Elevated Prothrombin Promotes Venous, but Not Arterial, Thrombosis in Mice

Maria M. Aleman, Bethany L. Walton, James R. Byrnes, Jian-Guo Wang, Matthew J. Heisler, Kellie R. Machlus, Brian C. Cooley, Alisa S. Wolberg

Objective—Individuals with elevated prothrombin, including those with the prothrombin G20210A mutation, have increased risk of venous thrombosis. Although these individuals do not have increased circulating prothrombotic biomarkers, their plasma demonstrates increased tissue factor–dependent thrombin generation in vitro. The objectives of this study were to determine the pathological role of elevated prothrombin in venous and arterial thrombosis in vivo, and distinguish thrombogenic mechanisms in these vessels.

Approach and Results—Prothrombin was infused into mice to raise circulating levels. Venous thrombosis was induced by electrolytic stimulus to the femoral vein or inferior vena cava ligation. Arterial thrombosis was induced by electrolytic stimulus or ferric chloride application to the carotid artery. Mice infused with prothrombin demonstrated increased tissue factor–triggered thrombin generation measured ex vivo, but did not have increased circulating prothrombotic biomarkers in the absence of vessel injury. After venous injury, elevated prothrombin increased thrombin generation and the fibrin accumulation rate and total amount of fibrin ≈3-fold, producing extended thrombi with increased mass. However, elevated prothrombin did not accelerate platelet accumulation, increase the fibrin accumulation rate, or shorten the vessel occlusion time after arterial injury.

Conclusions—These findings reconcile previously discordant findings on thrombin generation in hyperprothrombinemic individuals measured ex vivo and in vitro, and show elevated prothrombin promotes venous, but not arterial, thrombosis in vivo. (Arterioscler Thromb Vasc Biol. 2013;33:1829-1836.)

Key Word: arterial ▪ fibrin ▪ hyperprothrombinemia ▪ platelet ▪ prothrombin ▪ thrombosis ▪ venous

Arterial thrombosis and venous thrombosis/thromboembolism are traditionally regarded as distinct diseases with respect to their epidemiology and treatment strategies (reviewed by Liéffinger et al1 and Turpie et al2). The presence of certain nonoverlapping risk factors suggests that distinct features in the arterial and venous environments confer differential pathophysiology. Venous thrombosis is often associated with acquired or inherited plasma hypercoagulability and is thought to be triggered by expression of cell adhesion molecules and procoagulant activity on intact endothelium in low shear. In contrast, arterial thrombosis is typically associated with atherosclerotic plaque rupture and exposure of subendothelial cells and highly procoagulant material (tissue factor [TF] and collagen) to blood in high shear. Consequently, venous thrombi are high in erythrocyte and fibrin content, whereas arterial thrombi are platelet rich. Treatment strategies to minimize venous thrombosis/thromboembolism and arterial thrombosis (antiplatelet agents and platelet antagonists, respectively) have reduced efficacy versus1,3 supporting the premise that unique pathophysiological mechanisms promote thrombosis in veins and arteries.

Individuals with elevated prothrombin (hyperprothrombinemia), including those with the G20210A mutation in the prothrombin 3′-untranslated region,4,5 have ≈3-fold increased risk for venous thrombosis. In particular, the G20210A mutation is associated with ≈115% to 170% of normal prothrombin activity levels.4,9 This mutation is present in 1% to 4% of the general European population,7 making it the second most common genetic risk factor for venous thrombosis in the white population. In contrast, association of either elevated prothrombin or the G20210A mutation with arterial thrombosis is unclear.8,9 Although the G20210A mutation has been weakly associated with arterial disease,10 including coronary heart disease,11 ischemic stroke,12 and risk of myocardial infarction in young women13 and men,14 other investigations have failed to support these findings in patients with cerebral ischemia,15,16 myocardial infarction,17 or general arterial events.18 Patient heterogeneity may reconcile differences between these studies because arterial risk is increased in patients with additional risk factors (eg, smoking, hypertension, diabetes mellitus, and obesity)13,14; however, the independent association between
elevated prothrombin and arterial thrombosis is difficult to discern in a human cohort and remains unresolved.

The operant pathological mechanisms of hyperprothrombinemia in either venous or arterial vascular beds are also unknown. Individuals with elevated prothrombin do not have increased circulating prothrombin fragment 1.2 levels, suggesting thrombosis does not result from constitutive activation of coagulation. However, in vitro studies show that after coagulation activation, high prothrombin levels increase thrombin generation, induce activated protein C resistance, and promote formation of abnormal fibrin networks in clots that resist fibrinolysis. These findings suggest that elevated prothrombin levels promote thrombosis; however, this effect has never been directly demonstrated in vivo.

The objectives of the present study were to define the prothrombotic role(s) of elevated prothrombin in vivo and distinguishing pathological mechanisms differentiating these effects in venous and arterial thrombosis. We found that elevated prothrombin increased plasma thrombin generation ex vivo after TF-dependent initiation of coagulation, but did not activate coagulation or increase baseline thrombin generation in vivo in the absence of overt vascular injury. After vascular injury, elevated prothrombin increased in vivo thrombin generation, but did not increase the rate of platelet accumulation in either arterial or venous thrombi. Elevated prothrombin increased the rate of fibrin deposition and produced larger thrombi in models of venous thrombosis. In contrast, elevated prothrombin did not increase the rate of fibrin deposition or shorten the time to occlusion in models of arterial thrombosis. These data are the first to show that elevated prothrombin levels directly promote venous thrombosis in vivo, and show elevated prothrombin has little to no independent contribution to arterial thrombosis in the absence of additional risk factors.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Human Prothrombin Is Active in Murine Plasma
The in vivo hyperprothrombinemia model was developed by infusing mice with human prothrombin. Human prothrombin and the thrombin B chain have 81.4% and 88.8% amino acid identity with murine prothrombin and thrombin, respectively, and highly conserved substitutions in nonidentical residues. Human and mouse thrombin bind and cleave human and mouse fibrinogen, activate platelets to form aggregates with pseudopodia, bind murine thrombomodulin, and support activated protein C generation. To assess the ability of human (pro)thrombin to support thrombin generation in murine plasma, murine plasma was spiked with vehicle or murine or human prothrombin to 200% (final, endogenous plus spiked prothrombin) and thrombin generation was measured by calibrated automated thrombography in the absence and presence of 200 nmol/L murine thrombomodulin. Thrombin generation peaks were 49.4±7.2, 82.4±13.8, and 90.4±5.2 nmol/L (mean±SD; n=2–3) for plasma plus vehicle, plasma plus murine prothrombin, or plasma plus human prothrombin, respectively, and addition of murine thrombomodulin reduced the thrombin peaks by 73%, 74%, and 64%, respectively (Figure I in the online-only Data Supplement). These data demonstrate that human (pro)thrombin is compatible with the murine procoagulant and anticoagulant systems.

In the Absence of Vessel Injury, Elevated Prothrombin Does Not Activate Coagulation
Platelet-poor plasma from patients with elevated prothrombin demonstrates increased thrombin generation ex vivo; however, these patients do not have higher circulating prothrombin fragment 1.2 compared with age-matched controls. We hypothesized that elevated prothrombin does not independently cause thrombin generation in the absence of vascular injury, but increases thrombin generation after TF exposure. To test this hypothesis, we infused prothrombin (to 300% of normal levels; total prothrombin equals endogenous murine prothrombin plus infused human prothrombin) vehicle (HEPES-buffered saline [HBS], Control) into uninjured mice and measured prothrombin antigen by Western blotting, thrombin generation ex vivo by calibrated automated thrombography, and thrombin–antithrombin (TAT) complex levels by ELISA 12 hours after infusion. Human prothrombin still circulated in mice 12 hours after infusion and was strongly detected by the rabbit antihuman prothrombin antibody (Figure II in the online-only Data Supplement). Consistent with humans, thrombin generation was significantly elevated in platelet-poor plasma from prothrombin-infused mice compared with controls (49.5±2.1 versus 39.3±2.5 peak thrombin, respectively, mean±SEM; P<0.04; Figure 1A) after initiation of coagulation ex vivo. However, also consistent with that seen in humans, circulating TAT levels in mice with elevated prothrombin, even at this high level, were not elevated compared with controls (2.4±1.4 versus 3.8±4.0 ng/mL, respectively, mean±SEM; P=0.26; Figure 1B). These data reconcile outwardly discordant findings on thrombin generation in hyperprothrombinemic individuals measured ex vivo and in vitro by showing that elevated prothrombin does not increase baseline hemostatic idling in the absence of vascular injury but augments thrombin generation after a procoagulant trigger.

Elevated Prothrombin Accelerates Fibrin Deposition and Produces Larger Thrombi in Venous Thrombosis Models
To characterize the effect of elevated prothrombin on venous thrombosis in vivo, we first triggered thrombosis in the murine femoral vein via electrolytic injury and used intravital fluorescence detection to characterize the temporal and spatial contributions of elevated prothrombin to thrombus formation. The electrolytic injury model induces mural thrombus formation via iron-mediated injury that causes early platelet accumulation followed by fibrin accumulation (Figure 2A; Movies I and II in the online-only Data Supplement). Thrombus formation in this model is reduced by heparin, consistent with the sensitivity of venous thrombosis to thrombin generation. We tested 2 levels of prothrombin: 130% and 200% (final); these levels were chosen to approximate the mean and upper end of the pathophysiologic range. Neither prothrombin concentration significantly increased the rate or total amount of platelet...
accumulation in femoral vein thrombi (Figure 2B and 2D). However, the plasma prothrombin level showed a dose-dependent effect on fibrin accumulation in the vein. At 60 minutes, fibrin accumulation in mice infused to 130% and 200% prothrombin was 1.7 (P<0.06)- and 3.5 (P<0.002)-fold higher, respectively, than control mice (Figure 2C), and mice infused with 200% prothrombin exhibited a significantly (2.3-fold; P=0.006) increased fibrin accumulation rate than control mice (Figure 2D). Furthermore, in contrast to control thrombi that remained relatively localized to the thrombus induction site, thrombi in prothrombin-infused mice showed considerable downstream elongation of a mass containing both fibrin and platelets (Movies I and II in the online-only Data Supplement).

We also characterized the effect of elevated prothrombin on venous thrombosis in an inferior vena cava (IVC) ligation (stasis) model that triggers venous thrombosis via vessel distention, blood stasis, and dysfunction (exposure of vessel wall TF) of intact endothelium.32 For both control and prothrombin-infused mice, circulating TATs were significantly higher in mice that underwent IVC ligation than in mice that did not (Figure 3A), demonstrating activation of coagulation after vessel ligation. Circulating prothrombin was spiked to 300% so that levels remained elevated for the duration of thrombus formation (12 hours). After IVC ligation, TATs were >2-fold higher in prothrombin-infused mice compared with control mice (47.9±6.5 versus 21.2±4.5 ng/mL, respectively, mean±SEM; P<0.009; Figure 3A), suggesting elevated prothrombin augmented thrombin generation during venous thrombogenesis. After IVC ligation, all prothrombin-infused and control mice developed thrombi within 12 hours. Similar to that seen in the electrolytic injury model, thrombi in prothrombin-infused mice were significantly larger than thrombi in control mice (27.6 [26.1–43.6] versus 22.6 [9.5–27.7] mg, respectively, median [range]; P=0.01; Figure 3B), and in some mice extended into the iliac branches. Together, these data show that elevated prothrombin augments venous thrombus formation in vivo by increasing thrombin generation and intravascular fibrin deposition.

**Elevated Prothrombin Has Little to No Effect on the Time to Occlusion or Rate of Platelet Accumulation in Arterial Thrombosis Models**

We then tested the effect of elevated prothrombin in a second model of arterial thrombosis. The FeCl3 application/carotid artery model triggers arterial thrombosis via generation of reactive oxygen species and exposure of collagen, resulting in a platelet-rich thrombus.33–35 Because neither prothrombin concentration significantly increased either the rate or total amount of platelet accumulation, or the rate of fibrin accumulation in carotid artery thrombi (Figure 4B–4D). Elevated prothrombin increased fibrin deposition at 60 minutes (≈2-fold; Figure 4C), but this difference did not reach statistical significance (P=0.085).

We then tested the effect of elevated prothrombin in a second model of arterial thrombosis. The FeCl3 application/carotid artery model triggers arterial thrombosis via generation of reactive oxygen species and exposure of collagen, resulting in a platelet-rich thrombus.33–35 Because neither prothrombin concentration significantly altered arterial thrombus formation in the electrolytic model, we only tested the higher concentration of prothrombin (infusing to 200%, final) in the FeCl3/carotid artery model. Prothrombin infusion transiently elevated TAT levels (12.6±3.2 ng/mL), likely reflecting mild activation of coagulation from the venous infusion or trace (<0.004%) thrombin contamination in the prothrombin concentrate that was immediately inhibited by endogenous antithrombin (present in 1000-fold excess). This low thrombin level does not have any physiological effects.36,37 For both control and prothrombin-infused mice, circulating TATs were significantly higher in mice that underwent FeCl3 ligation than in mice that did not (Figure 4E), demonstrating activation of coagulation after vessel ligation. Circulating prothrombin was spiked to 300% so that levels remained elevated for the duration of thrombus formation (12 hours). After FeCl3 injury, TATs were >2-fold higher in prothrombin-infused mice compared with control mice (13.9±1.7 versus 23.5±2.6, for control and prothrombin-infused mice, respectively; P<0.007; Figure 4E). However, the absolute increase in TAT levels over baseline was similar in both control and prothrombin-infused mice,
suggesting arterial injury activated coagulation to a similar degree in both groups. Consistent with this observation, both control and prothrombin-infused mice developed stable, occlusive thrombi, and the time to vessel occlusion in prothrombin-infused mice was not different from controls (5.7 [4.3–40.0] versus 6.4 [4.5–8.5] minutes), respectively, median [range]; Figure 4F). Thus, elevated prothrombin did not significantly increase platelet or fibrin accumulation during arterial thrombus formation or shorten the time to artery occlusion. Together, these data show that although elevated prothrombin promotes venous thrombus formation, it does not significantly augment arterial (platelet dependent) thrombosis.

Discussion
Elevated prothrombin is a well-established risk factor for venous thrombosis, but its relationship to arterial thrombosis is unclear. Using state-of-the-art in vivo models of venous thrombosis and arterial thrombosis, we show that elevated prothrombin did not increase baseline prothrombotic markers in unchallenged mice, but did increase thrombin generation after venous injury. The presence of elevated prothrombin did not accelerate intravascular platelet accumulation after either venous or arterial injury. In venous thrombosis models, mice with elevated prothrombin exhibited an increased rate and amount of fibrin accumulation, thrombus extension and formation of thrombi with increased mass. However, in arterial thrombosis models, elevated prothrombin slightly (nonsignificantly) increased the total amount of fibrin deposited, but did not increase the rate of fibrin accumulation or shorten the time to occlusion. These findings suggest that elevated prothrombin has little to no independent contribution to arterial thrombosis, and are the first to show that elevated prothrombin levels directly promote venous thrombosis in vivo.

The choice of murine thrombosis model for investigating human thrombosis has been the subject of considerable debate because many models fail to recapitulate key aspects of the arterial and venous thrombogenic processes.38 An important strength of our study was the use of complementary arterial and venous models to delineate both kinetic processes and their consequences for thrombus composition. Complementary, integrated information from the 2 arterial models and 2 venous models reveals both common and vascular bed–specific processes operant in these vessels that are consistent with the histological appearance of arterial and venous thrombi isolated from humans. Arterial injury produced rapid platelet accumulation, whereas venous injury resulted in slower thrombus formation with fibrin accumulation. We used models that produced both occlusive (FeCl3/carotid and IVC) and nonocclusive (electrolytic injury) thrombi. Findings were consistent within vessels, but differed between arteries and veins, suggesting these models are sensitive to the unique physical and biochemical environments within the different vessels.

We detected both common and vascular bed–specific effects of elevated prothrombin on arterial and venous thrombosis. Elevated prothrombin significantly augmented endogenous thrombin generation in both the venous and arterial models (Figures 3A and 4E). However, elevated prothrombin did not shorten the time to arterial occlusion. This finding is consistent with a recent study showing that ApoE−/− mice expressing half of the prothrombin level of wild-type mice (FII−/+) are not...
Elevated prothrombin produces larger venous thrombi by increasing thrombin generation after inferior vena cava (IVC) ligation. A, IVC stasis was induced in mice infused with prothrombin (to 300%) or vehicle control. Twelve hours after ligation, blood was collected into citrate from just above the ligation site. Thrombin–antithrombin levels were measured from ligated (n=6–8/group) and uninjured mice (n=5/group) in parallel. Bars show mean±SEM. B, Twelve hours after ligation, the IVCs plus thrombi were excised and weighed. The box plot indicates medians and upper and lower quartiles (n=6–8/group).

**Figure 3.** Elevated prothrombin produces larger venous thrombi by increasing thrombin generation after inferior vena cava (IVC) ligation. A, IVC stasis was induced in mice infused with prothrombin (to 300%) or vehicle control. Twelve hours after ligation, blood was collected into citrate from just above the ligation site. Thrombin–antithrombin levels were measured from ligated (n=6–8/group) and uninjured mice (n=5/group) in parallel. Bars show mean±SEM. B, Twelve hours after ligation, the IVCs plus thrombi were excised and weighed. The box plot indicates medians and upper and lower quartiles (n=6–8/group).

Notably, although thrombin is a potent agonist for platelet activation, elevated prothrombin did not accelerate the rate of platelet accumulation in either arteries or veins (Figures 2D and 4D). These findings are consistent with our prior observation that 5% prothrombin is both necessary and sufficient to maximize platelet activation.19 Consequently, elevated prothrombin did not accelerate arterial occlusion, a platelet-dominated process. In contrast, the increased thrombin generation significantly increased fibrin deposition and, therefore, venous thrombosis, a fibrin-dominated process. We previously showed that elevated prothrombin also promotes the thrombin concentration-dependent formation of plasma clots with an abnormally dense fibrin network.19 Although fibrin network structure is difficult to assess in thrombi in the presence of cells, combined, these results suggest that in veins, elevated prothrombin promotes thrombosis with fibrin networks that have both increased mass and increased network density. Both properties are associated with increased clot stability in vitro and in vivo and have been correlated with increased thrombosis risk.40

It is interesting that although elevated prothrombin did not accelerate arterial occlusion after FeCl3 injury, elevated plasma factor VIII does.41–43 Both elevated prothrombin and elevated factor VIII increase thrombin generation in vitro and in vivo (Figure 3A; Machlus et al43). However, elevated factor VIII significantly shortens the lag time to platelet aggregation in vitro and trends toward increased platelet accumulation in vivo, whereas elevated prothrombin did not significantly change the rate of platelet aggregation in vitro or in vivo (Figures 2D and 4D). These data suggest elevated factor VIII modulates an early (amplification) phase of coagulation when thrombin levels are relatively low and platelet activation is taking place. Consequently, platelet-dependent arterial thrombosis models are sensitive to the effects of elevated factor VIII but not to elevated prothrombin. This observation is consistent with the premise that procoagulant factors have complementary, but distinct, roles in different phases of coagulation.45

Previous studies on the association between elevated prothrombin and arterial thrombosis (myocardial infarction or ischemic stroke) have been inconsistent, showing either no or a modest relationship.5–18 One explanation for these differences is that risk is present only in specific groups. Of interest are observations that relative risk increases when another identifiable cardiovascular risk factor is also present and seems higher than from either risk factor alone, suggesting an additive or synergistic interaction.13,14 A strength of our murine model in which prothrombin levels were acutely elevated in healthy wild-type mice is the clear absence of other risk factors. Nonetheless, the coexistence of additional known or unidentified risk factors may augment the positive associations detected in prior studies with human cohorts. For example, on an atherosclerosis-prone background (ApoE–/–), chronic plasma hypercoagulability (reduced ability to generate activated protein C in thrombomodulin-mutated mice, TMPro moi) increases atherogenesis and plaque formation, both of which are associated with atherothrombosis, and reduced prothrombin levels attenuate atherosclerotic lesion formation.39,40

This study has potential limitations. First, we used human prothrombin to increase circulating levels in the mouse. However, published studies25–29 and our data show that human prothrombin is stable in murine circulation and participates in murine pro- and anticoagulant pathways. Moreover, the infusion strategy enabled us to precisely control the level of circulating prothrombin. Second, the infusion model used in these experiments does not reflect pathological effects that chronic exposure to elevated prothrombin could have on the vasculature. Atherosclerotic disease reflects chronic vascular injury with occurrences of acute injury (plaque disruption and TF exposure). However, a major strength of the infusion model is that it enabled us to isolate and investigate the immediate, direct effects of elevated prothrombin on thrombus formation. These data on acute effects will be critical for interpreting findings from mice with genetically induced chronic plasma hypercoagulability (eg, factor V Leiden mice); comparison of short- and long-term exposure to hypercoagulability is likely to reveal interesting mechanisms that predispose these individuals to thrombosis. Third, the thrombosis models we used differed in methodologic aspects, including anesthesia and analgesia protocols. However, the observation that elevated prothrombin exhibited consistent effects in each of the venous and each of the arterial models suggests that the observed effects were not because of the methodologies but to the prothrombin level, itself. Finally, the arterial and venous models used in this study were sensitive to thrombus formation, but did not reflect additional effects elevated prothrombin may have on thrombus stability. For example, although groups have demonstrated increased activation of the thrombin activatable fibrinolysis...
inhibitor in plasma with increased prothrombin, we did not evaluate the long-term resistance of thrombi to fibrinolysis.

In summary, our findings demonstrate that elevated prothrombin does not trigger endogenous thrombin generation in the absence of vascular injury, suggesting that in lieu of a signal that initiates coagulation, plasma hypercoagulability is not independently prothrombotic. These data suggest that increased coagulation biomarkers (eg, fragment 1.2 or TATs) indicate vascular dysfunction that, when coupled to additional plasma prothrombotic potential, promote thrombosis. Our findings further show that elevated prothrombin increases thrombin generation after vascular injury. Elevated prothrombin does not accelerate platelet activation in either the artery or the vein, but significantly increases the rate and amount of fibrin deposition after venous injury. These findings are consistent with findings that elevated prothrombin is associated with venous thrombosis in humans, but is only weakly associated with arterial thrombosis in the absence of other risk factors. These results support the relevance of murine thrombosis models to studies of hypercoagulability-related thrombosis in humans. Integrating complementary in vivo models is a powerful approach to investigate the underlying mechanisms of hemostatic and thrombotic processes.

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References


**Significance**

Elevated plasma prothrombin, including that associated with the common G20210A prothrombin mutation, is associated with significantly increased thrombosis risk. Our findings demonstrate that elevated prothrombin does not trigger thrombin generation in the absence of vascular injury, suggesting that in lieu of a signal that initiates coagulation, plasma hypercoagulability does not independently cause thrombosis. However, after venous, but not arterial injury, elevated prothrombin accelerates fibrin accumulation and increases thrombus growth. These findings are the first to identify a causative mechanism by which elevated prothrombin promotes thrombosis in vivo. This study shows the importance of using integrated, complementary murine thrombosis models to understand hypercoagulability-related thrombosis in humans.
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METHODS
Proteins and materials
Human and murine prothrombin and human thrombin were from Haematologic Technologies, Inc. Murine thrombin was from Enzyme Research Laboratories. Human prothrombin was treated with inhibitors\(^1\), concentrated, and dialyzed into sterile 20 mM HEPES (pH 7.4)/150 mM NaCl (HBS). Prothrombin concentrate contained less than 0.004% thrombin (the lower level of detection). Murine thrombomodulin and protein C were from R&D Systems, Pefachrome PCa from Pentapharm, and hirudin from Calbiochem. Rhodamine 6G was from Sigma-Aldrich. Rabbit polyclonal antibodies against human fibrinogen (A0080) and human prothrombin (A0325) were from DAKO. Fluorescent secondary antibodies and protein labeling kits were from Invitrogen. Anti-fibrin antibody (59D8) was from a clone generously provided by Drs. Marschall Runge [University of North Carolina (UNC)] and Charles Esmon (Oklahoma College of Medicine).

Mice platelet-poor plasma (PPP) was prepared from blood drawn from the inferior vena cava (IVC) of 49 female C57BL/6 mice into 3.2% sodium citrate and processed by centrifugation (4,000xg, 20 minutes).

Thrombosis models
Procedures were approved by the UNC and Medical College of Wisconsin Institutional Animal Care and Use Committees. Mice (6-8 week old male C57BL/6) were purchased from Charles River Laboratories or Harlan SD. The left saphenous vein or jugular vein was exposed and catheterized as described.\(^3\)-\(^5\) Prothrombin or vehicle (HBS) was administered through the catheter on a per weight basis [blood volume (mL) is 7% of body weight (g), plasma is 50% of blood volume] 5 minutes before injury.

The electrolytic injury models were performed as described.\(^6,7\) Briefly, mice were anesthetized with intraperitoneal pentobarbital, and rhodamine 6G (platelet label), Alexa Fluor 647-labeled anti-fibrin (59D8), and prothrombin (to 200%, final) or saline were infused via a jugular vein branch 3-5 minutes before thrombus induction. Antibody 59D8 does not inhibit fibrin polymerization.\(^6\) Injury was induced with 30-second, 3-V direct current application via 140-μm diameter steel blunt-end needle on the carotid artery surface, or with a 30-second, 1.5-V direct current application via 70-μm blunt-end needle to the femoral vein surface. Vessels were
illuminated with beam-expanded green (532 nm) and red (650 nm) laser light. Fluorescent time-lapse video images were captured over 60 minutes with a low-light camera attached to a microscope at 100X magnification. Video images at 2-minute intervals were analyzed for relative fluorophore intensity (ImageJ 1.45s) within the thrombus zone and normalized for inter-animal comparisons. Rates of platelet and fibrin accumulation were determined by linear fit to 3 or more points exhibiting the maximal rate of increase.

The FeCl₃/carotid artery thrombosis model was performed as described.⁴,⁵ Briefly, mice were anesthetized with 1.5-2% isoflurane in oxygen and infused with prothrombin (to 200%, final) or vehicle (HBS) via left saphenous vein catheter 5 minutes before thrombus induction. The right common carotid artery was dissected and placed on parafilm. FeCl₃ (7.5%) was applied to the artery for 2 minutes. The artery was washed with warm saline and blood flow was monitored via Doppler transonic flow probe (Indus Instruments). The TTO was defined as the time between FeCl₃ administration and lack of flow for 60 consecutive seconds.

The IVC stasis model was performed as described.⁸,⁹ Briefly, mice were anesthetized with 1.5-2% isoflurane in 2% oxygen and prothrombin (to 300%, final) or vehicle was infused via tail vein injection. Following sterile laparotomy, the intestines were exteriorized, the IVC was dissected bluntly, and side branches were ligated with 8-0 Prolene suture and lumbar branches closed by cautery. The IVC was separated from the aorta by blunt dissection and completely ligated with 8-0 Prolene suture. After replacing the intestines, the muscle layer was closed with 5-0 vicryl suture and skin closed with 8-0 Prolene suture and skin glue. Mice recovered with analgesia (buprenorphine, 0.05 mg/kg subcutaneous). After 12 hours, blood was drawn from the IVC above the ligation site into 3.2% sodium citrate and processed to PPP by centrifugation at 5,000xg for 10 minutes. Thrombi were collected and weighed.

**Detection of circulating human prothrombin antigen**

PPP (1:10 dilution) was separated on Novex 10% Tris-Glycine gels and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR Biosciences). Primary antibodies were incubated overnight at 4°C. After washing three times with 10 mM phosphate (pH 7.4), 150 mM NaCl containing 0.1% Tween-20, membranes were incubated with fluorescently-labeled secondary antibodies (1:15,000 dilution) for 1 hour at room temperature. Membranes were then washed 3 times and scanned using an Odyssey® Infrared Imaging System (LI-COR Biosciences). Band intensity was quantified by densitometry.
Detection of circulating human prothrombin activity

The final level of circulating prothrombin was confirmed by clotting assay in separate, uninjured mice as described. Briefly, blood was drawn from the IVC 5 minutes after infusion into 3.2% sodium citrate and processed to PPP by centrifugation (5,000xg, 10 minutes). Murine PPP was mixed with human prothrombin-deficient PPP (5% murine PPP, 95% prothrombin-deficient PPP), and clotting was initiated with Kontact aPTT reagent (40%, final) and calcium chloride (10 mM, final). Clot formation was followed in a SpectraMax Plus 340 plate reader (Molecular Devices) and compared to a standard curve. The prothrombin level of mice infused to 300% prothrombin (final, endogenous murine plus infused human) was as expected (323±12% of normal activity, n=2).

Calibrated automated thrombography

Blood was drawn from the IVC into 3.2% sodium citrate and processed to PPP by centrifugation (5,000xg, 10 minutes). Thrombin generation was measured as described. Briefly, murine PPP (10 µL) was added to 30 µL HBS, and 10 µL of PPP Reagent Low or Calibrator Reagent. After 10 minutes at 37 °C, reactions were initiated by addition of 10 µL fluorogenic substrate/CaCl₂. Thrombin generation was detected on a Fluoroskan Ascent fluorometer (Thermo Labsystems) and evaluated using Thrombinscope software v3.0.0.29.

Measurement of circulating thrombin-antithrombin (TAT) complexes

TAT levels were measured by ELISA (Enzygnost TAT microELISA, Siemens Healthcare Diagnostics Inc.) using citrated PPP prepared from IVC blood draws. This kit detects mouse antithrombin in complex with either mouse or human thrombin, but is slightly (1.3-fold) more sensitive to human/mouse chimeric TAT complexes than mouse/mouse TAT complexes (data not shown).

Statistical Methods

Descriptive statistics (mean and standard deviation [SD] or standard error of the mean [SEM], as indicated) were calculated for each experiment. Analysis of variance (ANOVA) was used to identify significance in experiments with more than two dosing groups, with posthoc Student-Newman-Keuls tests for between-groups comparisons. Datasets with only two comparison groups (control and elevated prothrombin) were compared using a Student t test (normally-distributed data) or Wilcoxon-Mann-Whitney Rank Sum Test (non-normally distributed data) in Kaleidagraph version 4.1.3. P<0.05 was considered statistically significant.
REFERENCES for SUPPLEMENTAL SECTION


Elevated prothrombin promotes venous, but not arterial, thrombosis in mice
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Supplemental Figure I

Supplemental Figure I. Human prothrombin supports thrombin generation in murine plasma and mouse thrombomodulin reduces thrombin generation in mouse plasma spiked with human prothrombin. Thrombin generation was measured in the absence and presence of 200 nM thrombomodulin in A) murine PPP spiked with vehicle, B) murine PPP spiked to 200% with murine prothrombin, and C) murine PPP spiked to 200% with human prothrombin.
Supplemental Figure II. Human prothrombin circulates in mice 12 hours post-infusion. Twelve hours after infusion of HBS or prothrombin (to 300%, final), blood was drawn from the IVC into 3.2% sodium citrate and processed to PPP (n=4-5/group). PPP samples were separated by 4-12% SDS-PAGE, transferred to PVDF membranes, and probed with rabbit polyclonal anti-human prothrombin antibody. Band intensity was quantified by densitometry. Since human prothrombin is better detected by the rabbit polyclonal antibody, the blot readily indicates the presence of human prothrombin in these samples.
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Supplemental Video Legends

Supplemental Video I. Thrombus formation following electrolytic injury in the femoral vein of control mice. Mice were infused with saline (Control) and subject to electrolytic injury as described in the Methods. Video acquisition began 1 minute after initiation of the 30-second electrolytic injury. Flow is from left to right. Each video shows fluorescent platelet accumulation over the course of 60 minutes, followed by anti-fibrin accumulation in the same thrombus over the same time course. The dimensions of the field of view are 3.22 x 2.4 mm (width x height). The videos play as 3 time-lapse minutes per viewing second (20 seconds playing time for each 60-minute time-lapse series) at 18 frames per second. Note the dark spot in center of thrombus on lower left; this is pitted material from the site of electrolytic injury (approximately 75-micron-diameter contact zone of blunt needle).

Supplemental Video II. Thrombus formation following electrolytic injury in the femoral vein of prothrombin-infused mice. Mice were infused with prothrombin (200%, final) and subject to electrolytic injury as described in the Methods. Video characteristics are described in the legend to Supplemental Video 1, showing platelet accumulation over the 60-minute time course first, followed by fibrin accumulation over the same time course.

Supplemental Video III. Thrombus formation following electrolytic injury in the carotid artery of control mice. Mice were infused with saline (Control) and subject to electrolytic injury as described in the Methods. Video characteristics are described in the legend to Supplemental Video 1, showing platelet accumulation over the 60-minute time course first, followed by fibrin accumulation over the same time course.

Supplemental Video IV. Thrombus formation following electrolytic injury in the carotid artery of prothrombin-infused mice. Mice were infused with prothrombin (200%, final) and subject to electrolytic injury as described in the Methods. Video characteristics are described in the legend to Supplemental Video 1, showing platelet accumulation over the 60-minute time course first, followed by fibrin accumulation over the same time course.