The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions

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Summary
Developing novel anti-platelet therapies is an important clinical strategy for the prevention of arterial thromboses which cause heart attacks and most strokes. Thrombin activates platelets via protease-activated receptors (PARs), and PAR antagonists are currently under investigation as antithrombotics. Yet despite these clinical advances, the importance of PARs to platelet activation during thromboses formed under pathological conditions has not been investigated. To this end, we examined the role of PAR-dependent platelet activation in thrombus formation in the presence of elevated blood shear rates. We used two in vivo thrombosis models and an ex vivo whole blood flow approach in PAR4-/- mice, whose platelets are unresponsive to thrombin, to show that the contribution of PAR-mediated platelet activation to thrombosis is diminished at pathological blood shear rates as a direct result of decreased incorporation of thrombin-activated platelets into growing thrombi. Our ex vivo observations were replicated in human whole blood treated with a PAR1 antagonist. These results define a novel, shear-regulated role for thrombin/PAR-dependent platelet activation during thrombosis and provide important insights into the conditions under which PAR antagonists may best be used for the prevention of acute coronary syndromes.

Keywords
Blood shear, platelets, protease-activated receptors, thrombin, thrombosis

Introduction
Arterial thrombosis, manifesting predominantly as acute myocardial infarction or ischaemic stroke, is the single most common cause of morbidity and mortality in industrialised societies, accounting for ∼40% of all deaths in Western countries (1). Activated platelets and fibrin are the two essential components of arterial thrombi. Current pharmacotherapy, in the form of anti-platelet agents and anticoagulants, prevents platelet activation and fibrin formation, respectively, yet arterial thrombosis remains an enormous clinical problem. One key limitation of existing therapies is an inability to inhibit thromboses formed under pathological conditions. For example, the most commonly used anti-platelet and anticoagulant, aspirin and warfarin, respectively, both exhibit poor efficacy against arterial thromboses formed under high blood shear conditions, and concurrent administration does not significantly reduce rates of thrombosis without causing increases in major haemorrhagic events (2–6). Therefore, when considering novel anti-platelet approaches it is of particular importance to understand the impact of pathological conditions on the likely performance of any new agents.

Given the pivotal role of thrombin in the pathogenesis of arterial thrombosis, inhibition of thrombin-induced platelet activation may provide a successful approach for the prevention of acute coronary events (7, 8). Thrombin’s effects on platelets are mediated through the cleavage of membrane associated protease-activated receptors (PARs) (9, 10). In mice, PAR4 is the sole thrombin receptor capable of transducing a signal sufficient for platelet activation (11). PAR4-/- mice are protected against several experimental models of thrombosis yet do not exhibit spontaneous bleeding (11–15), indicating the potential of platelet PARs as targets for antithrombotic therapy in humans. Indeed PAR antagonists are currently being evaluated in phase 3 clinical trials for the prevention of arterial thrombosis (7, 8). Despite the clinical progression of PAR antagonists, little is known about the role of PAR-mediated platelet activation during pathological thrombus formation and the settings in which PAR antagonists are most likely to provide robust antithrombotic effects. Specifically, whilst numerous studies have shown markedly impaired in vivo thrombosis in PAR4-/- mice in small arteries or veins (12–15), few such studies have been performed in the large muscular arteries (16) in which pathological thrombosis occurs in humans, and no studies have directly addressed the impact
of the elevated blood shear rates present in many human pathologies which precipitate arterial thrombotic events (e.g. atherothrombosis). Therefore, we examined the importance of PAR-mediated platelet activation to in vivo thrombus formation under pathological blood flow conditions in large arteries and show that the contribution of PAR-mediated platelet activation to thrombus formation is diminished as blood shear rates are increased in both mice and humans. Our findings reveal a novel, shear-regulated role for thrombin-induced platelet activation during thrombus formation and provide insights into the use of PAR antagonists for the prevention of pathological, high-shear, arterial thrombosis.

**Materials and methods**

**In vivo models of thrombosis**

The Folts-like and electrolytic models of thrombosis were performed as previously described (17). Briefly, the Folts-like model used a suture tied around the carotid artery to cause a stenosis that decreased blood flow by ~50%. The segment of the artery under the stenosis was pinched with forceps five times and blood flow monitored until it reached 0 ml/minute (min). After 30 seconds (s) the site of stenosis was agitated to embolise the thrombi and restore...
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blood flow. The number of such cyclic flow reductions (CFRs) was recorded in PAR4+/+ or PAR4-/- mice over 40 min by a flow probe proximal to the suture/injury site. The electrolytic model used a constant current lesion maker to deliver 6 mA for 105 s to the carotid artery via a platinum electrode. In some experiments a suture was tied around the carotid artery distal to the electrode and tightened to reduce blood flow as required. In all cases, blood flow was allowed to stabilise for at least 15 min prior to further manipulation and was monitored for 30 min post-injury.

Analysis of platelet function

Aggregation of platelets in human platelet rich plasma, previously detailed (18), was used to assess the effect of the human PAR1 antagonist, SCH-79797 (Tocris, Ellisville, MO, USA). Agonists used included a PAR1-activating peptide (TFLLR; Auspep, Parkville, VIC, Australia), a PAR4-activating peptide (AYPGKF; Auspep) and ADP (Sigma, St Louis, MO, USA).

Ex vivo models of thrombosis

These experiments were carried out by pumping mouse whole blood, in the absence of anticoagulants, across a BSA (bovine serum albumin 2%, in saline)-blocked glass microslide (1 mm width x 0.1 mm height; internal diameter; Bio-scientific, Sydney, NSW, Australia) coated with type I collagen (0.25 mg/ml; bovine origin; Helena Laboratories). Blood was drawn directly from the inferior vena cava of anesthetised mice and pumped using a Harvard pump (Instrtech Laboratories, Plymouth Meeting, PA, USA) for 150 s. At the end of this period, the microslides were fixed via perfusion using 4% paraformaldehyde (PFA). The fixed microslides were stained with the membrane dye DiIC6 (1 μg/ml, Invitrogen, Carlsbad, CA, USA) and imaged using confocal microscopy, with images collected at 1 μm intervals. Thrombus volume (μm3/field) was determined by addition of the thrombus surface areas (μm2/field) using MetaMorph (MDS Analytical Technologies, Sunnyvale, CA, USA). Ten fields of view were analysed per microslide. In some experiments, hirudin (50 mg/kg, Refudran; Pharmion, Celegene Corp., Summit, NJ, USA) was injected intravenously (i.v.) into mice 10 min prior to the blood flow experiments. Similar experiments were performed in human whole blood which was collected in citrate (sodium citrate; 12.9% v/v) and then recalified (7 mM CaCl2) immediately prior to the blood flow experiment. The effect of PAR4-deficiency on thrombus propagation was examined by fluorescently labelling a subpopulation of platelets and examining the rate at which they were incorporated into growing thrombi. Platelets were isolated from PAR4+/+ or PAR4-/- mice and labelled with DiIC6 (1 μg/ml, Invitrogen). Labelled platelets (1.7 x 10^6) were then injected i.v. into mice of matching genotype and blood flow experiments carried out as described above. The number of fluorescently labelled platelets incorporated into thrombi was counted every 30 s.

Statistical analyses

Statistical significance (p < 0.05) was determined by either unpaired, two-tailed Student’s-t-test or one-way ANOVA with Dunnett’s test for multiple comparisons, as indicated. All statistical analyses were carried out using Prism software (v. 5.0, Graphpad, La Jolla, CA, USA).

Results

Protection against thrombosis in PAR4-/- mice is impaired by artery stenosis

We first examined in vivo thrombus formation in large, muscular arteries since they are more comparable to sites of pathological thrombosis in humans. We used a Folts’-like model of arterial thrombosis, in which a crush injury of a stenosed carotid artery (reduction in blood flow of ∼50% by narrowing the internal diameter of the carotid lumen by ∼80%) generates platelet-rich occlusive thrombi (17, 19, 20). Strikingly, there were no differences in occlusive thrombus formation between PAR4-/- mice and littermate PAR4+/+ controls (Fig. 1A). In contrast, pharmacological intervention with an inhibitor of αIIbβ3, GPI562 (1 mg/kg), abolished occlusive thrombus formation under the same conditions (Fig. 1B). Of note, thrombi formed in PAR4-/- mice were comprised of near-normal levels of platelets, indicating robust platelet activation and deposition in the absence of thrombin signaling in this model (Fig. 1C).

We next examined in vivo thrombosis in response to a different type of injury of the same vessel. We used an electrolytic injury model, which creates a more severe vascular injury than does the...
Figure 3: The contribution of PAR-dependent platelet activation to thrombus formation is reliant on blood shear rate. Unanticoagulated whole blood drawn directly from PAR4+/+ or PAR4-/- mice was flowed for 150 s through a glass microslide coated with collagen (0.25 mg/ml) at a shear rate of either 600 s⁻¹ or 1800 s⁻¹. A) The volume of thrombi deposited on the microslides was determined using confocal sectioning of thrombi fixed with PFA and stained with DiOC₆. Each data point is the average of 10 fields of view from one microslide (an individual mouse) and bars are mean ± SEM. *** p < 0.0001 (unpaired, two-tailed, t-test). B) Representative DIC and fluorescent (anti-GPIb antibody) images at the endpoint of each experimental group as shown in (A). Scale bar = 100 μM.

Folts'-type crush injury but remains dependent on both thrombin and platelets (17). In contrast to our observations with the Folts'-like model, PAR4-/- mice were markedly protected against electrolytic injury-induced thrombosis in the carotid artery when compared with littermate PAR4+/+ mice. All seven PAR4+/+ mice formed occlusive thrombi within 20 min post-injury compared to none of 7 PAR4-/- mice (Fig. 2A, B). However, the inclusion of a stenosis which reduced blood flow by ∼50% at the site of injury (similar to that employed in the Folts'-like model) markedly impaired the protection against thrombosis in PAR4-/- mice. All 14 PAR4+/+ and 12 out of 14 PAR4-/- mice occluded under these conditions (Fig. 2C) with no differences in blood flow through...
the injured arteries of PAR4+/+ and PAR4-/- mice over the 30 min observation period (Figure 2D). As observed in the Folts'-like model, αIIbβ3 inhibition abolished occlusive thrombus formation in stenosed vessels of both PAR4+/+ and PAR4-/- mice (Figure 2E, F) and these thrombi were comprised predominantly of platelets (Figure 2G). Therefore, we observed robust, platelet-rich, thrombosis in PAR4-/- mice in two distinct in vivo models of thrombosis, but only in the presence of significant artery stenosis during which marked increases in local blood shear rates occur.

PAR-mediated platelet activation during thrombosis is dependent on blood shear rate

To directly assess the impact of blood shear rate on thrombus formation, we established an ex vivo thrombosis assay in non-anticoagulated whole blood. In these experiments, blood was drawn directly from the venous circulation of anesthetised mice and passed over a thrombogenic surface of type I fibrillar collagen at controlled blood shear rates, and thrombus formation measured (21–23). At a shear rate of 600 s⁻¹ (approximating that in the arterial circulation) we observed more than a four-fold reduction in thrombus size in whole blood taken from PAR4-/- compared with PAR4+/+ mice (Figure 3A, B). In marked contrast, no differences were observed in thrombus size in blood taken from PAR4+/+ and PAR4-/- mice when blood was flowed at the elevated, pathological arterial shear rate of 1,800 s⁻¹ (Figure 3A, B).

The effects of PAR4-deficiency were next compared with those of thrombin inhibition. The reduction in thrombus size in PAR4-/- mice at arterial shear rates (600 s⁻¹) was reproduced by treating PAR4+/+ mice with doses of hirudin sufficient to prevent thrombin activity during the ex vivo blood flow experiments (50 mg/kg iv; Figure 4A and Suppl. Fig. 2, available online at www.thrombosis-online.com). As with PAR4-deficiency, hirudin treatment had no effect on thrombus volume in blood flow experiments carried out at 1,800 s⁻¹ (Figure 4A).

This shear rate-modulated involvement of PARs in thrombus formation was also observed when similar experiments were performed in human whole blood treated with the PAR1 antagonist, SCH79797 (10 μM; a concentration that provides selective impairment of PAR1-mediated platelet activation; Suppl. Fig. 1, available online at www.thrombosis-online.com). Specifically, the presence of a PAR1 antagonist significantly (p < 0.001) reduced the volume of thrombi formed at a shear rate of 600 s⁻¹, but had no effect on thrombus formation at a shear rate of 1,800 s⁻¹ (Figure 4B). In addition, the effects of PAR1 antagonism in human blood were

Figure 4: The contribution of thrombin-induced platelet activation to thrombus formation is dependent on blood shear rate in both mice and humans. Murine (A) or human (B) whole blood was flowed for 150 s through a glass microslide coated with collagen (0.25 mg/ml) at shear rates of either 600 s⁻¹ or 1,800 s⁻¹. The volume of thrombi deposited on the microslides was determined using confocal sectioning of thrombi fixed with PFA and stained with DiOC₆. A) Unanticoagulated whole blood was drawn from PAR4+/+ or PAR4-/- mice treated with hirudin (50 mg/kg i.v.) or vehicle (saline). B) Re-calculated human whole blood was pre-treated with either vehicle (DMSO) or SCH-79797 (10 μM). Each data point is the average of 10 fields of view from one microslide (an individual mouse or human) and bars are mean ± SEM. * p < 0.05, ** p < 0.01, *** P < 0.0001 (one-way ANOVA, with Dunnett’s test for multiple comparisons).
Figure 5: PAR-mediated platelet deposition into growing thrombi is dependent on blood shear rate. Unanticoagulated whole blood from PAR4+/+ or PAR4-/- mice that had been injected (i.v) with 1.7 x 10^8 DiIC12 labelled platelets of matching genotype was flowed for 150 s through a glass microslide coated with collagen (0.25 mg/ml) at shear rates of either 600 s\(^{-1}\) or 1,800 s\(^{-1}\) and the number of DiIC12 labelled platelets present in one field of view counted at 30 s intervals. A) Data is mean ± SEM of N = 5 – 8. * p < 0.05, (unpaired, two-tailed, t-test). B) Representative images of experiments shown in (A). Scale bar = 100 μM.
entirely reproduced by inhibition of thrombin activity with the anticoagulant, hirudin (800 U/ml; ▶ Fig. 4B). Therefore, despite differences in platelet size and shear forces experienced by platelets of mice and humans in these experiments, thrombin-dependent platelet activation was impaired at elevated blood shear rates in both cases, and to a similar extent.

We next examined whether the decrease in thrombus size in blood taken from PAR4−/− mice was due to a reduction in platelet number in the thrombus or otherwise (such as a change in overall thrombus structure). In experiments in which a tightly-controlled number of fluorescently-labeled platelets (1.7 x 10⁶) were infused i.v. into mice of matching genotype, a three-fold decrease in platelet number was observed in thrombi formed in blood from PAR4−/− compared with PAR4+/+ mice at the shear rate of 600 s⁻¹, but no differences were observed when blood from PAR4+/+ and PAR4−/− mice was flowed at 1,800 s⁻¹ (▶ Fig. 5A, B). Together, these results demonstrate that thrombin-mediated platelet activation and deposition into growing thrombi, via PARs, is dependent on blood shear rate in both mice and humans.

Discussion

Defining the mechanisms underlying pathological thrombus formation may lead to novel, safe, and effective antithrombotic approaches. Thrombin-induced platelet activation, via PARs, is important for thrombus formation (11–15) and PARs are key targets for novel anti-platelet therapy. Yet the role of PAR-dependent platelet activation in the formation of thromboses during pathological conditions, in particular in the presence of high blood shear, has not been examined. In this regard, we have uncovered a shear-regulation of PAR-mediated platelet activation during thrombus formation in both mice and humans. Given the clinical progression of PAR antagonists for use in the prevention of arterial thrombosis, these findings may have important implications for future antithrombotic strategies.

We used two distinct in vivo thrombus assays and an ex vivo whole blood flow approach to show that the contribution of PAR-mediated platelet activation to thrombosis is diminished at pathological blood shear rates. Despite initiating thrombosis in vivo with two distinct mechanisms of vascular injury, the protection against thrombosis routinely observed in PAR4−/− mice (11–13) was lost in the presence of vessel stenosis – and therefore presumably significantly greater blood shear rates at the site of thrombus formation – in both cases. While thrombus formation in the Folts-like and electrolytic-injury models used here are both known to be dependent on thrombin, the relative importance of thrombin in these two models may vary. In addition, the extent of vascular damage produced by the electrolytic injury is likely greater than that caused by the manual pinching performed in the Folts-like model, since occlusive thrombus formation is rarely achieved in the Folts-like model in unstenosed arteries. Yet despite these possible differences, similar shear-dependent effects were observed under each of the conditions examined in PAR4−/− mice, suggesting that the nature and extent of vascular injury does not markedly impact on the relative contribution of thrombin under high blood shear conditions. This hypothesis is further supported by our observation of a similar shear regulation in the ex vivo model, in which thrombosis is initiated primarily via collagen and the overall contribution of thrombin is low compared with most in vivo models.

The antithrombotic potential of PAR antagonists has long been recognised (9, 10). However, as with existing therapies, the efficacy of any novel agent is likely to be dependent on the context of the underlying pathology. Previous reports have demonstrated marked protection against thromboses in PAR4−/− mice initiated in arterioles (11, 13, 15) or veins (12), where blood shear rates are well below the pathological rates in stenosed carotid arteries of the present study. Our findings in both ex vivo and in vivo thrombosis models establish a novel, shear-dependent role for PARs in mediating platelet activation and deposition into growing thrombi, and suggest that PAR antagonists may experience limitations when used in isolation for the prevention of pathological arterial thrombi. This is very similar to the limitations currently noted when using thrombin inhibitors. Indeed, several studies have revealed that inhibition of thrombin activity decreased overall thrombus size at venous, but not arterial, blood shear rates (24–26). We have reproduced this observation in the present studies. In both mice and humans, inhibition of thrombin activity with hirudin markedly reduced thrombus formation at low shear rates (< 1,000 s⁻¹) but had no effect on thrombus formation at elevated shear rates (> 1,000 s⁻¹). In previous studies, this effect was thought to be due to decreased fibrin production and consequent impairment of thrombus stability (26). However, our findings extend on these earlier studies and suggest that thrombin-dependent platelet deposition is also impaired by elevated blood shear rates. Since hirudin did not produce additional effects on thrombus size over PAR4-deficiency, fibrin does not appear to contribute to thrombus growth or stability in our ex vivo model, implying that the assay is almost entirely platelet-dependent. Indeed, we directly observed a

What is known about this topic?

- Thrombin mediates platelet activation via protease-activated receptors (PARs).
- Platelet PARs are critical for arterial thrombosis in animal models performed in arterioles and veins under physiological blood flow conditions.
- PAR antagonists are in phase 3 trials for the prevention of arterial thrombosis.

What does this paper add?

- The contribution of PAR-mediated platelet activation to thrombus formation diminishes as blood shear rates increase in both mice and humans.
- Since pathological arterial thrombosis often occurs under high blood shear, the decreased role of PARs under such conditions may inform the use of PAR antagonists for the prevention of arterial thrombosis.
marked reduction in the number of platelets incorporated into PAR4-/- thrombi formed at low, but not high, blood shear rates.

Although human platelets express two thrombin-sensitive PARs, PAR1 and PAR4, and activation of either receptor is capable of inducing platelet activation (27), we were able to reproduce our findings in PAR4-/- mouse platelets when only PAR1 was inhibited pharmacologically in human whole blood. PAR1 is the major human platelet thrombin receptor and is capable of mediating platelet activation by low (sub-nanomolar) concentrations of thrombin (27). In the absence of PAR1 function, PAR4 in human platelets can also trigger full platelet activation but requires relatively high (10– to 30-fold higher) concentrations of thrombin to do so (27). Since hirudin treatment reproduced the effects of PAR1 antagonism in human whole blood experiments, residual platelet activation and deposition in this model is unlikely due to poor levels of pharmacological blockade or to the possible ‘backup’ function of PAR4 on human platelets. This finding raises questions regarding the function of human platelet PAR4 during thrombus formation, which will require the development of robust antagonists against human PAR4 in order to be addressed.

These findings of the present study may have important implications for the future use of PAR antagonists as antithrombotic agents. Two distinct PAR1 antagonists are currently in phase 3 clinical trials for the prevention of arterial thrombosis in at-risk individuals (7, 8, 28). Vorapaxar (SCH-530348) is a small molecule, high potency PAR1 antagonist which, when administered to patients in addition to standard anti-platelet therapy (clopidogrel and/or aspirin) in a phase 2 trial, showed no increase in major clinical bleeding endpoints (i.e. events requiring hospitalisation) but resulted in a non-statistically significant reduction in cardiovascular events versus standard anti-platelet therapy alone (29). A distinct human PAR1 antagonist, atopaxar (E-5555), showed similar results in phase 2 trials (28).

Shear forces are known to have direct effects on platelet aggregate formation (30, 31) and on markers of platelet activation (32). Yet regions of low blood flow/shear have also been recently shown to facilitate platelet aggregate formation (33), suggesting that the specific regulation of platelet function by blood shear forces remains unclear. Although the mechanism by which high blood shear rates limit the production and/or function of thrombin-dependent platelet activation observed in our studies remains unknown, recent work suggests the assembly of blood borne coagulation factors on the surface of activated platelets is limited under conditions of high blood flow/shear (34). Regardless of the mechanism involved, the clinical progression of PAR1 antagonists suggest it is now of interest to examine the potential interactions and possible synergies between existing anti-platelet agents with drugs that interfere with PAR-mediated platelet activation under such high shear pathological conditions.

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Conflict of interest

None declared.

References


