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Correction of the coagulation defect in hemophilia using a factor Xa variant with novel engineered protease function

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Abstract

Effective therapies are needed to control excessive bleeding in a range of clinical conditions. We describe a surprisingly useful approach to improve hemostasis *in vivo* using a variant of coagulation factor Xa (FXa^{I16L}). This conformationally pliant derivative is partially inactive due to a defect in transitioning from zymogen to protease^{1,2}. Using mouse models of hemophilia, we show that FXa^{I16L} has a prolonged half-life, relative to wild-type FXa and does not cause excessive activation of coagulation. Once clotting mechanisms are activated to produce its cofactor FVa, FXa^{I16L} is driven to the protease state and restores hemostasis in hemophilic animals upon vascular injury. Moreover, using human or murine analogs, we show that FXa^{I16L} is more efficacious than FVIIa which is used to treat bleeding in hemophilia inhibitor patients³. Because of its underlying mechanism of action, FXa^{I16L} may provide an effective strategy to enhance blood clot formation and act as a rapid pan-hemostatic agent for the treatment of bleeding conditions.

The formation of a blood clot following vascular injury is a vital host defense mechanism. This process is compromised in patients with congenital or acquired bleeding disorders including hemophilia A (HA; factor VIII (FVIII) deficiency) and hemophilia B (HB; factor

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Authorship Contributions

L.I. designed, coordinated, and performed experiments; R.T., P.M., G.P., H.K., A.S., J.L., and K.M.S. performed experiments; R.R.M. coordinated and performed experiments; V.C. designed and analyzed experiments; D.D.P. interpreted data; J.T., D.E., V.R.A., and R.M.C. designed experiments and analyzed data. L.I. and R.M.C. wrote the paper. All authors discussed the results and commented on the manuscript.

Conflict of Interest Statement

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IX (FIX) deficiency)⁴. FVIII and FIX function in the intrinsic pathway of coagulation to maintain normal hemostasis by affecting the conversion of FX to FXa. Membrane-bound FXa in the presence of its cofactor FVa (prothrombinase complex) converts prothrombin to thrombin, which activates platelets and converts fibrinogen to fibrin to form the thrombus⁵. Management of patients with HA and HB has dramatically improved over the past three decades⁶. However, development of inhibitory neutralizing alloantibodies (inhibitors) in response to infusion of FVIII or FIX protein remains a serious threat to disease related morbidity and mortality^{7,8}. Protein-based biopharmaceuticals that bypass the intrinsic pathway such as activated prothrombin complex concentrates (aPCCs; FEIBA) and recombinant FVIIa (FVIIa; NovoSeven) are viable strategies to re-establish hemostasis in these patients^{3,9}. However, neither is universally effective nor can completely normalize thrombin generation with some patients experiencing a variable response to either bypassing agent¹⁰. Furthermore, frequent high-dose infusions to stop bleeding result in costly treatment¹¹.

Existing bypassing strategies are directed towards enhancing FXa production to accelerate the formation of thrombin^{12,13}. FVIIa at pharmacological doses (90 µg/kg; 1.8 nmol/kg; ~25 nM) activates FX at sites of vascular injury where tissue-factor (TF) is exposed and activated platelets are abundant. In principle, infusion of FXa to increase the concentration of cell surface prothrombinase at the injury site would represent a more direct approach to enhance thrombin production. However, FXa is rapidly inactivated by circulating protease inhibitors resulting in a short half-life (<1–2 min) and can activate a range of procoagulant clotting factors possibly leading to pathological activation of coagulation¹⁴. These seemingly insurmountable limitations associated with the infusion of FXa have been borne out by findings in hemophilic animals^{15,16}.

Our approach to the problem is based on knowledge of the biochemistry of zymogen activation and protease formation in the S1 peptidase clan of chymotrypsin-like serine proteases¹⁷. Rather than attempting to enhance enzyme function, we recently developed FXa variants (e.g. FXa^{I16L} and FXa^{V17A}; numbered after chymotrypsinogen¹⁸) which have an impaired conformational transition from the zymogen to protease. In this family, the zymogen precursor is cleaved between Arg¹⁵-Ile¹⁶ liberating a new N-terminus (typically, Ile¹⁶-Val-Gly-Gly...) ¹⁷. After cleavage, the intermediate is in a zymogen-like state, which equilibrates to the protease state following insertion of the nascent N-terminus into a binding pocket forming a salt-bridge between Ile¹⁶ and Asp¹⁹⁴. Completion of this transition is critical for full expression of enzyme activity. For FXa, modification at Ile¹⁶ or Val¹⁷ perturbs the zymogen to protease transition¹. As a result, FXa^{I16L} and FXa^{V17A} have zymogen-like properties including an immature active site, decreased sensitivity towards plasma inhibitors and poor reactivity towards other physiological ligands¹. Notably, binding to FVa facilitates the transition to the active conformation and rescues the procoagulant activity of FXa^{I16L} and FXa^{V17A} resulting in normalized thrombin generation even in the context of hemophilic plasma^{1,2}. The current report examines whether zymogen-like FXa variants correct the hemostatic defect *in vivo* using murine models of HA and HB.

Hemophilic mice are useful models to probe the effectiveness of procoagulant therapeutics as they recapitulate aspects of the human disease. In a modified aPTT clotting assay (Fig.

1a), untreated HB mice have a prolonged clotting time compared to hemostatically normal wild-type (wt)-mice. Intravascular administration of human FXa^{I16L} (hFXa^{I16L}; 450 µg/kg or 9.8 nmol/kg, ~100 nM) corrects the prolonged aPTT in HB mice 10 min post infusion whereas PBS has no effect. The aPTT was shortened at 2 hrs and returned to baseline at 24 hrs post-infusion. Shortening of the aPTT with hFXa^{I16L} was dose-dependent as 225 µg/kg gave an intermediate result. In contrast to hFXa^{I16L}, administration of wt-hFXa in HB mice (450 µg/kg; n = 4) did not shorten the aPTT at any time point (not shown) likely due to its extremely short half-life (<0.5 min) in mouse plasma (Fig. 1b). The zymogen-like conformation protects hFXa^{I16L} in mouse plasma from inhibitors and extends the half-life by a factor of 30–60-fold (Fig. 1b). Estimated *ex vivo* half-lives in mouse plasma are approximately 5–10-fold shorter compared to human hemophilic plasma likely reflecting the increased activity of protease inhibitors as previously suggested¹⁹. Like HB mice, there is a dose-dependent shortening of the aPTT in HA mice treated with hFXa^{I16L} compared to PBS treated mice (Supplemental Fig. 1). Taken together, these data show that hFXa^{I16L} has an extended half-life compared to wt-hFXa and corrects the prolonged clotting time in both HA and HB mice. These latter findings indicate that sufficient amounts of FVa were generated during the coagulation assays to rescue the activity of hFXa^{I16L}.

Safety and the possibility of uncontrolled and systemic activation of coagulation is a major concern with any procoagulant protein therapeutic. Compared to PBS-treated animals, there was no major change in fibrinogen, platelets, or D-dimer levels over an extended time course with hFXa^{I16L} at 450 µg/kg (Fig. 1c–e). There was a transient increase in thrombin-antithrombin (TAT) 10 min post-infusion which returned to baseline at 30–60 min (Fig. 1f). Histopathological evaluation of lung, liver, kidney, and spleen from HB mice infused with hFXa^{I16L} revealed no evidence of treatment-related increases in fibrin deposition (not shown). To further evaluate the safety of hFXa^{I16L}, multi-day infusion experiments were performed in wt-mice (Balb/c). Animals were injected with hFXa^{I16L} at 450 µg/kg either once (n = 5) or twice (n = 5; injections spaced 4 hrs apart) daily for three consecutive days. Compared to pre-infusion controls or PBS-infused mice (n = 5), there were no statistically significant difference in platelets, fibrinogen, D-dimer, or TAT levels in mice treated with hFXa^{I16L} (not shown). Furthermore, hFXa^{I16L} was well tolerated as all mice survived the multi-dose infusion. A contributing factor to this favorable safety stem from its relatively poor reactivity towards a range of physiological ligands in the absence of circulating FVa^{1,2}.

To evaluate the efficacy of hFXa^{I16L} *in vivo*, blood loss was monitored following a hemostatic challenge. HB mice on the C57BL/6 (Fig. 2a) or Balb/c (Supplemental Fig. 2) background exhibit substantial blood loss following sectioning of the distal portion of the tail compared to normal mice. Infusion of hFXa^{I16L} in HB mice 5 min prior to injury reduced bleeding following tail clip in a dose-dependent manner. Treatment with the highest dose (450 µg/kg) reduced total blood loss to that seen with wt-mice whereas 225 µg/kg of hFXa^{I16L} was intermediate; there was no significant reduction in total blood loss at 113 µg/kg (Fig. 2a). Similar dose response results with hFXa^{I16L} were obtained in HA mice (Supplemental Fig. 3). Infusion of a second variant (hFXa^{V17A}; 450 µg/kg) with similar biochemical properties as hFXa^{I16L} into HB mice also decreased total blood loss to that obtained with normal mice (Supplemental Fig. 2). In contrast, HB mice infused with wt-

hFXa (450 µg/kg) showed no improvement in blood loss compared to HB controls (Supplemental Fig. 2). These data show that infusion of hFXa^{I16L} prior to injury is effective in reducing blood loss in hemophilic animals following a severe hemostatic challenge.

Since pro-hemostatic agents are generally administered following a bleeding episode, experiments examined whether hFXa^{I16L} is capable of reducing bleeding when infused after an injury (Fig. 2b). Following tail injury and an observation period of 2 min, hFXa^{I16L} or PBS were injected into mice via a pre-inserted jugular vein cannulus and total blood loss was monitored for an additional 10 min. Using this strategy, hFXa^{I16L} was very effective at decreasing bleeding in HB mice: 225 µg/kg and 450 µg/kg reduced total blood loss to that seen with wt-mice and 113 µg/kg showed partial reduction in blood loss. The increased effectiveness of hFXa^{I16L} when administered after tail injury (Fig. 2b) versus before (Fig. 2a) is likely due to inactivation of a proportion of the hFXa^{I16L} by circulating inhibitors prior to the injury.

Another useful test of therapeutic procoagulants is injury to the carotid artery using FeCl₃ as hemophilic animals do not generally form occlusive thrombi in this model²⁰. Following application of FeCl₃ (15%) to the surface of the carotid artery for 2 min, wt-mice or HB mice infused with hFIX after injury present with full occlusion characterized by interruption of blood flow (Table 1 and Supplemental Fig. 4). In contrast, no evidence of clot formation was detected in PBS-treated HB mice. To test hFXa^{I16L} in HB mice, FeCl₃-induced injury was monitored for 10 min to ensure that blood flow remained stable; subsequently hFXa^{I16L} was administered via a jugular vein cannulus. Using this model, hFXa^{I16L} rapidly induced complete vessel occlusion in all mice (n = 13) at 180–450 µg/kg (Table 1 and Supplemental Fig. 4). At a lower dose (90 µg/kg) transient and occlusive thrombi were observed. In a second method, hFXa^{I16L} (n = 5; 450 µg/kg) was administered to HB mice 10 min prior to the application of FeCl₃. Subsequent injury to the carotid artery resulted in complete occlusion in 5 out of 5 mice at 2.5 ± 1.1 min following injury (not shown). In these experiments, hFXa^{I16L} induced vessel occlusion more rapidly (~2 min) than untreated control mice (~10–14 min). We speculate that since infusion of hFXa^{I16L} bypasses both the intrinsic and extrinsic pathways the rate of thrombin formation would be expected to be much faster than in wt-mice. Consistent with this, introduction of hFXa^{I16L} into a thrombin generation assay using human hemophilic plasma substantially shortens the lag time or onset of thrombin generation compared to normal plasma without added FXa₂. Similarly, FVIIa and FEIBA also shorten the lag time in a thrombin generation assay²¹ and the time to occlusion following FVIIa administration in the FeCl₃ model is comparable to FXa^{I16L} (see Table 1).

The efficacy of hFXa^{I16L} was further assessed in the microcirculation through the kinetics of thrombus formation following laser injury to arterioles in the cremaster muscle. As shown in Fig. 3a, hFXa^{I16L} (10 µg/kg or 0.2 nmol/kg, ~2.5 nM) infused 5 min prior to vessel injury, restored platelet (red) and fibrin (green) accumulation over time comparable to wt-mice (see also Supplemental Movie 1 and 2). Quantitative analysis revealed that hFXa^{I16L} induced platelet accumulation was comparable to wt-mice whereas total fibrin deposition was reduced and slightly delayed (Fig. 3b,c). In contrast, little, if any, platelets or fibrin were detected in PBS-infused HB controls (Fig. 3a–c). At both 10 µg/kg (n = 3 mice/20 injuries)

and 90 $\mu\text{g}/\text{kg}$ ($n = 8$ mice/55 injuries) thrombus formation was observed 92% and 100% of the time, respectively. In addition, we explored the consequences of hFXa^{I16L} infusion after laser injury. As shown in Supplemental Movie 3, there is no detectable clot formation in HB mice following laser injury. However, subsequent infusion of hFXa^{I16L} (30 $\mu\text{g}/\text{kg}$) via a jugular vein cannulus rapidly resulted in platelet/fibrin accumulation at the previous injury site. Collectively, these data show that infusion of hFXa^{I16L} either before or after laser injury restores hemostasis at the microcirculation level. Interestingly, in this model hFXa^{I16L} was effective at a much lower dose (25–45-fold) than needed in the tail clip/ FeCl_3 models. While this could reflect sensitivity issues, it is also possible that differences in vessel size (~10-fold difference in diameter between carotid artery and arterioles), mechanism of clot formation, availability of FVa and/or the nature of the initiating injury could also contribute. Differences in efficacy between tail clip/ FeCl_3 and laser injury models has also been noted with FVIIa and when investigating how FV-Leiden influences the hemophilic phenotype^{20,22,23}.

Currently, FVIIa is the only recombinant bypassing biotherapeutic approved for treatment of bleeding episodes in hemophilia inhibitor patients³. It is generally thought that FVIIa at pharmacological doses binds to activated platelets and converts FX to FXa in a TF-independent manner, albeit at a very slow rate²⁴. This likely explains the need for frequent high dose infusions for an adequate hemostatic response³. The relative inefficiency of FVIIa in generating FXa at the platelet surface and the variability of procoagulant platelet populations from patient to patient may explain the difficulty in establishing a relationship between FVIIa plasma concentration and its hemostatic effect complicating optimal treatment²⁵. Furthermore, high treatment cost and lack of clinically useful laboratory assays to monitor therapeutic responses suggest a need to compare effectiveness of other hemostatic agents to FVIIa.

To begin to evaluate this, the efficacy of hFXa^{I16L} was compared to human FVIIa (hFVIIa) in the tail clip/ FeCl_3 injury models. Consistent with prior reports²⁶, high levels of hFVIIa were needed to reduce blood loss following tail injury in both HB (Fig. 2c) and antibody-induced HA animals (Supplemental Fig. 5). For hFVIIa treated mice, reductions in blood loss occurred between 3–5 mg/kg (60–100 nmol/kg, ~0.6–1.0 μM) representing an approximately 10-fold increase compared to hFXa^{I16L}. Similarly, high concentrations of hFVIIa were needed (3–5 mg/kg) to induce thrombus formation in HB mice in the FeCl_3 injury model (Table 1). Like hFXa^{I16L}, infusion of hFVIIa following injury to the carotid artery rapidly (2.5– 3.0 min) induced occlusive or transient thrombi.

While the effectiveness of hFXa^{I16L} versus hFVIIa is compelling, the overall findings must be interpreted cautiously since human proteins were used with the potential for suboptimal species compatibility between clotting factors. This is particularly true with hFVIIa as it binds weakly, if at all, to murine TF^{23,27}. To address this point, murine derivatives of FXa^{I16L} (mFXa^{I16L}) and FVIIa (mFVIIa) were expressed and purified. Recombinant mFVIIa has been recently shown functionally similar to hFVIIa except with respect to TF binding^{23,27}. Biochemical characterization of purified mFXa^{I16L} revealed that it has zymogen-like properties and can be rescued in assays where FVa is generated. For example, mFXa^{I16L} has low activity (2–4%) in the absence of FVa compared to wt-mFXa as assessed

by peptidyl substrate cleavage (Supplemental Fig. 6) yet the variant is as effective as wt-mFXa in a thrombin generation assay where FVa is generated *in situ* (Supplemental Fig. 7). Consistent with its zymogen-like properties, mFXa^{I16L} has a much longer half-life in murine HB plasma compared to wt-mFXa indicating that, like hFXa^{I16L}, it is partially protected from plasma-based inhibitors (Supplemental Fig. 8).

The efficacy of mFXa^{I16L} and mFVIIa in HB mice were compared in the tail clip/FeCl₃ injury models. As expected, mFXa^{I16L} was able to substantially reduce blood loss in the tail clip assay; interestingly the dose required for efficacy was 4-fold less for mFXa^{I16L} compared to the human protein. Only 100 µg/kg (2 nmol/kg, ~20 nM) reduced blood loss to that seen in wt-mice (Fig. 2d). Similarly, mFXa^{I16L} was also clearly effective in the FeCl₃ injury model with 5 out of 8 animals presenting with full occlusion at 50–100 µg/kg (Table 1). In contrast, high levels of mFVIIa were needed in both the tail clip (Fig. 2d) and FeCl₃ injury models (Table 1). For mFVIIa treated mice, reductions in blood loss occurred only at 5 mg/kg, representing an approximately 50-fold increase in therapeutic dose compared to mFXa^{I16L}. These within-species comparisons collectively indicate that FXa^{I16L} is superior as a hemostatic bypass compared to FVIIa in hemophilic mice using established injury models. Although results with FXa^{I16L} are certainly encouraging, additional parameters such as pharmacokinetic profile, dosing and safety will need to be assessed in a large animal model of hemophilia. Unlike the mouse model, the pharmacokinetics and dosing of FVIIa in hemophilic dogs is very similar to those parameters in humans. An important question in future experimentation is whether this 10–50-fold dose advantage of FXa^{I16L} versus FVIIa seen in mice translates to the hemophilic dog model.

The high dose of FVIIa needed to ameliorate the hemophilic phenotype in mice supports the notion that the platelet-surface, TF-independent procoagulant mechanism predominates²⁶. Unlike this mechanism, the efficacy of FXa^{I16L} absolutely requires not only procoagulant cellular surfaces but the availability of its cofactor, FVa. We speculate that activated platelets play a key overall role in this process as they provide the necessary procoagulant membrane surface and release relatively large quantities of FV/FVa from α-granules^{25,28}. Prior studies using isolated thrombin-activated platelets demonstrated that released platelet-derived FVa was sufficient to completely rescue the activity of hFXa^{I16L} leading to rapid prothrombin activation². The importance of activated platelets to the overall hemostatic potential of FXa^{I16L} was seen in additional thromboelastography experiments (Supplemental Table 1). In the absence of an added extrinsic (TF) or intrinsic pathway activator, the addition of hFXa^{I16L} with a direct platelet activator (ADP or collagen) to HB mouse whole blood completely normalized the coagulation parameters seen with hemophilic samples to values seen in normal blood. These data show that direct platelet activation with subsequent release of FV/FVa efficiently restores the activity of hFXa^{I16L} to correct altered whole blood coagulation in the context of hemophilia.

Knowledge of the biochemistry of the FX zymogen to protease transition has provided a novel approach to circumvent limitations inherent to wt-FXa that preclude its use as a therapeutic bypass for hemophilia. Our innovative strategy of employing rational mutagenesis to make FXa more zymogen-like and thereby reducing function has yielded a remarkably effective agent for modulating hemostasis in hemophilic mice. The plasticity of

zymogen-like FXa is central to this effectiveness, as enzyme function can be restored by FVa and activated cellular surfaces following vascular damage. This selective and localized enhancement of cell-surface prothrombinase rapidly produces thrombin at the injury site. The favorable safety profile of this variant in mice results from its extended half-life in plasma and poor reactivity towards other physiological substrates in comparison to wt-FXa. Any procoagulant therapeutic has the potential for thromboembolic complications and future studies in larger animals are needed. Furthermore, while the complexity of the immune response makes predictions difficult, it remains possible that an antibody response could be mounted against hFXa^{I16L} despite a conservative amino acid change. Of note, evaluation of a FVIIa variant with three amino acid substitutions reported no development of antibodies in a phase I clinical trial³⁰. Overall, the current data with FXa^{I16L} provide proof-of-concept for a new class of bypassing agent with high hemostatic potential to treat patients with bleeding disorders. These studies also provide a general protein engineering strategy to modulate, either directly or indirectly, the activity of other serine proteases of biologic and therapeutic importance.

METHODS

Reagents

All tissue culture reagents were from Invitrogen (Carlsbad, CA) except insulin-transferrin-sodium selenite which was from Roche (Indianapolis, IN). Human factor deficient plasmas from individual donors were purchased from George King Biomedical, Inc. (Overland Park, KS). Automated activated partial thromboplastin time reagent (aPTT; TriniClot or Sta-C.K.Preset) was from Tcoag (Wicklow, Ireland) or Stago (Parsippany, NJ). Adenosine diphosphate (ADP) and collagen (collagen fibrils, type I from equine tendons) was obtained from Chrono-log Corp. (Havertown, PA). All reagents necessary to perform rotational thromboelastography (ROTEM) were obtained from Pentapharm (Durham, NC). Preparation and characterization of recombinant wt-hFXa, hFXa^{I16L}, hFXa^{V17A}, and mFVIIa has been previously described^{1,23}. Recombinant wt-mFX and mFX^{I16L} were expressed in human embryonic kidney 293 (HEK293) cells and prepared essentially as described¹, except rather than employing immunopurification with an anti-FX antibody, the final purification step involved elution of mFX from a Poros HQ/20 column with a CaCl₂ gradient (0 to 20 mM).

Antibodies

Rat anti-mouse CD41 antibody (clone MWReg 30) prepared as a F_{(ab)2} fragment was from BD Bioscience (San Jose, CA). Mouse anti-human fibrin monoclonal antibody (clone 59D8) which cross reacts with mouse fibrin has been previously described^{31,32}. These antibodies were conjugated with Alexa₅₅₅ or Alexa₄₈₈ using the Alexa Fluor Protein Labeling Kit according to the manufacturer's instructions (Molecular Probes/Invitrogen, Carlsbad, CA). The mouse anti-human FVIII antibody, GMA-8015, was from Green Mountain Antibodies (Burlington, VT). Plasma levels of mouse TAT (Enzygnost TAT, Dade Behring, Marburg Germany), D-dimer (Asserachrom D-Di; Diagnostica Stago), and fibrinogen (Innovative Research) were measured by ELISA according to the manufacturer's guidelines.

Mice

Hemophilia B mice (Balb/c or C57BL/6) have been previously described and backcrossed for more than 5 generations onto the appropriate strain^{33,34}. Wild-type mice used in control experiments were either purchased from Jackson Laboratory (Bar Harbor, ME) or generated as littermates to HB mice (C57BL/6 background, heterozygous female×wild-type male breeding format). To experimentally induce an HA phenotype, C57BL/6 mice purchased from Taconic Farms (Germantown, NY) were injected with an anti-FVIII antibody (GMA-8015; 4.0 mg/kg). This is an inhibitory antibody directed against the FVIII A2 domain and cross reacts with FVIII from multiple species. For all experiments, mice were between 6–10 weeks of age and weighed 20 to 30 g. Experimental approval was obtained from the Children's Hospital of Philadelphia and Pfizer Institutional Animal Care and Use Committee.

Coagulation assays

Blood samples from mice obtained by tail-clipping were collected into one-tenth volume of 3.8% sodium citrate and immediately processed. Complete blood cell counts from individual mice were measured with an automated complete blood count analyzer with settings established for mouse blood (Hemavet, Drew Scientific Inc., Oxford, CT). Plasma was collected from whole blood following centrifugation and samples were evaluated fresh or snap frozen and stored at -80°C . The activated partial thromboplastin time (aPTT) on samples obtained from mice (wt or hemophilic) infused with FXa or PBS were assessed essentially as described³⁵. For *ex vivo* half-life determinations, FXa (20 nM, final) was incubated in diluted hemophilic mouse plasma (1:4, 1:5, or 1:8) at room temperature and at various time points aliquots of the reaction were further diluted (0.1 nM FXa, final) in 20 mM Hepes, 0.15 M NaCl, 0.1% polyethylene glycol 8000, pH 7.5 (dilution buffer). Residual FXa bypassing activity was assessed over time using a modified aPTT clotting assay. In a typical assay, 50 μl of human HB plasma was mixed with an equal volume of aPTT reagent followed by a 180 second incubation period at 37°C . A 50 μl mixture of FXa (0.1 nM) in dilution buffer was then added to the solution and coagulation was initiated following the addition of 50 μl of 25 mM CaCl_2 . Time to clot formation was measured using a Start4 coagulation instrument (Diagnostica Stago, Inc., Parsippany, NJ).

Chromogenic substrate hydrolysis

All kinetic measurements were performed in 20 mM Hepes, 0.15 M NaCl, 0.1% (w/v) PEG-8000, 2 mM CaCl_2 , pH 7.5 (assay buffer). The kinetic parameters of peptidyl substrate hydrolysis (Spectrozyme Xa; American Diagnostica, Stamford, CT) were measured using increasing concentrations of substrate (10–500 μM) and initiated with either wt-mFXa (2.0 nM) or mFXa^{I16L} (10 nM).

Rotational thromboelastography (ROTEM)

Whole blood (300 μl) from hemostatically normal wt or HB mice (Balb/c) was collected into citrate via tail clipping. Blood was transferred to a pre-warmed (37°C) ROTEM cup containing 0.2 M CaCl_2 (20 μl) and 20 μl of coagulation activator (INTEM reagent; ADP, 25 μM ; or collagen, 30 $\mu\text{g}/\text{mL}$). Bypassing protein or PBS (2 μl) to achieve the final

indicated concentration (Supplemental Table 1) was immediately added. Data derived from the ROTEM machine was collected over 90 min and analyzed using the manufacturers software.

Thrombin generation assays

Thrombin generation in platelet poor murine plasma was determined essentially as described with some modifications due to the higher activity of coagulation inhibitors in mouse plasma^{2,19}. Briefly, normal or HB murine (Balb/c) plasma (15 μ l) was mixed with a tissue-factor reagent (10 μ l; Technothrombin RB; 2 pM tissue factor/4.0 μ M phospholipid) followed by the addition of wt-mFXa or mFXa^{I16L} (25 μ l). The reaction was immediately initiated following the addition of Z-Gly-Gly-Arg-AMC in 15 mM CaCl₂ (50 μ l; 0.5 mM, final) and the concentration of thrombin (at 33°C) was determined as described².

Tail clip assay

Two different versions of the tail clip assay were employed. In the first, protein or PBS was initially infused via the tail vein approximately 5 min prior to injury. Mice were then anesthetized with isoflurane and then the tail was pre-warmed at 37°C and the distal portion transected at a diameter of 3 mm. The tail was then placed in a conical tube containing 10 mL or 14 mL of saline at 37°C and blood was collected for 10 min. In the second method, mice were anesthetized with isoflurane and then the tail was pre-warmed at 37°C and the distal portion transected at a diameter of 3 mm. The tail was then placed in a conical tube containing 14 mL of saline at 37°C and blood was collected for 2 min. Protein or PBS was then infused via a pre-inserted jugular vein cannulus. The injured tail was moved to a fresh tube of saline and blood was collected for an additional 10 min. Quantitative assessment of blood loss was determined by measuring total hemoglobin by absorbance at 575 nm following red cell lysis as described³⁶. For some experiments, hemoglobin content was determined using the QuantiChrom™ Hemoglobin Assay Kit (BioAssay Systems, Hayward, CA). For both determinations, quantitative assessment of hemoglobin content was converted to total blood loss (μ l) using appropriately established standard curves using each method.

Ferric chloride carotid artery model

Ferric chloride-induced injury was performed according to published procedures²⁰. Briefly, mice were anesthetized with pentobarbital and the carotid artery was exposed. A jugular vein cannula was established to allow for infusion of proteins. A miniature Doppler flow probe (Model 0.5VB; Transonic Systems, Ithaca, NY) was positioned around the exposed artery and baseline blood flow measurements were recorded. To induce an injury, a 2 mm² piece of Whatman #1 filter paper soaked in 15% FeCl₃ (0.92 M) was then applied to the adventitial surface of the artery for 2 minutes. The surface was then flushed with saline and blood flow was continuously monitored for up to 30 minutes. The time to carotid artery occlusion was defined as the time following application of FeCl₃ until the blood flow has decreased by >90%. In some experiments involving HB animals, an injury was induced with FeCl₃, blood flow was monitored for 10 minutes after which defined coagulation proteins were infused via a jugular vein cannula. Blood flow was monitored for an additional 20

minutes. In these experiments, the time to occlusion was defined as the time from infusion of clotting factors until the blood flow decreased by 90%.

Real-time *in vivo* imaging of thrombus formation

Evaluation of hemostasis following laser injury to the cremaster muscle has been previously described³⁷ and our specific experimental system has been detailed^{20,32}. Alexa₅₅₅-labeled rat anti- CD41 F_(ab)2 and Alexa₄₈₈-labeled anti-fibrin antibody were administered via a jugular vein cannula to label platelets and fibrin, respectively. Based on empirical studies, antibodies were infused into mice at 0.2 mg/kg for the anti-CD41 F_{ab} fragment and 0.1 mg/kg for the anti-fibrin antibody. Cremasteric arterioles of 30–50 μm were selected for study and vascular injury was induced using a pulse-nitrogen dye laser applied through the microscope objective. Brightfield and fluorescence images were collected over the course of 3–4 minutes at 4 frames/second and the data were analyzed with Slidebook 5 software (Intelligent Imaging Innovations, Denver, CO). The kinetics of clot formation was analyzed by determining median fluorescence intensity over time in approximately 18–20 thrombi from 3 animals/group.

Statistical analysis

All data are presented as mean ± SEM. An analysis of variance (ANOVA) followed by a Student-Newman-Keuls test was used to compare the treatment effects between groups. A *P* value of < 0.05 was considered statistically significant. For intravital experiments, data were considered nonparametric and presented as medians. In these experiments, the Wilcoxon rank sum test was used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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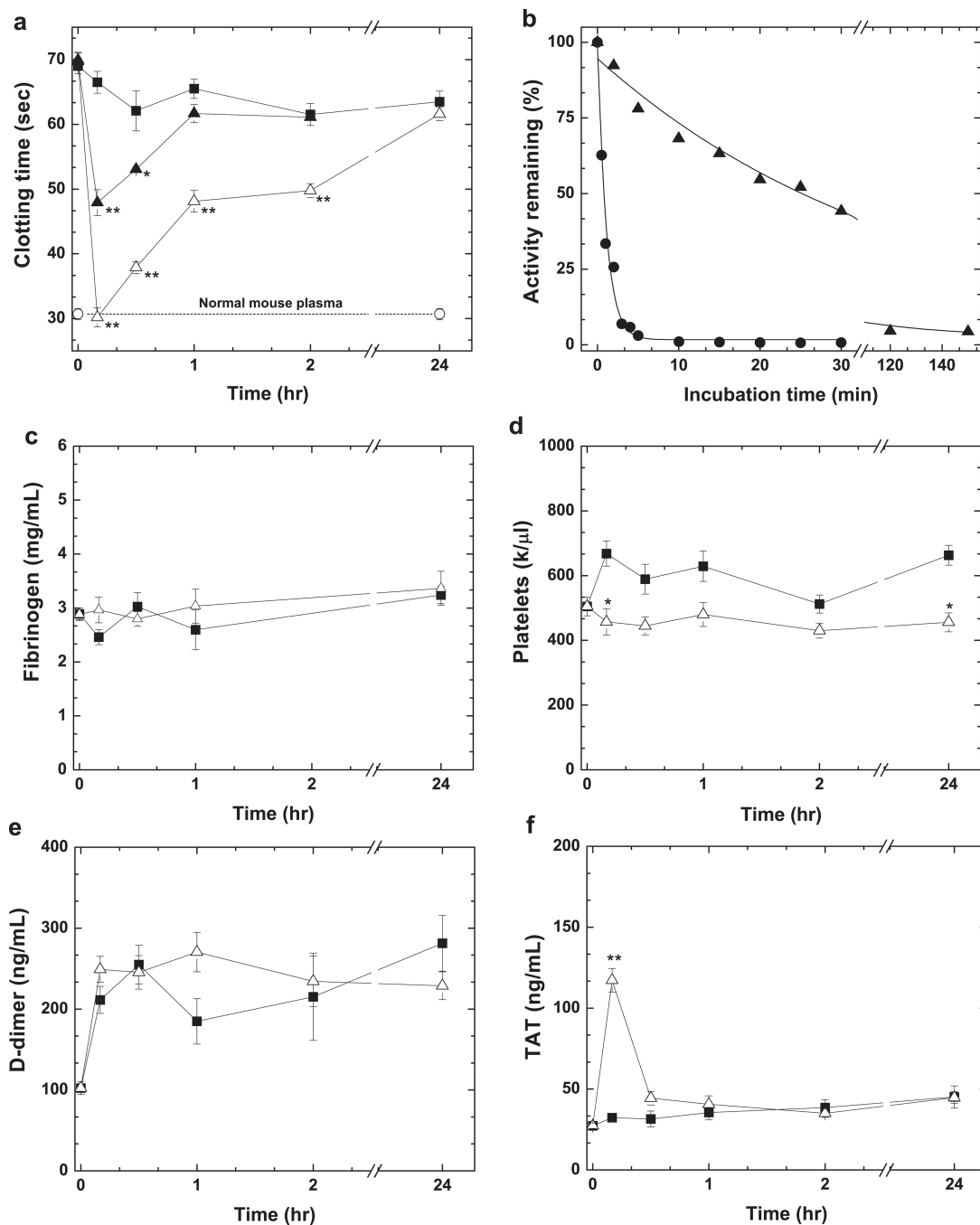
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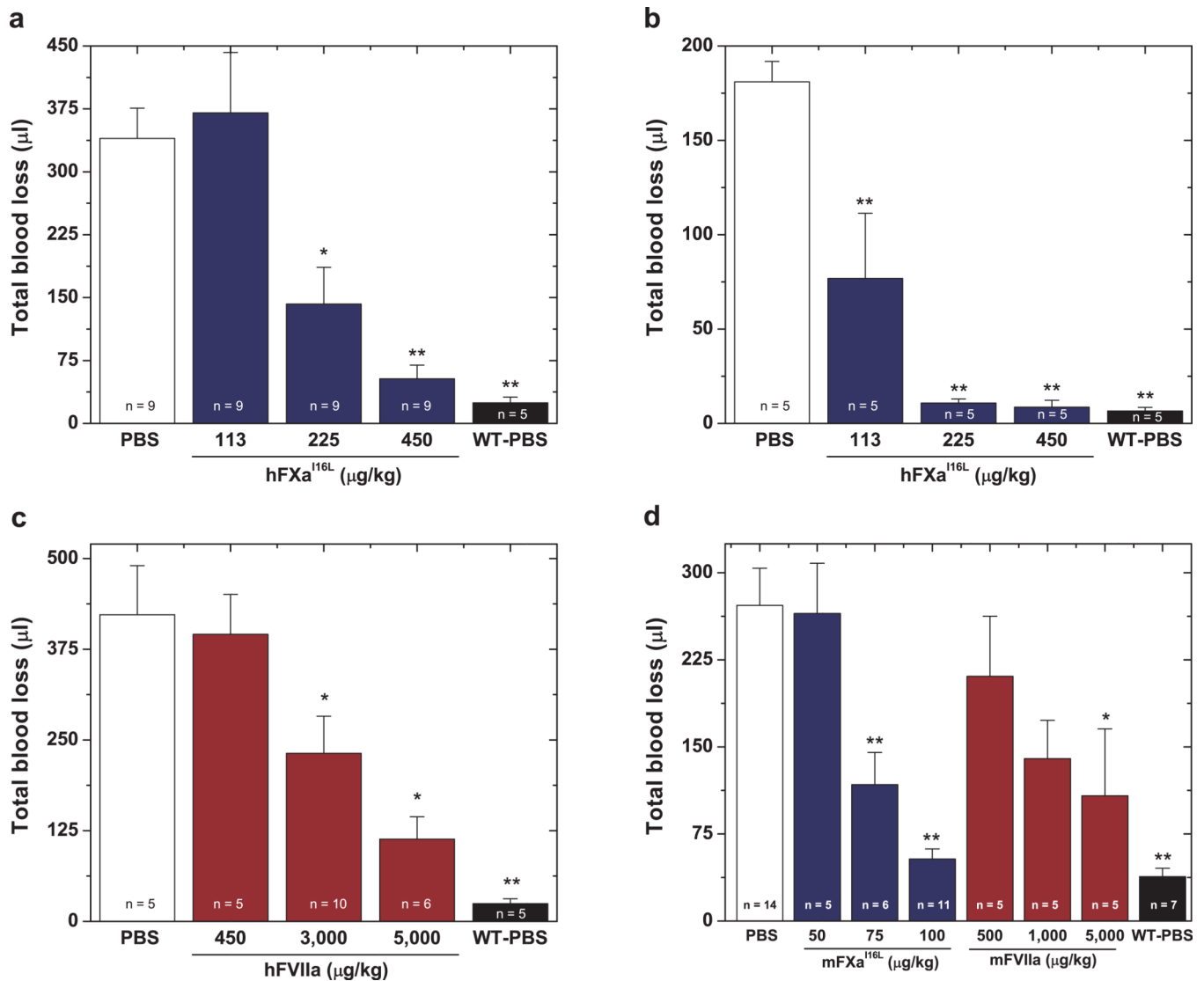
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**Figure 1.**

Effect of hFXa^{I16L} on coagulation parameters. In **a**, HB mice (Balb/c) were injected with PBS (■; n = 7) or hFXa^{I16L} (▲, 225 μg/kg, n = 4; △, 450 μg/kg, n = 9). At the indicated time intervals a modified one-stage aPTT was performed on processed mouse plasma. Clotting times for wt-mice (○, n = 7) is indicated. In **b**, wt-hFXa (●, 20 nM) or hFXa^{I16L} (▲, 20 nM) were incubated in diluted HB (Balb/c) mouse plasma and residual activity was assessed by clotting assay. The solid lines were drawn following analysis of data sets to a single exponential decay with fitted half-lives of: wt-hFXa, 0.15 ± 0.01 min;

hFXa^{116L}, 5.3 ± 0.46 min. These values have been appropriately adjusted for sample dilution. The data are representative of two to three similar experiments. In *c-f*, markers of coagulation activation were measured. HB mice (Balb/c) were injected with PBS (-■-, $n = 3-10$) or hFXa^{116L} (-△-, $450 \mu\text{g}/\text{kg}$, $n = 3-8$). At the indicated time intervals, levels of *c*) fibrinogen, *d*) platelets, *e*); D-dimer and *f*) TAT complex were measured. In panels *a* and *c-f*, all measurements are presented as mean \pm SEM. For statistical comparisons, treated animals are compared to HB-PBS controls at the same time point; ** $p < 0.001$; * $p < 0.05$.

**Figure 2.**

Blood loss following tail-clipping. In **a**, five minutes prior to injury, PBS (white column) or hFXa^{I16L} (blue columns) was administered to HB mice (C57Bl/6) via tail vein at the indicated dosage. Total blood loss (µl) was then measured following tail transection. In **b**, tail clipping was performed on wt-mice or HB mice (Balb/c) prior to protein infusion and blood was collected for 2 min (total blood loss at 2 min: wt-mice: 17 ± 5.4 µl; HB: 49 ± 7.7 µl). Subsequently, PBS (white column) or hFXa^{I16L} (blue columns) was administered to HB mice via a pre-inserted jugular vein cannulus at the indicated dosage; wt-mice also received PBS (black column). Blood was collected for an additional 10 min in a fresh tube of saline and total blood loss (µl) was measured. Data in **(b)** represent total blood loss subsequent to protein infusion. In **c**, five minutes prior to injury, PBS (white column) or hFVIIa (red columns) was administered to HB mice (C57Bl/6) via tail vein at the indicated dosage. Total blood loss (µl) was then measured following tail transection. In **d**, five minutes prior to injury, PBS (white column), mFXa^{I16L} (blue columns), or mFVIIa (red columns) was administered to HB mice (Balb/c) via tail vein at the indicated dosage. Total blood loss (µl)

was then measured following tail transection. In *a–d*, hemostatically normal mice on the appropriate strain infused with PBS (WT-PBS, black column) served as a control. In each panel, the number of animals per group is indicated and all measurements are presented as mean \pm SEM. For statistical comparisons, treated animals are compared to HB-PBS controls ** $p < 0.001$; * $p < 0.05$.

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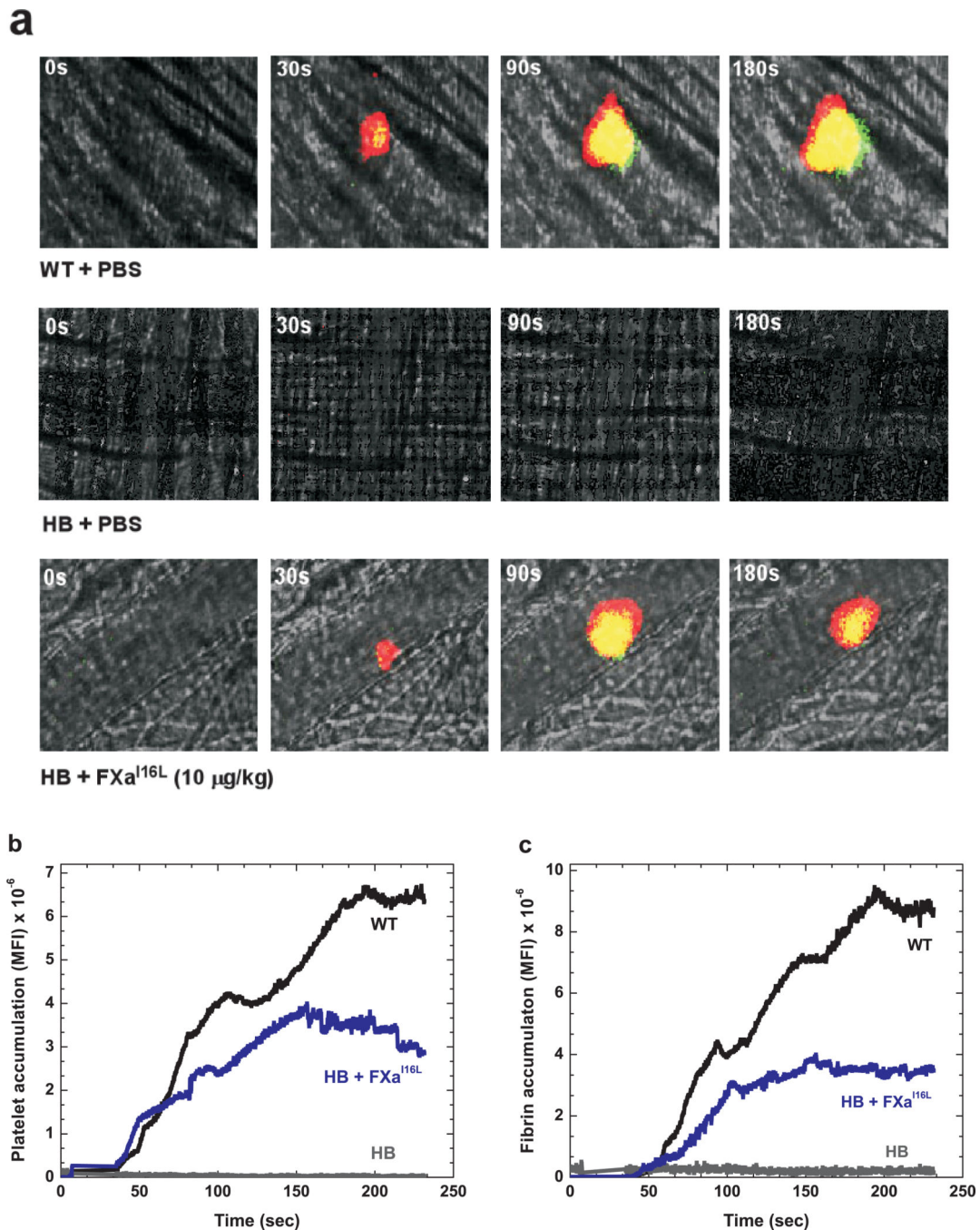


Figure 3.

Platelet and fibrin accumulation following laser-induced arteriole injury in wt-mice and hFXa^{116L} treated HB mice. In these experiments, PBS or protein was infused 5 min prior to vessel injury. In **a**, digital composite fluorescence and brightfield images of representative thrombi in wt-mice (+ PBS), HB (+PBS), and HB (+hFXa^{116L}, 10 µg/kg) mice prior to (0 sec) and 30, 90, and 180 sec after laser-induced injury of the blood vessel wall. Platelets (red) were detected by an Alexa₅₅₅-labeled rat anti- CD41 F(ab)₂ and fibrin (green) with Alexa₄₈₈-labeled anti-fibrin antibody; areas of overlap are depicted by yellow. (**b** and **c**)

Quantitative analysis of platelet and fibrin accumulation over time following laser injury in wt-mice (black; 20 thrombi; n = 3 mice), HB (grey; 20 thrombi; n = 3 mice), and HB + hFXa^{I16L} (blue; 20 thrombi; n = 3 mice; 10 µg/kg) treated mice. Median fluorescence intensity (MFI) for platelet (**b**) and fibrin (**c**) fluorescence are plotted versus time. For both platelet (**b**) and fibrin (**c**) accumulation, there is no statistically significant difference ($p > 0.1$) in MFI when comparing values between wt-mice and HB + hFXa^{I16L} at every 30 sec intervals by the Wilcoxon rank sum test. However, the MFI values (evaluated at 30 sec intervals) between wt-mice versus HB and HB + hFXa^{I16L} versus HB are significantly different ($p < 0.001$).

Table 1

Time to carotid artery occlusion following FeCl₃-induced injury.

Genotype	Sample	Dose	Total # of mice	No Occlusion	Transient Occlusion	Complete Occlusion	Time to Occlusion
		µg/kg	# of mice	# of mice	# of mice	# of mice	min
<i>Controls</i>							
WT (Balb/c)	PBS	--	7	0	0	7	10.1 ± 1.0
HB (Balb/c)	PBS	--	7	7	0	0	--
WT (C57BL/6)	PBS	--	4	0	0	4	11.9 ± 1.0
HB (C57BL/6)	PBS	--	4	4	0	0	--
HB (Balb/c)	hFIX	500	5	0	0	5	14.6 ± 1.1
<i>Experimental—Human Proteins</i>							
HB (Balb/c)	hFXa ^{U6L}	90	5	0	3	2	3.2 ± 0.6
HB (Balb/c)	hFXa ^{U6L}	180	3	0	0	3	2.4 ± 0.6
HB (Balb/c)	hFXa ^{U6L}	450	10	0	0	10	2.2 ± 0.2
HB (C57BL/6)	hFXa ^{U6L}	450	4	0	0	4	1.2 ± 0.1
HB (Balb/c)	hFVIIa	1,000	4	2	1	1	3.4 ± 0.1
HB (Balb/c)	hFVIIa	3,000	3	0	2	1	3.3 ± 0.3
HB (Balb/c)	hFVIIa	5,000	4	0	0	4	2.4 ± 0.2
<i>Experimental—Murine Proteins</i>							
HB (Balb/c)	mFXa ^{U6L}	50	3	0	2	1	2.6 ± 0.4
HB (Balb/c)	mFXa ^{U6L}	100	5	0	1	4	2.3 ± 0.4
HB (Balb/c)	mFVIIa	500	3	3	0	0	--
HB (Balb/c)	mFVIIa	3,000	7	5	0	2	2.4 ± 0.1
HB (Balb/c)	mFVIIa	5,000	4	0	0	4	2.5 ± 0.2

WT, hemostatically normal mice; HB, hemophilia B mice; PBS, phosphate buffer saline, FIX, factor IX.

The total number of mice per experiment is indicated. In HB mice, protein was infused 10 min after FeCl₃ exposure. Mice presenting with no occlusion, transient occlusion or complete occlusion of the carotid artery following FeCl₃ exposure is tabulated. Occlusion time measurements are presented as mean ± SEM and are derived from both transient and complete events. Representative Doppler blood flow *versus* time tracings can be found in Supplemental Fig. 4.