New Direction for WE Thrombin

Michael C. Berndt, Robert K. Andrews

An appealing strategy for developing therapeutic agents is to modify the design of human proteins, thereby taking advantage of target-specificity, multifunctionality of many proteins, and human compatibility—that is, provided any undesirable functional effects of the parent molecule can be selectively engineered away. In 2000, Cantwell & Di Cera reported a mutant form of the human serine protease, α-thrombin, a multifunctional enzyme involved in both pro-and antithrombotic pathways. This mutant, termed WE thrombin, containing Trp215Ala and Glu217Ala substitutions, still promoted formation of activated protein C (APC) that inhibits coagulation factors Va and VIIIa (antithrombotic), but insufficiently converted fibrinogen to fibrin (prothrombotic), and was an effective antithrombotic agent in primate models of thrombosis.1 But a number of observations suggested this was not the full story. For instance, at low WE thrombin concentrations, there was a greater antithrombotic effect than expected based on circulating APC levels and minimal systemic anticoagulation, and in addition, labeled WE thrombin was incorporated into a developing thrombus, suggesting it might be interacting with platelets.

In this issue of Arteriosclerosis, Thrombosis, and Vascular Biology, Berny and colleagues4 have provided further information (see Figure), revealing an unanticipated additional antithrombotic effect of WE thrombin, that is, binding to platelet glycoprotein (GP)IIb/IIIa (the major ligand-binding subunit of the GPib-IX-V complex),5–7 and inhibiting platelet adhesion to von Willebrand factor (vWF) under hydrodynamic flow and thrombus formation on a collagen matrix. The interaction of both wild-type and WE thrombin with GPIIb/IIIa involves the N-terminal ligand-binding region of GPIIb (residues 1 to 282), but with profoundly different functional consequences.

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The Role of Thrombin in Thrombosis
Thrombin has multiple roles in hemostasis and thrombosis.8 Control of these processes involves regulation of active thrombin generation at the business end of intrinsic (FXII-dependent) or extrinsic (tissue factor [TF]-dependent) coagulation pathways, thrombin inhibitors, and regulators of proteins downstream of thrombin activity. This extensive system of positive and negative feedback loops is essential for fine-tuning of thrombin-dependent events. The effect of thrombin on platelets is closely linked to its activity toward noncellular targets associated with thrombus formation. Thrombin activity is involved in converting fibrinogen to fibrin, activation of FXIII to FXIIIa (involved in fibrin cross-linking), activation of thrombin-activatable fibrinolyis inhibitor (TAFI), activating FXI (consolidation pathway), converting FV to FVa, and when associated with thrombomodulin, converting protein C to APC.1–4,8 FIXa-FXIIa and TF-FVIIa generate Fxa-FVa that converts prothrombin to thrombin.

Thrombin also activates platelets by different mechanisms. First, thrombin activates the G protein–coupled 7 transmembrane protease-activated receptor-1 or -4 (PAR-1 or PAR-4). PAR-1 activation is accelerated by thrombin binding to GPIIb/IIIa, the major ligand-binding subunit of the GPib-IX-V complex.5,6,8–10 Interestingly, thrombin can activate platelets by a second mechanism involving binding to GPIIb/IIIa, a process facilitated by proteolytic removal of GPV (a substrate for thrombin or metalloproteinases, ADAM-10 and -17).11–13 Platelets promote intrinsic and extrinsic coagulation pathways, and binding of thrombin to the ligand-binding N-terminal region of GPIIIa provides a mechanism for increasing the effects of thrombin activity, for example, by orientation of active thrombin with platelet-bound substrates including fibrinogen or FXI.5 The way in which thrombin recognizes different substrates, cofactors, or binding partners involves distinct exosites. Exosite I, or the fibrinogen recognition site, is centered on loop Arg67-Ile82, whereas exosite II, or the heparin-binding site, includes residues 93/97/101/115/167/178/179/184/186/188.11 How these sites control thrombin activity in thrombus formation is being revealed by structure-function analysis.1–5,8,10 Alanine scanning of thrombin also revealed residues outside these sites conferring binding partner recognition selectivity,14,15 opening the way for assessing the role of individual thrombin interactions in vivo and investigating pretherapeutic applications of mutant thrombin.1–4,16 Trp215Ala or Glu217Ala mutations of thrombin vastly decreased efficiency of fibrin production relative to near-normal binding to thrombomodulin and activation of protein C, with this effect maximized in a combined Trp215Ala plus Glu217Ala (WE) form of mutant thrombin.1–3,16 To date, unanswered, however, was how WE thrombin interacted with GPIIb/IIIa, either activating platelets via GPIIb/IIIa or PAR-1, or affecting interaction of GPIIb/IIIa with vWF or other ligands.

Current Study
The current study of Berny et al14 describes for the first time that mutant WE thrombin can bind platelet GPIIb/IIIa, but unlike wild-type thrombin, not activate platelets either directly via GPIIb/IIIa11,12 or indirectly by proteolysis of PAR-1.9,10 WE
thrombin also inhibited platelet tethering and rolling of platelets on immobilized vWF when whole blood was passed over the surface under shear-flow conditions. Another significant finding involves platelet spreading on immobilized wild-type thrombin. Human platelets or platelets from WAVE-1–deficient mice (WAVE-1 is an actin scaffolding protein) adhered and formed lamellipodia over 45 minutes at 37°C, whereas platelets from mice deficient in the cytoskeletal regulatory protein, Rac1, adhered and formed filopodia, but remained lamellipodial. Platelet adhesion to wild-type thrombin was also unaffected by soluble WE thrombin under static conditions. These experiments not only showed a dependence on Rac1 for platelet spreading on thrombin, but also showed a lack of platelet interaction with immobilized WE thrombin, prothrombin, PPACK-inhibited thrombin, or catalytically-inactive thrombin mutant, Ser195Ala. Active thrombin is emphatically essential for supporting platelet adhesion under static conditions or under flow conditions (300 s⁻¹), however in the latter case, soluble WE thrombin and the anti-GPIbα antibody, SZ2, significantly blocked platelet adhesion to wild-type thrombin.4 SZ2 has previously been mapped to the anionic, sulfated tyrosine-containing sequence of GPIbα (Asp269-Glu282) within the N-terminal ligand-binding domain (residues 1 to 282), and this sulfated region makes a major contribution to thrombin recognition, and interacts with thrombin exosite II.5–10

Together, these results suggest that WE thrombin is a novel antagonist for GPIbα binding to vWF. Crystal structures of a complex of wild-type thrombin and the N-terminal fragment of GPIbα show that thrombin can interact with this receptor in 2 orientations, involving different thrombin exosites, allowing 1 thrombin molecule to potentially cross-link (and activate) 2 GPIbα receptors on the same or adjacent platelets.5,17,18 Precisely how the mutant WE form of thrombin recognizes GPIbα is unclear. The thrombin-binding faces of GPIbα are distinct but partially overlap interactive surfaces of GPIbα N-terminal fragment and the vWF-A1 domain determined from crystalline structures under static conditions.5–7,19–21 However, functional studies using cross-species chimeras show a discrete anionic region of GPIbα becomes increasingly important for tethering to vWF as shear rate increases.7 This implies shear-dependent conformational changes could alter the functional binding state of GPIbα (or vWF-A1), but it remains to be seen whether the functional exposure of the anionic patch within the leucine-rich repeat domain5–7 regulates the interaction with thrombin or other ligands. In this respect, SZ2, which blocks binding of thrombin to GPIbα, also inhibits binding of P-selectin, and given WE thrombin is a GPIbα antagonist toward vWF ligand-binding A1 domain, it would also be interesting to assess its potential antiinflammatory role in blocking GPIbα-dependent platelet adhesion to activated endothelial cells (mediated by endothelial P-selectin) or leukocytes (mediated by the leukocyte integrin, α₅β₃, expressing an α₅ ligand-binding I-domain homologous to vWF-A1).5

Conclusions and Future Perspectives
The study showing WE thrombin is antithrombotic by acting as an antagonist of GPIbα-dependent adhesion to vWF, as well promoting APC but not fibrin production, enhances the potential use of WE thrombin for controlling thrombosis.1–4 Interestingly, the patho/physiological importance of the GPIbα–vWF interaction in thrombus formation at arterial shear rates in vivo or ex vivo in vitro is addressed in many past and recent studies.21–24 For example, depleting mouse platelets of the extracellular domain of GPIbα (in interleukin 4 [IL-4]–GPIbα cytoplasmic domain transgenic mice, where macrothrombocytopenia associated with GPIbα knockout is rescued), suggests a vital role for GPIbα in thrombus formation, but far less contribution from vWF (shown using vWF-deficient mice).22 This suggests that ligands for GPIbα other than vWF may be essential for stable thrombus formation. In this regard, in vivo or ex vivo studies with mouse and human platelets are consistent with vWF mediating initial, reversible platelet tethering at high shear rates preceding platelet activation and aggregation involving GPIb-IX-V associated receptors such as GPVI, Fc receptors, and integrins (mainly α₅β₃).21–24 With this in mind, future analysis should address whether WE thrombin associated with platelets/GPIbα is comparably efficient at generating APC, or more importantly, beneficially localizes this process to the developing thrombus surface.4 The combined antithrombotic effects of WE thrombin in coagulation, localized platelet-mediated coagulation, and platelet adhesion to vWF is likely to make a significant advance in understanding the functions and antithrombotic potential of this reagent in particular, and the approach of using engineered human proteins more broadly, including the unexpected (advantageous) consequences of this mutagenesis.

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None.

References


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