Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis

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Formation of fibrin is critical for limiting blood loss at a site of blood vessel injury (hemostasis), but may also contribute to vascular thrombosis. Hereditary deficiency of factor XII (FXII), the protease that triggers the intrinsic pathway of coagulation in vitro, is not associated with spontaneous or excessive injury-related bleeding, indicating FXII is not required for hemostasis. We demonstrate that deficiency or inhibition of FXII protects mice from ischemic brain injury. After transient middle cerebral artery occlusion, the volume of infarcted brain in FXII-deficient and FXII inhibitor–treated mice was substantially less than in wild-type controls, without an increase in infarct-associated hemorrhage. Targeting FXII reduced fibrin formation in ischemic vessels, and reconstitution of FXII-deficient mice with human FXII restored fibrin deposition. Mice deficient in the FXII substrate factor XI were similarly protected from vessel-occluding fibrin formation, suggesting that FXII contributes to pathologic clotting through the intrinsic pathway. These data demonstrate that some processes involved in pathologic thrombus formation are distinct from those required for normal hemostasis. As FXII appears to be instrumental in pathologic fibrin formation but dispensable for hemostasis, FXII inhibition may offer a selective and safe strategy for preventing stroke and other thromboembolic diseases.

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Ischemic stroke is a major cause of death and permanent disability in industrialized countries (1). Studies on the use of anticoagulant drugs in acute cerebral ischemia have shown no overall benefit, with decreases in lesion progression or stroke recurrence being offset by an increase in hemorrhage (2). Furthermore, long-term anticoagulation for prophylaxis to prevent thromboembolic events is inevitably associated with an increase in bleeding-related morbidity and mortality (3). Thus, it is highly desirable to identify novel targets for safe anticoagulation to treat stroke and other thrombotic disorders.

In the classic cascade or waterfall models of blood coagulation (4, 5), initiation of the complex process that culminates in fibrin formation in vitro can occur through either of two converging cascades, designated the extrinsic and intrinsic pathways. The factor VIIa–tissue factor (TF) complex comprises the extrinsic pathway (for reviews see reference 6), and deficiency of either factor VIIa or TF severely impairs blood coagulation in vivo (7, 8). On the other hand, hereditary deficiency of factor XII (FXII; Hageman factor), the protease that

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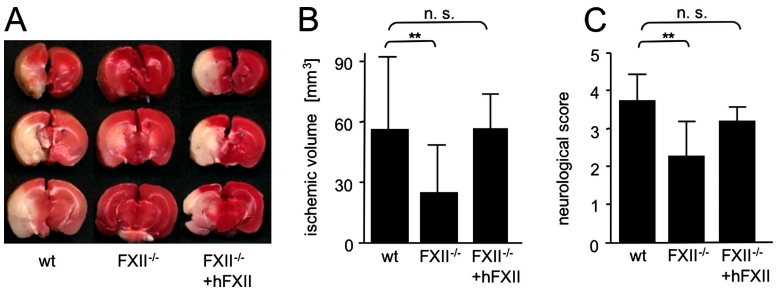

Figure 1. Infarct volumes and functional outcomes 24 h after focal cerebral ischemia in WT and FXII$^{-/-}$ mice, and in FXII$^{-/-}$ mice infused with human FXII. (A) Representative images of three corresponding coronal sections of WT (left), FXII$^{-/-}$ (middle), and FXII$^{-/-}$ mice reconstituted with human FXII (huFXII, 2 μg/g body weight i.v. 10 min before the MCAO; right) stained with TCC. (B) Brain infarct volumes in WT ($n = 18$), FXII$^{-/-}$ ($n = 18$), and FXII$^{-/-}$ mice reconstituted with huFXII ($n = 8$); **$P < 0.01$. (C) Neurological Bederson score assessed at day 1 after tMACO for WT ($n = 18$), FXII$^{-/-}$ ($n = 18$), and huFXII-treated FXII$^{-/-}$ animals ($n = 8$); **$P < 0.01$. n.s., not significant.

triggers the intrinsic pathway, is not associated with spontaneous hemorrhage or excessive injury-related bleeding in vivo (9, 10). These observations have led to revisions of the classic coagulation models that do not require FXII for fibrin formation (11).

We now demonstrate that deficiency or inhibition of FXII protects mice from ischemic brain injury in an experimental stroke model, without an increase in bleeding complications. Together with our previous findings that arterial thrombus formation triggered by artificial vessel injuries is defective in FXII-null mice (12), the data indicate that FXII inhibition may offer a selective and safe strategy for treatment or prophylaxis of vessel-occluding diseases. Furthermore, these novel findings suggest that the paradigm that pathologic thrombus formation is caused by dysregulation of the processes that normally prevent blood loss at a wound site may be incomplete and requires revision.

RESULTS AND DISCUSSION

To investigate the functions of FXII in hemostasis and thrombosis during ischemic stroke, we used FXII-deficient mice. Like their FXII-deficient human counterparts, FXII-null mice (FXII$^{-/-}$) develop normally and exhibit no spontaneous or injury-related hemorrhage, despite having very prolonged activated partial thromboplastin times (aPTT) clotting times (12) (a test of intrinsic pathway-initiated coagulation). Other studies of hemostasis, as well as cardiovascular characterization, did not reveal differences between WT and FXII$^{-/-}$ mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052458/DC1). As previous analyses of FXII$^{-/-}$ mice using chemical and mechanical vessel injuries in various arterial beds indicated defective thrombus stability (12), we assessed the contribution of FXII to the development of neuronal damage after transient cerebral ischemia in a model that depends on thrombus formation in microvessels downstream from a middle cerebral artery (MCA) occlusion (13, 14).

To initiate transient cerebral ischemia, a thread was advanced through the carotid artery into the MCA and allowed to remain for 1 h (transient MCA occlusion; tMCAO), reducing regional cerebral flow by >90% (8 ± 2% and 9 ± 2% of baseline in FXII$^{-/-}$ and WT mice, respectively). 15 min after removal of the thread, laser-doppler ultrasound revealed comparable recovery of MCA blood flow (FXII$^{-/-}$ 59 ± 8% and WT 59 ± 5%). 24 h after reperfusion, the infarct volumes in FXII$^{-/-}$ animals assessed by triphenyltetrazolium chloride (TTC) staining were dramatically reduced to <50% of the infarct volumes in WT mice (Fig. 1, A and B). The reduction in infarct size is functionally relevant, as the Bederson score assessing global neurological function (Fig. 1 C; $P < 0.01$) and the grip test that specifically measures motor function and coordination (FXII$^{-/-}$ 3.5 ± 0.5 and WT 1.9 ± 1.3; $P < 0.01$) were significantly better in FXII$^{-/-}$ mice than in WT mice.

To prove that the protective effect was specifically related to FXII deficiency and not to a secondary effect of the deficiency state, we reconstituted FXII$^{-/-}$ mice with human FXII in an attempt to "rescue" the WT phenotype. Intravenous infusion of human FXII (2 μg/g body weight) normalized the aPTT (29 ± 5 s for FXII$^{-/-}$ + human FXII compared with 27 ± 4 s for WT and 72 ± 20 s for FXII$^{-/-}$) and restored susceptibility to ischemic brain damage after tMCAO. Infarct volumes (56 ± 17 mm^3; Fig. 1 B) and neurological outcome parameters for FXII$^{-/-}$ mice treated with human FXII (Bederson score 3.2 ± 0.4; Fig. 1 C) were indistinguishable from WT mice. Importantly, FXII deficiency and FXII substitution did not affect normal injury-related coagulation as assessed by bleeding times in a tail amputation assay (FXII$^{-/-}$ 314 ± 78 s, FXII$^{-/-}$ + human FXII 301 ± 56 s, and WT 288 ± 81 s; $n = 12$ for each group).

Collectively, the rescue experiments provide strong evidence that the protection conferred by FXII deficiency in the tMCAO model is caused by the absence of plasma FXII. Furthermore, FXII may function similarly in mice and humans. Indeed, in a recent study of 21 patients with severe (homozygous) FXII deficiency no patient developed an arterial thrombotic event during a 15-yr follow-up period (15). However, larger epidemiologic studies are clearly needed to conclusively show a thromboprotective effect for FXII deficiency in humans.

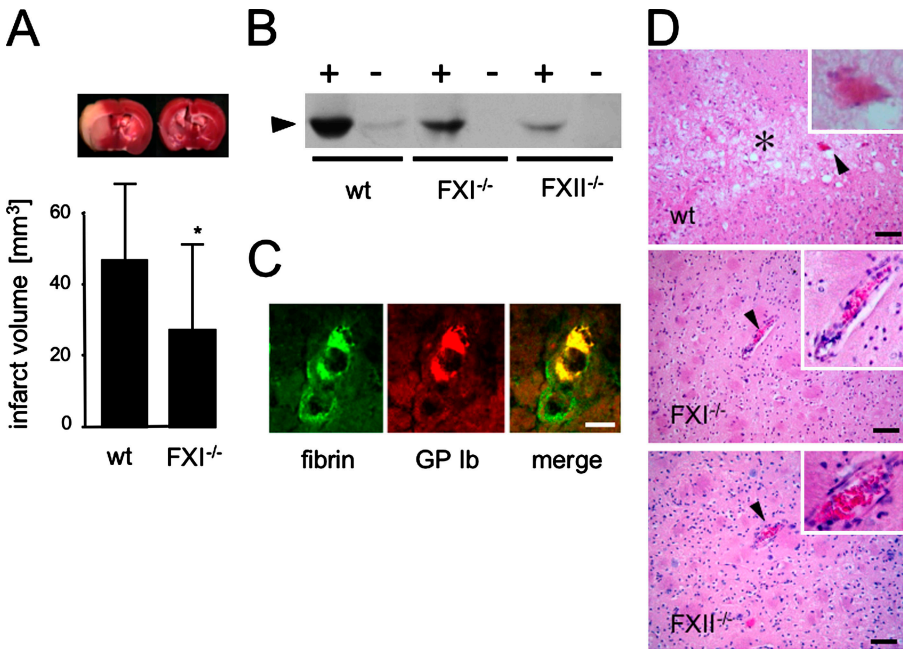

Figure 2. Comparison of FXII$^{-/-}$ and FXI$^{-/-}$ mice in the tMCAO model. (A) Representative TTC-stained coronal sections of FXI$^{-/-}$ and WT mice. Infarct volumes were assessed 24 h after tMCAO (WT, $n = 18$ and FXI$^{-/-}$, $n = 11$; *$P < 0.05$). (B) Accumulation of fibrin in the infarcted (+) and contralateral (−) hemispheres of WT, FXI$^{-/-}$, and FXII$^{-/-}$ mice. Fibrin formation 24 h after ischemia was analyzed by immunoblotting using the fibrin-specific antibody 59D8. (C) Immunohistochemical colocalization of fibrin (59D8 antibody) and platelets (anti-GP Ib antibody) in the lumens of microvessels after 24 h in the infarcted hemisphere of WT mice. Bar, 100 μm. (D) Hematoxylin and eosin–stained sections of corresponding territories in the ischemic hemispheres of WT, FXI$^{-/-}$, and FXII$^{-/-}$ mice. The dark arrows indicate blood vessels that are shown magnified in the inserts. Note the reduced microvascular patency in the ischemic areas of WT animals compared with FXI$^{-/-}$ and FXII$^{-/-}$ mice. Bar, 25 μm. The asterisk denotes the ischemic lesion.

In addition to its role in the intrinsic coagulation pathway, FXII may initiate or be involved in other proteolytic cascades, including the complement and kallikrein-kinin systems. The protease factor XI (FXI) is the primary substrate for activated FXII in the intrinsic pathway. Although FXII-deficient humans do not have a hemorrhagic condition, humans lacking FXI suffer from a mild to moderate bleeding disorder (16). If the prothrombotic effect of FXII in the tMCAO model is mediated through FXI activation, then FXI$^{-/-}$ mice should be protected from ischemic stroke similarly to FXII$^{-/-}$ animals. Indeed, in the tMCAO model brain infarct volumes were markedly diminished in FXI$^{-/-}$ mice compared with WT controls (27 ± 24 mm^3 and 46 ± 21 mm^3, respectively, $P < 0.05$; Fig. 2 A). These findings support the concept that activated FXII activates FXI during thrombus formation in WT mice, and are consistent with studies in primates and rabbits demonstrating that inhibiting FXI has antithrombotic effects (17, 18). In contrast to deficiencies of FXII or FXI, deficiency of factor IX, a substrate for both activated FXI in the intrinsic pathway and factor VII/TF (19), results in a severe bleeding disorder (hemophilia B) (20). Although a high degree of factor IX inhibition has been associated with hemorrhagic transformation in a murine stroke model, it does interfere with thrombus formation (21), supporting a decisive role for the intrinsic pathway in vessel occluding fibrin formation.

To confirm that FXII contributes to ischemic brain injury via activation of the intrinsic coagulation pathway, we measured fibrin formation in the infarcted hemisphere by quantitative immunoblot analysis of urea-insoluble tissue extracts. We used the antibody 59D8, which recognizes a neo-epitope exposed on fibrin following thrombin-dependent cleavage of fibrinogen, and which lacks cross-reactivity with the precursor protein (22). There was considerably reduced fibrin accumulation in the ischemic hemisphere of FXII$^{-/-}$ and FXI$^{-/-}$ mice compared with WT mice (Fig. 2 B). Immunohistochemistry consistently demonstrated intravascular fibrin deposits occluding vessels in WT mice and dramatically reduced fibrin deposits in FXII$^{-/-}$ and FXI$^{-/-}$ mice. Platelets were identified by glycoprotein (GP) Ib colocalization with fibrin clots in >80% of thrombi, suggesting that FXII-mediated fibrin formation and platelets synergize to form occluding thrombi in vessels distal to MCA occlusions (Fig. 2 C). Accordingly, histological sections of infarcted brain tissue from WT mice showed ischemic neurodegeneration associated with numerous occlusions of vessel lumina. In comparison, tissue injury in corresponding areas of FXII$^{-/-}$ and FXI$^{-/-}$ mice was markedly less severe than for WT mice, with a clear reduction in the number of occluded vessels (Fig. 2 D).

Cumulatively, our data suggest that protection from cerebral ischemia conferred by FXII deficiency is due to impaired microvascular thrombus formation caused by defective

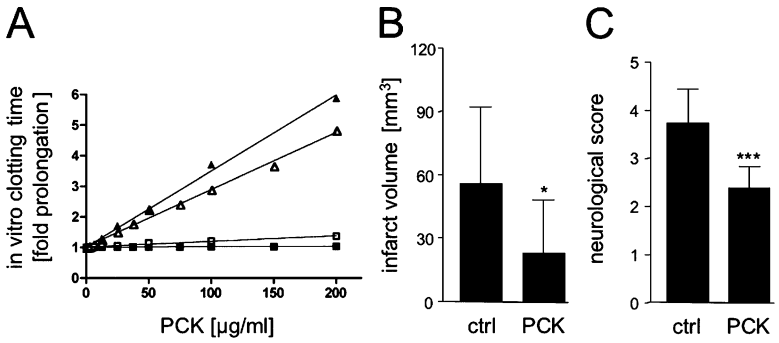

Figure 3. Inhibition of FXII activity inhibits clotting in vitro and thrombus formation in vivo. (A) Normal human plasma (open symbols) and WT mouse plasma (filled symbols) were incubated with increasing concentrations of PCK (1–200 μg/ml final concentration), an inhibitor that blocks FXIIa activity and activation. Clotting was initiated by adding kaolin and $CaCl_2$ (triangles) or TF (squares) to determine the aPTT and the PT, respectively. (B and C) PCK (8 μg/g of body weight) was infused intravenously into WT mice ($n = 8$) before tMCAO. 24 h after stroke, treated animals were analyzed and compared with untreated controls (ctrl, $n = 18$ per group). (B) Infarct volumes determined from TTC-stained sequential coronal sections (*$P < 0.05$) and (C) the neurological function assessed by the Bederson Score for PCK-treated and untreated mice (***$P < 0.0001$).

fibrin formation through the intrinsic pathway. Further analyses will be necessary to clarify the precise molecular mechanism for FXII activation in ischemic vessels and the additional contribution of other FXII-associated pathways, such as the complement and kallikrein-kinin systems, that have been shown to contribute to the pathology of cerebral ischemia (23, 24).

As congenital FXII deficiency protects mice from ischemic stroke, pharmacological targeting of FXII should provide similar protection. H-D-Pro-Phe-Arg-chloromethylketone (PCK) irreversibly inhibits the amidolytic activity of activated FXII (FXIIa) and plasma kallikrein-mediated activation of FXII (25, 26). In accordance with its effects on human plasma in vitro (Fig. 3 A, open triangles), PCK increased the aPTT in a dose-dependent manner when added to plasma from WT mice (Fig. 3 A, filled triangles) but did not prolong the prothrombin time (PT; a test for factor VIIa/TF–initiated activation of the extrinsic pathway of coagulation, filled and open squares). To test the protective potential of FXIIa inhibition, WT mice received PCK (8 μg/g body weight) before tMACO. PCK treatment prolonged the aPTT (61 ± 15 s) and reduced infarcted brain volume at 24 h compared with WT mice (23 ± 25 mm^3 and 56 ± 36 mm^3, respectively, $P < 0.05$; Fig. 3 B). PCK-treated mice showed a significantly better Bederson score (2.4 ± 0.4 for treated animals and 3.6 ± 0.8 for untreated animals, $P < 0.0001$; Fig. 3 C) and motor function (grip test score 3.7 ± 0.5 for PCK-treated animals vs. 1.9 ± 1.4 for untreated animals, $P < 0.01$). Western blot and immunohistochemical analyses of brain tissue using antibody 59D8 confirmed that PCK treatment had a striking effect on fibrin deposition, vessel occlusion, and ipsilateral brain damage after tMCAO compared with untreated control mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052458/DC1).

Dose–response analysis indicated that >4 μg/ml PCK was necessary to significantly inhibit fibrin formation, consistent with previous work showing that mice heterozygous for a FXII-null allele and WT mice exhibit similar arterial thrombus formation (12). Corroborating the concept that FXIIa inhibition is thrombo-protective, other FXII-blocking agents, such as anti-FXII antibodies and maize trypsin inhibitor, had similar effects to PCK on thrombus formation induced by arterial injury (unpublished data).

Although PCK infusion prolonged the aPTT of mice, normal hemostasis was not affected in a tail amputation assay (bleeding time 291 ± 134 s for treated animals and 278 ± 99 s for untreated animals). In comparison, the commonly used clinical anticoagulant heparin (0.2 U/g) prolonged the aPTT to an equivalent degree, but markedly prolonged the tail bleeding time to >20 min ($n = 14$; Fig. 4 A), indicating a substantial risk for hemorrhage. To further analyze the impact of FXIIa inhibition on the dynamics of infarct development and the risk of secondary hemorrhagic transformation, we used serial magnetic resonance imaging (MRI) on living mice. In $FXII^{-/-}$ mice and WT mice treated with PCK, areas of hyperintensity on T2-weighted images typical of acute cerebral infarction were <10% of the corresponding area in untreated WT mice 24 h after tMCAO ($P < 0.0001$; Fig. 4 B), confirming assessments with TTC staining. Importantly, follow-up MRI at days 3 and 7 post-tMCAO demonstrated that ischemic lesions in $FXII^{-/-}$ and PCK-treated WT mice always presented as hyperintense lesions on T2-weighted gradient echo images, an MRI protocol for detection of bleeding. Hypointense areas, which typically indicate hemorrhage, were absent in all animals, supporting the notion that FXII deficiency does not increase the risk of infarct or reperfusion-associated bleeding compared with WT control mice, even at more advanced stages of infarct development.

In summary, inhibition of FXII activity impairs pathological fibrin formation and salvages brain tissue during cerebral ischemia in mice. Importantly, neuroprotection through targeted inhibition of FXII did not increase bleeding during the acute infarct stage or in the recovery period. Therefore, the data of the current study and previous analyses using other

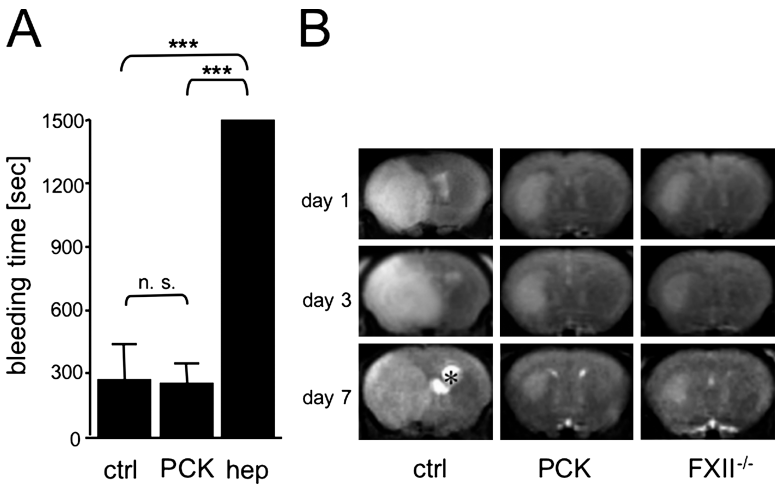

Figure 4. Inhibition of FXII activity does not affect normal hemostasis. (A) Tail bleeding times for PCK-treated (8 μg/g of body weight) and untreated control mice (ctrl; $n = 12$ per group; ***$P < 0.0001$). Heparin-infused (hep) mice are shown for comparison. (B) Serial coronal T2-weighted MRI brain images from untreated (ctrl), PCK-treated (8 μg/g of body weight), and FXII$^{-/-}$ mice at days 1, 3, and 7 after tMCAO ($n = 5$ per group). The asterisk indicates hydrocephalus of the left lateral ventricle as an indicator of infarct-related swelling.

models of arterial thrombosis (12) strongly support the evaluation of FXII as a novel pharmacological target for safe anticoagulation in humans. FXII inhibition may be useful when used prophylactically on a chronic basis, or for short-term therapy during procedures or conditions associated with increased risk of thrombosis, including vascular procedures and during surgery, without compromising hemostasis at a wound site. Finally, these findings challenge current concepts of coagulation by demonstrating that fibrin-generating mechanisms in pathologic vessel occlusion involve pathways distinct from those operating during normal hemostasis.

MATERIALS AND METHODS

Animals. All procedures and animal studies were approved by the Bezirksregierung of Unterfranken. FXII$^{-/-}$ and FXI$^{-/-}$ mice used in this study were described previously (12). 8–20-wk-old male and female mice backcrossed for not less than nine generations to C57BL/6J background were used for experiments, and WT littermate animals were used as controls. The peptide-based FXII inhibitor PCK was obtained from Bachem and administered intravenously.

Coagulation analysis. PT and aPTT clotting times were determined as described (12).

Detection of intracerebral fibrin. Tissue fibrin deposits were isolated as described, and the amount of fibrin β-chains normalized to identical cerebral tissue weight was determined by Western blot using the fibrin-specific mAb 59D8 (22).

Histopathological and immunofluorescence studies. Formalin-fixed brains embedded in paraffin (Histolab Products AB) were cut into 4-μm-thick sections and mounted. After removal of paraffin, tissues were stained with hematoxylin and eosin (Sigma-Aldrich). For immunohistochemistry, fibrin was detected using mAb 59D8, and platelets were detected using a mAb against GP Ib β (CD42c; Emfret). Images were generated using a Nikon E600 microscope (60× magnification) equipped with a Nikon C1 confocal laser scanning system.

Tail bleeding time. The bleeding time of mice was assessed as published earlier (12).

Murine stroke model. tMCAO was induced under inhalation anesthesia using the intraluminal filament (6021PK10; Doccol Company) technique (27). After 60 min, the filament was withdrawn to allow reperfusion. Laser Doppler flowmetry (Moor Instruments Ltd.) was used to monitor cerebral blood flow. For measurements of ischemic brain volume, animals were killed 24 h after induction of MCAO and infarctions were stained in 2% TTC. Brain infarct volumes were calculated and corrected for edema as described (28). To determine the effect of inhibition of FXII activity in WT mice, PCK (8 μg/g body weight) was administered intravenously immediately before tMCAO. In some experiments, human FXII (huFXII; American Diagnostics) was injected intravenously immediately before the experiment.

Neurological testing. Neurological function was assessed by two independent and blinded investigators 24 h after tMACO. Global neurological status was scored according to Bederson et al. (29). Motor function was graded using the grip test (30).

Stroke assessment by MRI. MRI was performed 24 h, and again 3 and 7 d after stroke on a 1.5 T unit (Vision; Siemens) under inhalation anesthesia. A custom made dual channel surface coil was used for all measurements (A063HACG; Rapid Biomedical). The MR protocol included a coronal T2-w sequence (slice thickness 2 mm), and a coronal T2-w gradient echo CISS sequence (Constructed Interference in Steady State; slice thickness 1 mm). M_t images were transferred to an external workstation (Leonardo; Siemens) for data processing, and were read blinded to group assignment. Infarct volumes were calculated by planimetry of the hyperintense area on high-resolution CISS images.

Statistics. Statistical analyses were made using the two-tailed student's t test. p values <0.05 were considered statistically significant. All results are given as mean ± 1 SD.

Online supplemental material. Fig. S1 shows that no obvious differences in the cardio- and cerebrovascular phenotype were observed between FXII$^{-/-}$ and WT mice. Fig. S2 gives evidence that treatment with the FXII inhibitor PCK reduces fibrin formation, intravascular fibrin deposition, and ischemic brain damage in the ischemic hemispheres

compared with control mice. Supplemental Materials and methods are also included. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052458/DC1).

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