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BRIEF REPORT

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Bleeding is increased in amyloid precursor protein knockout mouse

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Abstract

Background: Amyloid precursor protein (APP) is highly expressed in platelets. APP is the precursor to amyloid beta (Aβ) peptides that accumulate in cerebral amyloid angiopathy and plaques in Alzheimer disease. APP and its metabolites interact with many components of the coagulation system, and have both anticoagulant and pro-coagulant properties, but it is unclear if APP contributes to hemostasis in vivo.

Objectives: To determine whether APP contributes to hemostasis in mice, including when inhibitors of coagulation are administered.

Methods: Blood loss in APP knockout (KO) mice was measured in liver laceration and tail transection models of hemorrhage. Blood loss was also measured following tail transection in mice given an inhibitor of coagulation factor Xa (apixaban), platelet inhibitors (aspirin + clopidogrel), tissue-type plasminogen activator (t-PA), or the anti-fibrinolytic tranexamic acid (TXA).

Results and Discussion: Blood loss from liver lacerations was similar between APP KO mice and wild-type (WT) mice, but APP KO mice bled more from tail transections. When mice were challenged with aspirin + clopidogrel, the difference in bleeding between APP KO and WT mice was abrogated. In contrast, a difference in bleeding between the strains persisted when mice were treated with apixaban, t-PA, or TXA. Blood collected from APP KO mice and analyzed with thromboelastography had longer clotting times, and the clots were less stiff and more susceptible to fibrinolysis compared to blood from WT mice.

Conclusions: The absence of APP measurably increases bleeding in mice, which is consistent with a role for platelet-derived APP and Aβ peptides in hemostasis.

KEYWORDS

blood coagulation, cerebral amyloid angiopathy, fibrinolysis, hemorrhage, neurodegenerative diseases, thrombosis

Wilfred A. Jefferies and Christian J. Kastrup are co–senior authors.

Essentials

- Amyloid precursor protein (APP), the precursor of amyloid beta, is abundant in platelets.
- The role of APP in hemostasis in vivo is not clear from previous in vivo and in vitro studies.
- Mice lacking APP bled more than wild-type mice after tail transections.
- APP knockout mice bled more except with platelet inhibitors, suggesting a platelet-mediated mechanism.

1 | INTRODUCTION

Amyloid precursor protein (APP) is a type 1 transmembrane glycoprotein that is expressed in platelets.[1] On average, there are approximately 9000 molecules of APP per platelet, making APP one of the most abundant platelet proteins.[2] In humans, platelet-derived APP (platelet-APP) is the primary source of APP in the blood, accounting for more than 90% of circulating APP.[3] Platelet-APP and its metabolites, particularly amyloid beta (Aβ) peptides, have several activities involving both anticoagulant and procoagulant properties, but it is unclear whether APP contributes to physiological hemostasis.

Brain-derived APP (brain-APP) and platelet-APP are metabolic precursors of Aβ peptides. The accumulation of Aβ in brain parenchyma and cerebral vessel walls is correlated with the onset of Alzheimer disease (AD).[4] Membrane APP can be proteolytically processed by secretases in both amyloidogenic and nonamyloidogenic pathways, which release soluble Aβ and soluble APPβ, or peptide P3 and soluble APPα (sAPPα), respectively.[5] Platelets express the necessary proteases to cleave APP into these metabolites.[6] In platelets, APP, sAPPα, and Aβ are stored in alpha granules and released upon platelet activation and degranulation.[6,7]

Platelet-APP and brain-APP differentially affect reactions of the coagulation cascade. Due to differential splicing, platelet-APP, but not brain-APP, contains a Kunitz-type protease inhibitor (KPI) domain that inhibits multiple proteases, including chymotrypsin, and trypsin, and blood coagulation factors IXa, Xa, XIa, and the complex of factor VIIa with tissue factor.[8,9] Platelet-APP has previously been described as a cerebral anticoagulant.[9] Overexpression of platelet-APP, or intravenous administration of its KPI domain, has decreased cerebral thrombosis in mice.[10,11] Similarly, transgenic mice lacking the active KPI domain are prothrombotic and have shortened times of occlusion in the carotid artery and brain.[12] Mice deficient in APP (APP knockout [KO]) have 20% fewer but larger platelets with normal aggregation, secretion, and integrin αIIbβ3 inside-out activation.[13] APP KO mice also developed larger thrombi following inferior vena cava stenosis.[13] Additionally, these APP KO mice had elevated factor XIa, and shorter activated partial thromboplastin times, but not prothrombin times, in the presence of platelets, compared to wild-type (WT) mice. These same APP KO mice had elevated platelet-leukocyte aggregates and neutrophil extracellular traps.

Platelet-APP and its metabolites can also promote coagulation. Aβ increases clot formation in vitro through activation of coagulation factor XII.[14] Increased activation of the intrinsic coagulation pathway has also been observed in mouse models of AD and humans with AD.[15] Aβ peptides directly activate platelets, promote aggregation, and trigger thrombus formation.[16,17] Platelets release Aβ during thrombosis, and platelets can adhere to deposited Aβ.[18-20] Aβ interacts with fibrin to induce structural changes in the clot, forming plasmin-resistant blood clots.[21] Aβ is also a substrate for coagulation factor XIIIa.[22] Factor XIIIa can covalently crosslink Aβ to itself and to other platelet and coagulation proteins, and this can increase clot stiffness. Although Aβ can promote and stabilize clot formation in these ways, APP KO mice did not have an obvious bleeding phenotype in previous studies.[13] A significant difference in blood loss from a tail transection model between APP KO and WT mice was not previously detected; however, the mean tail bleeding time appeared to be over twice as long in APP KO mice.[13] Thus, it is not clear if APP contributes to hemostasis in vivo. Here, we extended these studies by examining hemostasis in multiple mouse models, by selectively inhibiting several aspects of coagulation in these models, and by analyzing coagulation of blood from APP KO mice using thromboelastography (TEG).

2 | METHODS

2.1 | Mouse experiments

All procedures were approved by the University of British Columbia Animal Care Committee and performed in accordance with the guidelines established by the Canadian Council on Animal Care. WT (C57Bl/6J) and APP KO (B6.129S7-App^{tm1Dbo}/J) mice were purchased from Jackson Laboratories (not littermates). To reduce variability in mouse bleeding models, mice were matched by weight (20-24 g) and sex matched to ensure equal proportions of males and females in each group.

2.2 | Liver laceration bleeding model

Mice were anesthetized via isoflurane inhalation, and livers were accessed via a 3-cm transverse incision. Two lacerations, each 2 mm long and 2 mm deep, were made on each liver using a 2-mm ophthalmic knife. Blood loss was quantified from each laceration independently. Blood was collected on preweighed filter paper immediately after injury until bleeding stopped. Filter papers (~2 cm × 2 cm) were arranged to line the site of puncture before incision. Each laceration bled for approximately 30 seconds. Blood loss was

compared by Mann-Whitney U test. To confirm that changes in filter paper mass correlated with the volumes of blood soaked, known volumes of fresh blood were soaked onto preweighed filter papers.

2.3 | Mouse tail clip bleeding model

Bleeding was monitored using the immersion method.[23] WT and APP KO mice were anesthetized via isoflurane inhalation and were kept at 37°C using a heating pad temperature probe. Mice then received intraperitoneal injections of apixaban (2 mg/kg, 100 µL, 30 minutes before injury; Eliquis, Bristol-Myers Squibb, Saint-Laurent, QC, Canada), aspirin + clopidogrel (100 mg/kg and 5 mg/kg, respectively, 100 µL, 60 minutes before; Sigma-Aldrich, St Louis, MO, USA), recombinant human tissue-type plasminogen activator (t-PA, 9 mg/kg, 150 µL, 5 minutes before; Tenecteplase, Genentech, San Francisco, CA, USA), tranexamic acid (TXA, 800 mg/kg, 250 µl, 20 minutes before; Sigma), or saline (50 µL phosphate buffered saline) as a control. Tails were transected 3 mm from the tip and were then immediately immersed in warm isotonic solution (citrated phosphate buffered saline [PBSC]) to collect shed blood and to monitor bleeding for 20 minutes. To quantify blood loss, the blood-PBSC solutions were treated with a solution that lyses red blood cells (1.5 M NH_4Cl, 0.1 M $NaHCO_3$, 0.01 M ethylenediaminetetraacetic acid; MilliporeSigma, Darmstadt, Germany) and incubated at room temperature for 10 minutes while gently inverting the mixture. The absorbance of each blood solution was measured at 590 nm (Tecan Genios plate reader) and converted to blood loss (microliters) using a standard curve with known amounts of mouse blood that was collected via intracardiac puncture. The calculated blood loss was normalized by the mouse body weight (microliters per gram) to account for the severity of blood loss with respect to the animal size. For all bleeding experiments, when comparing blood loss without correcting for body weight, 3 of the 4 comparisons that are significant remain significant. All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA, USA). Data sets were normally distributed within groups and were compared by unpaired *t* test.

2.4 | TEG analysis

Using a different group of mice separate from the bleeding experiments, clotting parameters of whole blood were evaluated at 37°C using a TEG Hemostasis Analyzer System 5000 (Haemoscope Corporation, Braintree, MA, USA). Citrated whole blood (10.9 mM sodium citrate final concentration) was collected by cardiac puncture and combined with $CaCl_2$ (13.6 mM), tissue factor (0.03 nM, MedCorp, Sao Paulo, Brazil), and t-PA (3.8 nM). Measurements began immediately after mixing all components together, and the experiment was run for 3 hours. Statistical analyses were performed using Prism 5 (GraphPad Software). Data sets were normally distributed within groups, and were compared by unpaired *t* test.

3 | RESULTS AND DISCUSSION

To extend and validate previous reports evaluating bleeding in APP KO mice under physiological conditions, we conducted liver laceration and tail clip models of hemorrhage. APP KO mice did not bleed significantly more following liver lacerations, but they bled 4-fold more than WT mice following tail clips ($P < .05$, Figure 1). This result extends a previous report in which bleeding times appeared to be increased 2-fold, though that study did not detect a statistically significant difference.[13]

To test if bleeding in APP KO mice was mediated by differences in thrombin generation, we treated both APP KO mice and WT mice with an inhibitor of coagulation factor Xa (apixaban, 2 mg/kg) and compared their blood loss. When mice were treated with apixaban, APP KO mice bled 3-fold more than WT mice ($P < .05$, Figure 2A). Due to high variability in these groups, we performed a Grubbs test for outliers, which excluded 1 data point in each group; the differences remained significant ($P = .02$) following exclusion. Apixaban significantly increased blood loss in WT and APP KO mice, confirming that thrombin generation was inhibited in both groups compared to untreated mice. The significant increase in bleeding between APP KO mice and WT mice with apixaban suggests that the bleeding phenotype is not primarily mediated by differences in thrombin generation, but does not rule out the possibility.

To determine if the increased bleeding by APP KO mice was mediated by platelets, we compared blood loss between APP KO and

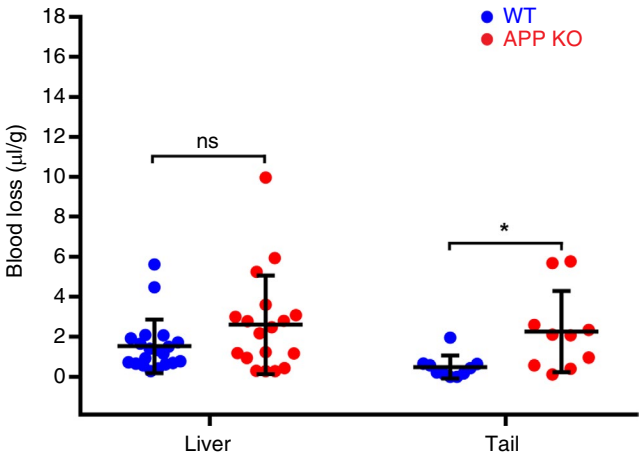

FIGURE 1 APP KO mice have a mild bleeding phenotype. Blood loss in the liver laceration model and tail transection model with WT and APP KO mice. Blood loss was normalized to body weight. In the liver laceration model, each marker indicates 1 of 2 bleeds per liver (n = 18 bleeds from 9 mice per group). In the tail transection model, each marker represents a single mouse (n = 10 mice). Error bars represent the mean ± SEM. * $P < .05$, ns indicates not significant ($P = .10$). APP, amyloid precursor protein; KO, knockout; ns, not significant; WT, wild type

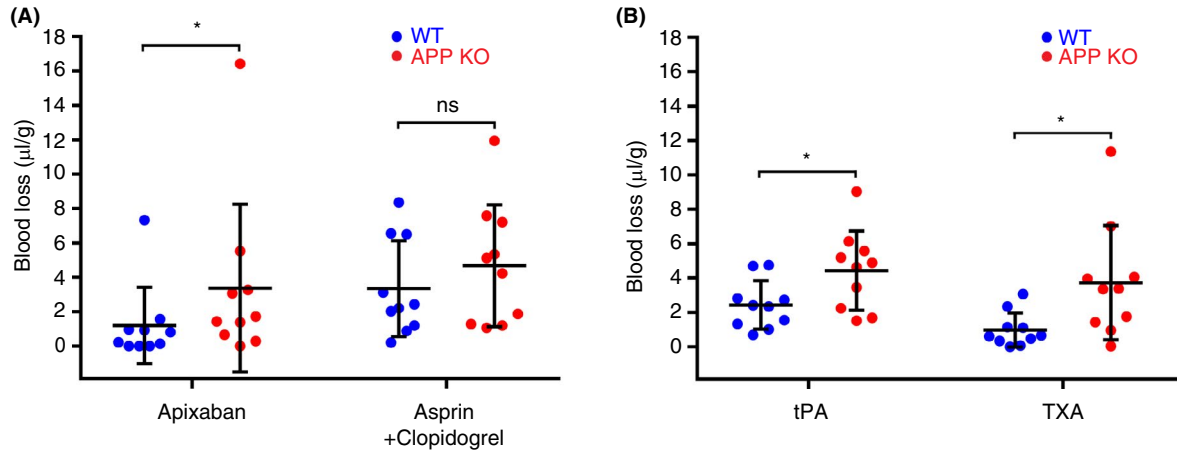

FIGURE 2 Platelet inhibitors abrogate the difference in the bleeding phenotype of APP KO mice, but apixaban, and pro- and antifibrinolytic treatments do not. (A) Blood loss following tail transection after treating mice with apixaban (2 mg/kg) and aspirin + clopidogrel (100 mg/kg and 5 mg/kg respectively). (B) Blood loss following tail transection after treating mice with t-PA (9 mg/kg) and TXA (800 mg/kg). n = 10. Error bars represent the mean ± SEM. * $P < .05$, ns indicates not significant ($P = .40$). APP, amyloid precursor protein; KO, knockout; SEM, standard error of the mean; t-PA, tissue-type plasminogen activator; TXA, tranexamic acid; WT, wild type

TABLE 1 Mean and median values with ranges for in vivo bleeding experiments

	WT		APP KO	
	Mean ± SEM[a]	Median (range)[a]	Mean ± SEM[a]	Median (range)[a]
Liver				
NT	1.52 ± 0.30	1.22 (0.28-5.62)	2.60 ± 0.58	2.33 (0.27-9.96)
Tail				
NT	0.48 ± 0.18*	0.32 (0-1.95)	2.26 ± 0.64*	2.08 (0.12-5.77)
Apixaban	1.19 ± 0.70*	0.51 (0-7.32)	3.37 ± 1.54*	1.57 (0-16.42)
Aspirin + clopidogrel	3.34 ± 0.88	2.32 (0.19-8.35)	4.68 ± 1.12	4.67 (1.06-11.94)
t-PA	2.42 ± 0.45*	2.37 (0.68-4.75)	4.43 ± 0.73*	4.76 (1.51-9.02)
TXA	0.98 ± 0.32*	0.63 (0-3.07)	3.73 ± 1.06*	3.37 (0.03-11.35)

Abbreviations: APP, amyloid precursor protein; KO, knockout; SEM, standard error of the mean; NT, no treatment; t-PA, tissue-type plasminogen activator; TXA, tranexamic acid; WT, wild type.

[a] All units in microliters per gram.

* indicates significant different between samples in row

WT mice treated with aspirin + clopidogrel, which inhibited platelet activation. When treated with aspirin + clopidogrel, APP KO mice did not bleed significantly more than WT mice (Figure 2A). This suggests the bleeding phenotype in APP KO mice is mediated by platelets.

To evaluate if APP KO mice and WT mice bled differently due to differences in fibrinolysis, we compared blood loss under hyper- and hypofibrinolytic conditions. When treated with t-PA, APP KO mice bled twice as much as WT mice ($P < .05$, Figure 2B). When treated with the antifibriolytic TXA, APP KO mice bled thrice as much as WT mice ($P < .05$). This suggests that the increased bleeding seen in APP KO mice is not primarily mediated by differences in fibrinolytic activity.

All mean and median values for the in vivo bleeding experiments are listed in Table 1. In some published mouse studies, liver and tail injuries cause similar blood loss.[23] In our liver laceration model, injuries were smaller and caused less severe capillary bed bleeds. APP KO mice bled more compared to WT in our tail transection model, which causes platelet-dependent arterial bleeding,[24] but not our liver laceration model. This suggests that platelet dysfunction may be a potential mechanism.[24] WT mice treated with apixaban, aspirin + clopidogrel, or t-PA bled significantly more than WT controls. TXA-treated WT mice also bled slightly more than controls, but the difference was not significant; this may be related to greater volume or tonicity of the injected TXA solution. It was expected that TXA-treated mice would not bleed less, since inhibition of fibrinolysis does not have a strong effect in arterial bleeding models.[24,25] Apixaban-treated APP KO mice bled significantly more than APP KO controls. While median blood loss in APP KO mice receiving aspirin + clopidogrel was twice that of APP KO controls, the difference was not significant; increased bleeding would suggest that inhibition of platelets by aspirin and clopidogrel can occur independent of APP. These

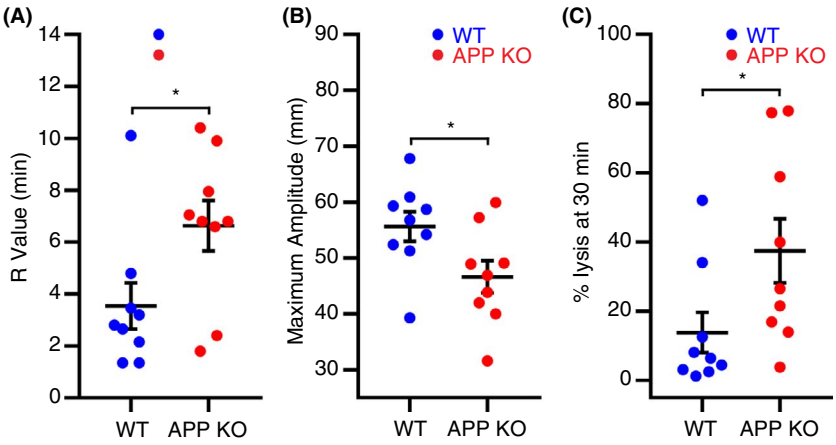

FIGURE 3 TEG analysis of whole blood demonstrating hemostatic abnormalities in APP KO mice. Graphs show (A) clot initiation time (R value), (B) clot strength (maximum amplitude), and (C) clot stability (percent lysis at 30 min, induced by 3.8 nM t-PA). n = 9 mice. Error bars represent the mean ± SEM. * $P < .05$. APP, amyloid precursor protein; KO, knockout; SEM, standard error of the mean; TEG, thromboelastography; t-PA, tissue-type plasminogen activator; WT, wild type

results do not fully exclude contributions of APP and Aβ to thrombin generation or fibrinolysis, as the tail transection model is more sensitive to changes in platelet activity compared to other aspects of hemostasis.[26]

We then used TEG to investigate if whole blood from WT and APP KO mice have different clot properties ex vivo. Citrated whole blood was collected from mice, and Innovin and t-PA were added to allow stable clots to form and lyse. TEG parameters of R-time (clot initiation time), maximum amplitude (MA; clot stiffness), and percent lysis at 30 minutes (susceptibility to fibrinolysis) were analyzed. Whole blood from APP KO mice had a 2-fold increased R-time, 16% decrease in MA, and 3-fold increase in percent lysis at 30 minutes ($P < .05$; Figure 3). This demonstrates that APP KO whole blood clots more slowly and forms weaker clots compared to WT whole blood. APP KO clots were more susceptible to fibrinolysis, likely because of weaker clot formation due to platelet inhibition, which is consistent with the tail bleed experiments. These results are also consistent with how platelet abnormalities are known to affect bleeding and TEG measurements.[27]

There are numerous studies examining the effect of APP and its metabolites on hemostasis and the characteristics of APP KO platelets. APP has anticoagulant properties through its KPI domain[9] and Aβ-mediated procoagulant properties through increased thrombin generation, platelet activation and aggregation, and resistance to fibrinolysis.[14-17,21] Platelets from APP KO mice have normal platelet aggregation, secretion, and αIIbβ3 signaling, but reduced platelet numbers and increased platelet size.[13] To corroborate these findings, we measured platelet concentration, aggregation, and secretion using APP KO mice, and the results were consistent with published values. Although we and other groups have not identified major differences in the coagulability of between APP KO and WT platelets ex vivo, here we have demonstrated a difference in vivo, likely related to the mechanisms previously published. Overall, APP and its metabolites can affect hemostasis in multiple ways, but in mice its procoagulant contributions to hemostasis are most distinct in arterial bleeds where platelet activity is critical.

In this study, we investigated the role of APP in hemostasis under physiological and challenged conditions. We found that APP plays a procoagulant role in primary hemostasis, and this role is mediated by platelets. APP possessing both procoagulant and anticoagulant properties is consistent with other components of hemostasis; for example, fibrin and thrombin are strong drivers of coagulation but also exhibit anticoagulant properties by inhibiting thrombin and activating protein C, respectively.[28,29] In conclusion, we found that APP KO mice have a consistent mild bleeding phenotype that is in part mediated by platelets, for which the specific mechanism remains to be validated.

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RELATIONSHIP DISCLOSURE

The authors declare nothing to report.

AUTHOR CONTRIBUTIONS

NM and JRB performed the animal experiments; AWS performed TEG assays; NM and CJK analyzed data; NM and CJK designed the study and wrote the manuscript; AWS, WSH, JRB, and WAJ provided critical revisions of the manuscript. All authors read and approved the manuscript.

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