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Thrombomodulin is required for the antithrombotic activity of thrombin mutant W215A/E217A in a mouse model of arterial thrombosis

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Abstract

Introduction—The thrombin mutant W215A/E217A (WE thrombin) has greatly reduced procoagulant activity, but it activates protein C in the presence of thrombomodulin and inhibits binding of platelet glycoprotein Ib to von Willebrand factor and collagen under flow conditions. Both thrombomodulin-dependent protein C activation and inhibition of platelet adhesion could contribute to the antithrombotic activity of WE thrombin.

Materials and Methods—To assess the role of thrombomodulin, we administered WE thrombin to thrombomodulin-deficient (TM^{Pro/Pro}) mice and measured the time to occlusive thrombus formation in the carotid artery after photochemical injury of the endothelium.

Results and Conclusions—Doses of WE thrombin 10 µg/kg prolonged the thrombosis time of wild-type mice (>1.6-fold), while doses 100 µg/kg only slightly prolonged the thrombosis time of TM^{Pro/Pro} mice. We conclude that thrombomodulin plays a predominate role in mediating the antithrombotic effect of WE thrombin in the arterial circulation of mice after endothelial injury. Thrombomodulin-independent effects may occur only when high doses of WE thrombin are administered.

Keywords

Thrombin; thrombomodulin; arterial thrombosis; mouse

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Authorship

C.P.V. designed and performed the experiments; H.W. provided TM^{Pro/Pro} mice; E.D.C. provided WE thrombin and APC; D.M.T. designed experiments and wrote the paper; all of the authors reviewed and approved the final manuscript.

Conflict of interest statement

E.D.C. is a consultant for Verseon. The other authors declare no competing financial interests.

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Thrombin is an extraordinary enzyme that has either procoagulant or anticoagulant activity depending on its environment.[1] When free in solution, thrombin promotes blood coagulation by cleaving specific peptide bonds in fibrinogen, factor V, factor VIII, and factor XI; it activates human platelets by cleaving protease-activated receptors (PAR1 and PAR4); and it stabilizes the fibrin clot by activating factor XIII. When bound to thrombomodulin on the surface of vascular endothelial cells, however, thrombin loses its procoagulant activity; gains the ability to activate protein C, which then degrades factors Va and VIIIa and thereby prevents further thrombin generation; and activates the carboxypeptidase thrombin-activatable fibrinolysis inhibitor.[2]

With the goal of developing novel antithrombotic agents, several groups have engineered thrombin variants that have reduced procoagulant activity but retain the ability to activate protein C in the presence of thrombomodulin.[3–5] The most successful of these is the thrombin mutant W215A/E217A (WE thrombin), which cleaves fibrinogen 19,000-fold and PAR1 1000-fold less efficiently (k_{cat}/K_m) than the wild-type enzyme and, therefore, is practically devoid of procoagulant activity.[3] By contrast, the k_{cat}/K_m for protein C in the presence of thrombomodulin is reduced only 7-fold. Administration of WE thrombin to baboons causes activation of endogenous protein C and inhibits acute vascular graft thrombosis without producing any sign of hemorrhage or other adverse events.[6] Interestingly, the amount of activated protein C detected in plasma after administration of WE thrombin is several-fold less than the amount of exogenous APC necessary to produce an equivalent antithrombotic effect.[7] This may be due to persistence of APC bound to the endothelial protein C receptor after in situ generation by WE thrombin, which is not recapitulated by exogenous administration of APC. Indeed, binding to the endothelial protein C receptor leads to underestimation of the total APC generated in the blood during endotoxemia.[8] On the other hand, this observation might be explained, in part, by the finding that WE thrombin interacts with platelet glycoprotein Ib and inhibits binding of platelets to von Willebrand factor and collagen under flow conditions.[9] Thus, both thrombomodulin-dependent protein C activation and inhibition of platelet adhesion could contribute to the antithrombotic activity of WE thrombin, and it is unclear which of the two mechanisms is more important. To clarify this issue, we determined the antithrombotic effect of WE thrombin administered to thrombomodulin-deficient mice.

Materials and Methods

Reagents

Human WE prethrombin-1 (W215A/E217A) was expressed in baby hamster kidney cells, activated to WE thrombin with prothrombinase or ecarin, purified to homogeneity, and characterized as described previously.[3] The active site concentration was determined by titration with hirudin and was found to be 95%. Purified human APC was a gift from the American Red Cross. Porcine intestinal heparin was obtained from Baxter.

Mice

Mice carrying the thrombomodulin E404P mutation (TM^{+/Pro}) were generated as described previously.[10] Animals used in the current study had been backcrossed (N>11) into the C57BL/6 strain and were then maintained as a breeding colony of homozygous TM^{Pro/Pro} mice. Wild-type C57BL/6 mice purchased from Taconic were used as controls.

Carotid artery thrombosis model

The internal carotid artery was isolated and illuminated with a 540 nm laser as described previously.[11] Thrombosis was initiated by injection of rose Bengal dye (50 mg/kg) into the lateral tail vein, and blood flow was monitored continuously. The occlusion time was

determined as the interval between injection of the dye and complete and stable (>5 min) cessation of flow. WE thrombin, APC, heparin or phosphate-buffered saline was administered by tail vein injection 10 min before initiation of thrombosis. Occlusion times are expressed as mean $\pm$ 1 SD. *P*-values less than 0.05 determined by the two-tailed Student's *t*-test are considered statistically significant. The experiments were approved by the Washington University Animal Studies Committee.

Results and Discussion

We investigated the relative importance of thrombomodulin in mediating the antithrombotic effect of WE thrombin by studying mice homozygous for the thrombomodulin E404P mutation (TM^{Pro/Pro}), which almost completely disrupts the ability of thrombomodulin to promote activation of protein C at physiologic thrombin concentrations (<1 nM).[10] TM^{Pro/Pro} mice have a mild hypercoagulable state characterized by increased fibrin deposition in several organs, but they do not spontaneously develop occlusive thrombi.

We used the photochemical dye rose Bengal, which causes oxidative damage to the endothelium, to induce thrombosis in the carotid artery. Wild-type mice formed stable occlusive thrombi in 63 $\pm$ 4 min, while TM^{Pro/Pro} mice formed thrombi in 46 $\pm$ 10 min (*P*=0.00017) (Fig. 1A). This result is consistent with previous experiments in which TM^{Pro/Pro} mice formed thrombi more rapidly than wild-type mice when carotid arterial injury was induced with FeCl₃. [12] When injected into wild-type mice, WE thrombin given at a dose of 10 μ g/kg or higher significantly prolonged the time to thrombotic occlusion (Fig. 1A). In previous work, a similar dose of WE thrombin (25 μ g/kg) was also found to improve neurologic outcome in wild-type mice after ischemic brain injury.[13] By contrast, WE thrombin had no effect on the thrombotic occlusion time of TM^{Pro/Pro} mice when given at a dose of 10 μ g/kg, and it had only a small effect at doses up to 1000 μ g/kg (Fig. 1A). WE thrombin was not tested in TM^{Pro/Pro} mice at a dose of 1 μ g/kg, because there was no difference in the occlusion times when these mice were given either 0 or 10 μ g/kg WE thrombin.

Fig. 1B compares the antithrombotic effect of WE thrombin to that of human APC. Although APC did not prolong the occlusion time at a dose of 10 μ g/kg, a 10-fold higher dose of APC (100 μ g/kg) had an effect similar to that of 10 μ g/kg WE thrombin (92 $\pm$ 10 min vs. 105 $\pm$ 17 min, respectively; *P*=0.18). Similar effects have been observed in a baboon model of arteriovenous shunt thrombosis, in which bolus administration of 11 μ g/kg WE thrombin reduced deposition of ¹¹¹indium-labeled platelets by 80% and was at least as effective as the administration of 450 μ g/kg APC.[6] Fig. 1B also shows that a dose of unfractionated heparin 125 μ g/kg is required to prolong the occlusion time to a degree comparable to that of 10 μ g/kg WE thrombin.

The carotid artery thrombosis model used in this study is sensitive to mutations or drugs that affect either coagulation or platelet function.[14] With regard to platelet function, both a platelet glycoprotein IIb/IIIa antagonist[15] and deletion of the platelet collagen receptor α 2 β 1 integrin[16] prolong the occlusion time. In other models of arterial thrombosis, molecules that inhibit binding of glycoprotein Ib to von Willebrand factor have strong antithrombotic effects.[17] Therefore, it is likely that the occlusion time in our experiments would have been prolonged if a major effect of WE thrombin is to block binding of platelet glycoprotein Ib to collagen or von Willebrand factor at a site of arterial injury. At doses of WE thrombin 100 μ g/kg, we observed a small but significant increase in the occlusion time over the baseline for TM^{Pro/Pro} mice (69 $\pm$ 18 min vs. 46 $\pm$ 10 min; *P*=0.014), which could result from inhibition of glycoprotein Ib or from some other mechanism, such as activation of protein C at high WE thrombin concentrations.

We conclude that the antithrombotic effect of WE thrombin in the arterial circulation of mice is predominantly mediated by thrombomodulin-dependent activation of protein C. Effects of WE thrombin that are independent of thrombomodulin may occur only when high doses of WE thrombin are administered.

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Abbreviations

PAR	protease-activated receptor
WE thrombin	mutant thrombin W215A/E217A
APC	activated protein C
TM^{Pro/Pro}	homozygous mutant thrombomodulin E404P

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Figure 1.

(A) Carotid artery occlusion times of mice given WE thrombin 10 min prior to initiation of thrombosis. Error bars represent the mean value $\pm$ 1 SD. * $P < 0.05$ vs. wild-type controls. ** $P < 0.05$ vs. TM^{Pro/Pro} controls (2-tailed t -test).

(B) Occlusion times of wild-type mice given WE thrombin (WE), activated protein C (APC) or heparin 10 min prior to initiation of thrombosis. C = control mice given an equivalent volume of phosphate-buffered saline. * $P < 0.05$ vs. wild-type controls.