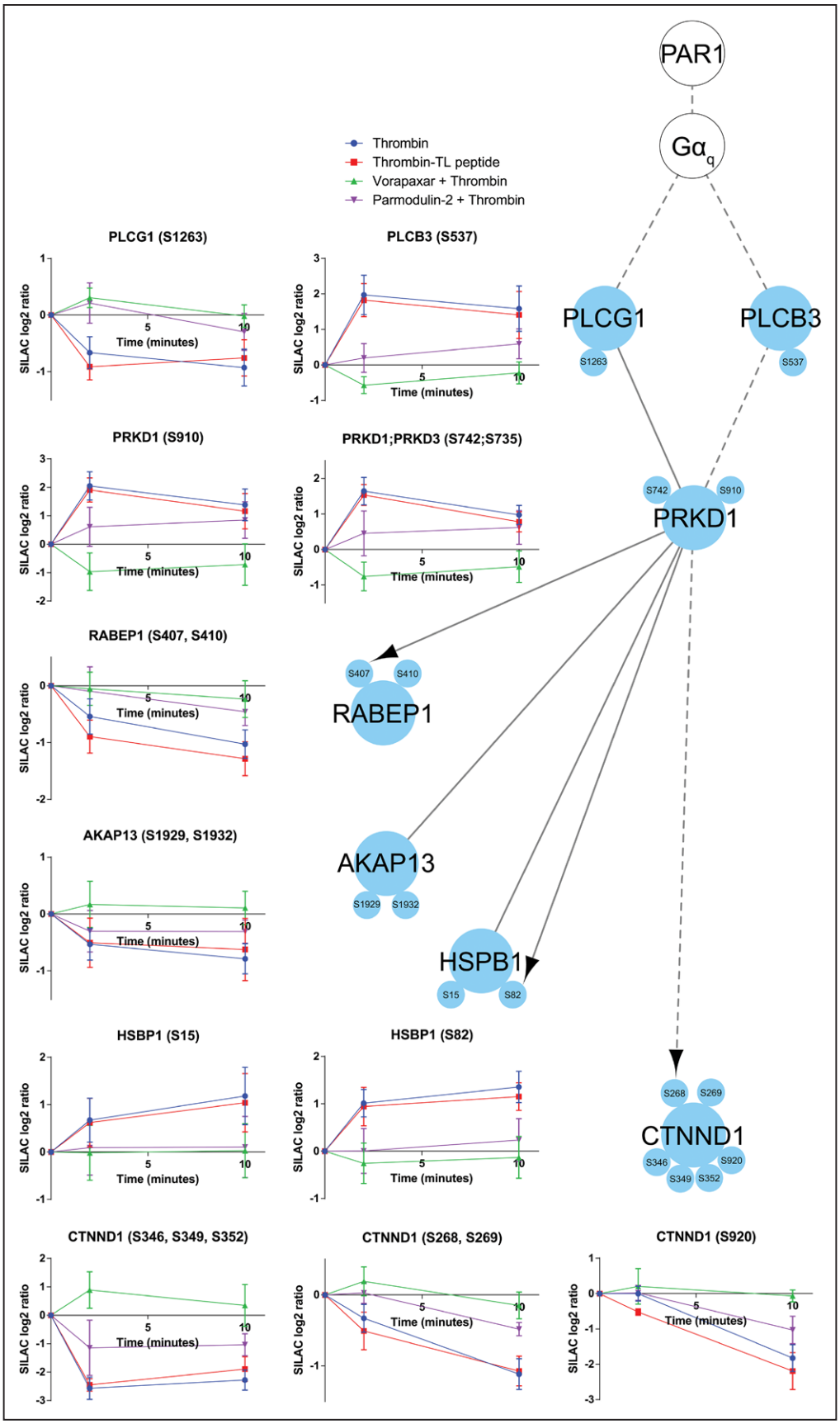

Figure 5. Visualization of PRKD1 (serine/threonine-protein kinase D1) interaction network. Gene names and localization of their phosphosites(s) are shown. A straight line visualizes protein–protein interactions from Biogrid, and kinase–substrate interactions from PhosphoSitePlus are visualized by an arrow. Proteins indicated as not-filled circles are added to the network. Dotted straight lines and dotted arrow (*Continued*)

In Contrast to Thrombin–TL Peptide, PR3–TL Peptide, MMP1–TL Peptide, NE–TL Peptide, and APC–TL Peptide Do Not Potently Induce Early Signaling in ECs

To study biased activation of differential PAR1 cleavage, we initially set out to study phosphoregulation by APC. APC is formed after cleavage of the zymogen protein C by thrombin[35]; therefore, hirudin is needed in all experiments to block residual thrombin.[16,36] Surprisingly, in our experimental conditions, hirudin itself had a stronger effect on EC phosphoregulation than APC (Figure VI in the online-only Data Supplement), preventing reliable phosphoproteomic analysis. Notably, activation of sphingosine 1-phosphate receptor-1 by SEW2871, which has been described to induce an increased endothelial barrier function,[37] showed distinct phosphoregulation (Figure VI in the online-only Data Supplement). Therefore, to study biased activation resulting from noncanonical cleavage of PAR1 by APC at Arg-46, we used the TL peptide that resembles APC cleavage of PAR1 and compared this with phosphoregulation by thrombin–TL peptide.[18] In addition, we took the opportunity to study biased activation of PAR1 by using the TL peptides that resemble PAR1 cleavage by PR3, MMP1, and NE, which have been shown to mimic the activation of PAR1 by the corresponding protease (Figure 6A and 6B).[19,20] For these 5 TL peptides, we performed a time-resolved phosphoproteomic analysis of stimulated blood outgrowth endothelial cells (Materials and Methods in the online-only Data Supplement). Mass spectrometry data were analyzed as described before, resulting in the identification of 9324 accurately localized (class I[32]) phosphosites, of which 477 (5.1%) have not been described before (PhosphoSitePlus 20 March 2017[33]) (Table V in the online-only Data Supplement). In total, 2319 phosphosite were quantified in both experiments, and 274 phosphosites, localized on 194 proteins, were found to be regulated in both experiments (Materials and Methods and Table VI in the online-only Data Supplement). Despite the different time points of stimulation between the thrombin/PAR1 inhibitor data set and the TL peptides data set, the quantified phosphosites show a large similarity (Figure IIB in the online-only Data Supplement). In contrast to thrombin–TL peptide, the noncanonical PAR1 TL peptides only induced limited early phosphoregulation (Figure 6C). Moreover, sites that were found to be regulated were surprisingly similar for each noncanonical TL peptide and mostly resembled the phosphorylation pattern of thrombin–TL peptide. Only a limited number of phosphosites showed opposing phosphoregulation by the noncanonical PAR1–TL peptides compared with thrombin–TL peptide (Figure 6D). In conclusion, these data show that the TL peptide that is formed after canonical cleavage of PAR1 by thrombin potently induces early phosphoregulation, whereas noncanonical PAR1–TL peptides only showed limited phosphoregulation in ECs.

Discussion

Thrombin-Induced Endothelial Signaling Is Mediated Exclusively Through PAR1

PAR1 is the predominant thrombin receptor on ECs; however, it has remained unclear whether PAR1 alone accounts for all thrombin effects in ECs. Dissecting the contribution of PAR1 in thrombin signaling in ECs is particularly relevant because PAR1 has become a therapeutic target for antithrombotic therapies. Here, we show that both orthosteric and allosteric PAR1 inhibitors not only specifically block thrombin–TL peptide–mediated platelet aggregation (Figure 1) and thrombin-mediated EC activation (Figure 2) but also block thrombin-induced signaling in ECs, albeit to a different extent (Figure 3). It has been shown that thrombin at high concentrations can directly activate PAR2.[7] However, we showed that in blood outgrowth endothelial cells, the thrombin-mediated endothelial barrier disruption is completely dependent on PAR1 activation (Figure 2D and 2E), suggesting that the specificity of thrombin may be context dependent. Using a complementary approach, we compared the possible biased activation of thrombin–TL peptide and thrombin cleavage of PAR1.[11] We showed that classical activation of PAR1 by thrombin and peptide activation by thrombin–TL peptide induce highly similar phosphoregulation in ECs (Figure 3), which implies that despite the difference in affinity of the TL formed after thrombin cleavage and the soluble thrombin–TL peptide,[23] similar signaling networks are activated. Taken together, our system-wide phosphoproteomics approach reveals that most, if not all, thrombin-mediated signaling in ECs is dependent on PAR1.

Mechanistically Distinct PAR1 Inhibitors Induce Biased Signaling

Orthosteric PAR1 inhibitors (vorapaxar and atopaxar) have been shown to increase the risk of bleeding, a side effect that has been linked to global inhibition of PAR1 on cells other than platelets, most notably ECs.[27] In the quest for a PAR1 antagonist that prevents coagulation without promoting bleeding, several allosteric PAR1 inhibitors have been developed that bind to the intracellular side of the receptor (pepducins[28,29] and parmodulins[14,15]). For parmodulin-2, it has been postulated that it promotes biased PAR1 signaling by preferentially blocking $G\alpha_q$- over $G\alpha_{12/13}$-mediated PAR1 signaling.[15] However, it remained unknown how parmodulin-2 blocks these different signaling networks. We found that vorapaxar and parmodulin-2 differentially inhibit thrombin-induced phosphoregulation (Figure 3). On the one hand, vorapaxar blocked virtually all thrombin-induced phosphoregulation in ECs, supporting the hypothesis that thrombin-induced phosphoregulation in ECs is dependent on PAR1 activation, whereas on the other hand, parmodulin-2 showed only partial inhibition (Figures 3 and 4). Notably, these remaining regulated phosphosites mostly contained phosphosites with a decreased abundance, suggesting

Figure 5 Continued. are added to the network based on literature. Interaction network was made using the PhosphoPath plug-in[55] from Cytoscape.[56] Graphs represent mean and SD for the MS-based quantifications of the indicated phosphosites (see Figure IC in the online-only Data Supplement for N values per stimulation condition). Mass spectrometry-quantifications of thrombin (10 nmol/L), thrombin-tethered ligand (TL) peptide, and 10 nmol/L thrombin in the presence of either vorapaxar or parmodulin-2 are shown; AKAP13 indicates A-kinase anchor protein 13; CTNND1, catenin δ-1; HSPB1, heat shock protein β-1; PAR1, protease-activated receptor-1; PLCG1, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ-1; and RABEP1, Rab GTPase-binding effector protein 1.

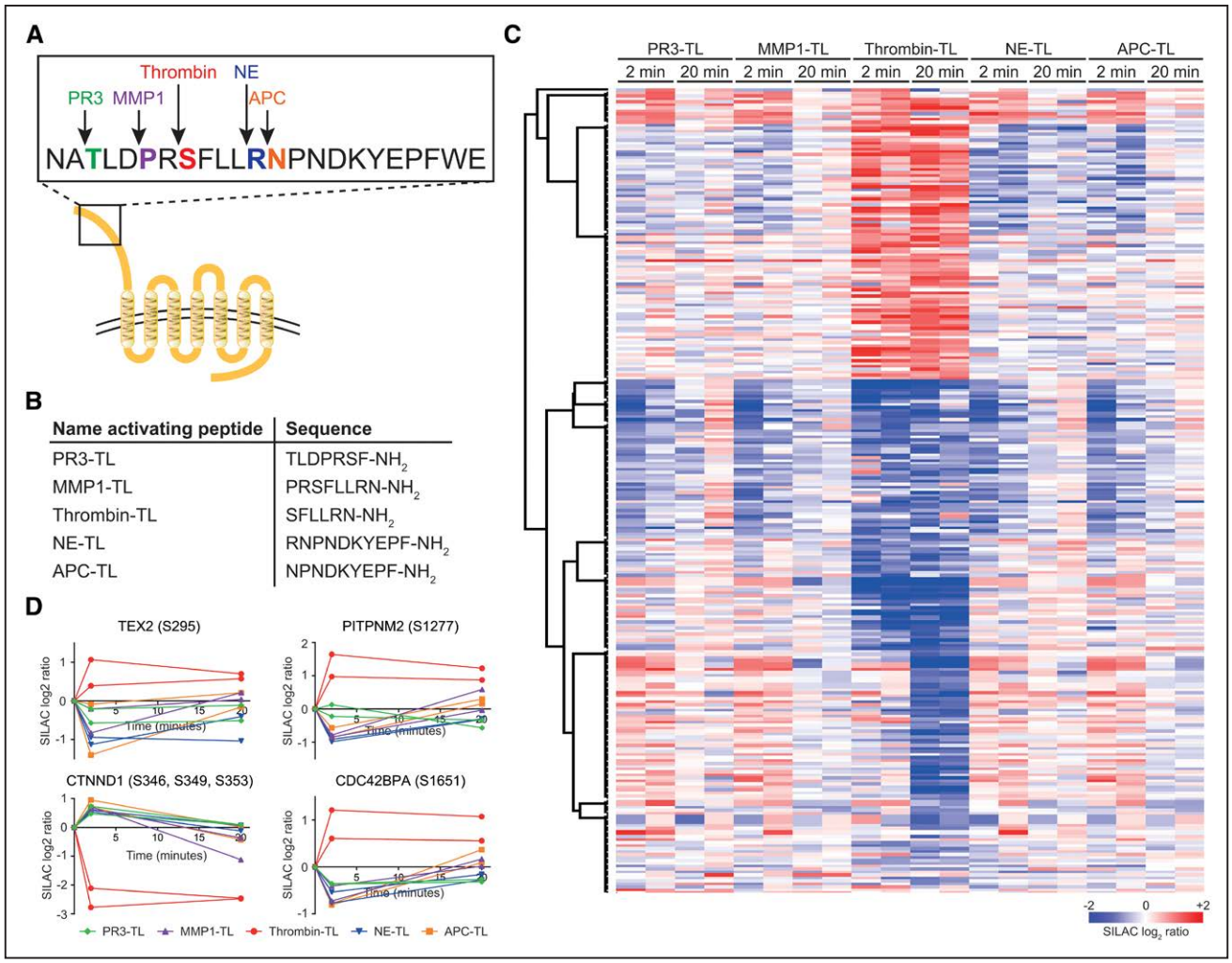

Figure 6. Only canonical PAR1 (protease-activated receptor-1) cleavage by thrombin generates a tethered ligand (TL) that potently induces early signaling in endothelial cells (ECs). **A**, Schematic representation of the PAR1 N terminus with the known cleavage sites of the proteases depicted; PR3 (neutrophil protease; Ala-36), MMP1 (matrix metalloproteinase-1; Asp-39), thrombin (Arg-41), NE (neutrophil elastase; Leu-45), and APC (activated protein C; Arg-46). **B**, Synthetic peptides that mimic the TL after cleavage of the indicated protease were used to mimic PAR1 activation by the corresponding protease. Starting amino acid of each TL peptide is indicated in **A**. **C**, Heatmap and hierarchical clustering (based on average Euclidean distance and preprocessed with k-means) based on the SILAC (Stable Isotope Labeling with Amino acids in Cell culture) $\log_2$ ratio of regulated phosphosites in the TL activation peptides data set (n=274). Blood outgrowth endothelial cells were stimulated with the indicated TL peptides (50 μmol/L). Heatmap colors (see legend) are based on the SILAC $\log_2$ ratios reported in Table VI in the in the online-only Data Supplement. **D**, Mass spectrometry-based quantifications of the indicated phosphosites, quantifications of both replicates are shown.

that parmodulin-2 may be unable to block a thrombin-activated phosphatase. PhosphoPath network analysis of the remaining regulated phosphosites showed that, despite the fact that there were phosphosites that were completely or differentially blocked (Figure V in the online-only Data Supplement), there was not 1 uniquely affected pathway (Figure IV in the online-only Data Supplement). However, detailed analysis of the $G\alpha_q$-mediated phosphorylation downstream of PRKD1 revealed that these processes were differentially inhibited (Figure 5; Figure V in the online-only Data Supplement). The early effectors downstream of $G\alpha_q$ are all completely inhibited by vorapaxar, whereas parmodulin-2 completely inhibits phosphorylation of 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ-1, but only partially inhibits phosphorylation of PLCB3 (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase β-3) and PRKD1 (Figure 5). These phosphosites include Ser-537 on PLCB3, which is located in the X-Y linker of PLCB3 that is close to the N-terminal coiled-coil domain of PLCB3 and the N terminus of $G\alpha_q$ in the crystal structure of the complex.[38] The location of this phosphosite suggests a link to activation of PLCB3 and would imply that PLCB3 activation is completely inhibited by vorapaxar and only partially by parmodulin-2. Some of the downstream effectors and the kinase-substrate–related phosphosites downstream of PRKD1 are completely inhibited by both PAR1 inhibitors, whereas the dephosphorylation of catenin δ-1, an interesting potential downstream target of PRKD1,[39,40] is differentially inhibited. Thrombin-specific phosphorylation of STAT3 on Ser-727[34] is completely inhibit by both PAR1 inhibitors (Figure 6B), which suggests that STAT3-mediated gene regulation is completely blocked. Taken together, vorapaxar and parmodulin-2 demonstrate distinct antagonistic properties for PAR1 on ECs. Our data corroborate the finding of

Aisiku et al[15] that parmodulin-2 seems to induce biased activation of PAR1 by thrombin. However, from our data, it seems that biased activation of PAR1 in the presence of parmodulin-2 is much more complex and not solely linked to a specific G-protein downstream of PAR1.

Biased Activation of PAR1: Canonical Versus Noncanonical Cleavage

To determine biased activation of PAR1 on ECs, we compared the phosphoregulation induced by thrombin at low and high concentrations as well as by the canonical thrombin–TL peptide and the noncanonical TL peptides PR3–TL, MMP1–TL, NE–TL, and APC–TL. In contrast to our expectations, these 4 noncanonical PAR peptides only showed limited early phosphoregulation (Figure 6), which was highly similar and mostly resembled thrombin–TL peptide–mediated phosphoregulation. This seems to be at variance with their hypothesized induction of biased signaling.[18–20] In addition, our data revealed only limited phosphoregulation by thrombin at low concentrations (Figure V in the online-only Data Supplement). Strikingly, the few phosphosites that remained regulated under these conditions mostly overlapped with those that were regulated by thrombin at high concentrations, suggesting that they represent the most thrombin-sensitive phosphosites (Figure V in the online-only Data Supplement). The same was true for the remaining regulated phosphosites in the presence of parmodulin-2, which explains the appearance of overlap in the principal component analysis (Figure 3A). In contrast, phosphosites that remained regulated in the presence of vorapaxar mainly showed opposing phosphoregulation. β-arrestin–mediated phosphoregulation by PAR1 has been described to play a role in the signal transduction of APC.[22] In our data set, we identified 1 phosphosite on β-arrestin-1, Ser-412, which has been described in the activation of the G-protein–coupled receptor 5-hydroxytryptamine receptor 4.[41] However, this phosphosites was not regulated in any of the stimulation conditions during the time course of our experiments. Because we only analyzed the signaling transduction in ECs in the first 10 to 20 minutes, and the barrier-protective effects of thrombin and APC have been shown to occur on a longer time scale (hours),[16–18,36] we may not have covered the barrier-protective phosphoregulation or the β-arrestin–dependent biased activation of PAR1. In addition, we cannot rule out that functional assays with respect to PAR1-mediated barrier-protective effects are more sensitive than our phosphoproteomics approach.

PAR1 Inhibition: More Than Platelets

Here, we show that the Food and Drug Administration–approved PAR1-directed antiplatelet drug vorapaxar virtually inhibits all thrombin-induced signaling in ECs. Notably, the vorapaxar concentration used in our study (100 nmol/L) lies within the range of steady state plasma levels observed in healthy individuals during phase I clinical trials.[42] In view of the putative protective role of PAR1 in maintaining endothelial barrier integrity, our data provide an possible explanation for the increased bleeding tendency of this drug.[27] Given the crucial role of endothelial PAR1, our study implies that phosphoproteomic studies may help in the drug design process by identifying the effects of PAR1-directed antiplatelet drugs in the endothelium, in a similar way as the use of quantitative mass spectrometry for profiling of kinase inhibitors.[43] Besides the role of PAR1 in coagulation, PAR1 has been linked to various other (patho)physiological conditions, including gastrointestinal and central nervous system diseases and cancer.[44] Within these diverse environments, PAR1 has also been studied as a drug target, as reviewed by Ramachandran et al.[44] Surprisingly, PAR1 has been identified as important marker for retention and recruitment of endothelial protein C receptor–expressing bone marrow hematopoietic stem cells.[45] In addition, diplopia has been observed in patients taking vorapaxar, showing the crucial role of PARs in the eye.[46] In summary, given the multiple signal pathways triggered by PAR1 in diverse tissues, it will be a challenge to design a PAR1 antagonist that will target only those pathways responsible for tissue pathology.

Future Directions

Here, we have shown that thrombin signals to ECs exclusively through PAR1. Other serine proteases of the coagulation cascade, like factor VIIa and factor Xa target not only PAR1 but also PAR2.[47,48] In addition, both PAR1 and PAR4 are important for thrombin-mediated platelet aggregation.[49] Although it has been described that PAR1, PAR2, and PAR4 activation can be linked to overlapping and distinct activation of G proteins and functional outcome,[50–52] to what extent all PARs induce similar signaling pathways is a subject for further studies. In addition, it remains an open question to what extent feed-forward signaling by thrombin-induced secreted agonist or proteins (including matrix metalloproteases[53,54] and sphingosine 1-phosphate[16]) contributes to the thrombin-induced signal transduction pathways. Unraveling the PAR-induced signaling pathways will help in dissecting the intimate interplay of the coagulation cascade and the vessel wall. This will aid in the understanding of PARs as a therapeutic target in the treatment of cardiovascular diseases, hemostatic disorders, and cancer.

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Disclosures

None.

References

1. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000;407:258–264. doi: 10.1038/35025229.
2. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991;64:1057–1068.
3. Hollenberg MD, Compton SJ. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol Rev*. 2002;54:203–217.

4. Martorell L, Martínez-González J, Rodríguez C, Gentile M, Calvayrac O, Badimon L. Thrombin and protease-activated receptors (PARs) in atherothrombosis. *Thromb Haemost*. 2008;99:305–315. doi: 10.1160/TH07-08-0481.
5. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arterioscler Thromb Vasc Biol*. 2006;26:1002–1007. doi: 10.1161/01.ATV.0000209501.56852.6c.
6. van den Biggelaar M, Hernández-Fernaud JR, van den Eshof BL, Neilson LJ, Meijer AB, Mertens K, Zanivan S. Quantitative phosphoproteomics unveils temporal dynamics of thrombin signaling in human endothelial cells. *Blood*. 2014;123:e22–e36. doi: 10.1182/blood-2013-12-546036.
7. Mihara K, Ramachandran R, Saifeddine M, Hansen KK, Renaux B, Polley D, Gibson S, Vanderboor C, Hollenberg MD. Thrombin-mediated direct activation of proteinase-activated receptor-2: another target for thrombin signaling. *Mol Pharmacol*. 2016;89:606–614. doi: 10.1124/mol.115.102723.
8. Kenakin T, Christopoulos A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov*. 2013;12:205–216. doi: 10.1038/nrd3954.
9. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. *Front Endocrinol (Lausanne)*. 2014;5:1–16.
10. Chen J, Ishii M, Wang L, Ishii K, Coughlin SR. Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J Biol Chem*. 1994;269:16041–16045.
11. McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedetto E, Hamm HE. Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J Biol Chem*. 2005;280:25048–25059. doi: 10.1074/jbc.M414090200.
12. Blackhart BD, Ruslim-Litrus L, Lu CC, Alves VL, Teng W, Scarborough RM, Reynolds EE, Oksenberg D. Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. *Mol Pharmacol*. 2000;58:1178–1187.
13. Vouret-Craviari V, Van Obberghen-Schilling E, Rasmussen UB, Pavirani A, Lecocq JP, Pouysségur J. Synthetic alpha-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. *Mol Biol Cell*. 1992;3:95–102.
14. Dockendorff C, Aisiku O, Verplank L, Dilks JR, Smith DA, Gunnink SF, Dowal L, Negri J, Palmer M, Macpherson L, Schreiber SL, Flaumenhaft R. Discovery of 1,3-diaminobenzenes as selective inhibitors of platelet activation at the par1 receptor. *ACS Med Chem Lett*. 2012;3:232–237. doi: 10.1021/ml2002696.
15. Aisiku O, Peters CG, De Ceunynck K, Ghosh CC, Dilks JR, Fustolo-Gunnink SF, Huang M, Dockendorff C, Parikh SM, Flaumenhaft R. Parmodulins inhibit thrombus formation without inducing endothelial injury caused by vorapaxar. *Blood*. 2015;125:1976–1985. doi: 10.1182/blood-2014-09-599910.
16. Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood*. 2005;105:3178–3184. doi: 10.1182/blood-2004-10-3985.
17. Bae JS, Kim YU, Park MK, Rezaie AR. Concentration dependent dual effect of thrombin in endothelial cells via Par-1 and Pi3 Kinase. *J Cell Physiol*. 2009;219:744–751. doi: 10.1002/jcp.21718.
18. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. *Blood*. 2012;120:5237–5246. doi: 10.1182/blood-2012-08-452169.
19. Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K, Covic L, Kuliopulos A. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell*. 2009;137:332–343. doi: 10.1016/j.cell.2009.02.018.
20. Mihara K, Ramachandran R, Renaux B, Saifeddine M, Hollenberg MD. Neutrophil elastase and proteinase-3 trigger G protein-biased signaling through proteinase-activated receptor-1 (PAR1). *J Biol Chem*. 2013;288:32979–32990. doi: 10.1074/jbc.M113.483123.
21. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: biased for translation. *Blood*. 2015;125:2898–2907. doi: 10.1182/blood-2015-02-355974.
22. Soh UJ, Trejo J. Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds. *Proc Natl Acad Sci U S A*. 2011;108:E1372–E1380. doi: 10.1073/pnas.1112482108.
23. Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, Green HF, Pandey A, Dror RO, Shaw DE, Weis WI, Coughlin SR, Kobilka BK. High-resolution crystal structure of human protease-activated receptor 1. *Nature*. 2012;492:387–392. doi: 10.1038/nature11701.
24. Morrow DA, Braunwald E, Bonaca MP, et al; TRA 2P–TIMI 50 Steering Committee and Investigators. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366:1404–1413. doi: 10.1056/NEJMoa1200933.
25. Magnani G, Bonaca MP, Braunwald E, Dalby AJ, Fox KA, Murphy SA, Nicolau JC, Oude Ophuis T, Scirica BM, Spinar J, Theroux P, Morrow DA. Efficacy and safety of vorapaxar as approved for clinical use in the United States. *J Am Heart Assoc*. 2015;4:e001505. doi: 10.1161/JAHA.114.001505.
26. Lee M, Saver JL, Hong KS, Wu HC, Ovbiagele B. Risk of intracranial hemorrhage with protease-activated receptor-1 antagonists. *Stroke*. 2012;43:3189–3195. doi: 10.1161/STROKEAHA.112.670604.
27. Ramachandran R. Developing PAR1 antagonists: minding the endothelial gap. *Discov Med*. 2012;13:425–431.
28. Zhang P, Gruber A, Kasuda S, Kimmelstiel C, O'Callaghan K, Cox DH, Bohm A, Baleja JD, Covic L, Kuliopulos A. Suppression of arterial thrombosis without affecting hemostatic parameters with a cell-penetrating PAR1 pepducin. *Circulation*. 2012;126:83–91. doi: 10.1161/CIRCULATIONAHA.112.091918.
29. Kuliopulos A, Covic L. Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. *Life Sci*. 2003;74:255–262.
30. Chackalamannil S, Wang Y, Greenlee WJ, Hu Z, Xia Y, Ahn HS, Boykow G, Hsieh Y, Palamanda J, Agans-Fantuzzi J, Kurowski S, Graziano M, Chintala M. Discovery of a novel, orally active himbacine-based thrombin receptor antagonist (SCH 530348) with potent antiplatelet activity. *J Med Chem*. 2008;51:3061–3064. doi: 10.1021/jm800180e.
31. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26:1367–1372. doi: 10.1038/nbt.1511.
32. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell*. 2006;127:635–648. doi: 10.1016/j.cell.2006.09.026.
33. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res*. 2015;43(Database issue):D512–D520. doi: 10.1093/nar/gku1267.
34. Waitkus MS, Chandrasekharan UM, Willard B, Haque SJ, DiCorleto PE. STAT3-mediated coincidence detection regulates noncanonical immediate early gene induction. *J Biol Chem*. 2013;288:11988–12003. doi: 10.1074/jbc.M112.428516.
35. Esmon CT. Molecular events that control the protein C anticoagulant pathway. *Thromb Haemost*. 1993;70:29–35.
36. Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood*. 2007;110:3909–3916. doi: 10.1182/blood-2007-06-096651.
37. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest*. 2001;108:689–701. doi: 10.1172/JCI12450.
38. Lyon AM, Dutta S, Boguth CA, Skiniotis G, Tesmer JJ. Full-length Gα(q)-phospholipase C-β3 structure reveals interfaces of the C-terminal coiled-coil domain. *Nat Struct Mol Biol*. 2013;20:355–362. doi: 10.1038/nsmb.2497.
39. Konstantoulaki M, Kouklis P, Malik AB. Protein kinase C modifications of VE-cadherin, p120, and beta-catenin contribute to endothelial barrier dysregulation induced by thrombin. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L434–L442. doi: 10.1152/ajplung.00075.2003.
40. Xia X, Mariner DJ, Reynolds AB. Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin. *Biochemistry*. 2003;42:9195–9204. doi: 10.1021/bi034597h.
41. Barthet G, Carrat G, Cassier E, et al. Beta-arrestin1 phosphorylation by GRK5 regulates G protein-independent 5-HT4 receptor signalling. *EMBO J*. 2009;28:2706–2718. doi: 10.1038/emboj.2009.215.
42. Kosoglou T, Reyderman L, Tiessen RG, van Vliet AA, Fales RR, Keller R, Yang B, Cutler DL. Pharmacodynamics and pharmacokinetics of the novel PAR-1 antagonist vorapaxar (formerly SCH 530348) in healthy subjects. *Eur J Clin Pharmacol*. 2012;68:249–258. doi: 10.1007/s00228-011-1120-6.
43. Médard G, Pachl F, Ruprecht B, Klaeger S, Heinzlmeir S, Helm D, Qiao H, Ku X, Wilhelm M, Kuehne T, Wu Z, Dittmann A, Hopf C, Kramer K,

Kuster B. Optimized chemical proteomics assay for kinase inhibitor profiling. *J Proteome Res*. 2015;14:1574–1586. doi: 10.1021/pr5012608.
44. Ramachandran R, Noorbakhsh F, Defea K, Hollenberg MD. Targeting proteinase-activated receptors: therapeutic potential and challenges. *Nat Rev Drug Discov*. 2012;11:69–86. doi: 10.1038/nrd3615.
45. Gur-Cohen S, Itkin T, Chakrabarty S, et al. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. *Nat Med*. 2015;21:1307–1317. doi: 10.1038/nm.3960.
46. Serebruany VL, Fortmann SD, Rao SV, Tanguay JF, Lordkipanidze M, Hanley DF, Can M, Kim MH, Marciniak TA. Vorapaxar and diplopia: possible off-target PAR-receptor mismodulation. *Thromb Haemost*. 2016;115:905–910. doi: 10.1160/TH15-11-0882.
47. Rana S, Yang L, Hassanian SM, Rezaie AR. Determinants of the specificity of protease-activated receptors 1 and 2 signaling by factor Xa and thrombin. *J Cell Biochem*. 2012;113:977–984. doi: 10.1002/jcb.23427.
48. Larsen KS, Ostergaard H, Olsen OH, Bjelke JR, Ruf W, Petersen LC. Engineering of substrate selectivity for tissue factor.factor VIIa complex signaling through protease-activated receptor 2. *J Biol Chem*. 2010;285:19959–19966. doi: 10.1074/jbc.M110.101030.
49. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*. 1999;103:879–887. doi: 10.1172/JCI6042.
50. Klarenbach SW, Chipiuk A, Nelson RC, Hollenberg MD, Murray AG. Differential actions of PAR2 and PAR1 in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases. *Circ Res*. 2003;92:272–278.
51. McCoy KL, Traynelis SF, Hepler JR. PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. *Mol Pharmacol*. 2010;77:1005–1015. doi: 10.1124/mol.109.062018.
52. Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem*. 2000;275:25216–25221. doi: 10.1074/jbc.M004589200.
53. Wang L, Luo J, He S. Induction of MMP-9 release from human dermal fibroblasts by thrombin: involvement of JAK/STAT3 signaling pathway in MMP-9 release. *BMC Cell Biol*. 2007;8:14. doi: 10.1186/1471-2121-8-14.
54. Koo BH, Han JH, Yeom YI, Kim DS. Thrombin-dependent MMP-2 activity is regulated by heparan sulfate. *J Biol Chem*. 2010;285:41270–41279. doi: 10.1074/jbc.M110.171595.
55. Raaijmakers LM, Giansanti P, Possik PA, Mueller J, Peeper DS, Heck AJ, Altelaar AF. PhosphoPath: visualization of phosphosite-centric dynamics in temporal molecular networks. *J Proteome Res*. 2015;14:4332–4341. doi: 10.1021/acs.jproteome.5b00529.
56. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498–2504. doi: 10.1101/gr.1239303.

Highlights

- Thrombin signaling in endothelial cells is mediated exclusively via PAR1 (protease-activated receptor-1).
- Classical and peptide activation of PAR1 initiate highly similar signaling pathways.
- The recently Food and Drug Administration–approved orthosteric PAR1-directed antiplatelet drug vorapaxar and the allosteric PAR1 inhibitor parmodulin-2 reveal differential antagonistic properties.
- In contrast to peptides that mimic noncanonical cleavage of PAR1, only the tethered ligand peptide that mimics canonical cleavage of PAR1 by thrombin potently induces early signaling in endothelial cells.