Paradigm of biased PAR1 activation and inhibition in endothelial cells dissected by phosphoproteomics

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Abstract

Objective: Thrombin is the key serine protease of the coagulation cascade and mediates cellular responses by activation of protease-activated receptors (PARs). The predominant thrombin receptor is PAR1 and in endothelial cells (ECs) thrombin dynamically regulates a plethora of phosphorylation events. However, it has remained unclear if 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 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 demonstrates that thrombin-induced EC phosphoregulation is mediated exclusively through PAR1, that thrombin and thrombin-TL 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 it will be a challenge to design a PAR1 antagonist that will target only those pathways responsible for tissue pathology.

Nonstandard Abbreviations and Acronyms

APC	Activated protein C
BOEC	Blood outgrowth endothelial cell
EC	Endothelial cell
GPCR	G-protein coupled receptor
MMP1	Matrix metalloproteinase-1
NE	Neutrophil elastase
PR3	Neutrophil protease 3
PAR	Protease-activated receptor
PLCB3	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3
PRKD1	Serine/threonine-protein kinase D1
STAT3	Signal transducer and activator of transcription 3
TL	Tethered ligand

Introduction

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 protease-activated receptors (PARs), a family of four G-protein coupled receptors (GPCRs): 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 alpha-granules, while thrombin activation of ECs leads to exocytosis of the von Willebrand Factor containing Weibel-Palade bodies and a decreased endothelial barrier function which promotes leukocyte extravasation.[3–5]

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

Like many GPCRs, PAR1 is subject to ligand-dependent preferential activation of downstream targets, a process known as biased signaling.[8] Several mechanisms have been described9, 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 beta-arrestins-mediated signaling.[21,22] The molecular mechanism of these distinct modes of biased PAR1 activation have 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 anti-thrombotic 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 FDA.[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 (non-canonical PAR1 cleavage), resulting in biased signaling in ECs.[9] The cytoprotective effects of activated protein C (APC) are mediated via PAR1 (cleavage at Arg-46)[18], and also Matrix metalloproteinase-1 (MMP1), Neutrophil elastase (NE) and Neutrophil protease 3 (PR3) 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 non-canonical cleavage of PAR1. Finally, thrombin itself has also been suggested to induce biased signaling in ECs depending on its concentration; at high concentrations (>100 pM) thrombin causes a barrier-disruptive response in ECs, whereas at low concentrations (50 pM) the effect of thrombin is believed to be barrier-protective.[16,17] While 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 results in highly similar signaling. Furthermore, thrombin at low concentrations initiates only limited phosphoregulation. Thrombin-mediated signaling in ECs is completely blocked by vorapaxar, while thrombin seems to induce biased activation of PAR1 in presence of parmodulin-2. Finally we show that, in contrast to the non-canonical 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 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 ± 1 ×

10^{-8} M (mean ± standard deviation (sd)), parmodulin-2: 7 ± 1 × 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, while 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 ± 1 × 10^{-8} M, parmodulin-2: 2 ± 1 × 10^{-6} M (Figure 2A,B,C). In addition, the role of other receptors than PAR1 in the thrombin-mediated endothelial barrier disruption was determined. Thrombin-mediated endothelial barrier disruption was completely blocked by vorapaxar, even at a thrombin concentration of 100nM (Figure 2D, E). 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 nM) and low (50 pM) 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 (BOECs) by using a three-way reverse SILAC labeling strategy (Supplementary Figure I and materials and methods). Time points of stimulation were chosen based on our previous study of thrombin signaling.[6] MS 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]) (Supplementary Table I). 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 (Supplementary Figure I, materials and methods and Supplementary Table II). The relatively low number of quantified class I phosphosites compared to 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 dataset was compared with our previously published thrombin dataset[6] (Supplementary Figure IIA). Although, for reasons mentioned above, the current dataset contains a lower number of quantified phosphosites compared to the published thrombin dataset (2553 vs. 7793 phosphosites), 816 new phosphosites were quantified. Notably, 87% of the thrombin-regulated phosphosites were found to be regulated in both datasets. Due to the short time course of our phosphoproteomic experiment, we expected that the proteomic content of the BOECs 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 five stimulation conditions, we performed a principal component analysis of the quantified phosphosites. The phosphoproteomes of ECs stimulated with 10 nM thrombin and thrombin-TL peptide clustered together and were clearly distinct from the those of ECs stimulated with 10 nM thrombin in the presence of vorapaxar or parmodulin-2 and ECs stimulated with 50 pM 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 nM thrombin and thrombin-TL peptide, followed by 10 nM thrombin in the presence of parmodulin-2, and only limited phosphoregulation was induced by 10 nM thrombin in the presence of vorapaxar and in ECs stimulated with 50 pM thrombin (Figure 3B). Regulated phosphosites included phosphosites with increased (241 sites; clusters 1-3) as well as 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 were 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 nM) and low (50 pM) 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 (Supplementary Figure III). 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 pre-incubation with vorapaxar or parmodulin-2 (Supplementary Tables III,IV), indicating that PAR1 inhibition alone does not induce (short term) protein expression differences nor 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 and Supplementary Table II). 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 vs 100 remaining regulated phosphosites, respectively). Remaining regulated phosphosites in presence of vorapaxar mainly

showed opposing phosphoregulation compared to thrombin alone (Figure 4A), while those that remained regulated in presence of parmodulin-2 mainly resembled thrombin-mediated phosphoregulation (Figure 4B). Notably, the latter mainly concerned dephosphorylation events. In order to provide insight into pathways which were blocked by the PAR1 inhibitors, we constructed a phosphopath interaction map (Supplementary Figure IV). No unique pathway was revealed that was differentially affected by vorapaxar and parmodulin-2 (Supplementary Figure IV). However, there were clearly phosphosites that were completely blocked (Supplementary Figure V-A) or differentially blocked (Supplementary Figure V-B) by the PAR1 inhibitors. Since it has been described that parmodulin-2 selectively blocks $G\alpha_q$- over $G\alpha_{12/13}$-mediated activation15, we reconstructed the $G\alpha_q$ pathway based on the interactors of Serine/threonine-protein kinase D1 (PRKD1) (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 beta-1 (HSPB1) and Rab GTPase-binding effector protein 1 (RABEP1) was completely inhibited by both PAR1 inhibitors, while phosphorylation of Catenin delta-1, another potential downstream target of PRKD1 showed differential inhibition. Thrombin-specific phosphorylation of the transcription factor Signal transducer and activator of transcription 3 (STAT3) on Ser-727[34] was completely inhibited by both PAR1 inhibitors (Supplementary Figure V-A). 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 which results in biased activation of PAR1 by thrombin.

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 (Supplementary Figure VI), 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 (Supplementary Figure VI). Therefore, to study biased activation resulting from non-canonical 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, B).[19,20] For these five TL peptides we performed a time-resolved phosphoproteomic analysis of stimulated BOECs (materials and methods). MS 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]) (Supplementary Table V). 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, Supplementary Table VI). Despite the different time points of stimulation between the thrombin/PAR1 inhibitor dataset and the TL peptides dataset, the identified phosphosites show a large similarity (Supplementary Figure IIB). In contrast to thrombin-TL peptide, the non-canonical PAR1 TL peptides only induced limited early phosphoregulation (Figure 6C). Moreover, sites that were found to be regulated were surprisingly similar for each non-canonical TL peptide, and mostly resembled the phosphorylation pattern of thrombin-TL peptide. Only a limited number of phosphosites showed opposing phosphoregulation by the non-canonical PAR1 TL peptides compared to 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, while non-canonical 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 since PAR1 has become a therapeutic target for anti-thrombotic 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 BOECs the thrombin-mediated endothelial barrier disruption is completely dependent on PAR1 activation (Figure 2D-E), 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 tethered ligand 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, atopaxar) have been shown to increase the risk of bleeding, a side effect which 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, while on the other hand, parmodulin-2 showed only partial inhibition (Figure 3, 4). Notably, these remaining regulated phosphosites mostly contained phosphosites with a decreased abundance, suggesting 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 (Supplementary Figure V), there was not one uniquely affected pathway (Supplementary Figure IV). However, detailed analysis of the $G\alpha_q$-mediated phosphorylation downstream of PRKD1 revealed that these processes were differentially inhibited (Figure 5, Supplementary Figure V). 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 gamma-1 (PLCG1), but only partially inhibits phosphorylation of PLCB3 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-terminus 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, while the dephosphorylation of Catenin delta-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 by 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 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 non-canonical 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 non-canonical TL peptides PR3-TL, MMP1-TL, NE-TL and APC-TL. In contrast to our expectations, these four non-canonical 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 (Supplementary Figure V). 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 (Supplementary Figure V). The same was true for the remaining regulated phosphosites in presence of parmodulin-2, which explains the appearance of overlap in the principal component analysis (Figure 3A). In contrast, phosphosites that remained regulated in presence of vorapaxar mainly showed opposing phosphoregulation. Beta-arrestin-mediated phosphoregulation by PAR1 has been described to play a role in the signal transduction of APC.[22] In our dataset we identified one phosphosite on beta-arrestin-1, Ser-412, which has been described in the activation of the GPCR 5–HT4R.[41] However, this phosphosites was not regulated in any of the stimulation conditions during the time course of our experiments. Since we only analyzed the signaling transduction in ECs in the first 10-20 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 nor the beta-arrestins-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 FDA approved PAR1-directed antiplatelet drug vorapaxar virtually inhibits all thrombin-induced signaling in ECs. Notably, the vorapaxar concentration used in our study (100 nM) 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 anti-platelet 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 FXa target not only PAR1 but also PAR2.[47,48] In addition, both PAR1 and PAR4 are important for thrombin-mediated platelet aggregation.[49] While 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.

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Highlights

- Thrombin signaling in endothelial cells is mediated exclusively via PAR1
- 'Classical' and 'peptide' activation of PAR1 initiate highly similar signaling pathways
- The recently FDA- approved orthosteric PAR1-directed anti-platelet drug vorapaxar and the allosteric PAR1 inhibitor parmodulin-2 reveal differential antagonistic properties
- In contrast to peptides that mimic non-canonical cleavage of PAR1, only the tethered ligand peptide that mimics canonical cleavage of PAR1 by thrombin potently induces early signaling in ECs

Figure legends

Figure 1. Vorapaxar and parmodulin-2 specifically inhibit thrombin-TL peptide-mediated platelet aggregation.(A) Platelets were pre-incubated for 10 minutes with indicated concentrations of vorapaxar, parmodulin-2, or vehicle (DMSO), followed by triggering platelet aggregation with 10 µM 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 pre-incubated for 10 minutes with indicated concentrations of vorapaxar, parmodulin-2, or vehicle (DMSO), followed by triggering platelet aggregation with the following agonists: thrombin-TL peptide (10 µM), PAR4 peptide (AYPGKF-NH2) (150 µM), collagen (2 µg/ml) or thromboxane A2 receptor agonist U46619 (5 µM). Data represent mean ± sd (n=3), * $P < .05$, *** $P < .001$ by two-way ANOVA with post hoc Tukey's multiple comparison test (Graphpad Prism version 6.04).

Figure 2. Vorapaxar and parmodulin-2 completely inhibit thrombin-induced endothelial barrier disruption. (A, B) BOECs were pre-incubated for 1 hour with indicated concentrations of vorapaxar (A), parmodulin-2 (B), or vehicle control (DMSO), followed by stimulation with 10 nM thrombin (1U/ml thrombin = 10 nM). Representative tracings

of transendothelial electrical resistance after addition of thrombin (t = 0 hours) are shown. Values were normalized per sample to the resistance 9 minutes prior to 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 pre-incubated for 1 hour with indicated concentrations of vorapaxar or vehicle control (DMSO), followed by stimulation with the indicated concentrations of thrombin (1U/ml thrombin = 10 nM). Values were normalized per sample to the resistance 9 minutes prior to addition of thrombin (t = 0 hours). Data represent mean ± sd (n=3). (E) Effect of vorapaxar pre-incubation 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).

Figure 3. Thrombin and thrombin-TL peptide induce highly similar temporal phosphoregulation. (A) Two components that capture 43.7% of the total variance of the BOEC phosphoproteome changes during the 5 different stimulation conditions (Thrombin alone (10 nM or 50 pM); thrombin-TL peptide; and 10 nM thrombin in the presence of either vorapaxar or parmodulin-2 (see legend)) are shown. Principal component analysis was performed on the quantified SILAC 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) Heat map 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 Supplementary Table II. (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.

Figure 4. Remaining regulated phosphosites reveal distinct antagonistic properties for vorapaxar and parmodulin-2 . Heat maps based on the SILAC log_2 ratio of regulated phosphosites by 10 nM thrombin (1U/ml thrombin = 10 nM) in the presence of (A) vorapaxar (35 phosphosites) or (B) parmodulin-2 (100 phosphosites). SILAC log_2 ratios of 10 nM thrombin alone and 10 µM 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 Supplementary Table II; ^, redundant

site, _1, _2 or _3 indicates if the quantification of the phosphorylation site was based on singly (_1), doubly (_2), or multiply (_3) phosphorylated peptides.

Figure 5. Visualization of PRDK1 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 are added to the network based on literature. Interaction network was made using the Phosphopath plug-in55 from Cytoscape.56 Graphs represent mean and sd for the MS-based quantifications of the indicated phosphosites (see Supplementary Figure I-C for N values per stimulation condition). MS-quantifications of thrombin (10 nM), thrombin-TL peptide; and 10 nM thrombin in the presence of either vorapaxar or parmodulin-2 are shown.

Figure 6. Only canonical PAR1 cleavage by thrombin generates a tethered ligand that potently induces early signaling in ECs. (A) Schematic representation of the PAR1 N-terminus with the known cleavage sites of the proteases depicted; Neutrophil Protease (PR3, Ala-36), Matrix Metalloproteinase-1 (MMP1, Asp-39), Thrombin (Arg-41), Neutrophil Elastase (NE, Leu-45) and Activated protein C (APC, Arg-46). (B) Synthetic peptides that mimic the tethered ligand 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) Heat map and hierarchical clustering (based on average euclidean distance and preprocessed with k-means) based on the SILAC $\log_2$ ratio of regulated phosphosites in the tethered ligand activation peptides dataset (n=274). BOECs were stimulated with the indicated TL peptides (50 µM). Heatmap colors (see legend) are based on the SILAC $\log_2$ ratios reported in Supplementary Table VI. (D) MS-based quantifications of the indicated phosphosites, quantifications of both replicates are shown.