Protection against thrombosis in mice lacking PAR3

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Abstract

The recent observation that knockout of protease-activated receptor-4 (PAR4) ablates thrombin signaling in mouse platelets and protects against ferric chloride-induced thrombosis of mouse mesenteric arterioles suggests that thrombin’s actions on platelets can play an important role in thrombosis. Complete ablation of thrombin signaling would be difficult to achieve in man because human platelets have two thrombin receptors that are each capable of mediating transmembrane signaling. However, it is possible that complete ablation of thrombin signaling in platelets is not necessary for an antithrombotic effect. In mouse platelets, PAR3 functions as a cofactor that binds thrombin and promotes productive cleavage of PAR4, and thrombin responses are decreased but not absent in Par3−/− platelets. We now report that Par3−/− mice were protected against ferric chloride-induced thrombosis of mesenteric arterioles and against thromboplastin-induced pulmonary embolism. Surprisingly, Par3−/− and Par4−/− mice showed similar degrees of protection in these models and similar prolongation of tail bleeding times. Thus even a partial decrease in mouse platelet responsiveness to thrombin protected against thrombosis and impaired hemostasis in some settings. These results demonstrate the importance of PAR3’s unusual cofactor function and underscore the relative importance of thrombin’s actions on platelets in vivo. They also suggest that PAR inhibition might be explored for the prevention or treatment of thrombosis in man.
Introduction

Thrombosis of the arteries that supply the heart, brain, and other vital organs remains a major cause of morbidity and mortality, and platelet activation plays a central role in this process. The coagulation protease thrombin is a potent activator of platelets, but multiple other agonists can serve this function. Thrombin’s actions on platelets are mediated by protease-activated receptors (PARs), and we recently showed that complete ablation of thrombin signaling in mouse platelets by knockout of PAR4 protected against ferric chloride-induced mesenteric arteriolar thrombosis. Thus despite the multiplicity of platelet agonists, thrombin’s actions on platelets can be important for thrombosis.

In human platelets, PAR1 and PAR4 both contribute to thrombin responsiveness. Substantial inhibition of thrombin signaling in human platelets can be achieved by blocking PAR1 function, but in the absence of PAR1 function, PAR4 can mediate platelet activation in response to high concentrations of thrombin. The presence of two thrombin receptors implies that complete inhibition of thrombin responses in human platelets would be difficult to achieve pharmacologically, but it is possible that the partial inhibition might be sufficient for an antithrombotic effect. To further explore the role of thrombin signaling in platelets in thrombosis and to determine whether partial inhibition of thrombin signaling in platelets might be sufficient to protect against thrombosis, we utilized Par3-/- mice. Mouse platelets use PAR3 and PAR4 instead of PAR1 and PAR4 to respond to thrombin. Moreover, rather than itself mediating transmembrane signaling, mouse PAR3 functions as a cofactor that promotes cleavage and activation of PAR4 on mouse platelets at low concentrations of thrombin. Unlike Par4-/- platelets, Par3-/- platelets do aggregate and secrete in response to thrombin but, compared to wild-type, such responses are markedly reduced at low concentrations of thrombin and delayed at high concentrations of thrombin. Our results suggest that PAR3’s unusual cofactor function is important in vivo, that partial attenuation of thrombin signaling in mouse platelets is protective in two models of thrombosis, and that PAR1 inhibition might be explored as an antithrombotic strategy in man.
Materials and Methods

Mouse lines.

PAR3-deficient mice and PAR4-deficient mice were mixed 50% 129SvJae and 50% C57Bl/6 background. Par3 refers to the gene encoding mouse PAR3 (also known as coagulation factor II receptor-like 2; F2rl2; gi: 6753801). Par4 refers to the gene encoding mouse PAR4 (coagulation factor II receptor-like 3; F2rl3; gi: 6679728). All experiments were performed with littermate offspring of heterozygote crosses.

Ferric chloride-induced mesenteric thrombosis.

This model was performed as described. Briefly, offspring from PAR3 heterozygote crosses were used between ages 24 and 31 days. Because sensitivity to ferric chloride injury decreases as the mice gain weight, litters that contained mice under 9 grams or over 16 grams were not studied. Mice were anesthetized with ketamine and xylazine, the mesentery exposed, and arterioles of 70-120 µm were selected for study. A 1 mm-wide strip of Whatman filter paper was soaked in saturated ferric chloride solution and applied to the surface of the mesentery overlying the arteriole of choice. Flow downstream of the filter paper was observed by videomicroscopy. Time from application to cessation of flow, defined as flow stoppage for > 30 s, was measured. Experiments were terminated at 30 min. Par3+/+, Par3+/− and Par3−/− mice were not distinguishable by any gross feature, and mice were not genotyped until the completion of each set of experiments (thus endpoints were determined blind to genotype).

Thromboplastin-induced pulmonary embolism.

We utilized a model similar to those recently described. The thromboplastin (ThromboMax plus Calcium; Sigma) used for these studies was from rabbit brain. The International Sensitivity Index (ISI) for the lot used was 1.78 and 1.73 as determined by the manufacturer by mechanical and
optical methods, respectively, on an Amelung AMAX 190 Plus Coagulation Analyzer. Each vial of thromboplasatin was resuspended in 40 ml of saline (instead of the 10 ml of water recommended by the manufacturer) and 5 µl/g of this solution was injected per mouse as below. This dose was chosen such that ~25% of wild-type female mice survived the protocol.

Female mice were used because preliminary studies revealed a gender difference in sensitivity to thromboplastin. Female littermate offspring from Par3+/- crosses, ages 21 to 25 days (10 – 18 g), were anesthetized with ketamine/xylazine. The abdomen was opened via a midline incision. Thromboplastin was injected intravenously via the inferior vena cava over ~3 s. Time to death (time to the onset of respiratory arrest that lasted at least 2 min) was recorded. Experiments were terminated at 30 min because, in preliminary studies, virtually all mortality occurred by this time. Genotypes were determined after the thrombosis studies were performed.

Two minutes after onset of respiratory arrest (but while the heart was still beating) or at the completion of the 30 min observation period, mice were perfused via the inferior vena cava with 0.5ml Evans' Blue solution (1% in saline). Lungs were excised en bloc, photographed, and fixed in 4% paraformaldehyde for later sectioning. Two independent observers, each blind to genotype and survival outcome, scored lungs for Evans' Blue perfusion defects using the scale shown (Fig. 2C). To assess the correlation of gross perfusion defects with intravascular thrombi, histological analysis was performed on three hematoxylin and eosin-stained sections of lung from each of five mice for each perfusion score. In addition, lungs from 9 wild-type and 9 Par3+/- mice were chosen at random for histological analysis. 5 fields (10X objective, 10X ocular) were chosen at random from within the left lobe in each section (one section per mouse) and the number of vessels > 40 µm in diameter in each field that contained any identifiable thrombus was counted. Thus 45 fields for each genotype were examined for the wild-type vs. Par3+/- comparison. Counts were performed blind to genotype.

Studies of the effect of PAR4 deficiency in this model were performed as above.
**Bleeding times.**

Tail bleeding times were performed as described\(^1\). Briefly, littermate offspring of Par3\(^{+/−}\) crosses, aged 24-31 days, were anesthetized with a mixture of ketamine and xylazine, and tails were transected 5mm from the tip with a scalpel blade. The amputated segment was saved for genotyping. The bleeding end was immersed in saline at 37\(^{°}\)C and time to cessation of flow (stoppage for >30 s) was measured. Assays were terminated at 10 min and were done blind to genotype. Note that this method differs from that used in the original PAR3 bleeding time study\(^3\), in which tails were transected only 0.5-1mm from the tip in awake mice.

**Statistics.**

The effects of genotype on fraction of arterioles patent (Fig. 1), mice surviving (Fig. 2), or tails bleeding (Fig. 3) as a function of time and at the end of the study were assessed using the Log-Rank and Chi-Square tests, respectively\(^8\).
Results

We first examined Par3−/− mice in the ferric chloride-induced thrombosis model (Fig. 1). Littermate offspring of Par3+/− crosses were examined blind to genotype; one arteriole was examined in each mouse. The endpoints were 1) time from application of ferric chloride to the arteriole to cessation of flow and 2) the number of arterioles still patent at the end of the experiment (30 min). In Par3+/+, Par3+/−, and Par3−/− mice, the median time to cessation of flow was 7.3, 5.1, and >30 min, respectively. Moreover, only 22% and 24% of the vessels were patent at 30 min in wild-type and Par3+/− mice, respectively, but 63% of arterioles remained patent in Par3−/− mice. This degree of protection conferred by PAR3 deficiency was surprising in that it was as profound as that previously seen with PAR4-deficiency1.

We next directly compared the effects of PAR3- and PAR4-deficiency in a second thrombosis model, that of thromboplastin-triggered pulmonary embolism (Fig. 2). Female offspring of Par3 or Par4 heterozygote crosses received an intravenous bolus of thromboplastin via the inferior vena cava. Endpoints were 1) time from thromboplastin injection to death, 2) number alive at 30 min, and 3) extent of lung perfusion defects. In Par3+/+, Par3+/−, and Par3−/− mice, the median time to death was 7.5, 7.0, and >30 min, respectively. Only 27% of wild-type and 32% of Par3+/− mice were alive 30 min after thromboplastin infusion vs. 67% of Par3−/− mice alive at this time (Fig. 2A). Results with PAR4-deficient mice were similar. In Par4+/+, Par4+/−, and Par4−/− mice the median time to death was 5.8, 14.4, and >30 min, respectively, and 27% of wild-type, 45% of Par4+/− mice and 85% of Par4−/− mice were alive 30 min after thromboplastin injection (Fig. 2B).

In addition to the hard endpoint of death, we assessed the degree of vascular occlusion in the lungs of thromboplastin-challenged mice by perfusion with Evans' Blue solution and by histology (Fig. 2C-F). Normal lungs turned completely blue upon perfusion, lungs with complete occlusion of the pulmonary arteries remained pink, and lungs with segmental or lobar defects showed a mixed pattern (Fig. 2C). As expected, perfusion defect score (Fig. 2C,D) was inversely correlated with
survival (data not shown) and positively correlated with the number of intravascular thrombi seen in histological sections (for example, see Fig. 2E,F). Par3\(^{-/-}\) mice showed better gross lung perfusion than wild-type (Fig. 2D). Moreover, histological examination of sections from wild-type lungs revealed thrombi in 1.6 ± 0.2 vessels over 40 μm in diameter per 100X field (or 72% of vessels that size). By contrast, sections of Par3\(^{-/-}\) lungs revealed thrombin in only 0.7 ± 0.2 of >40 μm vessels per 100X field (or 38%) (\(P<0.001\); Fig. 2E,F). In addition to being less frequent, thrombi in Par3\(^{-/-}\) lungs were usually non-occlusive, while those in wild-type lungs were usually occlusive (Fig. 2E,F). Thus the protection against death seen in Par3\(^{-/-}\) mice was correlated with protection against pulmonary vascular occlusion and with the presence of fewer thrombi.

The similar levels of protection against thrombosis associated with PAR3 vs. PAR4 deficiency was curious in light of our initial characterization of hemostasis in these mice\(^1,3\). While neither PAR3- nor PAR4-deficient mice were anemic or showed signs of spontaneous bleeding, no prolongation of bleeding times was found in Par3\(^{-/-}\) mice\(^3\) but remarkable prolongation was found in Par4\(^{-/-}\) mice\(^1\). However, the PAR3 study had used a less stringent test of hemostasis than the PAR4 study, and re-examination of bleeding times in Par3\(^{-/-}\) mice using the method of the later PAR4 study indeed revealed a clear defect in hemostasis (Fig. 3). Median bleeding times were 1.6 min for both Par3\(^{+/+}\) and Par3\(^{+/-}\), and >10 min for Par3\(^{-/-}\) mice. None (0%) of the Par3\(^{+/+}\) mouse tails and only one (3%) of the Par3\(^{+/-}\) tails continued to bleed at 10 min, the arbitrary time for termination of each experiment. By contrast, 69% of Par3\(^{-/-}\) mouse tails were still bleeding at 10 min. These data are similar to those previously obtained in PAR4-deficient mice\(^1\) and suggest that even a partial decrease in thrombin signaling in platelets can impair hemostasis if the challenge is sufficiently severe.
Discussion

The finding that PAR3 deficiency yielded protection in two mouse models of thrombosis and prolonged tail bleeding times is, to our knowledge, the first demonstration that PAR3 function is important in vivo. In an in situ hybridization survey, PAR3 expression was detected in megakaryocytes (where it is co-expressed with PAR4) but not in blood vessels themselves. This restricted expression pattern suggests that the effect of PAR3 deficiency on thrombosis and hemostasis is likely due to its effects on the thrombin responses in platelets rather than other cell types. The mechanism by which PAR3 promotes platelet activation is unusual among G protein-coupled receptors in that it functions as a cofactor or co-receptor for thrombin rather than as a bonafide transmembrane signaling molecule. Our results provide the first evidence that PAR3’s peculiar cofactor role in thrombin signaling is important for platelet function in vivo.

The observation that even the partial attenuation of thrombin signaling caused by PAR3-deficiency had dramatic effects in two thrombosis models — effects similar to those seen with the complete ablation of platelet thrombin responses caused by PAR4-deficiency — is perhaps surprising and emphasizes that thrombin's actions on platelets are important despite the many potentially redundant pathways and processes involved in thrombosis. How is it that PAR3-deficiency and PAR4-deficiency have similar effects in these models? In contrast to wild-type platelets, stimulation of Par3-/- platelets with low concentrations of thrombin (1 and 3 nM) yielded almost no secretion and aggregation. At higher thrombin concentrations (10 or 30 nM), secretion and aggregation were only variably diminished but were reliably and substantially delayed compared to those seen in wild-type platelets. Thus it is possible that the concentration of thrombin acting at the platelet surface is in the 1-3 nM range in these models and simply insufficient to activate platelets that lack PAR3. Alternatively, the local concentration of thrombin may be higher, but rapid secretion and aggregation responses to thrombin may be necessary for occlusive thrombi to form in
these models. In this case, the delay in activation of Par3−/− platelets seen even at high concentrations of thrombin may be key.

How is it that attenuated thrombin signaling in platelets protects against thrombosis given the many potentially redundant pathways involved? In general, the decision to form a clot or a thrombus reflects a change in the kinetic balance of multiple positive and negative regulatory pathways10. In the thromboplastin model, it is likely that thrombin is the initiator of platelet activation, and it is possible that thrombin plays an initiating role in the ferric chloride injury model. In this case, diminished or delayed direct platelet aggregation by thrombin and/or diminished or delayed release of ADP6,11 and other amplifiers of platelet activation may slow platelet incorporation into thrombi sufficiently to account for the protection against thrombosis conferred by PAR3-deficiency. Interruption of a positive feedback loop between platelet activation and thrombin generation might also contribute to the relatively profound effect of PAR3 deficiency in thrombosis models. Activated platelets and platelet-derived microparticles provide a favorable surface for the assembly of coagulation factor complexes; this platelet procoagulant activity contributes to hemostasis by accelerating local thrombin generation10,12,13. The weaker, slower signaling seen in Par3−/− platelets may hobble this important amplification loop and slow thrombin production, tipping the balance in favor of coagulation inhibitors and yielding less platelet activation, delayed or diminished local fibrin formation, and smaller or less stable thrombi. These hypotheses may be testable by more detailed analysis in mouse models and by ex vivo clotting studies.

The observation that PAR3-deficiency in mice protects against thrombosis but has a relatively mild effect on hemostasis raises the question of whether PAR1 inhibition should be considered as a possible antithrombotic strategy in humans. In both PAR1-inhibited human platelets and PAR3-deficient mouse platelets, residual platelet responses to thrombin appear to be mediated by PAR4, and, perhaps as a consequence, thrombin responses are remarkably similar2,3. Human and mouse platelets normally aggregate and secrete their granule constituents in response to 1-3 nM
thrombin; these responses are virtually absent in both PAR1-inhibited human platelets and PAR3-deficient mouse platelets. At 10-30 nM thrombin, PAR1-inhibited human platelets and PAR3-deficient mouse platelets do aggregate and secrete, but such responses are delayed and variably diminished. For example, time to half-maximal ATP secretion in response to 30 nM thrombin was ~5 s in normal human and mouse platelets but was ~20 s in both PAR1-inhibited human and PAR3-deficient mouse platelets. PAR1 inhibition in human platelets and PAR3 deficiency in mouse platelets also had similar effects on thrombin-induced increases in cytoplasmic calcium (E.J.W. and S.R.C., unpublished). Such functional similarities suggest that, to the extent that mouse thrombosis models are relevant to man, blockade of PAR1 might be sufficient to achieve an antithrombotic effect despite the fact that human platelets have two thrombin receptors. A small non-human primate study supports this hypothesis: administration of a polyclonal PAR1 antiserum reduced or abolished platelet-dependent cyclic flow variations in carotid artery in four African green monkeys. While the latter study suggests that thrombin signaling in platelets might play a relatively important role in primate as well as mouse thrombosis models, there are, of course, noteworthy differences between these models and human thrombotic diseases. The ferric chloride-induced thrombosis model followed injury-induced thrombosis in an arteriole, not arterial thrombosis overlying a ruptured atherosclerotic plaque. The primate study cited above examined normal, not atherosclerotic, arteries. The mouse pulmonary embolism model used intravenous thromboplastin; how this relates to embolism of deep venous thrombi caused by stasis or trauma in man is unknown. We hope that the current genetic study in mice will, in the context of the previous primate study and our present knowledge of species differences in platelet thrombin receptors, stimulate efforts to develop potent small molecule PAR1 antagonists or avid blocking monoclonal antibodies. Such reagents will be required to permit more robust exploration of the possible utility of PAR1 inhibition for the prevention and treatment of thrombosis in relevant models.
References


Figure 1. Effect of PAR3-deficiency on ferric chloride-induced thrombosis in mesenteric arterioles. Offspring of PAR3 heterozygote crosses were studied. Shown is the fraction of arterioles remaining patent as a function of time after ferric chloride application. The mice were not genotyped until the completion of each experiment. Par3 genotypes and number of mice studied for each genotype are indicated at right. The effect of genotype on patency was significant by Log-Rank test ($P<0.02$), and the fraction of vessels patent at 30 min in Par3 −/− mice vs. +/- or +/+ mice was different by Chi-Square ($P<0.035$).
Figure 2. Effect of PAR3- vs. PAR4-deficiency on thromboplastin-induced pulmonary embolism and death. Female offspring of PAR3 (A) or PAR4 (B) heterozygote crosses were studied blind to genotype. (A,B) Time from thromboplastin injection to death during a 30 min observation period. Data are expressed as the fraction of mice alive as a function of time. The number of mice studied for each genotype is indicated at right. The effect of genotype on survival was significant by Log-Rank test (A, \( P < 0.04 \); B, \( P < 0.005 \)), and the fraction of \( Par3^{-/} \) mice and \( Par4^{-/} \) mice that survived for 30 min was significantly greater than the fraction of wild-type mice (+/+) that survived (A, \( P < 0.05 \); B, \( P < 0.005 \), Chi-squared test). (C,D) Mice in (A) were perfused with Evans' Blue 2 min after onset of respiratory arrest or at 30 min for those mice that survived the study; lungs were then excised, photographed, and scored for perfusion defects. (C) Key for perfusion defect scores. Zero indicates no perfusion defect (entirely blue) and four a complete lack of perfusion (entirely pink). (D) Perfusion defect scores by \( Par3 \) genotype (mean +/- s.e). The effect of genotype was significant by one way ANOVA, and \( Par3^{+/+} \) and \( -/- \) groups were different by t-test with Bonferroni correction (\( P < 0.01 \)). (E,F) Representative histological sections of lungs ranked with perfusion defect scores of 4 (E) and 0 (F) (genotypes happen to be wild-type in E and \( Par3^{+/} \) in F). Note frequent occurrence of occlusive intravascular thrombi in E (arrow heads) compared with partially occluded and unobstructed vessels in F (arrow heads). Scale bars = 100 µm.
Figure 3. Effect of PAR3-deficiency on bleeding time. Tail bleeding times were performed in a manner that provides a relatively strong hemostatic challenge (Methods). Offspring of PAR3 heterozygote crosses were studied. Shown is the fraction of tails still bleeding as a function of time after tail transection. Mice were genotyped after the bleeding times were performed. Genotypes and the number of mice of each genotype studied are indicated at right. The effect of genotype on bleeding time was significant by Log-Rank test ($P<0.0001$), and the fraction of tails still bleeding at 10 min in $Par3^{-/-}$ mice group was different from that in $Par3^{+/+}$ and $Par3^{++/+}$ mice by Chi-Square ($P<0.0001$).
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