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RESEARCH PAPER

Safety and efficacy of targeting platelet proteinase-activated receptors in combination with existing anti-platelet drugs as antithrombotics in mice

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BACKGROUND AND PURPOSE

Developing novel anti-platelet strategies is fundamental to reducing the impact of thrombotic diseases. Thrombin activates platelets *via* proteinase-activated receptors (PARs), and PAR antagonists are being evaluated in clinical trials for prevention of arterial thrombosis. However, one such trial was recently suspended due to increased bleeding in patients receiving a PAR_1 antagonist in addition to anti-platelet drugs that most often included both aspirin and clopidogrel. Therefore, it remains unclear how to best manipulate PARs for safe antithrombotic activity. To address this, we have examined potential interactions between existing anti-platelet drugs and strategies that target PARs.

EXPERIMENTAL APPROACH

We used *in vivo* mouse models in which interactions between various anti-platelet strategies could be evaluated. We examined the effects on thrombosis and haemostasis in PAR_4–/– mice (platelets unresponsive to thrombin) treated with therapeutic doses of either aspirin or clopidogrel.

KEY RESULTS

Using a model in which occlusive thrombosis occurred in PAR_4–/– mice or wild-type mice treated with aspirin or clopidogrel, PAR_4–/– mice treated with either anti-platelet agent showed marked protection against thrombosis. This antithrombotic effect occurred without any effect on haemostasis with aspirin, but not clopidogrel. Furthermore, specifically targeting thrombin-induced platelet activation (via PARs) improved the therapeutic window of non-specifically inhibiting thrombin functions (via anticoagulants).

CONCLUSIONS AND IMPLICATIONS

Our results indicate that PAR antagonists used in combination with aspirin provide a potent yet safe antithrombotic strategy in mice and provide insights into the safety and efficacy of using PAR antagonists for the prevention of acute coronary syndromes in humans.

Abbreviations

AYPGKF, Ala-Tyr-Pro-Gly-Lys-Phe-NH_2; PAR, proteinase (protease)-activated receptor

Introduction

Arterial thrombosis, manifesting predominantly as acute myocardial infarction or ischaemic stroke, is the single most common cause of morbidity and mortality in industrialized societies, accounting for approximately one third of all deaths in Western countries (American Heart Association, 2006; Roger *et al.*, 2011). 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 (Roger *et al.*, 2011). A key limitation of existing therapies is the propensity towards pathological bleeding when multiple agents are administered in combination. For example, concurrent administration of the most commonly used anti-platelet and anticoagulant, aspirin and warfarin, respectively, does not significantly reduce rates of thrombosis without causing an increase in major haemorrhagic events (CARSC Investigators, 1997; Hurlen *et al.*, 2002; 2002; Becker, 2002; Fiore *et al.*, 2002; Andreotti *et al.*, 2006). As a consequence, the current treatment recommendation for primary or secondary prevention of cardiovascular events in acute coronary syndrome is anti-platelet therapy alone (Becker *et al.*, 2008). However, the most commonly used anti-platelet agents, aspirin and clopidogrel, prevent only ~15 and 17% of lethal cardiovascular events, respectively (CAPRIE Steering Committee, 1996; Antithrombotic Trialists' Collaboration, 2002). In combination, these two agents are more effective than when either is used alone, but at the expense of increased bleeding (Antithrombotic Trialists' Collaboration, 2002). Therefore, when considering novel anti-platelet approaches, it is of particular importance to develop strategies that will increase antithrombotic efficacy without increasing bleeding when the new agent is combined with existing therapies.

Given the pivotal role of thrombin in the pathogenesis of arterial thrombosis, inhibition of thrombin-induced platelet activation may be a successful approach for the prevention of acute coronary events (Morrow *et al.*, 2009). Thrombin's effects on platelets are mediated through the cleavage of membrane-associated proteinase-activated receptors (PARs) (Coughlin, 2000; Hamilton, 2009). In mice, PAR_4 is the sole thrombin receptor capable of transducing a signal sufficient for platelet activation (Sambrano *et al.*, 2001). PAR_4–/– mice are protected against several experimental models of thrombosis yet do not exhibit spontaneous bleeding (Sambrano *et al.*, 2001; Weiss *et al.*, 2002; Camerer *et al.*, 2004; Hamilton *et al.*, 2004; Vandendries *et al.*, 2007), 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 (Morrow *et al.*, 2009). However, the TRA-CER trial of the PAR_1 antagonist vorapaxar was recently stopped because increased intracranial bleeding was detected in patients receiving vorapaxar who had a previous history of stroke (Tricoci *et al.*, 2012). A second trial examining vorapaxar for secondary prevention of atherothrombotic events was continued but modified to exclude patients with a previous incidence of stroke. In these trials, vorapaxar was added to standard of care anti-platelet medication that included both aspirin and clopidogrel in ~97% of cases (Becker *et al.*, 2009), such that the specific drug combination responsible for causing bleeding was unknown. As a result, there is much to learn regarding how to best manipulate thrombin signalling in platelets to improve the efficacy and safety of anti-platelet therapy. To directly address these topical issues, we examined the importance of PAR-mediated platelet activation in *in vivo* thrombus formation and the effect of concurrent administration of existing anti-platelet agents in order to provide insights into the efficacy and safety of combining PAR antagonists with existing anti-platelet agents. Our findings suggest that PAR antagonists in combination with aspirin will provide a safe and effective approach for the prevention of arterial thrombosis in humans.

Methods

Mice

Mice used in these studies were either proteinase-activated receptor 4-deficient (PAR_4–/–) (Sambrano *et al.*, 2001), or littermate or age-, sex- and weight-matched wild-type (PAR_4+/+) controls. All 173 mice used in this study were backcrossed ≥10 generations onto a C57Bl/6 genetic background (i.e. ≥99.8%). Mice were maintained on a 12 h light/dark cycle with food and water *ad libitum* and all studies were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. For the *in vivo* and *ex vivo* haemostasis and thrombosis experiments described next, mice were treated with aspirin (200 $mg \cdot kg^{-1}$; Solprin, Reckitt Benckiser, Slough, UK) or its vehicle (volume matched 0.9% normal saline, Baxter, Vienna, Austria), clopidogrel (3 or 20 $mg \cdot kg^{-1}$; Plavix, Sanofi Winthrop, Paris, France) or its vehicle [0.9% normal saline for clopidogrel at 3 $mg \cdot kg^{-1}$; 5% (w v^{-1}) gum arabic for clopidogrel at 20 $mg \cdot kg^{-1}$], or hirudin (2, 5, 10, or 20 $mg \cdot kg^{-1}$; Refludan, Celgene, Summit, NJ, USA) or its vehicle (volume matched 0.9% normal saline). Aspirin and clopidogrel were administered p.o. at 24 and then 2 h before experimentation. Hirudin was administered i.v. 10 min prior to experimentation.

The results of all studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

In vivo *thrombosis model*

Mice were anaesthetized using sodium pentobarbitone (~60 $mg \cdot kg^{-1}$, i.p.; Virbac Animal Health, Milperra, NSW, Australia), and anaesthesia was monitored using pedal reflex. Lignocaine (1%, Xylocaine; Astra Pharmaceuticals, North Ryde, NSW, Australia) was used for local anaesthesia at the site of surgery. The left carotid artery was exposed via blunt dissection and dissected clear of the vagus nerve and surrounding tissue. A flow probe (0.5 mm i.d.) linked to a flow metre (TS420, Transonic Systems, Ithaca, NY, USA) was placed around the artery and blood flow ($mL \cdot min^{-1}$) was recorded using PowerLab Chart software (v. 5.0, AD Instruments, Colorado Springs, CO, USA). All mice were allowed to stabilize for at least 15 min following surgery before the experiment proceeded. The electrolytic model of thrombosis was performed essentially as previously described (Sturgeon *et al.*, 2006). A constant current lesion maker (Model D.C.LM5A, Grass

Instruments, West Warwick, RI, USA) was used to deliver 18 mA for 2 min to the carotid artery via a platinum electrode – the minimal current required to reliably produce a stable occlusive thrombotic event in untreated, wild-type mice. In some experiments, a suture was tied around the carotid artery, distal to the flow probe, and tightened to cause a stenosis that decreased blood flow to ~50% of the starting carotid blood flow. In all cases, blood flow was allowed to stabilize for at least 15 min before further manipulation and was monitored for 30 min post-injury.

In vivo *haemostasis model*

Haemostasis was assessed in mice by using the template tail bleeding time method (Schoenwaelder *et al.*, 2011). A scalpel blade was used to make a standardized incision (5 mm long and 1 mm deep) 10 mm from the tip of the tail of anaesthetized mice heated to 37°C using a heat pad. The wound site was then blotted every 30 s until no blood was detected and the time to cessation of bleeding was recorded.

Analysis of platelet function

Aggregation of washed platelets was used to assess the effect of drug treatment on platelet function. The preparation of platelets was as previously described (Maxwell *et al.*, 2004). Agonists used included a PAR_4-activating peptide (AYPGKF, 100 μM; Auspep, West Melbourne, Victoria, Australia), arachidonic acid (AA, 100 and 200 μM; Helena Laboratories, Beaumont, TX, USA) and ADP (10 and 20 μM; Sigma, St Louis, MO, USA).

Statistical analyses

Statistical significance ($P < 0.05$) was determined by either Student's unpaired, two-tailed *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

Development of an in vivo *thrombosis model resistant to PAR_4-deficiency or to pretreatment with clinically relevant doses of existing anti-platelet agents*

The electrolytic injury model we used in these studies delivered the minimal current required to induce a stable, platelet-rich, occlusive thrombus in 100% of untreated wild-type mice. Using this model, we first showed that PAR_4–/– mice were markedly protected against electrolytic injury-induced thrombosis in the carotid artery when compared with littermate PAR_4+/+ mice. All four PAR_4+/+ mice formed occlusive thrombi within 20 min post-injury compared with none of the four PAR_4–/– mice (Figure 1A,B). Similarly, pretreatment of wild-type mice with either of the most commonly used anti-platelet agents, aspirin or clopidogrel, also conferred striking protection against thrombosis in this model (Figure 1A,B). We confirmed that platelets isolated from mice treated with aspirin or clopidogrel showed the expected, clinically relevant, levels of impaired response to AA (Kuster and Frolich, 1986) and ADP (Denninger *et al.*, 1999), respectively. Pretreatment of mice with the $P2Y_{12}$ ADP receptor antagonist clopidogrel (3 or 20 $mg \cdot kg^{-1}$ p.o. at –24 and –2 h) inhibited maximal platelet aggregation in response to ADP by ~60% (3 $mg \cdot kg^{-1}$) and 80% (20 $mg \cdot kg^{-1}$) (Figure 1C). Aspirin (200 $mg \cdot kg^{-1}$ p.o. at –24 and –2 h) was used at the minimum dose required to reliably abolish AA-induced aggregation of isolated platelets (Figure 1D). Neither treatment affected aggregation responses to a PAR_4-activating peptide (AYPGKF; Figure 1C,D). Therefore, we used doses of 3 $mg \cdot kg^{-1}$ for clopidogrel and 200 $mg \cdot kg^{-1}$ for aspirin in subsequent *in vivo* experiments because they most accurately mimicked the level of platelet function inhibition achieved in humans following standard clinical doses of each of these anti-platelet agents.

In order to be able to examine potential interactions between anti-platelet strategies, we modified this *in vivo* thrombosis assay such that it became resistant to treatment with either aspirin or clopidogrel, or to PAR_4 deficiency (Lee *et al.*, 2012). The inclusion of a stenosis which reduced blood flow by ~50% at the site of injury markedly impaired the protection against thrombosis in PAR_4–/– mice. All PAR_4+/+ and PAR_4–/– mice occluded under these conditions ($n = 6$ each; Figure 2A) with no differences in blood flow through the injured arteries between PAR_4+/+ and PAR_4–/– mice (Figure 2B). In addition, we also observed robust thrombosis in this model in wild-type mice treated with either aspirin or clopidogrel. Occlusive thrombi occurred in all six vehicle-treated wild-type mice and in five out of six of each of the aspirin- or clopidogrel-treated mice (Figure 2C,D). Therefore, this *in vivo* mouse thrombosis model enables interactions between relevant anti-platelet strategies to be examined.

Existing anti-platelet agents act synergistically with PAR_4-deficiency to provide improved antithrombotic effects

To examine potential interactions between the two most commonly used anti-platelet drugs and targeting PARs, we next examined thrombosis initiated in stenosed carotid arteries (~50% reduction in blood flow) in PAR_4–/– mice treated with aspirin (200 $mg \cdot kg^{-1}$) or clopidogrel (3 $mg \cdot kg^{-1}$). The stable occlusive thrombi which was observed in all six vehicle-treated PAR_4–/– mice examined was strongly prevented by the addition of either aspirin or clopidogrel (Figure 3). The level of protection provided by each anti-platelet drug in PAR_4–/– mice was similar, with 0/6 occlusive thrombi and a similar level of blood flow recorded through the injured arteries in both treatment groups. Of note, treatment of PAR_4+/+ mice with a combination of aspirin and clopidogrel provided a similar level of protection against thrombosis in this model (Figure 3C,D). These findings suggest a synergy between targeting platelet PARs and existing anti-platelet strategies, and indicate that such combinations provide a potent anti-thrombotic effect.

Improved antithrombotic efficacy without impairment of haemostasis with aspirin, but not clopidogrel

To determine the comparative safety of inhibiting PAR-dependent platelet activation in combination with aspirin or clopidogrel, we assessed haemostasis using the gold-standard

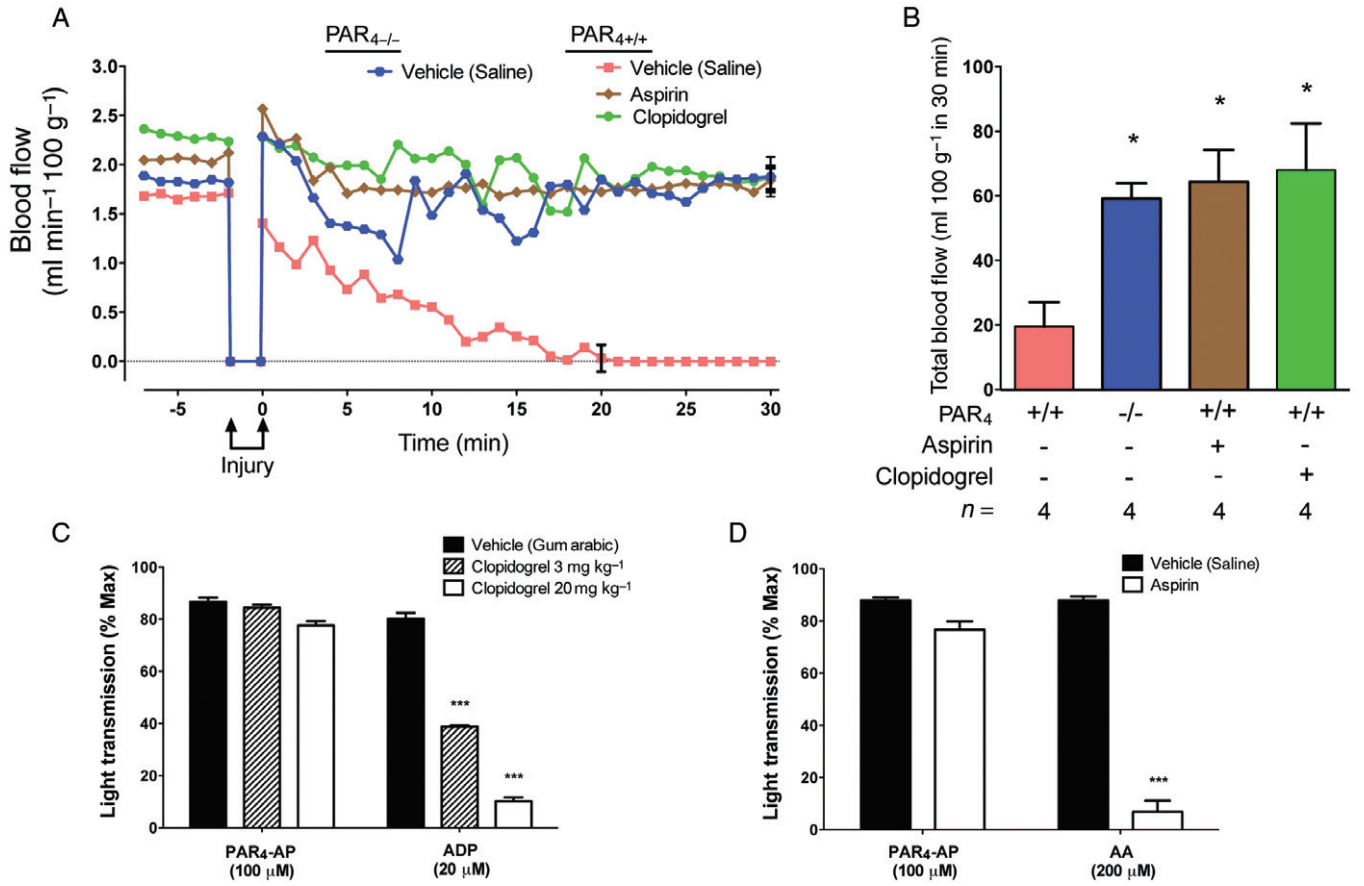

Figure 1

PAR$_4$-deficiency, aspirin or clopidogrel provide marked protection against *in vivo* thrombosis in mouse carotid arteries. *In vivo* thrombosis in PAR$_4$+/+ mice in the absence and presence of the anti-platelet drugs aspirin (200 mg·kg^{-1}) or clopidogrel (3 mg·kg^{-1}) as well as PAR$_4$–/– mice. Electrolytic injury of carotid arteries was induced under stasis by a current of 18 mA for 2 min. (A) Body weight-adjusted blood flow rates were continuously recorded from 5 min before to 30 min after injury. (B) Body weight-adjusted total blood flow over the 30 min post-injury period. Data are mean ± SEM; *$P < 0.05$ (one-way ANOVA, with Dunnett's test for multiple comparisons vs. vehicle-treated PAR$_4$+/+ mice). (C,D) Aggregation of platelets isolated from PAR$_4$+/+ mice treated with either clopidogrel (3 or 20 mg·kg^{-1}) or aspirin (200 mg·kg^{-1}) induced by ADP (20 μM), arachidonic acid (AA, 200 μM) or a PAR$_4$-activating peptide (AYPGKF, 100 μM; PAR$_4$-AP). The maximum response induced by each treatment is presented. Data are mean ± SEM; $n = 3$; ***$P < 0.001$ (one-way ANOVA with Dunnett's test for multiple comparisons vs. vehicle-treated PAR$_4$+/+ mice).

approach in mice – that of tail bleeding time. As previously observed, either PAR$_4$ deficiency (Sambrano *et al.*, 2001) or treatment with aspirin or clopidogrel (Momi *et al.*, 2005) caused marked prolongation of the bleeding time in the most commonly used mouse model of haemostasis, which involves tail transection (data not shown). Therefore, to analyse any additive and/or synergistic effects on haemostasis, we used the template tail bleeding time model – a comparatively insensitive assessment of bleeding in which neither aspirin nor clopidogrel affected the haemostatic potential of PAR$_4$+/+ mice (Figure 4A). Using this model, pretreatment with clopidogrel, but not with aspirin, caused a significant prolongation in the bleeding time of PAR$_4$–/– mice: all eight PAR$_4$–/– mice pretreated with clopidogrel continued to bleed 15 min after the incision, at which time the assay was terminated (Figure 4A). In contrast, no difference in bleeding time was observed in PAR$_4$–/– mice in the absence or presence of aspirin treatment (Figure 4A). These results suggest that the improved antithrombotic activity comes at the expense of impaired haemostasis when PARs are targeted in combination with clopidogrel, but not with aspirin.

Specifically targeting PAR-mediated platelet activation increases the therapeutic window over anticoagulation

Using the same approach to examine efficacy (antithrombotic activity) and safety (impact on haemostasis) as outlined previously, we next directly compared the effects of specific inhibition of thrombin-induced platelet activation (PAR$_4$–/–) with non-specific inhibition of thrombin activity using the direct thrombin inhibitor, hirudin. In order to make a useful comparison, we first determined the dose of hirudin that, in the presence of aspirin (200 mg·kg^{-1}), caused a similar haemostatic effect to that recorded in aspirin-treated PAR$_4$–/– mice (5 mg·kg^{-1}; Figure 4A,B: arrows). Hirudin administered at this dose (5 mg·kg^{-1}) was sub-maximal for *in vivo* thrombin

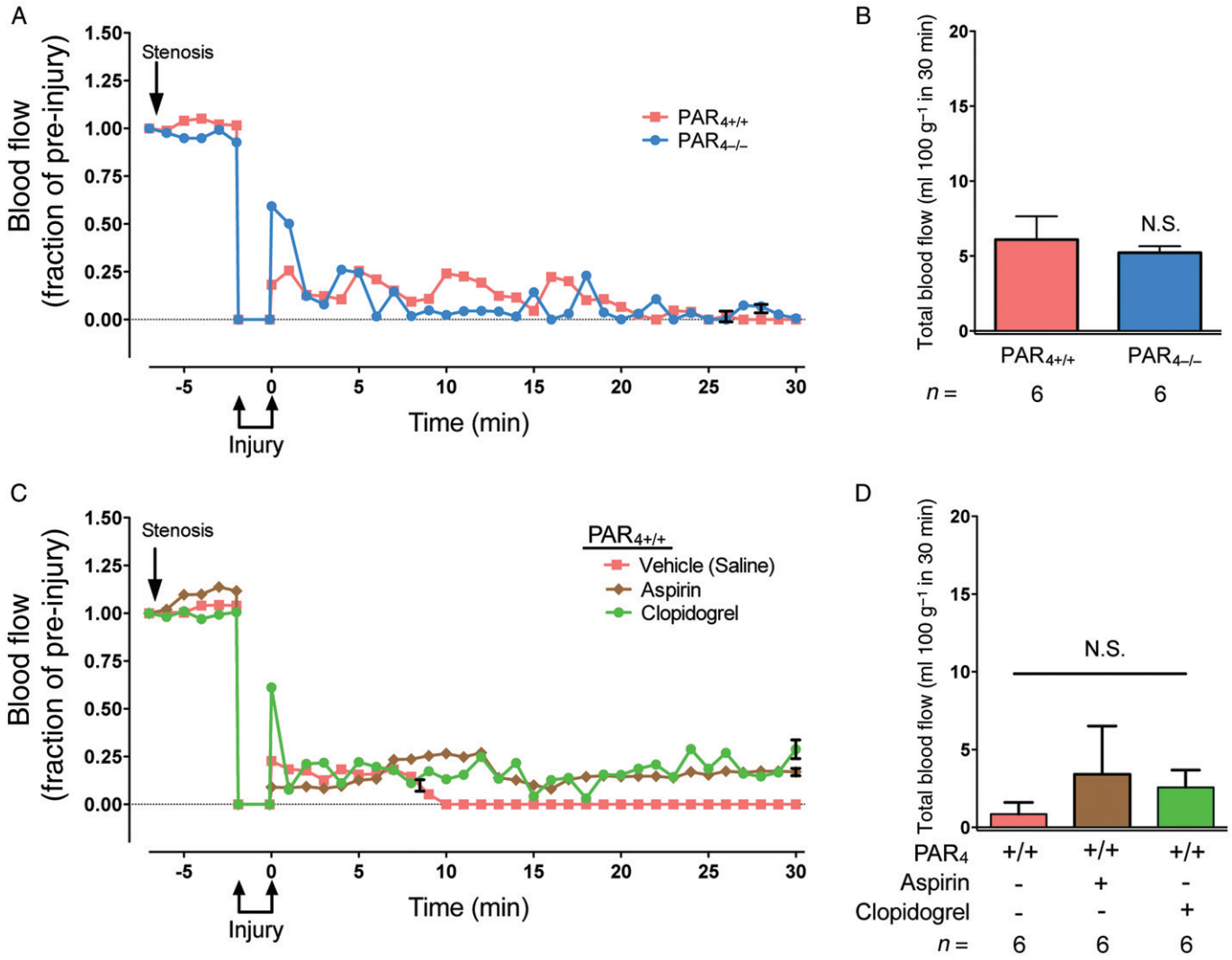

Figure 2

Development of an *in vivo* thrombosis model resistant to PAR_4 deficiency or to pretreatment with aspirin or clopidogrel. *In vivo* thrombosis in (A) PAR_4+/+ versus PAR_4–/– mice and (B) PAR_4+/+ mice in the absence and presence of the anti-platelet drugs aspirin (200 mg·kg^{-1}) or clopidogrel (3 mg·kg^{-1}). Electrolytic injury was induced under stasis by a current of 18 mA for 2 min in arteries with a stenosis in which blood flow was reduced to ~50% of control levels. (A,C) Body weight-adjusted blood flow rate and (B,D) body weight-adjusted total blood flow. Vehicle is saline. Data are mean ± SEM; N.S., not significant.

inhibition, as a twofold increase in hirudin dose prolonged the tail bleeding time in these experiments (Figure 4B). Despite the similar effect on haemostasis in the template tail bleeding assay, we observed significantly greater protection against *in vivo* thrombosis in aspirin-treated PAR_4–/– mice than in aspirin- and hirudin-treated wild-type mice (Figure 5A,B). Furthermore, during the course of these *in vivo* thrombosis studies, we also observed markedly more surgical bleeding in aspirin- and hirudin-treated mice when compared with aspirin-treated PAR_4–/– mice (Figure 5C). Specifically, the extent of bleeding in aspirin- and hirudin-treated mice was so severe that almost all mice in this group failed to survive the experiment without active management of the wounds at the site of surgery to prevent excessive blood loss. In contrast, PAR_4–/– mice treated with aspirin did not require such intervention and lost very little blood during the surgery. Together, these findings suggest that, when used in combination with aspirin, specifically targeting thrombin-induced platelet activation via PARs improves the therapeutic window of the current strategy of non-specifically inhibiting the actions of thrombin with anticoagulants (i.e. inhibiting all actions of thrombin, including both fibrin formation and PAR-dependent platelet activation) for the prevention of platelet-dependent arterial thrombosis.

Discussion and conclusions

Developing novel anti-platelet strategies for the prevention of arterial thrombosis is a key clinical priority. Thrombin-

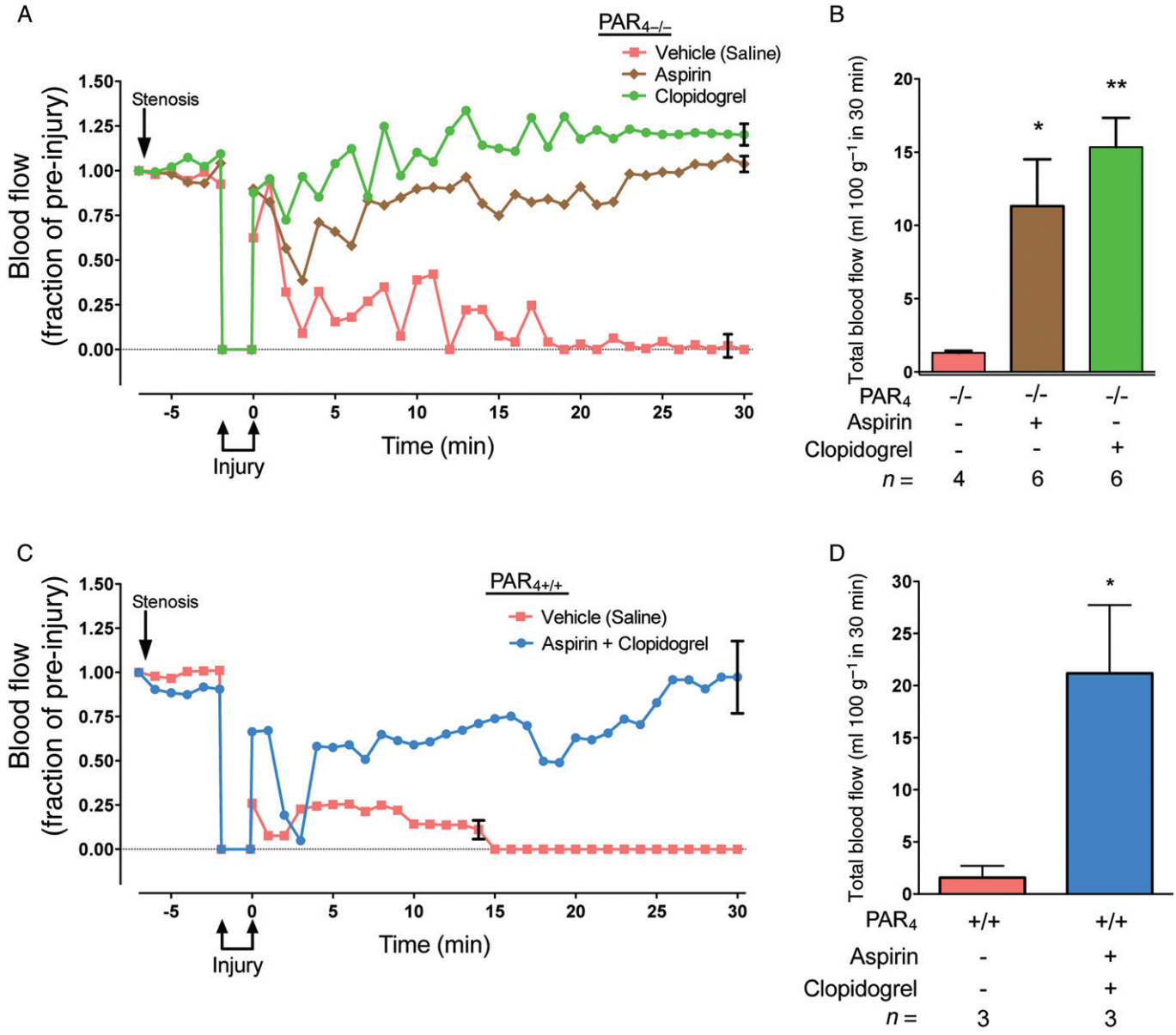

Figure 3

Aspirin and clopidogrel act synergistically with PAR_4 deficiency to provide robust antithrombotic activity. *In vivo* thrombosis in (A) PAR_4–/– and (B) PAR_4+/+ mice in the absence and presence of the anti-platelet drugs aspirin (200 mg·kg^{-1}) and/or clopidogrel (3 mg·kg^{-1}). Electrolytic injury was induced under stasis by a current of 18 mA for 2 min in arteries with a stenosis in which blood flow was reduced to ~50% of control levels. (A,C) Body weight-adjusted blood flow rate and (B,D) body weight-adjusted total blood flow. Vehicle is saline. Data are mean ± SEM; *$P < 0.05$; **$P < 0.01$ (one-way ANOVA with Dunnett's test in (B), Student's unpaired *t*-test in (D)).

induced platelet activation, via PARs, is important for thrombus formation (Sambrano *et al.*, 2001; Weiss *et al.*, 2002; Camerer *et al.*, 2004; Hamilton *et al.*, 2004; Vandendries *et al.*, 2007), and antagonists of platelet PARs are leading candidates for such therapy. Indeed, two distinct PAR_1 antagonists are currently undergoing assessment in Phase 3 clinical trials. Vorapaxar (previously known as SCH-530348) is a small molecule, highly potent PAR_1 antagonist which, when administered to patients in addition to standard anti-platelet therapy (clopidogrel and/or aspirin) in a Phase 2 trial resulted in a non-statistically significant reduction in cardiovascular events versus standard anti-platelet therapy alone (Becker *et al.*, 2009). A distinct human PAR_1 antagonist, atopaxar (previously known as E-5555), showed similar results in Phase 2 trials (Goto *et al.*, 2010) and is also currently being evaluated in Phase 3 trials for the primary and secondary prevention of cardiovascular events (Morrow *et al.*, 2009; TRA*CER Executive and Steering Committees, 2009). However, the recent decision to suspend the TRA-CER trial of vorapaxar due to increased bleeding in patients receiving this drug in

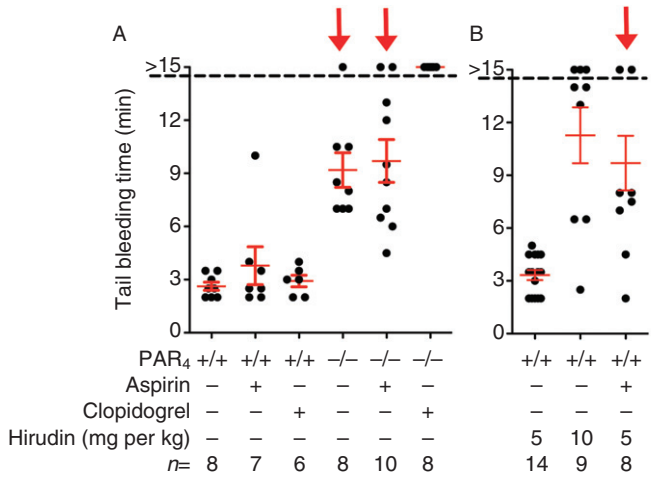

Figure 4

Clopidogrel, but not aspirin, affects haemostasis when in combination with PAR_4-deficiency. (A) Tail bleeding times in PAR_4+/+ and PAR_4–/– mice treated with vehicle (saline), aspirin (200 mg·kg^{-1}) or clopidogrel (3 mg·kg^{-1}), and/or (B) hirudin (5 or 10 mg·kg^{-1}), as determined using the template method. Individual data points are shown. (Note partial obstruction of $n = 8$ data points in the clustered group of PAR_4–/– mice treated with clopidogrel.) Bars are mean ± SEM. Note the similar bleeding time in untreated PAR_4–/– mice (A), aspirin-treated PAR_4–/– mice (A), and aspirin- and hirudin-treated PAR_4+/+ mice (B) (arrows). *In vivo* thrombosis was examined in these three groups in Figure 5.

combination with standard of care anti-platelet therapy has underscored the lack of knowledge regarding potential interactions between current anti-platelet therapies and drugs that target PARs. To directly address this knowledge gap, we examined the efficacy (antithrombotic activity) and safety (bleeding risk) of combining each of the two most commonly used anti-platelet drugs, aspirin and clopidogrel, with blocking platelet PAR function using *in vivo* mouse models.

To perform these studies, we used a well-characterized *in vivo* thrombosis model in which platelet-rich, occlusive thrombi are reliably and reproducibly generated in response to the vascular injury created by passing current across the artery (Sturgeon *et al.*, 2006). Although PAR_4 deficiency provides robust protection against thrombosis in a variety of mouse models performed under physiological blood flow and blood vessel conditions (Sambrano *et al.*, 2001; Weiss *et al.*, 2002; Hamilton *et al.*, 2004; Vandendries *et al.*, 2007; Cornelissen *et al.*, 2011), we first confirmed that a similar protection also occurs following electrolytic injury of mouse carotid arteries. We next modified this model such that thromboses became resistant to individual anti-platelet approaches so that additive and/or synergistic effects of combination strategies could be examined. Specifically, the inclusion of a stenosis at the site of thrombus formation (~80% decrease in cross-sectional luminal area) abolished the antithrombotic effects of PAR_4 deficiency as well as that provided by either aspirin or clopidogrel. Of note, such conditions of artery stenosis are frequently present at the sites of thrombosis in human pathologies, most notably the rupture of mature atherosclerotic plaques. Therefore, the *in vivo* thrombosis model employed here may provide an appropriate approach to examine combination therapies, in part by more closely representing the blood flow conditions present during pathological thrombosis in the large arteries of humans.

Using this *in vivo* mouse model of thrombosis, we have shown that either of the most commonly used anti-platelet agents, aspirin or clopidogrel – at doses which cause clinically relevant levels of platelet inhibition – provide a potent antithrombotic setting when used in combination with PAR_4 deficiency. The development of novel anti-platelet agents that work well in combination with existing drugs is a key requirement for any future antithrombotic strategies. In this regard, our findings in mouse models suggest an important synergy between PAR-dependent platelet activation mechanisms and those targeted by existing anti-platelet agents (i.e. clopidogrel, which inhibits the $P2Y_{12}$ platelet receptor for ADP, and aspirin, which inhibits the synthesis of thromboxane A_2). However, this antithrombotic efficacy occurred without additional bleeding liability when PAR_4 deficiency was combined with aspirin, but not with clopidogrel. This pharmacological finding is consistent with the observation that mice deficient in both PAR_4 and $P2Y_{12}$ receptors have markedly impaired haemostasis (Cornelissen *et al.*, 2011). In contrast, we have shown that aspirin treatment of PAR_4–/– mice had very little impact on haemostasis, despite a powerful antithrombotic effect with this combination. These findings may inform the possible bleeding risk in humans, particularly as all trials of PAR_1 antagonists are conducted with patients receiving the test drug in addition to standard of care that most often includes both clopidogrel and aspirin. In the abandoned TRA-CER trial, it is still not known whether the increased bleeding observed in patients receiving vorapaxar clustered to those receiving triple anti-platelet therapy, or whether those patients not receiving clopidogrel were spared pathological bleeding. Our findings suggest that such analyses of trials of PAR antagonists are warranted.

PAR antagonists inhibit thrombin-induced platelet activation (Kahn *et al.*, 1999; Ahn *et al.*, 2000; Derian *et al.*, 2003; Chackalamannil *et al.*, 2005; Wu and Teng, 2006). Anticoagulants non-specifically inhibit the production and/or function of thrombin. When the prevention of platelet-dependent arterial thrombosis is the goal, one key advantage proposed of PAR antagonists over anticoagulants is that they may preserve haemostatic function by sparing the non-PAR functions of thrombin – most notably that of fibrin formation, but also feedback activation of coagulation factors. The current studies directly tested this hypothesis in mouse models. We compared the safety and efficacy of specifically targeting thrombin-induced platelet activation (PAR_4–/– mice) with that of non-specific inhibition of thrombin activity with an anticoagulant (hirudin) and showed that targeting PARs provided a safer and more effective therapeutic approach for the prevention of arterial thrombosis. It should be noted that, as is the case clinically, hirudin was used in these studies at doses that do not completely neutralize thrombin activity *in vivo* (5 mg·kg^{-1}). This was evident from the lack of haemostatic effect in mice treated with hirudin at 5 mg·kg^{-1}, but not at ≥10 mg·kg^{-1}. In contrast to the lack of specific effects on thrombin activity afforded by anticoagulants, the specific

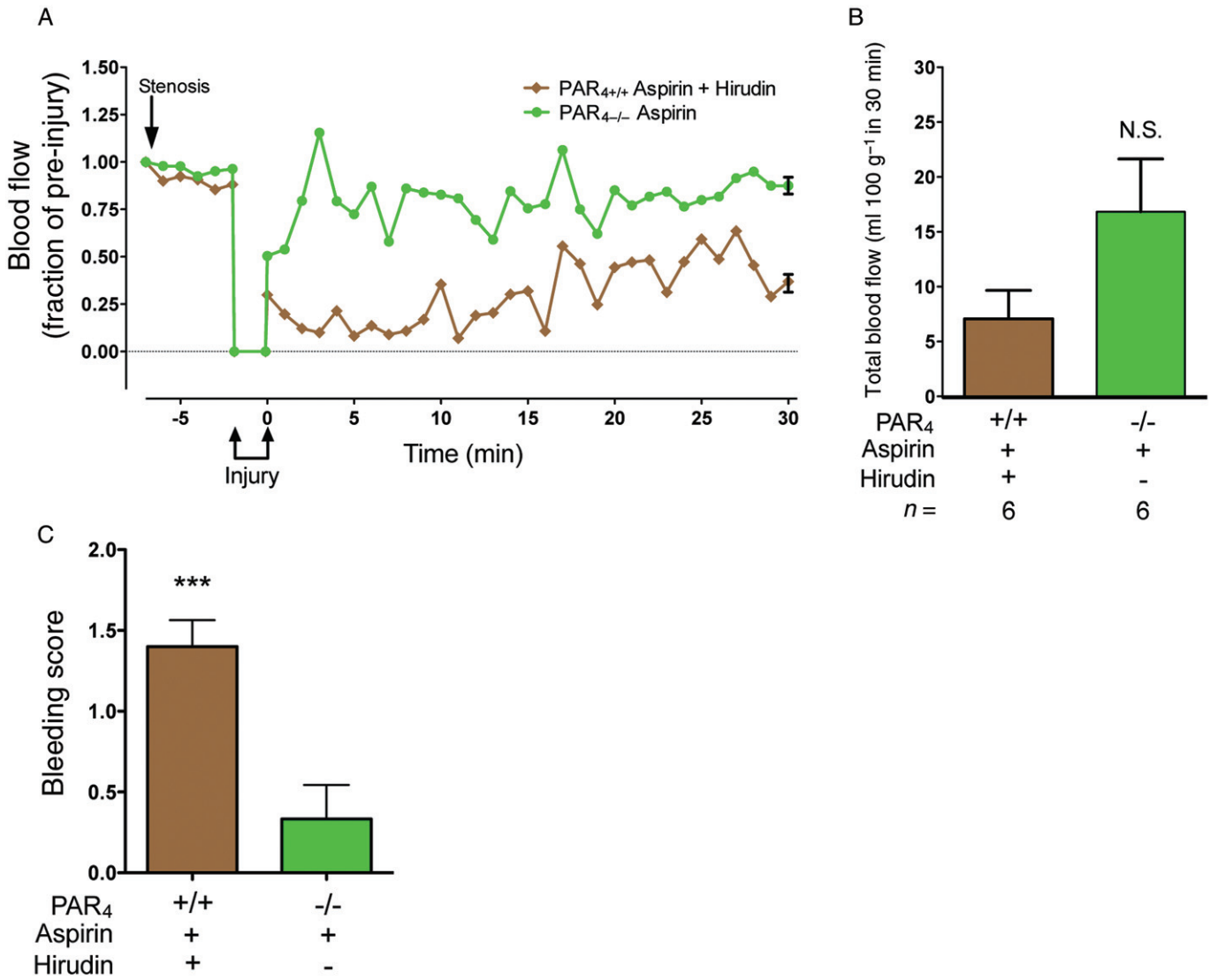

Figure 5

Increased therapeutic window in PAR_4–/– mice treated with aspirin compared with mice treated with hirudin and aspirin. *In vivo* thrombosis in aspirin-treated PAR_4–/– mice versus aspirin- and hirudin-treated PAR_4+/+ mice. Electrolytic injury was induced under stasis by a current of 18 mA for 2 min in arteries with a stenosis in which blood flow was reduced to ~50% of control levels. (A) Body weight-adjusted blood flow rate and (B) body weight-adjusted total blood flow. (C) Surgical bleeding scores. 0 = no/limited bleeding; 1 = bleeding requiring intervention in order for the experiment to proceed; 2 = bleeding causing death. Vehicle is saline. Data are mean ± SEM. N.S., not significant. ***$P < 0.001$ (Student's two-way, unpaired *t*-test).

effect of PAR_4 deficiency is that platelets from PAR_4–/– mice are unresponsive to thrombin. As such, our studies have compared the effects of completely blocking thrombin-induced platelet activation with partially inhibiting all of thrombin's actions. It remains to be seen whether the clinical strategy of pharmacological PAR_1 blockade will provide a similar advantage. On this point, it is also worth noting that human platelets express two thrombin-sensitive PARs, PAR_1 and PAR_4, and activation of either receptor is capable of inducing platelet activation (Kahn *et al.*, 1999). In the presence of pharmacological blockade of PAR_1 function in human platelets, PAR_4 can also trigger full platelet activation but requires relatively high (10- to 30-fold higher) concentrations of thrombin to do so (Kahn *et al.*, 1999). Therefore, whether our findings in PAR_4–/– mice (thrombin-induced platelet responses absent) accurately reflect the situation in human platelets when only PAR_1 is inhibited pharmacologically (thrombin-induced platelet responses impaired but not abolished) remains to be seen. Regardless, it is well recognized that combination anticoagulant/anti-platelet therapy, most commonly as warfarin plus aspirin, impairs haemostasis without providing additional antithrombotic effects for the prevention of platelet-rich arterial thrombosis (Hurlen *et al.*, 1997; 2002; Becker, 2002; Fiore *et al.*, 2002). Therefore, our studies strongly suggest that PAR antagonists represent a more suitable alternative to anticoagulants for the prevention

of thrombin-dependent platelet activation in the setting of arterial thrombosis.

In summary, our findings further support the notion that inhibition of thrombin-dependent platelet activation, via PAR antagonists, is a promising support strategy for preventing thrombosis during conditions such as acute coronary syndromes. These observations using *in vivo* mouse models may have relevance for the potential safety and efficacy of using PAR antagonists for the prevention of arterial thrombosis in humans.

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Conflict of interest

None.

References

Ahn H-S, Foster C, Boykow G, Stamford A, Manna M, Graziano M (2000). Inhibition of cellular action of thrombin by *N*3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7*H*-pyrrolo[3,2-*f*]quinazoline-1,3-diamine (SCH 79797), a nonpeptide thrombin receptor antagonist. Biochem Pharmacol 60: 1425–1434.

American Heart Association (2006). Heart Disease and Stroke Statistics: 2006 Update. American Heart Association: Dallas, TX.

Andreotti F, Testa L, Biondi-Zoccai GG, Crea F (2006). Aspirin plus warfarin compared to aspirin alone after acute coronary syndromes: an updated and comprehensive meta-analysis of 25,307 patients. Eur Heart J 27: 519–526.

Antithrombotic Trialists' Collaboration. (2002). Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Br Med J 324: 71–86.

Becker RC (2002). Antithrombotic therapy after myocardial infarction. N Engl J Med 347: 1019–1022.

Becker RC, Meade TW, Berger PB, Ezekowitz M, O'Connor CM, Vorchheimer DA *et al.* (2008). The primary and secondary prevention of coronary artery disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 133 (6 Suppl.): 776S–814S.

Becker RC, Moliterno DJ, Jennings LK, Pieper KS, Pei J, Niederman A *et al.* (2009). Safety and tolerability of SCH 530348 in patients undergoing non-urgent percutaneous coronary intervention: a randomised, double-blind, placebo-controlled phase II study. Lancet 373: 919–928.

Camerer E, Duong DN, Hamilton JR, Coughlin SR (2004). Combined deficiency of protease-activated receptor-4 and fibrinogen recapitulates the hemostatic defect but not the embryonic lethality of prothrombin deficiency. Blood 103: 152–154.

CAPRIE Steering Committee (1996). A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 348: 1329–1339.

CARSC Investigators (1997). Randomised double-blind trial of fixed low-dose warfarin with aspirin after myocardial infarction. Lancet 350(9075): 389–396.

Chackalamannil S, Xia Y, Greenlee WJ, Clasby M, Doller D, Tsai H *et al.* (2005). Discovery of potent orally active thrombin receptor (protease activated receptor 1) antagonists as novel antithrombotic agents. J Med Chem 48: 5884–5887.

Cornelissen I, Palmer D, David T, Wilsbacher L, Concengco C, Conley P *et al.* (2011). Roles and interactions among protease-activated receptors and P2ry12 in hemostasis and thrombosis. Proc Natl Acad Sci U S A 107: 18605–18610.

Coughlin SR (2000). Thrombin signalling and protease-activated receptors. Nature 407: 258–264.

Denninger MH, Necciari J, Serre-Lacroix E, Sissmann J (1999). Clopidogrel antiplatelet activity is independent of age and presence of atherosclerosis. Semin Thromb Hemost 25 (Suppl. 2): 41–45.

Derian CK, Damiano BP, Addo MF, Darrow AL, D'Andrea MR, Nedelman M *et al.* (2003). Blockade of the thrombin receptor protease-activated receptor-1 with a small-molecule antagonist prevents thrombus formation and vascular occlusion in nonhuman primates. J Pharmacol Exp Ther 304: 855–861.

Fiore LD, Ezekowitz MD, Brophy MT, Lu D, Sacco J, Peduzzi P (2002). Department of Veterans Affairs Cooperative Studies Program Clinical Trial comparing combined warfarin and aspirin with aspirin alone in survivors of acute myocardial infarction: primary results of the CHAMP study. Circulation 105: 557–563.

Goto S, Ogawa H, Takeuchi M, Flather MD, Bhatt DL (2010). Double-blind, placebo-controlled Phase II studies of the protease-activated receptor 1 antagonist E5555 (atopaxar) in Japanese patients with acute coronary syndrome or high-risk coronary artery disease. Eur Heart J 31: 2601–2613.

Hamilton JR (2009). Protease-activated receptors as targets for antiplatelet therapy. Blood Rev 23: 61–65.

Hamilton JR, Cornelissen I, Coughlin SR (2004). Impaired hemostasis and protection against thrombosis in protease-activated receptor 4-deficient mice is due to lack of thrombin signaling in platelets. J Thromb Haemost 2: 1429–1435.

Hurlen M, Abdelnoor M, Smith P, Erikssen J, Arnesen H (2002). Warfarin, aspirin, or both after myocardial infarction. N Engl J Med 347: 969–974.

Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 103: 879–887.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010) NC3Rs Reporting Guidelines Working Group.Br J Pharmacol 160:1577–1579.

Kuster LJ, Frolich JC (1986). Platelet aggregation and thromboxane release induced by arachidonic acid, collagen, ADP and platelet-activating factor following low dose acetylsalicylic acid in man. Prostaglandins 32: 415–423.

Lee H, Sturgeon SA, Jackson SP, Hamilton JR (2012). The contribution of thrombin-induced platelet activation to thrombus growth is diminished under pathological blood shear conditions. Thromb Haemost 107: 328–337.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Maxwell MJ, Yuan Y, Anderson KE, Hibbs ML, Salem HH, Jackson SP (2004). SHIP1 and Lyn kinase negatively regulate integrin alpha IIb beta 3 signaling in platelets. J Biol Chem 279: 32196–32204.

Momi S, Pitchford SC, Alberti PF, Minuz P, Del Soldato P, Gresele P (2005). Nitroaspirin plus clopidogrel versus aspirin plus clopidogrel against platelet thromboembolism and intimal thickening in mice. Thromb Haemost 93: 535–543.

Morrow DA, Scirica BM, Fox KA, Berman G, Strony J, Veltri E *et al.* (2009). Evaluation of a novel antiplatelet agent for secondary prevention in patients with a history of atherosclerotic disease: design and rationale for the Thrombin-Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2 degrees P)-TIMI 50 trial. Am Heart J 158: 335–341. e333.

Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM *et al.* (2011). Heart disease and stroke statistics – 2011 update: a report from the American Heart Association. Circulation 123: e18–e209.

Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR (2001). Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature 413: 74–78.

Schoenwaelder SM, Jarman KE, Gardiner EE, Hua M, Qiao J, White MJ *et al.* (2011). Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. Blood 118: 1663–1674.

Sturgeon SA, Jones C, Angus JA, Wright CE (2006). Adaptation of the Folts and electrolytic methods of arterial thrombosis for the study of anti-thrombotic molecules in small animals. J Pharmacol Toxicol Methods 53: 20–29.

TRA*CER Executive and Steering Committees (2009). The Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRA*CER) trial: study design and rationale. Am Heart J 158: 327–334. e324.

Tricoci P, Huang Z, Held C, Moliterno DJ, Armstrong PW, Van de Werf F *et al.* (2012). Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. N Engl J Med 366: 20–33.

Vandendries ER, Hamilton JR, Coughlin SR, Furie B, Furie BC (2007). Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. Proc Natl Acad Sci U S A 104: 288–292.

Weiss EJ, Hamilton JR, Lease KE, Coughlin SR (2002). Protection against thrombosis in mice lacking PAR3. Blood 100: 3240–3244.

Wu CC, Teng CM (2006). Comparison of the effects of PAR1 antagonists, PAR4 antagonists, and their combinations on thrombin-induced human platelet activation. Eur J Pharmacol 546: 142–147.